Optogenetic and pharmacogenetic dissection of the Melanin-Concentrating-Hormone (MCH) system: implications for sleep-state modulation

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April 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy, Ph.D.

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Abstract

Sleep is a fundamental physiological process and sleep-like states have been described in nearly every animal studied to date. Despite the existence of distinct sleep-wake states, our understanding of the mechanism of sleep regulation remains incomplete. The present work focused on a particular hypothalamic neuronal population which expresses melanin-concentrating hormone (MCH). The MCH peptide is thought to have a role in the promotion of sleep based on the findings that MCH-expressing neurons are active during sleep and that administration of MCH into the ventricles dramatically increases both Rapid-Eye-Movement (REM) and, to a lesser extent, non-REM (NREM) sleep. However, all of the evidence supporting a functional role for the MCH system in sleep stems from *in vitro* and *in vivo* techniques that have spatial and temporal limitations and involve possible compensatory mechanisms. The present work aimed at clarifying the action of MCH neurons and their co-expressed neurotransmitters on sleep.

In our first study, we used optogenetic tools in newly-generated Tg(*Pmch-Cre*) mice and found that acute optical activation of MCH neurons (using ChETA or SSFO) at the onset of REM sleep extended REM sleep duration whereas MCH neuronal stimulation at NREM sleep onset promoted transition from NREM to REM sleep. In contrast, acute silencing of MCH neurons (eNpHR3.0 or ArchT) reduced the frequency and amplitude of the hippocampal theta rhythm without affecting REM sleep duration. *In vitro* activation of MCH neuron terminals induced $GABA_A$-mediated inhibitory post-synaptic currents (IPSCs) in wake-promoting neurons of the tuberomammillary nucleus (TMN), while *in vivo* activation of MCH neuron terminals in the TMN or medial septum also prolonged REM sleep episodes. Collectively, these results suggest that activation of MCH neurons maintains REM sleep, possibly through inhibition of arousal circuits in the mammalian brain, while their inhibition induced a NREM-to-REM sleep transitional state.

Our second study investigated the role of MCH peptide and GABA transmitter, which are thought to be both released by MCH neurons, during acute and semi-chronic optogenetic activation of MCH cells. We used newly-generated Tg*(Pmch-Cre):R1*$^{-/-}$ mice, Tg(*Pmch-Cre*):Vgat$^{flox/flox}$ and Tg(*Pmch-Cre): R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice along with

administration of MCH-R1 antagonist SNAP 7941. Our study revealed that acute activation of MCH neurons selectively consolidates REM sleep state whereas semi-chronic activation of the same neurons favors both NREM and REM sleep. The absence of either MCH-R1 or Vesicular GABA Transporter (VGAT) in MCH neurons, or both, raises REM sleep mean duration closer to a ceiling value in control condition, masking the REM sleep promoting effect during acute stimulation. Semi-chronic activation of MCH neurons revealed that MCH peptide likely mediates the NREM sleep promoting effect whereas REM sleep promoting effect might involve additional neurotransmitters beside GABA neurotransmission. Together, our results suggest that GABA produced by MCH neurons is playing a crucial role in the basal control of REM sleep. Importantly, these results have confirmed that the mode of MCH activation (acute vs. semi-chronic) has a different effect on sleep, possibly due to the release of various neurotransmitters acting on various targets with different timescales.

In the third study, we investigated the effect of pharmacogenetic activation of MCH neurons in the lateral hypothalamus and found that DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) activation of MCH neurons during the resting period (i.e., light period of the light/dark cycle) specifically increased REM sleep whereas a similar activation paradigm during the active period (i.e., dark period of the light/dark cycle) enhanced wakefulness and possibly arousal. These results suggest that subpopulations of MCH neurons exist, and that their modulation of behavior, including sleep, depends on the circadian phase and the physiological and homeostatic need of the animal.

Collectively, our results causally demonstrated that MCH neurons are involved in REM sleep regulation, at least during the resting phase of the sleep-wake cycle. Acute activation of MCH neurons significantly promotes REM sleep although this effect is not dependent on MCH peptide release, whereas GABA from MCH neurons is possibly involved in the regulation of basal REM sleep. Our results further suggest that MCH peptide is implicated in the modulation of NREM sleep following semi-chronic activation of MCH soma, consistent with the slow mechanism of action of peptides in the brain.

Together with studies from others, our work suggests the existence of MCH neurons subpopulations that would co-release different peptides/neurotransmitters. It is very likely that those different neurotransmitters (MCH, GABA, Nesf-1 and CART) would affect distinct sleep-wake targets and modulate the sleep-wake cycle differently.

Résumé

Le sommeil est un besoin physiologique indispensable et nécessaire à tous les animaux. Cependant les mécanismes sous-jacents le contrôlant restent méconnus. Le travail effectué durant cette thèse se concentre sur une population neuronale de l'hypothalamus qui exprime l'hormone de mélano-concentration (MCH). De nombreuses études ont mis en évidence l'implication de ce neuropeptide dans la promotion du sommeil. En effet, les neurones à MCH sont actifs seulement pendant le sommeil et l'administration de MCH dans les ventricules augmente considérablement les durées de sommeil lent (SL) et paradoxal (SP). Toutefois, les études supportant un rôle du système MCH dans l'élévation du sommeil utilisent souvent des techniques *in vivo* et *in vitro* qui possèdent une résolution spatiale ou temporelle limitée ou encore impliquant des mécanismes de compensations. Le travail effectué durant cette thèse a comme objectif de clarifier l'action des neurones à MCH ainsi que des neurotransmetteurs exprimés au sein de ces neurones dans la régulation du sommeil.

Dans la première étude, nous avons utilisé l'optogénétique sur des souris nouvellement crées et avons montré que la stimulation aigue des neurones à MCH (utilisant les opsines ChETA et SSFO) au début d'un épisode de SP prolonge la durée moyenne de cet état tandis que la stimulation optogénétique de ces neurones au début d'un épisode de SL favorise la transition vers le SP. Au contraire, l'inhibition aigue des neurones à MCH (utilisant les opsines eNpHR3.0 et ArchT) réduisent la fréquence et l'amplitude du rythme hippocampique theta sans affecter la durée moyenne du SP. L'activation *in vitro* des terminaisons des neurones à MCH induit un courant post-synaptique inhibiteur qui est GABA-dépendant sur les neurones du noyau tubéromammillaire (TMN), un noyau favorisant l'éveil. L'activation *in vivo* des terminaisons MCH-ergique au niveau du TMN ou du septum median prolonge également les durées des épisodes de SP. Ces résultats suggèrent que l'activation des neurones à MCH consolide les durées de SP possiblement par l'inhibition des circuits contrôlant l'éveil du cerveau de mammifères, alors que leur inhibition induit un état se rapprochant de l'état de transition depuis le SL vers le SP.

Notre seconde étude s'est intéressee au rôle de la MCH et du GABA, co-exprimés et libéré par les neurones à MCH, pendant la stimulation optogénétique aigue et chronique des neurones à MCH. Nous avons généré des lignées de souris Tg*(Pmch-Cre):R1*$^{-/-}$, Tg(*Pmch-Cre*):Vgat$^{flox/flox}$ and Tg(*Pmch-Cre*):R1$^{-/-}$:Vgat $^{flox/flox}$ que nous avons optogénétiquement stimulé en parallèle avec l'administration d'un antagoniste de MCH-R1, SNAP 7941. Notre étude révèle que l'activation aigue des neurones à MCH consolide l'état de SP spécifiquement tandis que leur activation semi-chronique favorise le SL en plus du SP. D'un coté, l'absence de MCH-R1 et/ou du transporteur vésiculaire du GABA (VGAT) au sein des neurones à MCH élève la durée moyenne du SP à une valeur s'approchant ou atteignant un plateau chez les animaux contrôle, ce qui a pour effet de masquer l'élévation des quantités de SP durant l'activation optogénétique. En plus de la transmission GABAergique, l'activation semi-chronique des neurones à MCH démontre que la MCH est impliqué dans l'effet promoteur du SL alors que l'effet promoteur du SP serait modulé par d'autres neurotransmetteurs. Dans l'ensemble, nos résultats suggèrent que le GABA exprimé au sein des neurones à MCH joue un rôle important dans le contrôle des quantités de SP. Il est important de souligner que nos résultats confirment que le mode d'activation des neurones à MCH (aigue ou semi-chronique) va promouvoir des états de sommeil différents, certainement dû à la libération de différents neurotransmetteurs/peptides agissant sur des structures cibles variées avec une échelle de temps différente.

Dans notre troisième étude, nous avons regardé l'effet de l'activation pharmacogénétique d'une population de neurones à MCH localisé dans l'hypothalamus latéral. Nous avons trouvé que l'activation de cette population neuronale pendant la période de repos (c'est-à-dire la période claire) a pour effet de promouvoir spécifiquement le SP tandis que cette même manipulation effectuée pendant la période d'activité (c'est à dire la période sombre) stimule l'éveil. Ces résultats suggèrent que les neurones à MCH peuvent réguler le comportement, incluant le sommeil, selon la phase circadienne et les besoins physiologiques et homéostasiques de l'animal.

L’ensemble de nos résultats démontrent de manière causale que les neurones à MCH sont très fortement impliqués dans la régulation du SP, tout au moins au cours de la phase claire du cycle veille-sommeil. L’activation aigue des neurones à MCH fortement augmente les quantités des SP bien que cet effet ne soit pas dû à la libération de la MCH, alors que le GABA des neurones à MCH est probablement impliqué dans la régulation du SP.

En accord avec l’action durable des effets induits par les peptides, la MCH est impliquée dans la modulation du SL suite à un mode d’activation semi-chronique des corps cellulaires des neurones à MCH. Nos travaux, avec les travaux d’autres équipes, suggèrent fortement l’existence de sous-population de neurones à MCH se définissant soit par les neurotransmetteurs/peptides qu’ils expriment (MCH, GABA, Nesf-1 and CART) ou soit par les structures sur lesquelles ils agissent.

Acknowledgements

Many have accompanied me throughout the years and have allowed me to get to this point. It is clear that my stay at the Douglas would not have been as enjoyable without their help, support and continued presence. I will try here to give them proper credit.

First among them is my supervisor Antoine Adamantidis (PhD.). He has given me the opportunity to learn and grow in the work environment he created. He trusted me since the very beginning, even when the lab did not exist yet, and I am very grateful to him. Among other things, he gave me the opportunity to pioneer *in vivo* optogenetics at the Douglas and everything I accomplished since then is for a great part thanks to him. In his laboratory I have enjoyed scientific freedom and been exposed to a range of techniques and topics that have considerably expanded my scientific horizons. He was always present to hear my difficulties and a little meeting in his office or on Skype was all that was needed to put me back on the right track. I am certain that his current and future students in Bern will be as lucky as I was here.

Antoine has gathered around him a group of very talented people with whom I had the chance of sharing the last few years. Many of which I now consider friends and some have become even a bit more. They include, in order of appearance Richard Boyce (MSc.), Stephen Glasgow (PhD), Carolina Gutierrez-Herrera (PhD), Jessica Colby-Milley (MSc.), Raphael Lavoie (MSc.) and Sean Reed (MSc.). You are all part of what made the Tidis lab a great learning experience.

I also find it appropriate to thank all the people from the animal facility staff at the Douglas who cared for all of my animals. The well-being of our mice was the result of their work and was directly beneficial to my research. Thank you for the animals!

For the past years, I have been surrounded by incredible people who made my stay at the Douglas a great adventure to remember. These people helped me in the everyday life in the lab but also outside the lab during the good and bad days. I am thinking particularly, but not only, of Richard, Marie-Eve, Adeline, Greg, Béné, Jill, Jenn, Caro and Geneviève. My experience here would have been less enjoyable without you.

I also must at this point acknowledge my previous mentors. I would like to thank the people who surrounded me during my Master in France: my previous supervisor, Patrice Fort (Ph.D.) and all of the Luppi group who have given me essential foundations and perspectives to pursue my research on sleep and MCH neurons. To this day we continue to work together. I also would like to thank Nicolas Cermakian (Ph.D.) who was very understanding and supportive of my choices at the beginning of my stay at the Douglas.
I wish to thank my supervisory committee members Barbara Jones (Ph.D.), Sylvain Williams (Ph.D.), Thien Thanh Dang-Vu (M.D./Ph.D.) and my IPN mentor Joe Rochford (Ph.D.), who have taken their time to offer me guidance and counsels. Lastly, thanks to my examiners and defense committee who are currently reading this work. I eagerly await your comments and questions regarding this work.

Finally, I also wish to thank my family, especially my parents and my brother who were and remain very supportive with my work and always trusted me with any decision I was making.

Contribution of Authors

Chapter 2: Optogenetic identification of a rapid-eye-movement (REM) sleep modulatory circuit in the hypothalamus.

The majority of the data and analysis presented in chapter 2 were done by the author with the following exceptions. Stephen Glasgow performed all the *in vitro* data collection and analysis including the verification of the opsin efficiency upon optogenetic activation of MCH cells and the stimulation of Cheta-expressing MCH fiber to the TMN. Carolina Gutierrez Herrera performed the fluorescent *in situ* hybridization and data analysis for the quantification of the co-expression of GAD67 mRNA in MCH neurons. Richard Boyce created a MATLAB script in order to detect slow theta in the sleep recording during the REM sleep silencing of MCH neurons. Sean Reed and the author performed unit recordings in anesthetized MCH: Cre animals.

Chapter 3: Role of MCH peptide in the modulation of sleep-wake cycle

All experiments and analysis presented in chapter 3 were performed by the author.

Chapter 4: Pharmacogenetic excitation of MCH peptide across the sleep-wake cycle

All experiments and analysis presented in chapter 4 regarding the pharmacogenetic activation of MCH neurons was performed by the author. However, this study is part of a project done in collaboration with Christophe Varin (Msc) and Patrice Fort (PhD) in Lyon (France), who are looking at the pharmacogenetic silencing of MCH neurons on sleep, although this data is not reported here.

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Foreword: Overview and organization

The current work investigated the role of Melanin-Concentrating-Hormone (MCH) neurons and their co-expressed neurotransmitters on sleep. Before the start of this project, the MCH neuron population was recognized as a general sleep-promoting factor but its precise role in the regulation of particular sleep stages, namely non-Rapid-Eye-Movement (NREM) sleep and/or REM sleep remained unclear. Accordingly, in the introductory section, I will present a brief overview of the circuits that are presently known to regulate sleep and arousal, as well as a description of the MCH system and its role in regulating several physiological functions, including sleep.

In the second chapter, I will describe the effect of optogenetic activation and inhibition of MCH neurons during sleep specific states. This method allowed us to appreciate the effect of acute activation of MCH neurons, which was impossible to do with more traditional methods such as pharmacology or knock-out/knock-in mice. We also looked at the effect of optogenetic activation of MCH neural projections to different arousal centers.

In the third chapter, I present the results of a series of optogenetic experiments in various transgenic mice lines I generated. The purpose of these experiments is to better understand the role of MCH peptide in the sleep promoting effect. I used mice with disrupted MCH transmission (absence of functional receptor for MCH, MCH-R1), mice with disrupted GABAergic transmission (absence of vGAT in MCH neurons) and triple transgenic mice with an absence of both MCH-R1 and vGAT. I performed acute and semi-chronic stimulation on all of these mouse lines in order to assess the possibility that neurotransmitters (such as GABA) and peptides (such as MCH) can act preferentially under different stimulation parameters.

Finally, in the fourth chapter, I took advantage of new advances in pharmacogenetic technology to confirm the effect of chronic activation of MCH neurons across the sleep wake cycle in order to determine whether effects of MCH neurons are modulated by the underlying circadian rhythm.

Chapter 1: Introduction

Sleep or sleep-like states have been demonstrated throughout the animal kingdom from invertebrates to vertebrates, reflecting the primary and essential biological need for sleep (Hendricks et al., 2000; Cirelli and Tononi, 2008). In higher vertebrates, the sleep-wake cycle is a succession of three vigilance states: wakefulness, non-REM sleep (NREM, also called slow wave sleep) and REM sleep for ‘‘rapid eye movement’’ sleep (also called paradoxical sleep). The mammalian sleep cycle consists of an orderly progression through the sleep states. Following NREM sleep episode, animals shift to either wakefulness (and then back to NREM sleep) or a REM sleep state. REM sleep episodes often terminate with a transition to wakefulness. These vigilance states are studied by recording brain activity using the electroencephalogram (EEG) along with muscular tone using electromyogram (EMG) recordings. The neural underpinnings of the sleep-wake cycle involve interactions between arousal systems located mainly in the posterior hypothalamus and in the brainstem reticular formation and sleep-promoting areas located in the hypothalamus, and brainstem. More recently, a small number of studies have suggested that the posterior hypothalamus contains a unique contingent of melanin-concentrating hormone (MCH) neurons that is critically involved in the regulation of REM sleep. However, mostly due to technical limitations, the modulatory role of the MCH system on sleep and wakefulness remained unclear. The objective of my studies was to demonstrate that MCH neurons act as a sleep-promoting system that specifically regulates the REM sleep state. To test this hypothesis, I combined optogenetic, pharmacological and pharmacogenetic modulation of MCH neurons with EEG/EMG recordings in a genetically engineered mouse model. But before going into the results and to better understand the context of this work, I will first provide some introductory information on the sleep-wake cycle regulation and the MCH system.

1-1 Sleep-wake cycle

The sleep-wake cycle results from the interactions between sleep-promoting areas, such as the hypothalamus and brainstem, and arousal systems, including the posterior hypothalamus, the basal forebrain and the brainstem. Distinct neuronal populations within these structures contain neurotransmitters and/or neuromodulators such as glutamate, histamine, acetylcholine, noradrenaline, serotonin and neuropeptides. Their respective actions on the sleep-wake cycle are summarized below.

1-1-1 Wakefulness

Wakefulness is characterized by cortical activation, which is evident on electroencephalograms (EEG) recorded from the surface of the cortex, and behavioral arousal, which is evident on the electromyogram (EMG) by high-amplitude activity in the postural muscles, particularly in the neck. Wakefulness is associated with a low-voltage/fast-frequency EEG (also known as "activated" or "desynchronized", although this latter term should be avoided since synchronized oscillations do occur during wake states) and with high muscle tone (see Fig. 1-1).

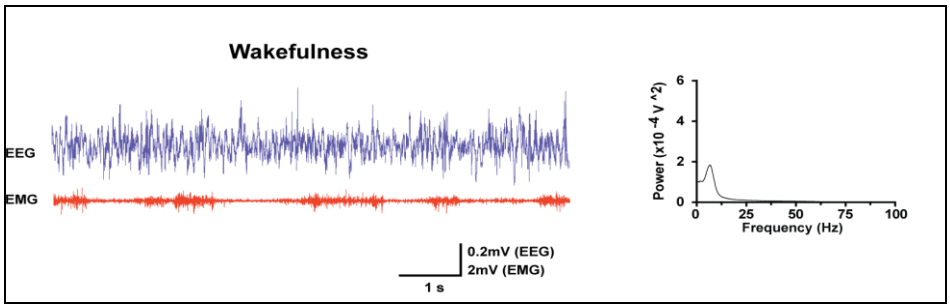

Figure 1-1: **EEG and EMG during arousal** in freely-behaving male mouse. *Left,* representative EEG/EMG recording trace of a period of wakefulness indicated by fast EEG cortical oscillation and muscle tone. *Right*, power spectrum (0 - 100 Hz) of EEG signals during wakefulness.

Early experiments performed by Moruzzi and Magoun showed that electrical stimulation of the midbrain reticular formation in anesthetized cats caused the appearance of an “activated” EEG similar to that seen during wakefulness (Moruzzi and Magoun, 1949). These findings led to the important concept of the “ascending reticular activating system (ARAS),” a network (reticulum) of nerve fibers ascending from the

brainstem, which through multiple intermediary sites causes activation of the forebrain during wakefulness and REM sleep.

The ARAS consists of dorsal and ventral pathways. Axonal tracing studies coupled with histochemical or immunohistochemical visualization of particular neurotransmitter systems revealed the anatomical pathways transferring brainstem activity to the cerebral cortex (Jones, 2003). Single-unit recordings and indirect measures of neuronal activity (using immunohistochemical detection of the immediate early gene product Fos) defined the neurons in these areas whose activity is correlated with wakefulness or sleep (Jones, 2003). Two main pathways have been identified : a dorsal route through the thalamus and a ventral route, through the hypothalamus and the BF (Jones, 1993) (Fig. 1-2).

The dorsal pathway is largely direct to the cerebral cortex to stimulate cortical activation. Glutamatergic nuclei from the reticular formation (Steriade and Glenn, 1982; Steriade et al., 1988; Cornwall et al., 1990; Newman and Ginsberg, 1994) and cholinergic cell groups located in the lateral dorsal tegmental (LDT) and pedunculopontine tegmental (PPT) regions of the pons (Jones and Beaudet, 1987; Steriade et al., 1988) project to the thalamic nuclei which in turn activate the entire cerebral cortex (Nauta, 1946; Saper and Loewy, 1980; Macchi et al., 1986). Surprisingly, experiments in rodents (Buzsaki et al., 1988; Fuller et al., 2011) and cats (Villablanca and Salinas-Zeballos, 1972) showed that very large lesions of the thalamus appear to have very little effect on cortical activation and the sleep-wake cycle in general, aside from a loss of sleep spindles, suggesting that the dorsal pathway is not absolutely necessary. However, a complete and selective ablation of thalamus is hard to achieve with lesion techniques, and it remains possible that a small thalamic projection remained after these lesions that was sufficient to maintain function.

The ventral pathway is mainly indirect to the cerebral cortex as it passes through neurons in the midbrain, posterior/lateral hypothalamus, and basal forebrain (BF) (Jones, 1993), which in turn project to the cortex. The ascending fibers arise from the brainstem and include projections from the reticicular formation, the parabrachial nuclei (PB, glutamate), LDT/PPT, LC, raphe, and dopaminergic (periaqueductal gray - PAG)

neurons. These systems synapse onto glutamatergic, histaminergic, and orexinergic/ hypocretinergic (Hcrt) neurons in the posterior/lateral hypothalamus (Jones, 2005). All of these systems converge onto caudal BF cholinergic, GABAergic, and glutamatergic neurons that project to and activate the neocortex (Dringenberg and Vanderwolf, 1998; Detari et al., 1999; Semba, 2000; Jones, 2003). A branch of this system innervates the rostral BF theta rhythm generator. Direct projections to the cortex and the nonspecific thalamic nuclei also arise from brainstem noradrenergic and serotonergic neurons (See Fig. 1-2).

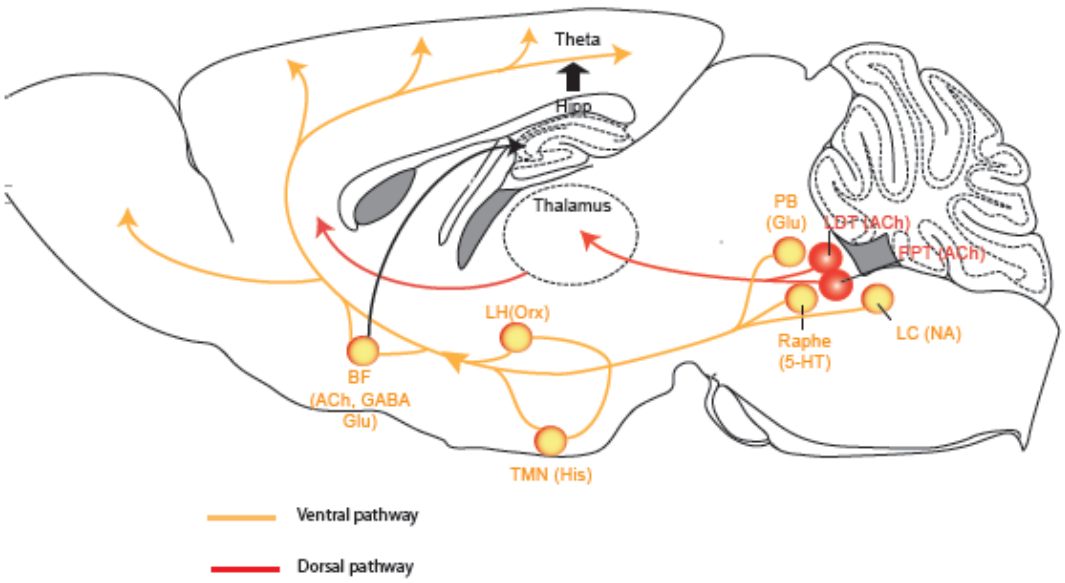

Figure 1-2: Ascending activation of the cortex. The ascending reticular activation system (ARAS) reaches the cortex through a ventral pathway (Hypothalamus, BF) and a dorsal pathway, the thalamic relay. Abbreviations: 5-HT, serotonin; Ach, Acetylcholin; BF, Basal forbrain; Glu, Glutamate; His, Histamine; Hipp, hippocampus; LDT, laterodorsal tegmental nucleus;LC; Locus coeruleus; LH, Lateral Hypothalamus; PB, Parabrachial nucleus; PPT, pedunculopontine tegmental nucleus; TMN, tuberomammillary nucleus.

In contrast to the thalamic lesions discussed above, a recent study showed that large lesions of the BF, or of the brainstem PB, which provides one of the major brainstem glutamatergic input to the BF, led to a comatose state in rats (Fuller et al., 2011), whereas, as discussed above, thalamic lesions had little effect. This suggests that

projection from PB, relayed by BF to the cerebral cortex, may be critical in behavioral or cortical arousal. However, these results should be carefully interpreted since lesions in the PB often lead to respiratory effects (Kaur et al., 2013), which can also contribute to the comatose effect observed.

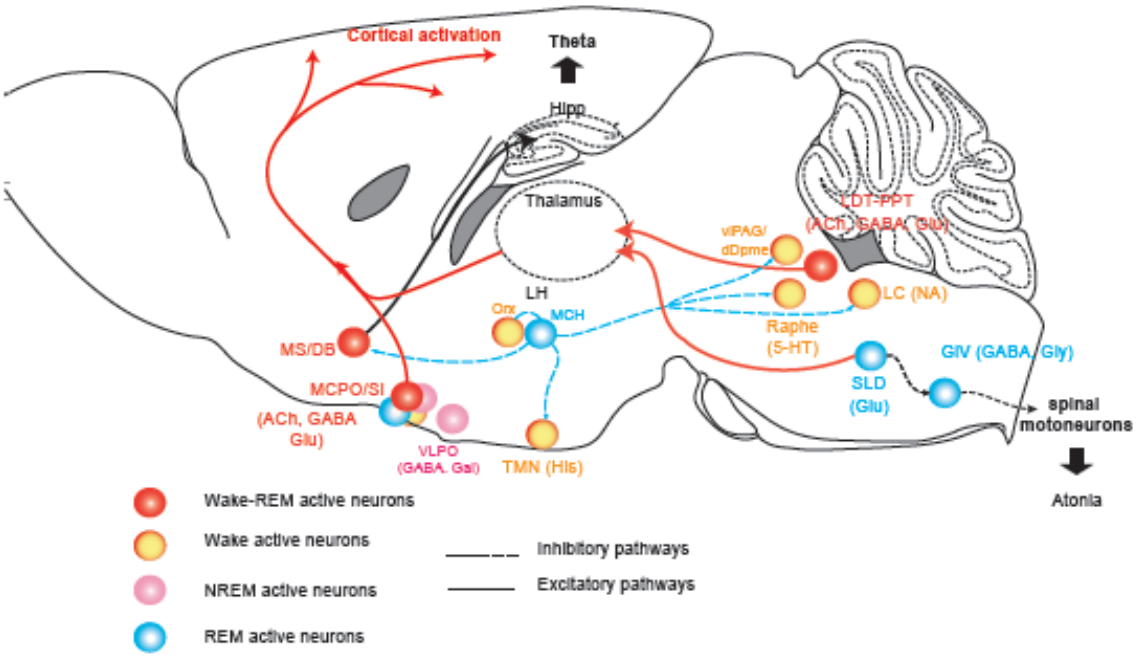

Figure 1-3: Sagittal schematic view of a rodent brain depicting the main structures involved in sleep-wake regulation with their chemical neurotransmitters and pathways by which they influence cortical activity or behavior across the sleep-wake cycle (with an emphasis on MCH cells). REM active neurons can be found in the BF, the SLD, GiV and LH. Note the numerous targets of MCH neurons. Abbreviations: 5-HT, serotonin; Ach, Acetylcholine; BF, Basal forebrain; GiV, gigantocellular medullary nucleus ; Glu, Glutamate; His, Histamine; Hipp, hippocampus; LDT, laterodorsal tegmental nucleus;LC; Locus coereleus; LH, Lateral Hypothalamus; MCH, Melanin-Concentrating Hormone; MCPO/SI, magnocellular preoptic nucleus/substantia innominata; MS/DB, Medial spetum/Diagonal Band of Broca; PB, Parabrachial nucleus; PPT, pedunculopontine tegmental nucleus; SLD, Sub-latero dorsal Nucleus; TMN, tuberomammillary nucleus;vPAG/dDPMe, Ventrolateral periaqueducal gray/ deep mesencephalic reticular nucleus; VLPO, Ventro Lateral Preoptic Area.

Wake-promoting neurons can discharge either only during wakefulness or during both Wake and REM sleep (also characterized by cortical activation). Neurons which are active during wakefulness and silent during both NREM and REM sleep send ascending

projections to the cortex, which stimulate fast cortical activity, but also send descending projections to the spinal cord, which stimulate movement and enhance postural-muscle tone, as typical of behavioral arousal (Sprague and Chambers, 1954). These neurons include NA from the LC, 5-HT from the raphe, Histamine neurons from the TMN, Orx from the LH and some putative glutamate neurons from the BF (McGinty and Harper, 1976; Aston-Jones and Bloom, 1981; Lee et al., 2004; Lee et al., 2005a; Mileykovskiy et al., 2005; Takahashi et al., 2006; Wu et al., 2012) (See Fig. 1-3).

On the other hand, neurons which discharge in association with fast EEG activity (gamma: 20-60 Hz), cease firing with delta activity (NREM sleep oscillation, 0.5-4 Hz), are active during W and REM sleep and send predominantly ascending projections to the cortex but not to motoneurons. These neurons include ACh, glutamate and some GABA neurons from the BF and LDT/PPT (el Mansari et al., 1989; Lee et al., 2004; Lee et al., 2005b; Boucetta et al., 2014) (See Fig. 1-3).

1-1-2 NREM sleep

During NREM sleep, the EEG is dominated by high-voltage slow-waves (Delta: 0.5 - 4Hz) reflecting a high degree of neuronal synchronization associated with low muscle tone (See Fig. 1-4). Slow oscillations results from the rhythmic change between hyperpolarisation and depolarisation of thalamocortical neurons. Delta rhythm is generated intrinsically by thalamic relay neurons as a result of their ability to generate burst firing in the delta frequency range due to their intrinsic membrane properties (Jahnsen and Llinas, 1984b, a; Leresche et al., 1990; McCormick and Pape, 1990a; Soltesz et al., 1991). Indeed, the interplay between their

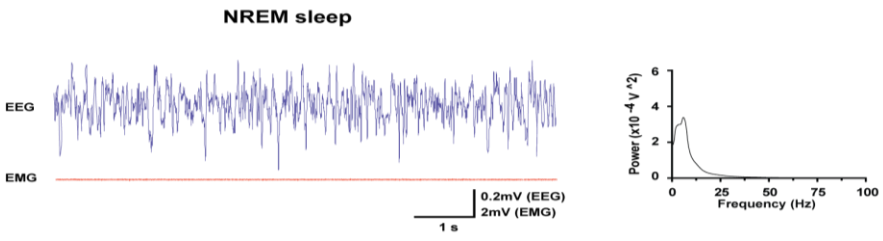

Figure 1-4: **EEG and EMG recording during NREM sleep** in freely-behaving male mouse. *Left,* representative EEG/EMG recording trace of a period of NREM sleep indicated by slow EEG cortical oscillation and no muscle tone. *Right*, power spectrum (0 - 100 Hz) of EEG signals during NREM sleep showing the predominance of the delta band (0.5-4 Hz) at that stage.

low-threshold Ca^{2+} current (I_T) and hyperpolarization activated cation current (I_h) (McCormick and Pape, 1990b) change their firing from tonic to bursting mode when membrane potential of the thalamic relay neurons are hyperpolarised. As such, the delta oscillation may be observed during NREM sleep when thalamic relay neurons are hyperpolarized sufficiently to deinactivate I_T (McCormick and Pape, 1990a; Leresche et al., 1991; Soltesz et al., 1991; Dossi et al., 1992) by the increased withdrawal of excitatory inputs (from ARAS system: mainly cholinergic and aminergic). *In vivo* recordings from thalamocortical neurons revealed that high-frequency bursts of action potentials occur at delta frequencies interspersed with silent periods (McCarley et al., 1983; Steriade et al., 1991; Amzica et al., 1992; Dossi et al., 1992; Nunez et al., 1992), a pattern which can be abolished by brain stem cholinergic stimulation (Steriade et al., 1991; Amzica et al., 1992; Nunez et al., 1992). These slow oscillations are not involved in NREM sleep generation *per se* but are responsible for the cortical synchronisation of this state.

The involvement of the preoptic area and adjacent basal forebrain in sleep regulation has been recognized since Constantin von Economo reported that lesions of this region cause prolonged insomnia in humans (von Economo, 1926). Nauta (1946) demonstrated that preoptic–basal forebrain lesions cause insomnia in rats (Nauta, 1946) , and Sterman and Clemente (Sterman and Clemente, 1962a, b) demonstrated similar effects in cats. These experimental studies used electrolytic or mechanical lesions that not only destroyed neuronal cell bodies but also damaged fibers of passage. More recent studies using chemical toxins to ablate the neuronal cell bodies in this area in rats and cats have confirmed the production of insomnia (Szymusiak and McGinty, 1986a; Sallanon et al., 1989; John and Kumar, 1998) which identified a specific population of neurons in the ventrolateral preoptic area (VLPO)(Sherin et al., 1996).

The onset of NREM sleep correlates with the neuronal discharge in the ventrolateral preoptic area (VLPO) of the anterior hypothalamus and the activation of cortico-thalamic loops. VLPO contains sleep-active neurons which increase their discharge selectively at sleep onset (Szymusiak and McGinty, 1986b, 1989) (See Fig. 5). The VLPO cells contain GABA/Galanine and project to serotonergic, noradrenergic and cholinergic cell

groups in the brainstem reticular formation and histaminergic neurons in the TMN (Tuberomammillary nucleus) (Szymusiak et al., 2007). Therefore, it has been proposed that activation of VLPO neurons induces inhibition of the wake-promoting neuronal cells associated with the ARAS.

Although animals with lesions of the VLPO show a marked (~60-70%) decreased of delta power and a 50-60% decrease in NREM sleep quantities (Lu et al., 2000), NREM sleep is not completely abolished, suggesting that additional circuitry for NREM sleep promotion and maintenance must exist. Indeed, recent studies reveal that GABAergic/Glycinergic containing neurons from the medullary parafacial zone (PZ) promote NREM sleep (Anaclet et al., 2012) by inhibiting glutamatergic neurons from the parabrachial neurons, which in turn project to and release synaptic glutamate onto cortically projecting neurons of the magnocellular basal forebrain (Anaclet et al., 2014). Thus, it has been postulated that PZ neurons can trigger and modulate slow wave activity. Interestingly, the VLPO and PZ do not seem to send projection to each other (Sherin et al., 1998; Chou et al., 2002) so they might operate with synergy to modulate NREM sleep. Furthermore, several studies showed the presence of NREM sleep active neurons distributed through the preoptic area and basal forebrain (Hassani et al., 2009b; Sakai, 2011), which may also contribute to the regulation of NREM sleep.

In addition, NREM sleep can also be regulated by other homeostatic modulators, such as adenosine and prostaglandins, which accumulate during wakefulness and would favor sleep initiation (Hayaishi and Urade, 2002; Gallopin et al., 2004). Production of adenosine, a sleep promoting neuromodulator, is coupled with metabolic activity, which is higher during wakefulness as compared to sleep (Maquet, 1995; Porkka-Heiskanen et al., 1997). Acting through A1 receptors, adenosine has inhibitory effects on neurons in several wake promoting cell populations, including cholinergic forebrain and Hcrt neurons, that promote sleep (Greene and Haas, 1991; Rainnie et al., 1994; Portas et al., 1997; Basheer et al., 1998; Liu and Gao, 2007). In addition, several studies also suggest that adenosine may directly activate a subset of the VLPO sleep promoting neurons at sleep onset by acting on postsynaptic A_{2A} receptor to promote sleep (Satoh et al., 1996; Scammell et al., 2001; Gallopin et al., 2005). Conversely, adenosine

receptor antagonists (e.g., caffeine, which is a potent behavioral stimulant) suppress sleep (Landolt et al., 1995; Bennett and Semba, 1998; Huang et al., 2005). Interestingly, the specific neurons on which caffeine acts to produce arousal have been located in the shell region of the nucleus accumbens and this involves blockade of A_{2A} receptors (Lazarus et al., 2011).

1-1-3 REM sleep

REM sleep is characterized by theta activity (6-9Hz) in the EEG associated with muscular atonia (See Fig. 1-5).

Pioneering studies in the 60's performed on cats showed that REM sleep persists after removal of brain regions located rostral to the brainstem suggesting that areas involved in REM sleep generation lie within the pons (Jouvet, 1965). Further experiments showed that glutamatergic neurons of the sublaterodorsal nucleus (SLD) were critical for the onset of REM sleep (Boissard et al., 2003; Sapin et al., 2009).

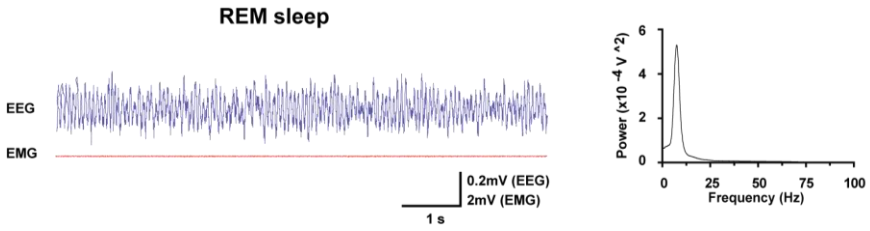

Figure 1-5: **EEG and EMG recording during REM sleep** in freely-behaving male mouse. *Left,* representative EEG/EMG recording trace of a period of REM sleep indicated by fast EEG cortical oscillation associated with no muscle tone. *Right*, power spectrum (0 - 100 Hz) of EEG signals during REM sleep, showing the predominance of the theta band (6-9 Hz) at that stage.

Following the localization of REM sleep generating nuclei, enhancement of cholinergic tone or stimulation of cholinergic receptors in the pontine reticular formation of cats and dogs was found to cause REM like states leading to the "cholinergic hypothesis" of REM sleep generation (Reid et al., 1994; Kubin, 2001). In agreement with this hypothesis, it was shown that a proportion of cholinergic neurons in the LDT/PPT were Fos positive after carbachol injection-induced REM in cats (Shiromani et al., 1996) or during recovery after REM sleep deprivation in rats (Maloney et al., 1999). However, other studies using carbachol administration in freely moving rats described either a

moderate REM sleep enhancement compared to cats (Gnadt and Pegram, 1986; Velazquez-Moctezuma et al., 1989; Bourgin et al., 1995) or no effect (Deurveilher et al., 1997), contradicting this model. Overall, the question of whether activation of cholinergic neurons and inhibition of monoaminergic neurons (that are silent during REM sleep) are essential for the induction of REM sleep remains unresolved (McCarley and Hobson, 1975; Hobson and McCarley, 1977). An alternative but related hypothesis is that cholinergic and monoaminergic neurons are not absolutely required for REM sleep generation but instead facilitate it (Brown et al., 2012).

Recently, there has been increased interest in the role of GABAergic and glutamatergic neurons in the control of REM sleep. Indeed, it has been hypothesised that REM-ON (i.e., neurons that are active during REM sleep) GABAergic nuclei (such as the ventral and lateral PAG (vlPAG) and the dorsal paragigantocellular reticular nucleus (DPGi)) initiate and/or maintain REM sleep by inhibiting REM-OFF neurons (i.e., neurons that are silent during REM sleep) such as monoaminergic neurons or vlPAG GABAergic REM-OFF neurons (Luppi et al., 2012). Additionally, SLD neurons would trigger muscle atonia through their descending projection to the glycinergic and GABAergic neurons of the ventral and alpha gigantocellular (Giv) nuclei inhibiting spinal motoneurons (Luppi et al., 2012). Recently, hypothalamic neuronal populations, including MCH neurons and neurons located in the extended VLPO, have been proposed to regulate the REM sleep state (Verret et al., 2003; Saper et al., 2005b; Clement et al., 2012).

1-2 Hypothalamic regulation of the sleep-wake cycle

The hypothalamus has long been known as a brain region that modulates energy homeostasis, food intake, temperature, goal-oriented and reward behaviors (Bernardis and Bellinger, 1996; Saper et al., 2001; Saper et al., 2005a). Growing evidence indicates that the hypothalamus also plays a role in the regulation of the sleep-wake cycle. Single-unit recordings have identified hypothalamic neurons that are strongly activated during wakefulness, NREM or REM sleep (Steininger et al., 1999; Alam et al., 2002; Koyama et al., 2003; Goutagny et al., 2005), suggesting the existence of neuronal populations with sleep- and wake-promoting properties. However, their chemical nature

remained unknown until recently. In the next section we will describe the main hypothalamic neuromodulators involved in arousal promotion (the orexins/hypocretins, GABA and histamine) and the ones implicated in the promotion of sleep (GABA, galanine and MCH).

1-2-1 Orexins / hypocretins

Three independent studies identified Hcrts (de Lecea et al., 1998) (Hcrt-$_{1, 2}$, also known as Orexins-$_{A, B}$ (Sakurai et al., 1998)) as wake-promoting neuronal populations since their activity was found to be silent during NREM and REM sleep, low during quiet wakefulness and higher during attentive or active waking (Lee et al., 2005a; Mileykovskiy et al., 2005; Takahashi et al., 2008). Orexin neurons project to the entire brain and provide heavy innervations to modulate other wake-promoting brain regions such as the LC and TMN (Peyron et al., 1998; Carter et al., 2010).

Orexins have been implicated in regulating arousal since loss of orexin neurons (Hara et al., 2001) or orexin peptide (Chemelli et al., 1999) is associated with narcolepsy, a sleep disorder characterized by excessive daytime sleepiness, sleep fragmentation, wakefulness instability, hypnagogic hallucinations, sleep-onset REM sleep episodes and cataplexsy (sudden loss of muscle tone). Human narcoleptics with cataplexy have low to negligible levels of orexin-A in the cerebrospinal fluid (Nishino et al., 2000; Mignot et al., 2002) and post-mortem tissue examination revealed a massive loss of the orexin neurons in the brain of narcoleptic patients (Peyron et al., 2000; Thannickal et al., 2000; Dauvilliers et al., 2003). Furthermore, stimulation of orexin neurons with optogenetic or Designer Receptors Exclusively Activated by Designer Drugs (DREADD) increases waking (Adamantidis et al., 2007; Carter et al., 2010; Sasaki et al., 2011) while inhibition increases time spent in NREM sleep (Sasaki et al., 2011; Tsunematsu et al., 2011). Altogether, these results point out the important arousal-promoting effect of orexin.

1-2-2 Histamine

Neurons of the tuberomammillary nucleus (TMN) are the only neuronal source of histamine in the CNS (Haas et al., 2008) and their activity was found to be maximal

during attentive waking, lower during quiet waking and silent during sleep (Takahashi et al., 2006). Histamine plays a critical role in arousal and in maintaining vigilance. Drugs that blocks histamine receptor H1 signaling (commonly called anti-histaminic) are often sedating, and mice with disrupted histamine signaling have reduced arousal during the dark period and when placed in a novel environment (Parmentier et al., 2002; Haas et al., 2008; Thakkar, 2011). TMN neurons project widely throughout the CNS, notably innervating wake-promoting brain regions (Panula et al., 1989), and histamine activates neurons in the LC, raphe nuclei, thalamus, basal forebrain, and cortex (Haas et al., 2008). TMN neurons also project heavily to sleep-active ventrolateral preoptic nucleus (VLPO) neurons, which reciprocally innervate the TMN (Sherin et al., 1998; Steininger et al., 2001; Chou et al., 2002). Recent study found that in VLPO the response to histamine was indirect, most likely via a GABAergic interneuron (Williams et al., 2014). This mutual inhibition is predicted to stabilize behavioral states while allowing transitions between each state.

1-2-3 GABA/Glycine

On the other hand, GABAergic and glycinergic cells in the anterior hypothalamus have sleep-promoting properties since they are active (according to c-Fos expression studies) during both NREM and REM sleep (Gallopin et al., 2000; Lu et al., 2002; McGinty et al., 2004). GABAergic neurons located in VLPO and MnPN (median preoptic nucleus) areas have also been shown to be involved in this inhibition during NREM sleep while those located in the extended VLPO exert an inhibitory influence during REM sleep (Lu et al., 2002). In addition, a large portion (more than 50%) of the GABAergic neural population from the lateral hypothalamus (LH) is active only during sleep states. Among these sleep-active neurons more than 80% are active only during REM sleep (Hassani et al., 2010), demonstrating that the LH contains both sleep-active and wake-active neuronal populations. Recently, neurons producing Melanin-Concentrating Hormone (MCH) peptide (Kawauchi et al., 1983; Vaughan et al., 1989) have been proposed to promote sleep, in particular REM sleep (Verret et al., 2003; Modirrousta et al., 2005; Hassani et al., 2009a).

1-3 Discovery of MCH system

1-3-1 MCH peptide

The MCH peptide was found to be expressed in the hypothalamus of many vertebrate groups, the most well-established structural, expression and functional studies being in teleosts and mammals. The peptide was initially discovered, and incidentally named, on the basis of its striking pigment aggregation property in the skin melanophores from fish, opposing the pigmentary dispersing effect of α-MSH. In fishes, MCH was characterized as a prominent neurohypophysial 17 AA-long peptide (Kawauchi et al., 1983). The ability of MCH to aggregate melanin was not found confined to teleost fishes since holostean fishes also respond to this peptide. However, there is no evidence that MCH plays a major role in color change other than in neopterygians, suggesting therefore that its skin lightening function evolved later and uniquely in a group ancestrals to teleosts and holosteans (Baker, 1993; Kawauchi and Baker, 2004). Immunochemical studies using an antisalmon MCH antiserum indicated the existence of a MCH-like factor in the rat (Zamir et al., 1986; Nahon, 1994). The rat peptide was biochemically isolated from rat hypothalamic extracts in 1989 (Vaughan et al., 1989). Rat and salmon MCH are both cyclic

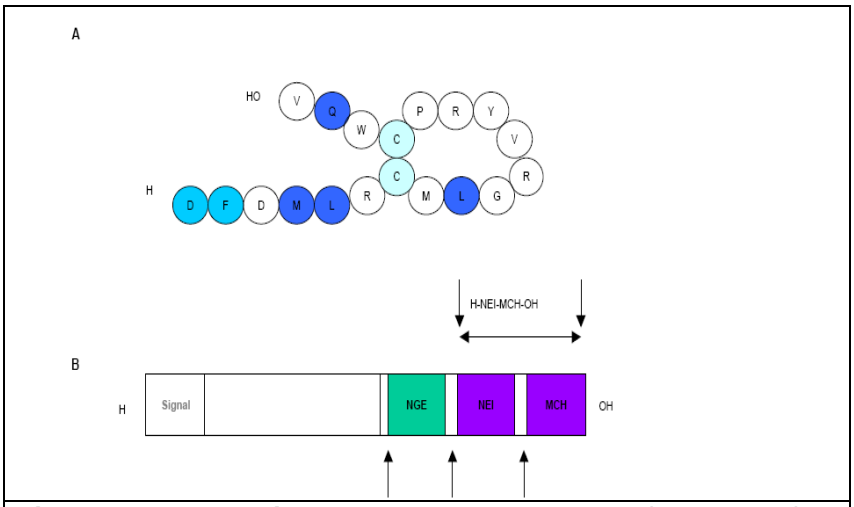

Figure 1-6: MCH and prepro-MCH structures. A. The rat MCH is a cyclic 19 amino acid peptide. Amino acid residues that differ from the fish sequence are dark blue and additional residues for the mammalian peptide are mid blue. The disulfide bridge is light blue. The peptide is matured from a prepro-MCH protein by pro-hormone convertase proteolysis. The precursor, which contains a signal peptide, could also release NEI and possibly NGE (B). Basic cleavage sites are indicated by arrows. MCH and NEI have been biochemically characterised, whilst NGE has not. Adapted from Hervieu (Hervieu, 2003).

neuropeptides and display strong sequence identity conserved within the loop structure of the molecule. Cloned mammalian forms of MCH (mouse, rat, human) display a strong amino acid sequence identity (Fig. 1- 6).

MCH is generated by the cleavage of a prepro-MCH (ppMCH) peptide precursor of 165 amino acids in length. Additional peptides are also generated by this cleavage including neuropeptide EI (NEI) and neuropeptide GE (NGE; (Bittencourt and Celis, 2008)). Anecdotally, the amidated C-terminal tail of NEI led to the MCH neuronal population being called the α-2 system, or 'second α-MSH' system. In addition, alternative splicing of the MCH precursor gene leads to the expression of a different precursor which encodes two other bioactive peptides: MCH gene-overprinted peptides MGOP-14 and MGOP-27 (Toumaniantz et al., 1996). An additional transcriptional product of the MCH gene is transcribed from the antisense strand partially overlapping with the MCH gene locus and is named antisense RNA overlapping MCH (AROM) gene. This gene produces multiple products through alternative splicing but their function remains unknown (Borsu et al., 2000).

1-3-2 Anatomy

In mammals, MCH-expressing neurons (~12000 neurons in rat brain (Modirrousta et al., 2005)) are restricted to neurons localized in the lateral hypothalamus and the Zona Incerta (ZI) (Kawauchi et al., 1983; Skofitsch et al., 1985; Bittencourt et al., 1992; Saito and Nagasaki, 2008). MCH neurons project to many brain regions throughout the central nervous system (Bittencourt et al., 1992).

These include brain structures involved in the sleep-wake cycle such as the thalamus, septum, TMN of the posterior hypothalamus, the preoptic area of the anterior hypothalamus, VTA, PAG, LC, the nucleus pontis oralis (NPO in the cat) or SLD in rodent, LDT-PPT nuclei and DR, cortex, hippocampus and nucleus accumbens (Bittencourt et al., 1992; McGregor et al., 2005; Elias et al., 2008; Torterolo et al., 2009) (See Fig. 1-7).

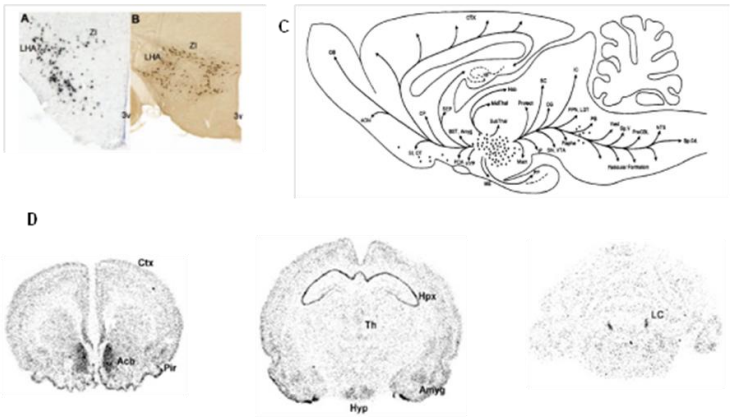

Figure 1-7: MCH and MCH-R1 localisation in the rat brain. A. In situ hybridization showing wide distribution of MCH mRNA-expressing neurons in the lateral hypothalamus and ZI of the mouse. B. Immunohistochemical detection of MCH-expressing neurons reveals a similar distribution pattern throughout the LH and ZI, from Pissios, 2006 (Pissios et al., 2006). C. MCH efferent projections are sent throughout the brain (Bittencourt et al., 1992). D. In most brain regions there is a good correspondence between MCHR mRNA distribution and that of MCH-immunoreactive fibers (Saito et al., 2001a). ACB: Nucleus accumbens; AON: Anterior olfactory nucleus; Cb: Cerebellum; CP: Caudate-putamen; CTX: Neocortex; GRN: Gigantic reticular nuclei; HDB: Horizontal limb of the diagonal band; hi: Hippocampus; IC: Inferior colliculus; LHA: Lateral hypothalamic area; LL: Lateral lemniscus; MCH: Melanin-concentrating hormone; MDRN: Medullary reticular nuclei; MV: Medial vestribular nuclei; NTS: Nucleus of the solitary tract; OB: Olfactory bulb; OT: Olfactory tube; Pn: Pons; PRN: Pontine reticular nuclei; PSV: Principal sensory trigeminal; SC: Superior colliculus; SPV: Nucleus of the trigeminal nerve; SUB: Subiculum; VP: Ventro–posterior thalamus complex. LHA: Lateral hypothalamus area; 3V: third ventricle.

Interestingly, MCH peptide expression has been found in tanycytes, cells that are specialized in the transport of substances from the CSF to neural parenchyma, suggesting that MCH is absorbed from the CSF and subsequently liberated to act upon neurons of remote nuclei (Torterolo et al., 2008).

1-3-3 MCH receptors

In rats and mice, MCH-R1 is the only functional receptor. MCH-R1 binds MCH with nanomolar affinity and couples to Gi, Gq and Go proteins to activate multiple intracellular signaling pathways (Hawes et al., 2000). Through coupling to intracellular

effectors, MCH-R1 decreases the activity of adenylate cyclase, increases the levels of intracellular calcium, activates ERKs, and interacts with intracellular proteins (Bachner et al., 1999; Chambers et al., 1999; Lembo et al., 1999; Shimomura et al., 1999). In addition to MCH-R1, a second receptor, MCH-R2, has been found to be functional in humans, rhesus monkeys, dogs and ferrets, but not in rodents (Tan et al., 2002). Both MCH-R1 and MCH-R2 receptors are expressed predominantly in the brain. They are both widely distributed, but the mRNA profiles show noticeably higher contributions from limbic areas such as amygdala, hippocampus, parahippocampal gyrus, in a number of cortical regions, as well as areas related to the control of sleep and wakefulness (Saito et al., 2001a; Saito and Nagasaki, 2008). The lower relative levels of MCH-R2 mRNA in pituitary and hypothalamus compared with MCH-R1 suggests that MCH-R2 may be involved in physiological processes other than feeding or neuroendocrine modulation (Hill et al., 2001). Interestingly, MCH-R2 mRNA has also been found in peripheral organs such as adipose, lymphocytes, pancreas, prostate and intestine (Hill et al., 2001).

1-4 Functional consideration of MCH system

As a hypothalamic neuronal population, MCH neurons receive and integrate inputs from many neuronal populations from both the brainstem and the forebrain and in turn, respond to them by acting on multiple targets. In the next section, we will summarize what is known about the synaptic action of MCH peptide on postsynaptic targets and how MCH neurons are modulated by neuromodulaters. But first, we will describe the neurochemical content of MCH-expressing neurons, since other neuropeptides and neurotransmitters in addition to MCH peptide itself have been described to be colocalized in MCH neurons.

1-4-1 Neuropeptides co-localized in MCH neurons

In addition to the neuropeptides derived from the ppMCH, other neurotransmitters/modulators have been described to co-localize with MCH. Elias et al. have demonstrated that the anorexinergic cocaine- and amphetamine-regulated transcript (CART), is co-expressed in 95% of the MCH neurons of the zona incerta and in 70% of MCH-positive neurons of the LH of the rat (Elias et al., 2001). MCH neurons that co-localize with CART send ascending projections toward the septum and hippocampus, whereas the non-CART MCH neurons send descending projections toward the brainstem and spinal cord (Hanriot et al., 2007). In addition, all MCH neurons express the hypothalamic molecule Nesfatin-1 (Fort et al., 2008) that also suppresses feeding (Oh et al., 2006). Recently, utilizing MCH immunohistochemistry and *in situ* hybridization, we and others demonstrated that approximately 85% of MCH neurons express the glutamic acid decarboxylase (GAD)-67 (Elias et al., 2008; Sapin et al., 2010; Jego et al., 2013). Accordingly, transcriptional analysis indicated that MCH neurons can be GABAergic as demonstrated by the presence of GAD-65 and GAD-67 (Harthoorn et al., 2005; Meister, 2007). Interestingly, some MCH neurons also contain glutamate (Abrahamson et al., 2001) and express the excitatory amino-acid transporters EAAT3 (Collin et al., 2003) and vesicular glutamate transporters vGLUT1 and vGLUT2 (Harthoorn et al., 2005). Recently, it was reported that 94% of MCH neurons express VGLUT2 (Chee et al., 2015) but surprisingly not the vesicular GABA transporter VGAT (Chee et al., 2015; Jennings et al., 2015), suggesting that MCH neurons could also be excitatory.

Significant amount of data indicate that most MCH neurons have an inhibitory phenotype, as evidenced by the expression of inhibitory markers. However, the possibility that MCH neurons can activate other neurons is gaining more and more consideration, adding a level of complexity to MCH neurons action.

1-4-2 Synaptic effect of MCH

Studies in lateral hypothalamic neurons *in vitro* have shown a predominantly inhibitory effect of MCH both at pre- and post-synaptic levels (Gao and van den Pol, 2001). Hence, MCH inhibited the synaptic activity of glutamate, and to a lesser degree, GABA inputs to lateral hypothalamic neurons. In addition, MCH reduced the amplitude of glutamate-evoked inward currents and the amplitude of miniature excitatory currents, indicating an inhibitory modulation of postsynaptic glutamate receptors. MCH also reduced the miniature excitatory currents frequency, indicating that MCH reduced the release probability or the number of release site at pre-synaptic glutamate terminals. Importantly, these MCH actions were eliminated when LH neurons were treated with pertussis toxin (PTX), which blocks the Gi/Go protein. Furthermore, MCH attenuated each of the 3 types of voltage dependant Calcium channels L-, N-, and P/Q-type but has no effect on potassium and sodium voltage-dependant currents (Gao and van den Pol, 2001, 2002).

MCH and the orexigenic peptide neuropeptide Y (NPY), a peptide produced from the arcuate nucleus of the hypothalamus, showed no direct effect on membrane potential, input resistance, action potential width, or after-hyperpolarization potential of MCH cells, but inhibited voltage-dependent calcium currents, indicating that MCH neurons expressed both MCH and NPY receptors (Gao et al., 2003). Interestingly, approximately 60% of MCH neurons co-expressed MCH-R1 (Parks et al., 2014a), however this finding was contradicted by a recent study looking at MCH-R1 distribution using transgenic mice expressing the Cre recombinase in MCH-R1 expressing cells, revealing no expression of MCH-R1 on MCH cells (Chee et al., 2013).

Outside the LH, it has been reported that MCH modulates the activity of a subpopulation of septal vGluT2-GnRH neurons which are sensitive to a peptide called Kisspeptin. MCH inhibits this particular subset of cells via a direct post-synaptic Ba^{2+}-sensitive K^{+} channel mechanism involving MCH-R1 (Wu et al., 2009). Approximately 50% of MCH neurons express KissR1, Kisspeptin receptor, indicating that Kisspeptin is likely to regulate the activity of the MCH system in return (Parks et al., 2014a). Then, the MCH inhibitory effect would prevent the activation by Kisspeptin, a potent actor in triggering

puberty and maintaining fertility (Kauffman et al., 2007; Chan et al., 2009), and would contribute to maintaining an appropriate equilibrium between energy balance and reproduction (Wu et al., 2009). Another study showed that MCH, through activation of MCH-R1, can reduce the activity of the medium spiny neurons in the nucleus accumbens shell by reducing the amplitude of AMPA receptor-mediated synaptic events, and action potential firing of medium spiny neurons through G protein gated K^+ (GIRK) channel activation (Sears et al., 2010). Through MCH inhibitory action on the nucleus accumbens shell, the influence of cortical excitatory inputs would be blunted, resulting in a decrease of the food intake (Sears et al., 2010).

Few studies have so far consistently reported inhibitory actions of MCH peptide through pre and post synaptic modulation of their targets. However, additional work would be needed, since the MCH peptide action on many targets, especially arousal promoting centers, are still poorly understood.

1-4-3 Effect of neurotransmitters and factors on MCH cells

Transmitters from the arousal system including noradrenaline, serotonin and Acetylcholine (through a muscarinic receptor) have an inhibitory action on MCH neurons. The effect of noradrenaline is mediated through α2 receptors and decreases the frequency of EPSCs while increasing the frequency of IPSCs (van den Pol et al., 2004). Interestingly, dopamine could also act on these α2 receptors to hyperpolarize MCH cells (Alberto et al., 2011) and also D1 and D2 like receptors (Conductier et al., 2011). Acetylcholine was shown to increase pre-synaptic activity through an activation of muscarinic receptors (van den Pol et al., 2004) and particularly to increase GABA release on MCH neurons (Jo et al., 2005). Serotonin induces hyperpolarization of MCH neurons with no significant effect on post synaptic current frequency (van den Pol et al., 2004). Histamine, a potent arousal modulator, inhibits MCH neurons by activating postsynaptic H3R. This effect is mediated through G protein-dependent inwardly rectifying potassium (GIRK) channels (Parks et al., 2014b). Taken together, these results suggest that neurons regulating arousal will inhibit MCH neurons (and other

sleep centers) in order to maintain wakefulness. Surprisingly, MCH neurons are excited by orexin directly and indirectly by enhancing glutamate release from excitatory neurons making synapses on MCH neurons (van den Pol et al., 2004; Huang et al., 2007). In turn, MCH inhibits orexin neurons, an effect not seen in MCH-R1 KO mice (Rao et al., 2008). However, a recent study revealed that orexins neurons inhibited action potential firing in most MCH neurons, an effect that required the $GABA_A$ receptor, suggesting that orexin neurons can either release GABA or, more likely, activate local GABA neurons which will in turn inhibit MCH neurons (Apergis-Schoute et al., 2015).

MCH neurons express functional glutamate and GABA receptors (Gao et al., 2003) and show robust responses to ionotropic glutamate receptors. Accordingly, both AMPA and NMDA activation depolarize these neurons and generate burst of spikes (van den Pol et al., 2004). The activation of group I metabotropic glutamate receptor also excites MCH neurons through post-synaptic excitation of the Na^+/Ca^{2+} exchanger and inhibition of K^+ currents. (Huang and van den Pol, 2007). In addition, mGluR1 activation mediates a long lasting potentiation of NMDA responses and increases spontaneous excitatory synaptic currents in MCH neurons by pre-synaptic mechanisms (Huang and van den Pol, 2007).The $GABA_A$ agonist muscimol hyperpolarizes MCH neurons (van den Pol et al., 2004).

Cannabinoids depolarize and increase spike frequency of MCH neurons by pre-synaptic attenuation of GABA release from nearby hypothalamic GABA neurons by activating CB1 receptors (Huang et al., 2007). This effect is consistent with the role of MCH neurons and cannabinoids at promoting sleep.

Interestingly, MCH neurons respond to physiological concentrations of glucose by a depolarization associated with an increase in membrane resistance (Burdakov et al., 2005).

Together, these data support the view that MCH neurons may integrate different inputs, including neurotransmitters promoting arousal or metabolism.

1-5 Main physiological functions of the MCH system in the brain

Given the widespread expression of MCH-R1, it is not surprising that MCH affects several behaviors in rodents. The LH area, where MCH neurons are located, is an integrative region that participates in homeostatic control of vital functions regulated by the hypothalamus in mammals (See Fig. 1-8). These include energy homeostasis, sleep-wake cycle regulation, motor activity, motivation and reward (Bernardis and Bellinger, 1996).

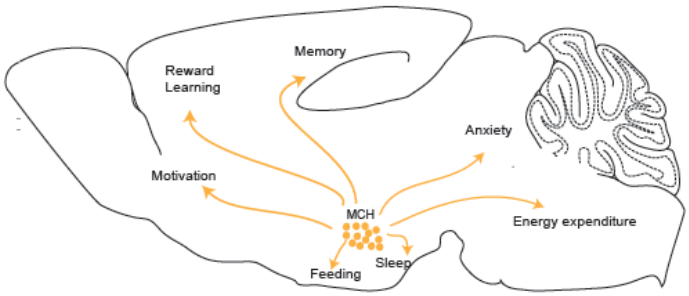

Figure 1-8: Schematic illustration of MCH system physiological functions

Note that the effect of MCH in a given physiological functions is not the result of the modulation of only one target. The arrow represented here are for an illustration matter to represent diffuse projection of MCH throughout the brain.

1-5-1 Feeding and Metabolism

Acute infusion of MCH into the lateral ventricles induces feeding in rodents and fasting induces an up-regulation of MCH mRNA expression in mice (Qu et al., 1996), rat (Herve and Fellmann, 1997) and human (Gavrila et al., 2005). In addition, MCH mRNA levels are also elevated in the obese leptin-deficient ob/ob mice, suggesting that leptin, the satiety hormone, negatively regulates MCH expression (Qu et al., 1996). Accordingly, mice deleted for the prepro-MCH gene have a reduced body weight due to hypophagia and have an increase rate of metabolism : increase in temperature, heart rate, higher metabolic rate (Shimada et al., 1998; Kokkotou et al., 2005). However, as the prepro-MCH gene also encodes for the NEI, NGE, and MGOP peptides as well as the nuclear factor AROM, it cannot be concluded that the observed phenotype is entirely due to the

MCH absence alone. In response to fasting, MCH KO mice exhibit marked hyperactivity, accelerated weight loss and an exaggerated decrease in REM sleep, suggesting that these mice have a profound inability to adapt appropriately to changes in energy balance (Willie et al., 2008). Conversely, transgenic mice in which MCH is over-expressed are hyperphagic and manifest obesity as well as insulin resistance (Ludwig et al., 2001). Importantly, KO mice for MCH-R1 are lean due to their hyperactivity, have altered metabolism but in contrast to MCH KO mice, are hyperphagic (Chen et al., 2002; Marsh et al., 2002). These results suggest that MCH might control energy expenditure by regulating locomotion/arousal and food intake.

Interestingly, MCH neurons respond to physiological concentrations of glucose by a depolarization (Burdakov et al., 2005). Since MCH neurons are promoting sleep (see the following section) and suppress metabolism, the excitatory action of glucose on MCH neurons might promote resting and energy conservation when body resources are high. Interestingly, MCH release is increased after feeding in human (Blouin et al., 2013).

1-5-2 Stress, mood and anxiety

The presence of MCH fibers and receptors in the limbic system tends to suggest that MCH system could play a role in the regulation of mood and emotion. Systemic infusion of an MCH-R1 antagonist (SNAP 7941), decreases anxiety and depression in rats in a forced swim test, by decreasing the time of immobility in the water (Borowsky et al., 2002; Georgescu et al., 2005). Similarly to SNAP 7941, two orally administrated MCHR1 antagonists also induce anxiolytic and antidepressant effects in rats on elevated plus mazes and increase social interaction among unfamiliar rats (Chaki et al., 2005).

In addition, Gonzalez et al. have provided a role for MCH in the modulation of amine release, thereby reducing serotonergic activity and inhibiting dopamine release (Gonzalez et al., 1997). MCH, when applied to the DRN, produces an anxiogenic

behavior evaluated in the forced swimming test, which is prevented by the antidepressant fluoxetine, a selective serotonin reuptake inhibitor, as well as by nortriptyline, a noradrenergic antidepressant. In contrast, the immunoneutralization of MCH within the DRN induced an antidepressive effect, while the microinjection of a specific MCHR-1 antagonist into this nucleus prevents the pro-depressive response elicited by MCH (Lagos et al., 2011b; Urbanavicius et al., 2014). Furthermore, MCH reduces the activity of serotonergic neurons of the DRN (Devera et al., 2014). Together, these studies suggest that MCH modulates serotonergic activity within the DRN and is involved in the pathophysiology of depressive disorders.

The above-mentioned data suggest that MCH has an anxiogenic effect. However, there are conflicting reports on behavioral effects of MCH and MCH-R1 antagonist on anxiety. Indeed, several reports also showed that MCH produces anxiolytic effects when injected ICV on the elevated plus made or the Vogel's punished drinking test (Monzon and De Barioglio, 1999; Kela et al., 2003). Furthermore, behavioural studies showed that MCH antagonises the anxiogenic effects of the α-MSH and NEI on grooming and locomotor activities in the rat (Sanchez et al., 1997).

The fact that both stimulating and inhibitory actions of MCH on the stress axis are reported could appear to be confusing. It is impossible at the present time to reconcile the differences between the studies outlined above. However, several variables could affect the outcome, such as the different species used in these studies (rats vs. mice), the various tests used to score behaviors (open field test, elevated plus maze, social interaction, etc.), the different routes of compounds administered (ICV vs. oral administration), the type of compounds (antagonists for MCHR1 vs. MCH), and, the use of various genetic mouse lines of MCH-R1 deficiency (Pissios et al., 2006).

1-5-3 Learning and memory

Consistent with its modulation of learning and memory processes, high levels of MCH-R1 mRNA expression have been found in the CA1 of the hippocampus, subiculum,

cerebral cortex, amygdala and the shell of the nucleus accumbens (Hervieu et al., 2000; Saito et al., 2001b; Borowsky et al., 2002).

MCH-R1 KO mice show impaired memory retention when tested in an inhibitory passive avoidance paradigm although no difference in anxiety levels. In addition, intracellular recordings of CA1 pyramidal neurons in hippocampal slices from MCH-R1 KO mice show significantly decreased NMDA response associated with a decrease in NMDA R1 subunit in the CA1 region (Adamantidis et al., 2005). Given the importance of the hippocampus and NMDA receptor in learning and memory processes, it would not be surprising that MCH could influence these circuits.

Furthermore, infusion of MCH in the amygdala increases memory retention in emotion-dependant tasks (Monzon et al., 1999; Varas et al., 2002), suggesting that MCH can modulate emotional components of learning and memory consolidation.

1-5-4 Reward

Although MCH synthesis is restricted to the hypothalamus, MCH-R1 is expressed throughout mesolimbic reward circuitry (Chung et al., 2009a). Ventral striatal nucleus accumbens shell (AcbSh) medium spiny neurons received particular attention (Georgescu et al., 2005; Chung et al., 2009b; Sears et al., 2010), given the strong expression of MCH-R1 in this particular region. Analysis of neuronal firing in the AcbSh during operant behaviors for palatable substances reveals reduced action potential firing in a majority of neurons, which can then permissively gate appetitive and feeding behaviors (Taha and Fields, 2006). Accordingly, MCH injection in the AcbSh decreases the excitability of this region (Sears et al., 2010), and leads to an increase in food intake (Georgescu et al., 2005). AcbSh neurons express both dopamine D1 and D2 receptors and motivated behaviors require concomitant D1 and D2 receptor signaling (Schmidt and Pierce, 2006). Interestingly, MCH potentiate dopamine response *in vitro* and *in vivo* and chronic blockade of the MCH system attenuates motivation for cocaine, whereas acute blockade inhibits cocaine self-administration, as well as cue- and cocaine-induced

reinstatement (Chung et al., 2009b). Recently, it was shown that optogenetic activation of MCH neurons during intake of the artificial sweetener sucralose increased striatal dopamine levels and inverted the normal preference for sucrose over sucralose (Domingos et al., 2013). These results suggest that the MCH system is a powerful potentiator of the dopaminergic system in the striatum and can modulate reward processes.

In agreement with this, Sherwood et al. also reported a critical role for MCH-R1 in conditioned incentive learning, but not on its incentive motivational value. This can be interpreted as if MCH would enhance the value of conditioned reinforcers leading to facilitated learning (Sherwood et al., 2012).

1-6 Circadian regulation of MCH (and orexins)

Sleep-wake cycle alternates in 24h cycles as a result of interactions between a circadian timing system and the homeostasis drive for sleep (Borbely, 1982; Dijk and Czeisler, 1994). The circadian pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, exerts control over sleep/wake cycles (Edgar et al., 1993; Moore, 1996). It has been shown that SCN sends projections to MCH and orexin neurons and might regulate their circadian expression (Abrahamson et al., 2001; McGregor et al., 2011). As mentioned earlier, neurons containing orexin and MCH are intermingled with each other in the perifornical and lateral hypothalamus. Each is a separate and distinct neuronal population, but they share the same general territory and projections. They also fired in a reciprocally manner across the sleep wake cycle (Hassani et al., 2009a) and they have opposite effects on most targets (Gao and van den Pol, 2001; Bayer et al., 2005). CSF levels of MCH in rats are highest during the day when the rats are sleeping whereas orexin levels are highest at night (Pelluru et al., 2013), which is consistent with their action on the sleep/wake cycle (sleep promoting and arousal promoting effect respectively). In humans, extracellular levels of MCH are increased at sleep onset and after eating while orexin levels are high during behaviors that require a high level of arousal (anger, emotion or social interaction) (Blouin et al., 2013).

These results suggest that MCH and orexin neurons can be modulated by a direct SCN circadian pacemaker influence, in addition to other structures, especially the ARAS system.

1-7 MCH system and sleep states regulation

The restricted localization of MCH neurons (LH and ZI) and their widely distributed axonal projections place them in an ideal position to integrate peripheral inputs, such as metabolic and sleep homeostatic afferents, into coherent behavioural outputs including goal-oriented behavior and arousal. In the past few years, several studies investigated the role of MCH neurons in sleep regulation. These studies can be classified in three distinct parts according to the experimental approach used. These include correlative, lack-of-function and gain-of-function studies and are summarized in the next sections.

1-7-1 Correlative evidence

Unit recording studies across the sleep wake-cycle reported the existence of neurons in the lateral hypothalamus that discharge specifically during REM sleep (Alam et al., 2002; Koyama et al., 2003; Goutagny et al., 2005; Hassani et al., 2010). Hassani et al. provided crucial data uncovering the pattern of discharge of identified MCH neurons across the sleep-wake cycle in head-restrained rats (Hassani et al., 2009a). Using a juxtacellular recording technique, these authors demonstrated that MCH positive neurons were silent during wake but increased their spiking as the transition to NREM sleep was made before spiking maximally during REM sleep. Although the spiking rates of MCH neurons during REM sleep are low when averaging spike number over the total duration of REM sleep episode, interspike intervals (ISI) analysis revealed that MCH neurons can discharge at high-frequency rate (up to 60 Hz) during doublets. In line with these results, Verret found that 25% of the activated lateral hypothalamic neurons (using c-Fos as a marker of neuronal activation) during hypersomnia evoked after 72h of REM-specific deprivation were expressing MCH and 58% of the MCH cells were Fos

positive (Verret et al., 2003). Collectively, these studies suggest that MCH neurons are likely to modulate the REM sleep state. However, the data do not allow to draw firm conclusions due to several technical limitations including low temporal resolution for c-Fos labelling and a limited ability to extract neural network data or discharge profile of possible REM-sleep silent MCH cells from unit recordings.

1-7-2 Lack of functional evidence

Studies using different antagonists of the MCH receptor (compounds A and B) have reported conflicting results, showing either a reduction of sleep concomitant with an increase in wakefulness (Ahnaou et al., 2008) or no effect on sleep (Able et al., 2009). Importantly, it should be noted that these different antagonists possess variable affinity for other receptors making it difficult to interpret the specificity of MCH-R1 involvement in sleep regulation (Chung et al., 2011). Studies using knockout (KO) mice for either MCH-R1 or ppMCH show that these animals have altered sleep architecture. MCH KO mice slept less during both the light and dark phase and also were hypophagic, lean and had increased energy expenditure under baseline conditions (Shimada et al., 1998; Astrand et al., 2004; Willie et al., 2008). In response to fasting these animals exhibited a marked activity, accelerated weight loss, and an exaggerated decrease in REM sleep compared to wild-type littermates. However, following 6h of total sleep deprivation, these animals had a normal sleep rebound (Willie et al., 2008). This data support a role for the MCH system in vigilance state regulation in response to a challenge in energy homeostasis. In contrast, MCH-R1 KO animals on a 129/Sv genetic background presented a mild hypersomniac phenotype during their spontaneous sleep-wake cycle compared to wild-type animals (Adamantidis et al., 2008). However, a more recent study using MCH-R1 KO mice with a pure background (C57BL/6J) showed that overall the mice were more awake at the expense of NREM sleep (Ahnaou et al., 2011). Compensatory mechanisms or the different genetic background used in the two studies could explain these conflicting results. A recent report showed that temporally controlled ablation of MCH cells by cell- specific expression of diphtheria toxin A increased

wakefulness and decreases NREM sleep without affecting REM sleep (Tsunematsu et al., 2014).

Collectively, these results indicate that the MCH system is sensitive to metabolic state and is important for the maintenance of a normal sleep-wake cycle.

1-7-3 Gain of function evidence

Intraventricular microinjection of MCH (Verret et al., 2003) or its infusion in the DR (Lagos et al., 2009), produced an increase of both NREM and REM sleep and reduced the amount of wakefulness. This suggests that MCH acts as a global sleep-promoting factor. However, infusion of MCH in the SLD, the LC and basal forebrain (BF) produced a specific increase in REM sleep amounts associated with a decrease in wakefulness (Torterolo et al., 2009; Lagos et al., 2012; Monti et al., 2014). These results are in agreement with previous unit recording studies (Hassani et al., 2009a) indicating that MCH can specifically modulate REM sleep states. In contrast, microinjection of MCH in the preoptic area induced an increase in NREM sleep with no change in REM sleep amount suggesting that MCH is also able to modulate the NREM sleep state (Benedetto et al., 2012).

1-8 Rationale and Aims

Previous studies point to a role for the MCH system in the regulation of sleep states. However, all of the evidence supporting a functional role for the MCH system in sleep stems from *in vitro* and *in vivo* techniques (agonist infusion, gene overexpression, lesion, antagonist injection, mouse models, cell ablation, single unit recording, immunodetection of immediate early gene) that have several limitations including low spatial and temporal resolution and possible compensatory mechanisms. Indeed, the restricted spatial distribution of the MCH neurons in the LH and dorsal ZI offers unique opportunity to manipulate the activity of the entire MCH population selectively during NREM or REM sleep episodes *in vivo* by using optogenetic technology. Since I started this study, work from others groups also looked at the regulatory role of MCH neurons in sleep using similar techniques (Konadhode et al., 2013; Tsunematsu et al., 2014) and their results will be reported and discussed further in the discussion of this thesis.

The goal of our first study, described in the Chapter 2, was to investigate whether MCH neurons specifically modulate REM sleep state or if it can also modulate NREM sleep. Our hypothesis was that MCH neurons comprise a REM sleep state regulator since, as previously reported, MCH neurons tend to fire maximally during that state (Hassani et al., 2009a). To do so, we used various opsins for both optogenetic activation and silencing of MCH neurons cell bodies during sleep-specific state in genetically-engineered mouse models (Tg(*Pmch-Cre)*, Tg(*Pmch-Cre*-R1$^{-/-}$) combined with *in vivo* electrophysiology (EEG/EMG).

In the second study, described in Chapter 3, we looked at the contribution of GABA and MCH peptide co-localized in MCH neurons in the regulation of REM sleep. In order to distinguish the effect of GABA from the effect of MCH peptide, we employed different protocols of activation of MCH cells such as acute stimulation (e.g. optogenetic state specific stimulation) and semi-chronic-stimulation (e.g. 1h of semi-chronic activation). The rationale behind this idea is that neuropeptides such as MCH, might requires a different pattern of stimulation than the one used to release fast neurotransmitters such as GABA. These various patterns of activation will be tested in genetically-engineered

mouse models (Tg(*Pmch-Cre)*, Tg(*Pmch-Cre*-R1$^{-/-}$), Tg(*Pmch-Cre-Vgat*$^{flox/flox}$)) using optogenetics combined with pharmacology (MCH receptor1 antagonist; SNAP 7941) and *in vivo* electrophysiology (EEG/EMG).

In the third study, described in Chapter 4, we looked at the pharmacogenetic (Designer Receptors Exclusively Activated by Designer Drugs DREADD) activation of MCH neurons at different time across the sleep-wake cycle. Chronic activation of MCH might favor the release of peptide and we will test this hypothesis by combining DREADD activation combined with SNAP 7941 administration.

Chapter 2:

Optogenetic identification of a rapid-eye-movement (REM) sleep modulatory circuit in the hypothalamus. *

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* **Jego S, Glasgow SD, Herrera CG, Ekstrand M, Reed SJ, Boyce R, Friedman J, Burdakov D, Adamantidis AR (2013) Optogenetic identification of a rapid eye movement sleep modulatory circuit in the hypothalamus. Nat Neurosci 16:1637-1643.**

2-1 Introduction

Rapid Eye Movement (REM) sleep, or paradoxical sleep (Jouvet, 1962), is characterized by rapid eye movements, muscle atonia and prominent theta rhythm in the hippocampus and cortex (Saper et al., 2010). Pioneer studies initially located the REM sleep "generator(s)" within the pons (Jouvet, 1962), where reciprocal inhibition between cholinergic neurons and serotonergic neurons from raphe nuclei and noradrenergic neurons from locus coeruleus were originally hypothesized to gate REM sleep (Hobson et al., 1975; McCarley and Hobson, 1975; McGinty and Harper, 1976; Aston-Jones and Bloom, 1981; Pace-Schott and Hobson, 2002). More recently, neurons producing the neurotransmitter GABA (**γ**-aminobutyric acid) and glutamate were integrated to the brainstem circuitry critical for the onset of REM sleep (Boissard et al., 2003; Verret et al., 2003; Lu et al., 2006; Sapin et al., 2009). In addition, the activity of neurons outside the brainstem also correlates with REM sleep state (Luppi et al., 2013), including neurons located in the anterior (Lu et al., 2002) and lateral (Verret et al., 2003; Sapin et al., 2010; Clement et al., 2012) parts of the hypothalamus. Collectively, these studies suggested a role for the lateral hypothalamus (LH) networks in regulating REM sleep states, however, causal evidence is missing.

In this study, we investigated the role of neurons producing the melanin-concentrating hormone (MCH) peptide in regulating REM sleep state. Pharmacological infusion of the MCH peptide (Verret et al., 2003; Monti et al., 2013) and selective activation of a subset of MCH neurons (Konadhode et al., 2013) induced both NREM and REM sleep. MCH neurons are strongly activated during a homeostatic sleep rebound induced by a prolonged sleep deprivation (Verret et al., 2003; Modirrousta et al., 2005; Hanriot et al., 2007; Clement et al., 2012), and discharge maximally during REM sleep, but were silent during NREM and wake episodes (Hassani et al., 2009a). However, mice with a genomic deletion of the MCH (Willie et al., 2008) and the MCH receptor 1 (MCH-R1)(Adamantidis et al., 2008) gene showed discrepant phenotype. Thus, it remains unclear whether MCH neurons are causally involved in the regulation of REM sleep.

Therefore, to characterize the role of the MCH neurons in regulating REM sleep state, we generated a new transgenic Tg(*Pmch-Cre)* driver mouse model and took advantages of recently-developed activating and silencing opsins to manipulate the activity of LH MCH neurons with high temporal resolution in behaving mice.

2-2 Methods

2-2-1Generation of Tg(*Pmch-Cre)* transgenic mice.

To restrict Cre expression to MCH neurons, we used a BAC clone containing the full-length pro-melanin-concentrating hormone (*Pmch)* gene (RP23-129A21) with upstream and downstream flanking sequences of 108kb and 89kb, respectively. BAC DNA was prepared and electroporated into E.coli strain SW102 as required (Warming et al., 2005). An NLS-Cre PolyA construct (pML78, Mark Lewandowski, National Cancer Institute) was targeted to replace the ATG translational start codon of *Pmch* exon 1 and correct insertion was verified by PCR and sequencing. 5' recombineering homology: TGAAAGTTTTCATCCAATGCACTCTTGTTTGGCTTTATGCAAGCATCAAA

3' recombineering homology: CTGCAGAAAGATCCGTTGTCGCCCCTTCTCTGGAACAATACAAAAACGAC (See Fig. 1a). All DNA fragments used for recombineering were generated with the FastStart High Fidelity PCR System (Roche). The modified BAC insert was excised by NotI digestion, purified and used for pronuclear injection.

Heterozygous Tg(*Pmch-Cre)* mice were maintained on a C57BL/6 genetic background by intercross breeding. Tg(*Pmch-Cre)* males were used for sleep recordings and optogenetic stimulations, whereas both sexes were used for *in vitro* electrophysiology. No detectable effects on phenotype were found. Mice were housed in individual custom-designed polycarbonate cages at constant temperature (22 ± 1 °C), humidity (30–50%), and light-dark cycle (12 h light-dark cycle, starting at 8 AM). Food and water were available *ad libitum*. Animals were treated according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

2-2-2 Characterization of Tg(*Pmch-Cre)* transgenic mice

To determine the specificity of the Cre recombinase expression, Tg(*Pmch-Cre*) were crossed with Rosa-CAG-LSL-td Tomato-WPRE (line Ai9, Jackson Laboratories). Quantification of co-localization was performed on adjacent sections containing the LH as described below. Some ectopic expression was found in the septum, the cortex and the cerebellum.

2-2-3 Plasmid and viral targeting

Ef1α-DIO-eNpHR3.0-EYFP, Ef1α- DIO-ChETA- EYFP, Ef1α-DIO-SSFO (stabilized step function opsins)-EYFP or control Ef1α-DIO-EYFP plasmids were kindly provided by Dr K. Deisseroth and packaged in adeno-associated (AAV) DJ serotype (Grimm et al., 2008) at Vollum Vector Core. As reported previously (Adamantidis et al., 2007), viruses were stereotactically infused (0.8 µl total at 50 nl/min) bilaterally in the LH (anteroposterior (AP), -1.50 mm; mediolateral (ML), ± 0.9 mm; dorsoventral (DV), 5.35 mm) of Tg(*Pmch-Cre)* mice anesthetized with isoflurane (5% induction, 1% maintenance). Tg(*Pmch-Cre) m*ice were randomly assigned to viral injection. Viral transduction efficiencies of MCH neurons were similar for all viruses.

2-2-4 In vitro electrophysiology

Slice preparation. P15-18 Tg(*Pmch-Cre)* mice were stereotactically injected into the LH (AP, -1.45 mm; ML, +/- 0.5 mm; DV, 5.35 mm). 3-5 weeks after virus transduction, mice were deeply sedated using isoflurane, decapitated, and the brain was rapidly removed and cooled in ice-cold high-sucrose cutting solution containing (in mM): 252 Sucrose, 26 $NaHCO_3$, 2.5 KCl, 4 $MgCl_2$, 1.25 KH_2PO_4, 0.1 $CaCl_2$, 10 Glucose. All animals were sacrificed during the light phase and the slices were used during the afternoon. Coronal slices were cut using a vibratome (VT1000S, Leica), and allowed to recover for ~1.5 h at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM): 124

NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose saturated with 95% O_2 and 5% CO_2 (pH ~7.3; 300-310 mOsm). In TMN experiments, minimal concentrations of 4-amino-pyridine (4-AP; 10 µM) were added to the perfusion solution to increase the reliability of the evoked IPSC (Nagode et al., 2011). Coronal brain slices were transferred to the recording chamber, and perfused continuously with oxygenated ACSF (1.5-2 ml/min) maintained at 22 ± 0.5°C. Cellular morphology and fluorescence were visualized with an upright fluorescent microscope (BX51WI, Olympus) equipped with a 40x water-immersion objective, differential interference contrast optics, IR DIC and a near-infrared fluorescence camera (EXi Blue, QImaging). Histaminergic neurons were identified based on previously published electrophysiological characteristics (Schone et al., 2012) and immunohistochemical analyses (see below).

2-2-5 Electrophysiological recordings and data analysis

Micropipettes were prepared from borosilicate glass capillaries (1.0 mm OD, 0.58 mm ID) using a horizontal puller (P-97, Sutter Instr.), and had tip resistances between 3-8 MΩ. Somatic whole-cell current and voltage clamp recordings from MCH neurons were obtained using patch recording pipettes containing (in mM): 120 K-gluconate, 20 KCl, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 7 phosphocreatine di-Tris, 2 $MgCl_2$, 0.2 ethylene glyco-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4 Na^{2+}-ATP, 0.3 GTP-Tris (pH adjusted to 7.20 - 7.26 using KOH; 275-285 mOsm). In TMN experiments, voltage clamp recordings were obtained in patch recording pipettes containing (in mM): 70 K-gluconate, 70 KCl, 2 NaCl, 2 $MgCl_2$, 10 HEPES, 5 QX-314, 1 EGTA, 4 MgATP, 0.3 GTP, and 0.1% biocytin (pH: 7.2-7.26, 275-285 mOsm). Inhibitory postsynaptic currents were routinely recorded in the presence of the AMPAR antagonist, CNQX (20 µM), and low concentrations of 4-AP (10 µM) (Nagode et al., 2011). *In vitro* electrophysiological data was discarded if intrinsic cell properties were more than 3SDs outside group mean (ie. resting membrane potential, input resistance) and / or if recording stability changed by >15%, as measured through series resistance throughout the course of the experiment (mean: 17 ± 1 MΩ). Axopatch

700B amplifier was used for all current-clamp and voltage-clamp recordings, and signals were digitized (Axon Instr., Digidata 1322A) and sampled at 20 KHz for storage on hard-disk using pClamp 10.2 software package. Current-clamp recordings were filtered at 10 kHz, and voltage-clamp data were filtered at 2 kHz.

For optical stimulation, square pulses of blue (473 nm) or yellow (593 nm) light, respectively, were delivered using lasers (Laserglow) connected to a 200 µm optical fiber and triggered via a built-in TTL circuit controlled by Clampex 10.3 software package. Light intensity was tested before each experiment, and was calibrated to emit 30-40 mW from the tip of the optical fiber.

Electrophysiological characteristics of MCH neurons were quantified using Clampfit 10.3 software package. Action potential measurements were derived from the first spike in response to a depolarizing intracellular current injection (typically 40-60 pA), and action potential amplitude was calculated from resting membrane potential. Action potential duration and afterhyperpolarizations were calculated from action potential threshold, and input resistance and rectification ratio was calculated in response to a -100 pA and -200 pA hyperpolarizing current pulse, respectively, from a holding voltage of -60 mV. Inhibitory postsynaptic current frequency and amplitude analyses were quantified in Clampfit 10.3 using event threshold detection using predefined criteria (a_{crit} > 7 pA) over three non-consecutive 5 s samples of baseline and stimulation conditions, and all detected events were subsequently visually validated. Membrane potentials reported here were corrected off-line for the liquid junction potential, estimated at ~7.6 mV using JpCalc (Axon Instruments).

2-2-6 *In vivo* optrode recording

Simultaneous optical stimulation and extracellular recording of LH neurons from Tg(*Pmch-Cre)* mice transduced with AAVdj-ChETA-YFP were carried out using glass pipette recording, as described elsewhere (Tsai et al., 2009). Briefly, recordings were conducted while gradually lowering the pipette (loaded with 0.5 KCl) in small increments

to the LH area. The optical fiber was coupled to a 473 nm laser (~ 40 mW). Single unit recordings were recorded in urethane-anesthetized animals. Data were band-pass filtered at 300 Hz low/3 kHz high using an extracellular amplifier (Cygnus technologies) and acquired on a computer using Spike 2 software.

2-2-7 Pharmacological manipulations

Drugs were stored in frozen stock solution, and diluted to final concentrations in ACSF prior to recordings. In TMN experiments, bicuculline methiodide (10 µM) was used to block $GABA_A$ receptors. All salts and powders were purchased from Sigma-Aldrich, except for 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-(±)-2-amino-5-phosphonopentanoic acid (AP-5), and bicuculline methiodide (10 µM) (Ascent Scientific).

2-2-8 Surgery

As described previously (Adamantidis et al., 2007), 9-weeks Tg(*Pmch-Cre*) and Tg(*Pmch-Cre*):MCH-$R1^{-/-}$ animals were chronically implanted with bilateral fiber implants above the LH (Doric Lenses; AP, 1.40; ML, 0.95 mm; DV, 4.6 mm) and EEG/EMG connector secured to the skull with C&B Metabond and dental cement (Patterson dental). EEG and EMG signals were recorded from 2 pairs of electrodes inserted on the skull and the neck muscle, respectively. After 2 weeks of recovery from surgical procedure and 1 additional week of

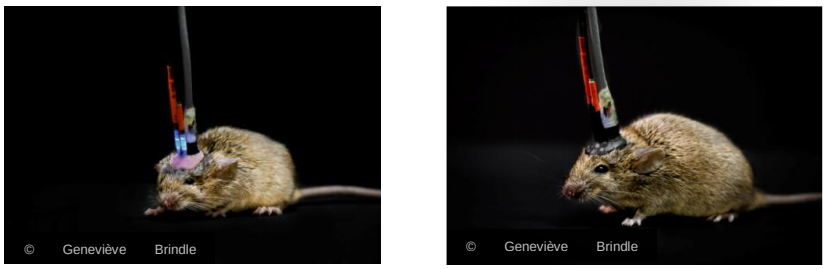

Figure 2-1: Tg(*Pmch-Cre*) mice implanted for polysomnographic recording and for bilateral optogenetic stimulation above the hypothalamus. Left: Animal when the light is on. Right: Animal when the light is on but after the application of the black nail polish. Note that the absence of light refraction.

habituation to the EEG-EMG recording set up, optical patch cords and zirconia sleeves (ID=1.25; Doric Lenses) were connected permanently to the fiber implants. Black nail polish and black furcation tubing were used to blackout light refraction from the implant and the patch cord, respectively, during the optogenetic stimulation (Fig. 2-1).

For circuit mapping experiments, a bilateral optical fiber implant was implanted above the TMN (AP,-2.18mm, ML,+/-1.12 mm and DV, -5.25mm; n = 4). A unilateral optical fiber implant with a 6° angle was implanted above the medial septum (AP,0.86 mm, ML,0.60 mm and DV, -3.62mm ; n = 6). A unilateral optical fiber implant with a 12° angle was implanted above the dorsal raphe (AP,-4 mm, ML,1 mm and DV, -3.40 mm; n = 4). Position of optical fibers was verified for each animals included in this study (see Supplementary Fig. S8). Animal with no viral expression, abnormal sleep-wake cycle (3SD outside group mean) or optical fiber implants outside the target area were discarded from the study. Based on these criteria, 4 ChETA, 2 EYFP, 2 NpHR3.0 transduced Tg(*Pmch-Cre*) animals and 1 ChETA transduced Tg(*Pmch-Cre*):MCH-R1$^{-/-}$ animals were excluded from the study.

2-2-9 Polysomnographic Recording

All sleep recordings took place mainly during the light phase (12-7 PM; light onset: 8 AM). EEG and EMG signals from electrodes were amplified (Grass Instruments) and digitized at 512 Hz (Vital Recorder, Kissei Comtec) as previously described (Adamantidis et al., 2007; Carter et al., 2010). Polysomnographic recordings were scored and analyzed using sleep analysis software (SleepSign for Animal, Kissei Comtec). All scoring was performed manually in 5-s epochs as previously described (Franken et al., 2001; Adamantidis et al., 2007). Briefly, we defined wakefulness as desynchronized low-amplitude EEG and high tonic EMG activity with phasic bursts; NREM sleep as synchronized, high-amplitude, low-frequency (0.5–4 Hz) EEG and highly reduced EMG activity compared with wakefulness with no phasic bursts; REM sleep as having a pronounced theta rhythm (6-9 Hz) and a flat EMG. Data collection and analysis were not performed blind to the conditions of the experiments.

2-2-10 Spectral EEG/EMG analysis

EEG power spectra were computed for 6 to 15 stimulated events per animal (> 20 s duration). Power spectra and time-frequency power spectra were calculated in Matlab with the Chronux toolbox (Bokil et al., 2010) using a 5-s sliding window (0.062 s increments). To normalize the data, all power spectral densities at the different frequency ranges, that is, delta (0.5–4 Hz), theta (6–9 Hz), alpha (9–12 Hz), sigma (12-20 Hz), low gamma (20-60 Hz) and high gamma (60-100 Hz) were expressed as relative values to the total power of the same event. EMG power was calculated based on a 30-100 Hz range frequency.

2-2-11 Unbiased automatic detection of an increase of power in the 3-5 Hz frequency band during REM sleep

We automatically detected epochs when the EEG power in the 'slow theta' range was increased based on a detection threshold established during the baseline recording period (Fig. 2-11 g-j). The analysis was performed as follows:

1) 'theta' = 6-9 Hz and 'slow theta'= 3-5 Hz were defined and kept constant for every mouse included in analysis.

2) For each animal, the average 'slow theta'/'theta' power ratio was calculated for all REM epochs (5 s duration each) occurring during baseline recording at the corresponding time period (12-7 PM). Spectral power of each 5 s REM epoch was calculated in Matlab using the Chronux toolbox (Bokil et al., 2010) and the 'slow theta/'theta' ratio for each epoch was obtained from the summed spectral power from the specified 'slow theta' frequency range divided by that of the 'theta' frequency range. The average 'slow theta/'theta' power ratio and standard deviation for all baseline REM epochs was then calculated. The threshold 'slow theta'/'theta' power ratio for each mouse used for subsequent analysis during REM inhibition experiments (below) was defined as the average baseline REM 'slow theta/'theta' power ratio + 2 SDs.

3) For REM silencing experiments, the ‘slow theta’/’theta’ power ratio was calculated for each REM 5-s epoch as described for baseline recordings. Each epoch was identified as having a ‘slow theta’/’theta’ power ratio above or below the defined threshold calculated as described in step 2. Thus, for each mouse the percentage of detected relative to non-detected epochs was quantified and average power spectra values were calculated from all non-detected or detected epochs.

2-2-12 Optical stimulation

All optical stimulations (5-ms pulses; 1-20 Hz) were generated by a waveform generator (Master 9) that triggers two blue-diode lasers (473 nm, LaserGlow). Acute optical stimulation consistently started ~10 s after the onset of a stable NREM or REM sleep event. For REM sleep, pulses of 1 Hz or 20 Hz started 5 s after the onset of REM sleep and terminated at the next wake event (Fig. 2-2).

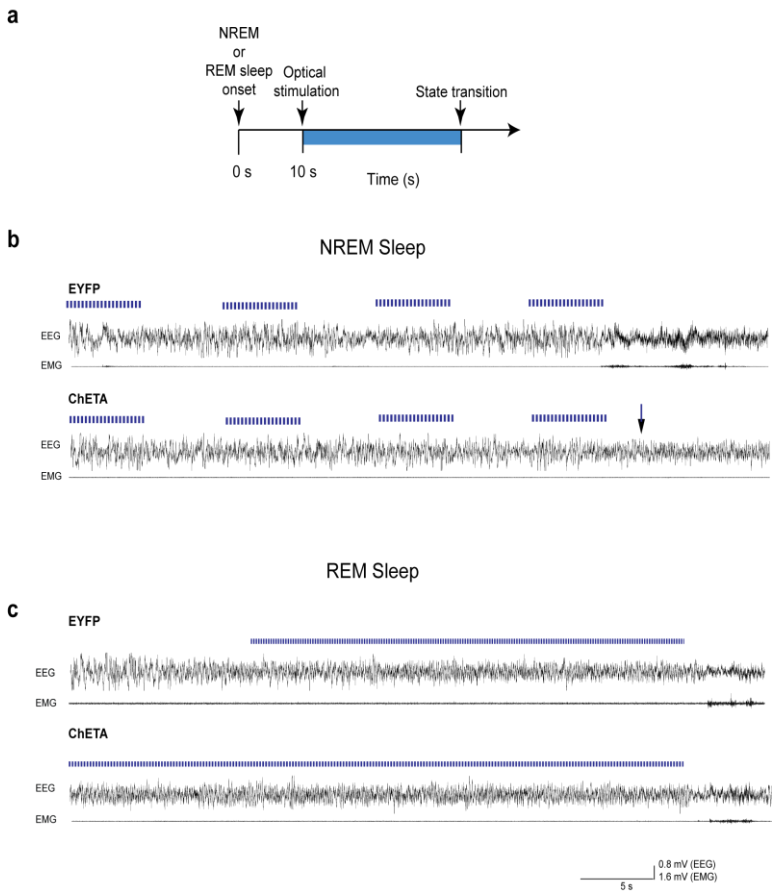

Figure 2-2. NREM and REM sleep optogenetic stimulation protocols.

a. timeline of NREM and REM sleep-specific stimulation experiments. **b,** Representative EEG/EMG recordings showing optical stimulation of ChETA (lower trace) and EYFP (control, upper trace) animals time-locked to NREM sleep episode. Note the transition from NREM to REM sleep occurring in ChETA animals after blue light stimulation. Horizontal blue bar (dashed) represents optical stimulations. **c,** Representative EEG/EMG recordings showing optical stimulation of ChETA (lower trace) and EYFP (control, upper trace) animals time-locked to REM sleep episode. Note that optical stimulation of EYFP animal starts at the onset of REM sleep (i.e., after the transition from NREM to REM sleep is complete). Wakefulness signals the termination of REM sleep. Horizontal blue bar (dashed) represents optical stimulations.

Optical stimulations (1-20 Hz) were randomly applied within a given sleep state and each Tg(*Pmch-Cre*) mouse was stimulated during both NREM and REM sleep. For the stimulation of MCH projections in the MS, TMN or DR, 20 Hz stimulations were used. For NREM sleep experiments, we chose to discard micro-arousals (< 1s) during NREM sleep while stimulating and terminated stimulation only after the onset of a REM or a wake event > 5 s. To circumvent overstimulation of MCH neurons during prolonged NREM sleep period, 5 ms light-pulses (1 Hz or 20 Hz) were delivered for 5 s every 10 s and this cycle was repeated until the animal exhibited a state transition (Fig. 2-2). For SSFO experiment, we applied 50 ms blue light pulses every 10 s over the entire sleep

episode and single 50 ms yellow light pulse was used to deactivate SSFO (See Fig. 2-9 a). For silencing experiment, constant light delivery was provided by a yellow-light laser (593 nm; LaserGlow) split into two patch cords.

2-2-13 Immunohistochemistry

Mice were deeply anesthetized with ketamine/xylazine/acepromazide (100, 16 and 3 mg per kg, respectively, intraperitoneal injection) and perfused transcardially with 1× PBS-heparine 0.1%, pH 7.4, followed by 4% paraformaldehyde in PBS. Brains were extracted, postfixed overnight in the same fixative at 4 °C, and cryoprotected in PBS-Azide 0.1 %/30 % sucrose for an additional 24 h at 4 °C.

For double immunolabelling (Fig. 2-4 b, d), 25-µm thick brain sections from virally-transduced mice were washed (PBST, 10 min, room temperature), incubated in a blocking solution (PBST/4% bovine serum albumin (BSA), 1 h, room temperature) and then in a goat anti-proMCH (SC14509, Santa Cruz Biotech.) at 1:1000 and in a rabbit anti-YFP (Ab290, Abcam) at 1:5000 in block solution (BSA 4%) at 4 °C for ~48 h. After 3 × 10-min washes in PBST, sections were incubated in a solution of Alexa 555 donkey anti-goat IgG secondary antibody (1:1000; A21432, Invitrogen) or Alexa 488 Donkey anti-rabbit IgG secondary antibody (1:1000, A21206, Invitrogen) in blocking solution (BSA 4%, 1 h, room temperature). Sections were washed 3 x 10min in PBST, mounted and coverslipped with Fluoromount-G (Southern Biotech.) and quantified for MCH-YFP colocalization in adjacent LH sections.

To verify that histamine was expressed in recorded TMN neurons, patch pipettes were filled with 0.1% biocytin (Sigma). Coronal brain slices were transferred to 4% PFA in PBS for >24 hrs and incubated for 30 min in 0.3% PBST for 1 h, rinsed 5x (5 min/wash) in 0.1% PBST, then further incubated in 0.1 % PBST with 1 % BSA for 1h. Slices were then transferred to the same solution containing 1% BSA with rabbit anti-histidine decarboxylase (1:500, Acris EUD2601) at 4º C. Slices were then washed (5x 10 min in 0.1% PBST), and incubated in a secondary antibody (anti-rabbit IgG Alexa 555 and

Strepdavidin-B conjugated with Alexa 649) for 1.5 h at room temperature, washed (5 x 10 min in PBS), and mounted on microscope glass.

2-2-14 In situ hybridization and immunocytochemistry

Tg(*Pmch-Cre*) mice were deeply anesthetized with 5% isoflurane and decapitated. Brains were extracted, quickly frozen in isopentane at -30° C, and stored at -80° C for at least 24h. Coronal brain sections (16 µm) were mounted onto glass slides and stored at -80° C. LH sections were transferred into cold 4% PFA for 20 min, followed by 5 x 5 min in 0.1 M PBS, and then incubated in PBS containing 0.3 % H_2O_2 for 10 min at room temperature, followed by 5 x 5 min washes in PBS. Acetylation was induced using 0.1M TEA buffer containing 0.25% acetic anhydride for 10 min, followed by 5 x 5 min wash in PBS. Sections were then dehydrated dried for 10 min, and placed in a humid chamber saturated with formamide. Sections were incubated in a pre-hybridization buffer (40 % formamide, 10 mM Tris-HCl, pH 8.0, 200 *µ*g/ml yeast tRNA, 10 % dextran sulfate, 1x Denhardt's solution, 600 mM NaCl, 1 mM EDTA, pH8.0) for 2 h at 60° C, and subsequently transferred to a hybridization buffer including the antisense- or sense-GAD67 riboprobe (1:1000) for 12h at 60° C, followed by SSC buffer washes under gentle agitation: 5 min in 5X SSC; 1 min in 2X SSC; 30 min in 0.2X SSC buffer containing 40 % formamide at 60° C; 5 min in 0.2X SSC at room temperature, and 5 min in 0.1M PBS. Sections were then blocked with 4% BSA and 0.5% Blocking Reagent (Roche) in PBS for 1 h at room temperature, and subsequently incubated in PBS containing goat anti-proMCH at 1:1000 for 72 h at 4° C, followed by 5 x 5 min washes in PBS. Slides were subsequently incubated in anti-DIG-POD (Roche) at 1:1000 overnight at 4° C, followed by 5 x 5 min washes in PBST. Antibodies were detected by incubation of sections using the TSA-Plus-cy5-fluorescein system (Perkin Elmer) for 10 min, followed by 5 x 5 min washes with PBS, and subsequent incubation in 0.1 M PBS containing 1:200 anti-goat Alexa Flour 488 for 1 h. Sections were then washed 5 x 5 min in PBS, coverslipped with Fluoromount-G before visualization with a

fluorescent microscope. Quantification of colocalization was performed on sections containing LH-MCH cells.

2-2-15 Microscopy

Non-confocal images were collected on Axio Observer Carl Zeiss fluorescent microscope using fluorescent reflected light and confocal images were collected on a LSM 710 Carl Zeiss confocal microscope. Digital images were minimally processed using Image J, Adobe Photoshop CS3 software or Zen to enhance brightness and contrast. All digital images were processed in the same way between experimental conditions to avoid artificial manipulation between different datasets.

2-2-16 Statistical analysis

Experimental sample size was defined based on previous studies (Adamantidis et al., 2007; Schone et al., 2012). No statistical methods were used to pre-determine sample sizes. Statistical analyses of electrophysiological properties and synaptic responses were assessed using repeated measures ANOVAs, two-tailed paired t-tests, and significant effects were investigated using pairwise multiple comparisons using Student-Newman-Keuls method for parametric data, unless otherwise indicated. *In Vitro* data were tested for normality of the distribution using Kolmogorov-Smirnov test (with a Lilliefors' correction). Statistical analysis of optical stimulations during sleep was assessed using repeated ANOVAs or two-tailed t test as mentioned throughout the text. *In Vivo* data distribution was assumed to be normal but this was not formally tested. Data are presented as mean ± SEM. All data were analyzed using Prism 5.0 (GraphPad Software), Clampfit 10.3 (Axon Instruments), and Sigmaplot 11 (Systat).

2-3 Results

2-3-1 Genetic targeting of LH MCH neurons

To target optogenetic actuators to MCH neurons located in the lateral hypothalamus and the zona incerta area (LH-ZI), we generated a Tg(*Pmch-Cre*) mouse using bacterial artificial chromosome (BAC) technology in which the Cre recombinase gene is driven by a ~ 108 kb MCH gene promoter (Fig. 2-4 a). To assess the selectivity of this transgene, Tg(*Pmch-Cre*) mice were crossed with cre-dependent tdTomato reporter (Rosa-CAG-LSL-tdTomato-WPRE) and 83.6 ± 4.1 % of MCH-immuno-positive cells were found to express TdTomato in the LH, whereas 97.9 ± 0.4 % of TdTomato cells expressed MCH peptide (Fig. 2-4 b). After stereotactic injection of Cre-inducible adeno-associated virus AAVdj-EF1-DIO-ChETA-EYFP (Atasoy et al., 2008; Tsai et al., 2009; Gunaydin et al., 2010) into the LH-ZI of Tg(*Pmch-Cre*) mice, we found that 87.67 ± 2.96 % of MCH-immunopositive cells co-expressed ChETA-EYFP (1672/1912 cells, 3 sections per animal, *N* = 6 animals), and MCH immunoreactivity could be detected in 89.65±1.08% of ChETA-EYFP expressing cells (1672/1852 cells, 3 sections per animal, *N* = 6 animals) (Fig. 2-4 d). No EYFP fluorescence was observed in wildtype (Cre-negative) animals following virus transduction, confirming that the expression was selective for MCH-cre neurons. ChETA-EYFP expression persisted as long as six months, and we observed dense ChETA-EYFP-containing MCH terminals within the hypothalamus, septum, raphe nucleus, locus coeruleus and cortex (Fig. 2-3), consistent with previous characterizations (Bittencourt et al., 1992).

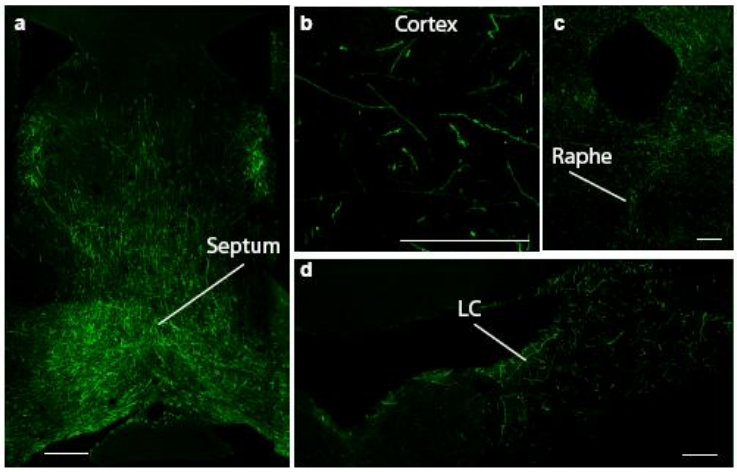

Figure 2-3

Photomicrographs of coronal brain sections from a Tg(Pmch-Cre) animal injected with AAVdj-ChETA-EYFP in the LH-ZI area.

Pictures show dense EYFP-expressing MCH neuron projection terminating within sleep-related structures including the medial septum (a), neocortex (b), raphe nucleus (c), and the locus coeruleus (d). Abbreviations used: LC: Locus coeruleus. Scale bar: 200 µm.

To test the response of ChETA-expressing MCH neurons to optical stimuli, we performed whole-cell patch-clamp recordings in acute hypothalamic slices (Fig. 2-4 e-j). As previously reported for wild-type MCH neurons (van den Pol et al., 2004; Burdakov et al., 2005), ChETA-EYFP-expressing MCH neurons were not spontaneously active and retained typical resting membrane potential and input resistance (-61.7 ± 1.4 mV and 428.0 ± 63.1 MΩ, respectively; Fig. 2-4 e). Blue light illumination (473 nm) evoked inward photocurrents (-158.2 ± 25.8 pA; Fig. 2-4 f, g), and brief pulses of light (1-10 ms) evoked single action potentials (Fig. 2-4 h) at frequencies up to 20 Hz with high fidelity (Fig. 2-4 i, j).

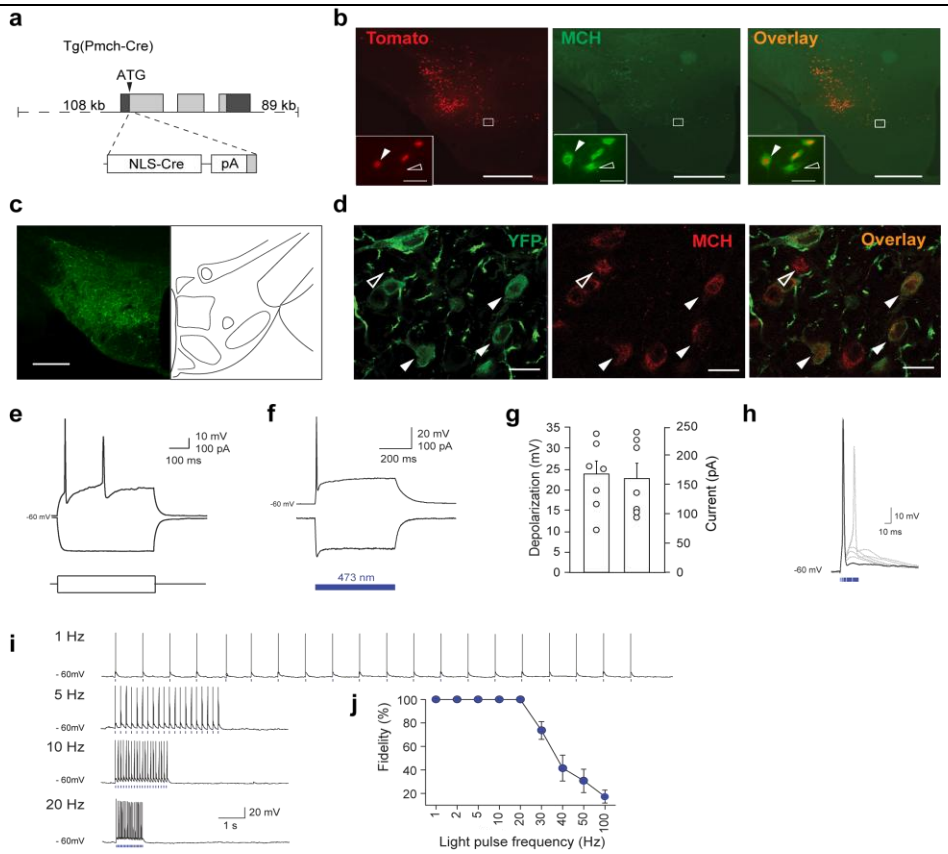

Figure 2-4. Selective targeting and functional virus expression in MCH neurons.

a, Generation Tg(*Pmch-Cre*) mice using BAC technology. **b,** Photomicrograph of a brain section from [Tg(*Pmch-Cre*) X *R26*^{tom}] mouse showing colocalization of tdTomato-positive cells (red) with MCH immuno-positive cells (green). Scale bar: 500 µm. *Inset*, magnification of hypothalamus area. White arrowheads represent MCH+/Cre+ cells. Open arrowhead represent MCH+/Cre– cell. Scale bar: 50 µm. **c,** Photomicrograph of ChETA-EYFP expression in the hypothalamus. Scale bar: 500 µm. **d,** Photomicrographs of ChETA-EYFP expression (green) within MCH neurons (red). White arrowheads represent MCH-positive cell expressing ChETA-EYFP. Open arrowhead represents non-transfected MCH neurons. Scale bar: 20 µm. **e,** MCH neurons transfected with AAVdj-ChETA-EYFP show typical firing-rate adaptation of wild-type MCH neurons in response to excitatory current injection (100 pA). **f,** ChETA-expressing MCH neurons show robust depolarization and spiking (top) upon 500-ms illumination (473 nm light) in current-clamp mode. This depolarization coincided with inward current in voltage-clamp mode (bottom). **g,** Quantification of voltage depolarization and inward currents of ChETA-expressing MCH neurons upon blue light illumination (*n* = 7 cells in 7 different slices, n=6 animals). **h,** Brief pulses of 473-nm light evoke single action potentials in ChETA-expressing MCH neurons. Note that pulse width > 20 ms typically resulted in spike doublets. **i,** Voltage responses of MCH cell shown in (**e**) to 20 pulses of blue light delivered at various frequencies (1-20 Hz). Blue bars represent 5-ms light pulses. **j,** Group data showing ChETA-expressing MCH neurons fidelity response to light pulses at frequencies up to 20 Hz (*n* = 7cells in 7 different slices, n=6 animals).

Extracellular recording in anesthetized animals confirmed that optogenetic stimulation of LH MCH neurons evoked spike waveforms similar to spontaneous waveforms (Fig. 2-5).

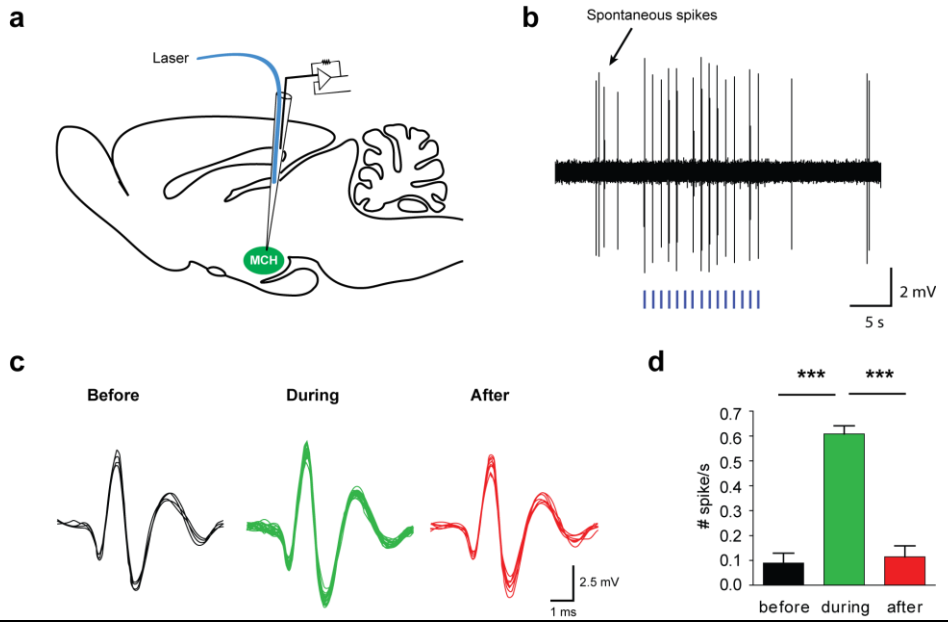

Figure 2-5. MCH neurons transfected with AAVdj-ChETA-EYFP increase their firing rate in response to blue light pulses.
a, Schematic of the optrode recording system. Note that the optic fiber is placed in the glass pipette. **b**, In vivo extracellular recording of ChETAexpressing MCH neuron in the LH showing tonic firing evoked by 1 Hz light pulse trains. Note the spontaneous firing rate of MCH neuron in the absence of light. **c**, Extracellular waveforms recorded from putative MCH neurons before (black), during (green) and after (red) optical stimulation of LH area. Note the strong similarity between spontaneous and light-evoked waveforms. **d,** Quantification of the discharge of MCH neurons before, during and after the light stimulation. MCH neurons firing was significantly increased by the 1Hz light pulse trains (n = 4 cells, 4 animals, 12 optical stimulations). Number of spike per second are represented as mean ± SEM. ***: $p < 0.001$ compared to the firing evoked by light pulse trains, one-way ANOVA within subject design, followed by Tukey post-hoc test.

2-3-2 Activation of MCH neurons stabilizes REM sleep

In mammals, sleep is not homogeneous, but consists of a progression through various states, including NREM and REM sleep. After the onset of NREM sleep, animals switch to either wake or REM sleep states (Pace-Schott and Hobson, 2002). REM sleep episodes typically terminate by a transition to wakefulness. To probe the role of MCH neurons in different stages of sleep, we transduced Tg(*Pmch-Cre*) mice with AAVdj-EF1-DIO-ChETA-EYFP (ChETA) or AAVdj-EF1-DIO-EYFP (control) viruses and implanted electroencephalographic (EEG) and electromyographic (EMG) electrodes, as

well as bilateral optical fibers above the LH-ZI area. Genetic and viral manipulations did not disrupt spontaneous sleep-wake cycles (Fig. 2-6 a) and experiments were conducted during the second half of the light phase (12-7 PM), when REM sleep episodes are most frequent.

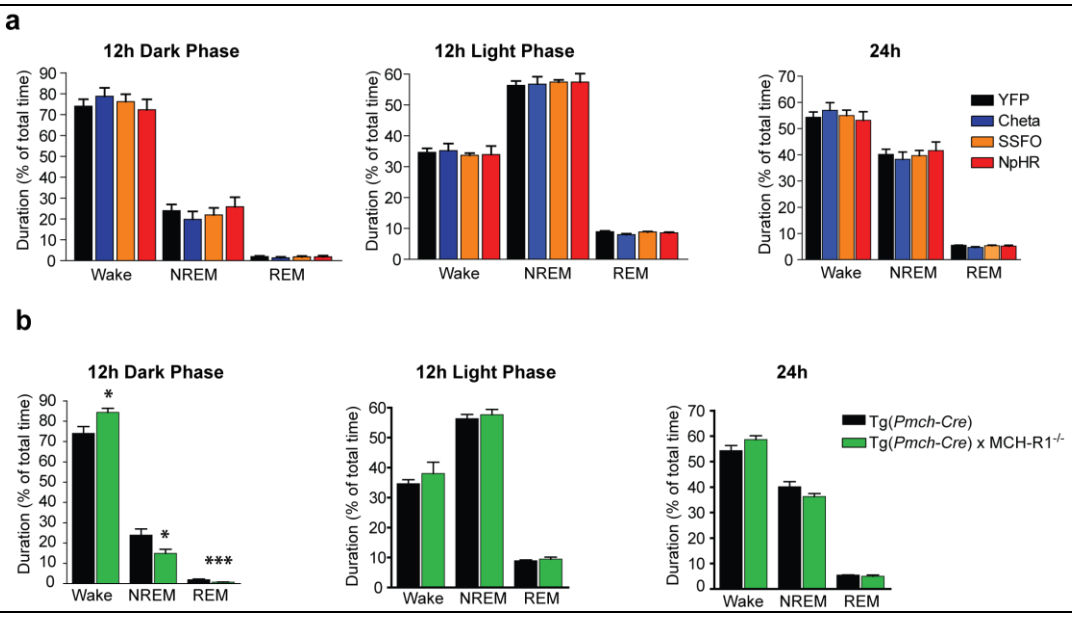

Figure 2-6 Spontaneous sleep-wake cycle of Tg(Pmch-Cre) and [Tg(Pmch-Cre) XMCHR-1-/-]. **a**, Spontaneous duration of wake, NREM and REM sleep (expressed as a percentage of time) of Tg(Pmch-Cre) transduced with ChETA-EYFP (blue, n = 8), SSFO-EYFP (orange, n=4),NpHR3.0-EYFP (red,n = 6) virus, and their EYFP controls (black, n = 8) during the light and dark phases, and 24h of the light/dark cycle. No significant differences were found between Tg(Pmch-Cre) animals expressing the opsin and their controls demonstrating that expression of the opsin in MCH neurons does not alter the spontaneous sleep-wake cycle of the mice. **b**, Spontaneous duration of wake, NREM and REM sleep (expressed as a percentage of time) of Tg(Pmch-Cre) animals (n = 8) compared to [Tg(Pmch-Cre) X MCHR-1-/-] animals (n = 8) showing a hyperactive phenotype during the dark phase for the double transgenic animals associated with a significant decrease of both NREM sleep and REM sleep. No significant differences during the light-dark phases and the 24h were found. Mean durations are represented as mean ± SEM. *: $p < 0.05$, ***: $p < 0.001$ one-way ANOVA between subject design for viral transduction, followed by Tukey post-hoc test or unpaired two-tailed t test.

To investigate the effects of MCH cell activity on NREM and REM sleep, optical stimuli were started at the onset of each sleep state, and stopped upon termination of each sleep state, as detected by real-time EEG/EMG analysis in freely-moving animals. Note that optogenetic stimulation patterns (1-20 Hz) were consistent with spontaneous discharge of MCH neurons during REM sleep (Hassani et al., 2009a). We found that optogenetic stimulation of MCH neurons at 20 Hz or 1 Hz at the onset of NREM sleep

episodes did not affect NREM sleep episode duration (p = 0.59 and 0.82 respectively; Fig. 2-7 a), but increased the probability of NREM-to-REM sleep transitions (by ~80% and ~71%, respectively, in ChETA compared to control animals; p= 0.048 and 0.039 respectively; Fig. 2-7 b).

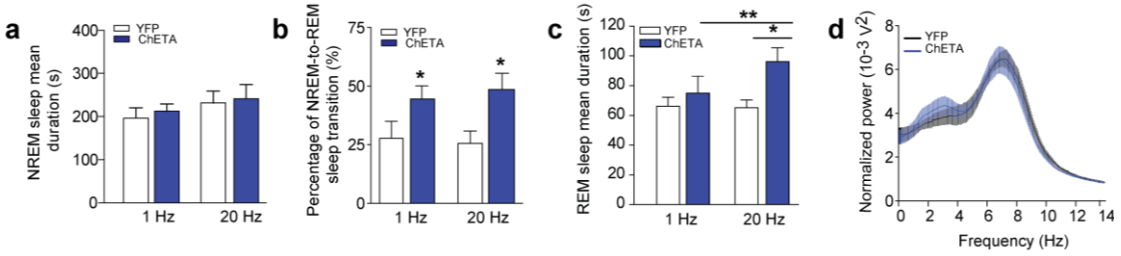

Figure 2-7. Optogenetic activation of MCH neurons extends REM, but not NREM sleep duration.

a, Mean duration of NREM sleep upon optogenetic stimulation at 1 Hz and 20 Hz of control (white) and ChETA-EYFP group (blue) animals (n = 6 per group; > 15 stimulations per frequency and per animal). **b,** Percentage of successful NREM-to-REM sleep transitions upon optogenetic stimulation during NREM sleep shown in (a). Data are shown as a percentage of total number of NREM-to-REM sleep transitions on the total number of stimulation during NREM sleep (n = 6 animals per group). **c,** Mean duration of REM sleep episodes upon optogenetic stimulation at 1 Hz and 20 Hz of control (white) and ChETA-EYFP (blue) animals (n = 6 per group). Data analysis is based on an average of at least 15 stimulations per frequency and per animal during REM sleep episodes. **d,** Power spectrum analysis of REM sleep episodes upon 20 Hz optogenetic stimulation of control (black) and ChETA-EYFP (blue) animals (n = 6 per group).

In contrast, we found that optical 20 Hz stimulation of MCH neurons at the onset of REM sleep episodes significantly extended their duration (+ 47 %) in ChETA compared to control animals (p =0.014; Fig. 2-7 c), while 1 Hz stimulations had no effect (p = 0.49; Fig. 2-7 c). Importantly, the EEG power spectrum of REM sleep (p = 0.45; Fig. 2-7 d) and the muscular tone during NREM and REM sleep remained unchanged during optical activation of MCH neurons (YFP: 0.44 ± 0.007 mV^2 vs. ChETA: 0.42 ± 0.006 mV^2; p = 0.07 and YFP: 0.45 ± 0.020 mV^2 vs. ChETA: 0.40 ± 0.013 mV^2; p = 0.13, respectively).

To rule out the possibility that the observed effects of MCH neurons on REM sleep were due to potentially unnatural synchrony among ChETA-driven MCH neurons, we utilized the ChR2 mutant step-function opsin (SSFO) (Berndt et al., 2009). Activation of SSFO

with a single blue light pulse increases excitability of targeted cells through sustained depolarization, until SSFO is turned off (typically by a yellow light pulse) (Berndt et al., 2009). Animals were transduced with AAVdj-DIO-SSFO-EYFP in the LH-ZI area of Tg(*Pmch-Cre*) with similar efficiency as for ChETA virus.

Using whole-cell voltage-clamp recordings, we found that SSFO-mediated activation of MCH neurons (50-ms light pulses width at 0.1 Hz) induced prolonged inward currents that were rapidly terminated by a single pulse of yellow light (50 ms-pulse width; Fig. 2-9 a). This produced a significant membrane depolarization that could be maintained for several minutes (> 5 min).

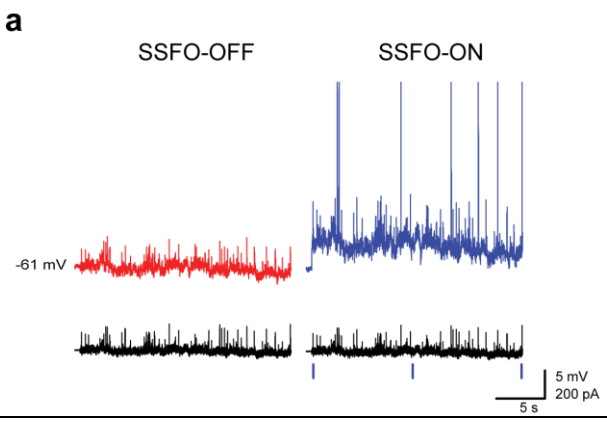

Figure 2-8 Activation of SSFO in MCH neurons leads to increased excitability in response to a replayed current trace.

a, To assess whether SSFO activation resulted in increased excitability, a series of EPSCs and IPSCs in an identified MCH neuron were recorded. The current trace (black) was then replayed to MCH cells transfected with SSFO before (red) and during (blue) activation with a brief pulse of blue light (50 ms pulse width). **b**, Spike waveforms from MCH neurons before (red) and during (blue) SSFO activation are similar. **c**, Average data show that activation of SSFO resulted in a 4.5-fold increase in the number of spikes elicited during replay compared to baseline (control : 2.5±1.3 spikes, SSFO: 11.3±0.9 spikes, p = 0.0008, n = 4, at least 3x30 s sweep per cell). *** p<0.001, paired two-tailed t-test.

To further assess the hyperexcitability of MCH neurons upon SSFO activation, we use intracellular current injection to “replay” a previously recorded current trace while recording the spontaneous synaptic inputs to MCH neurons in whole-cell current-clamp mode. We found that upon SSFO activation, MCH neurons significantly increased their

firing activity by ~ 4.5 fold as compared to the de-activated state (p = 0.0008) (Fig. 2-8 a,c). Importantly, SSFO-induced spikes waveforms were indistinguishable from spontaneously occurring spikes (Fig. 2-8 b).

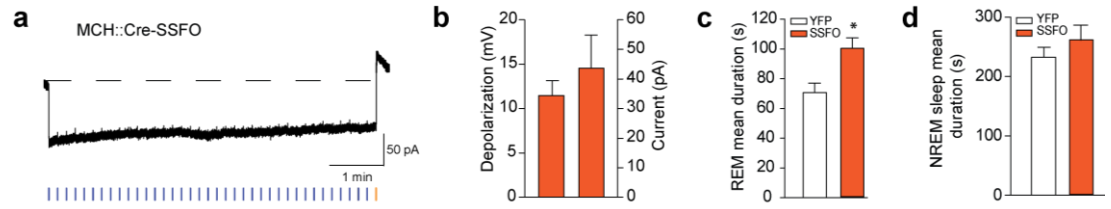

Figure 2-9. SSFO activation of MCH neurons extends REM sleep duration.

a, Whole-cell voltage clamp recording from a MCH neuron expressing SSFO-EYFP shows a prolonged inward current upon blue light pulse (50 ms delivered every 10 s) that is terminated by a 50-ms yellow pulse. **b,** Quantification of membrane depolarization and inward currents of SSFO-expressing MCH neurons upon optogenetic stimulation (*n* = 8 cells in 5 slices, n = 3 animals). **c, d,** Quantification of REM (g) and NREM (h) sleep mean duration upon optogenetic stimulation (50 ms blue pulse delivered every 10 s; termination: 50 ms yellow pulse, see Methods) of EYFP and SSFO-EYFP animals (n = 4 per group). Data analysis is based on an average of at least 10 stimulations per frequency in each mouse during REM and NREM sleep episodes. Mean duration are represented as mean ± SEM. *, $p<0.05$, **, $p<0.01$ two-way mixed factorial ANOVA between stimulation condition and viral transduction, followed by Tukey post-hoc test or unpaired two tailed *t* test

Next, we used SSFO to study whether increasing excitability of MCH cells would extend REM sleep duration in freely-moving animals. Indeed, we found that optical activation of SSFO-expressing MCH neurons during REM sleep significantly increased REM duration (+ 41 %, p =0.022; Fig. 2-9 c), while it had no significant effect during NREM sleep (p = 0.40; Fig. 2-9 d). Together with our previous results (Fig. 2-7 c), these experiments provide evidence that activation of MCH selectively extends the duration of REM, rather than NREM sleep episodes.

2-3-3 Silencing of MCH neurons slows REM sleep theta rhythm

To assess whether activity of MCH neurons is necessary for natural REM sleep, we employed halorhodopsin (eNpHR3.0) (Han and Boyden, 2007; Zhang et al., 2007; Gradinaru et al., 2010) to transiently and reversibly silence MCH neurons. We targeted the expression of eNpHR3.0-EYFP to MCH neurons by injecting AAVdj-DIO-eNpHR3.0-EYFP in the LH-ZI area of Tg(*Pmch-Cre*). Using patch-clamp recordings performed in acute hypothalamic slices, we found that yellow illumination (593 nm) resulted in outward photocurrents (steady-state mean: 138.2 ± 48.4 pA; Fig. 2-10 a, b) and a strong hyperpolarization (27.4 ± 6.4 mV; Fig. 2-8 a, b) in eNpHR3.0-expressing MCH neurons.

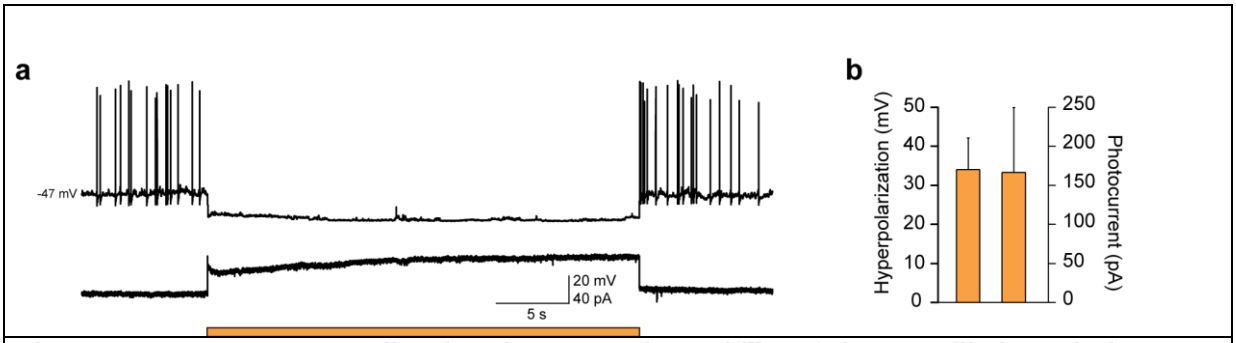

Figure 2-10. MCH neurons silencing decreases the stability of theta oscillations during REM sleep.
a, ArchT-expressing MCH neurons show a persistent outward current (bottom) and concomitant hyperpolarization (top) upon 30 s constant yellow illumination in voltage and current clamp, respectively. Note that cells were depolarized to above threshold potentials using constant steady-state current injection to elicit spiking. **b**, Quantification of membrane hyperpolarization (Mean : 34.0 ±8.1 mV) and outward currents (166.2±83.4 pA) of ArchT-expressing MCH neurons upon optical silencing (n=5 cells in 4 slices across 2 animals).

To study the effect of a rapid and reversible silencing of MCH neurons on REM sleep, we bilaterally delivered continuous yellow illumination (593 nm) to the LH-ZI area at the onset of REM sleep episodes. We found that optogenetic stimulation of MCH neurons during REM sleep did not significantly reduce its duration (eNpHR3.0: 67,2 ± 4.4 s vs EYFP: 60,2 ± 5.2 s, p = 0.337), but rather induced a shift in the dominant theta peak frequencies towards slower oscillations (Fig. 2-11 c,d for representative traces). This

was characterized by a significant reduction of 6-9 Hz theta power concomitant to an increase of 3-5 Hz slow theta power (p = 0.003; Fig. 2-10 g), that occurred 8.31 ± 3.72 s after the light onset. Delta rhythms (0.5 - 4 Hz) remained unchanged during silencing of MCH neurons (eNpHR3.0: 0.28 ± 0.015 vs EYFP: 0.24 ± 0.016, p = 0.094). Similar to ChETA and SSFO experiments, muscle tone was not affected by optogenetic stimulation of MCH neurons (eNpHR3.0: 0.15 ± 0.06 vs EYFP: 0.24 ± 0.12, p = 0.481). These results were further confirmed with the use of archaerhodopsin (ArchT) (Chow et al., 2010), a light-driven proton pump (p = 0.017; Fig. 2-11 h)).

Indeed, we found that the number of slow theta oscillations was significantly increased (by ~ 10 times) during optogenetic silencing of MCH neurons in eNpHR3.0 compared to control animals ($p = 0.010$; Fig. 2-11 h). Importantly, the observed slow theta oscillations were not artificial because they occurred during spontaneous REM sleep, although less frequently, in all recorded animals, as detected using an unbiased waveform recognition algorithm (Fig. 2-11 i, j). Taken together, these results show that silencing of MCH neurons decreases theta peak frequency (i.e., REM sleep quality), rather than duration, of REM sleep episodes.

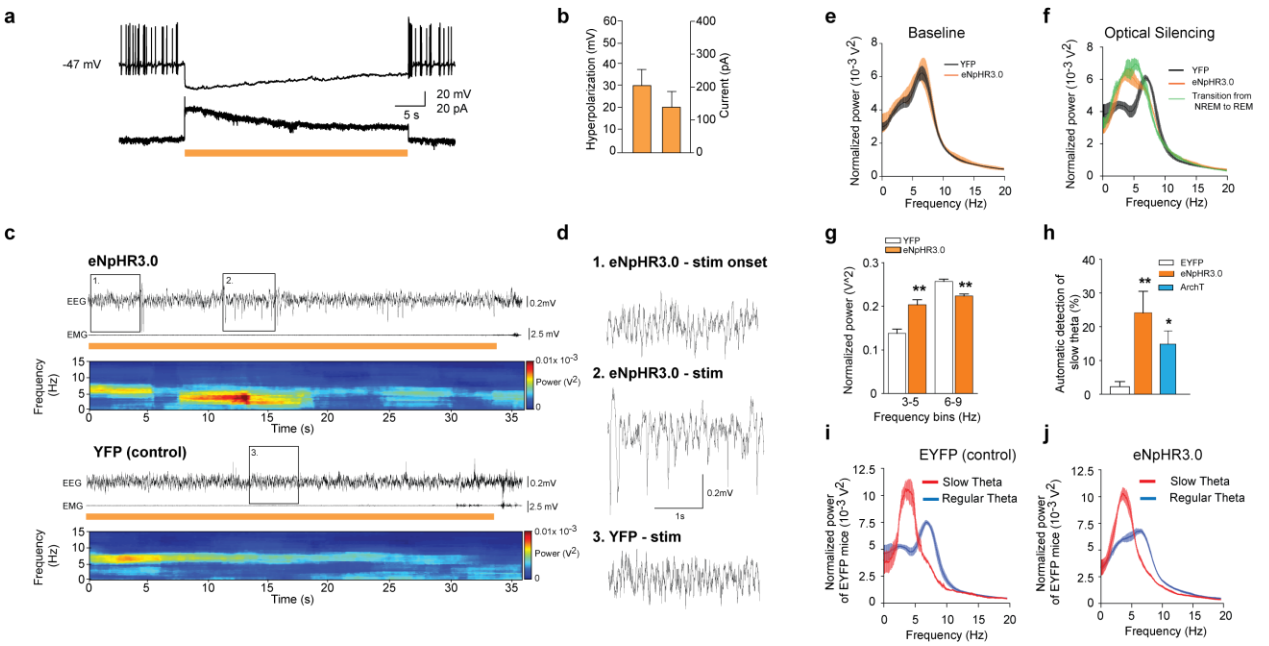

Figure 2-11. Optogenetic silencing of MCH neurons induced shift in the dominant theta peak frequencies towards slower oscillations

a, eNpHR3.0-expressing MCH neurons show a persistent hyperpolarization (top) and outward current (bottom) upon 30-s constant yellow light illumination in current and voltage clamp, respectively. **b,** Quantification of membrane hyperpolarization and outward currents of eNpHR3.0-expressing MCH neurons upon optogenetic silencing (n= 8 cells in 5 slices, n=3 animals). **c,** Representative EEG/EMG recordings and EEG power spectrum during optogenetic silencing (horizontal yellow bars) in eNpHR3.0-YFP (top) and EYFP (bottom) animals during REM sleep. **d,** Magnification of the boxes in figure (**c**). Note the high amplitude slow theta oscillations few seconds after the onset of optogenetic silencing in eNpHR3.0 animals. **e-f,** Average spectral distribution of relative cortical EEG power densities during baseline (**e**) and optogenetic silencing (**f**) in EYFP and eNpHR3.0-YFP animals (*n* = 5 per group; > 15 stimulations per frequency per animal). The green line represents the power spectrum of transition from NREM to REM sleep. Note the similarity with the power spectrum resulting from the MCH neurons silencing. **g,** Quantification of slow theta oscillations shown in (**f**) upon optogenetic silencing of control and eNpHR3.0-YFP animals (*n* = 5 per group). Normalized power densities are represented as mean ± SEM. *, $p<0.05$, **$p<0.01$, unpaired two-tailed *t* test. **h**, automatic detection of slow theta oscillations during optogenetic silencing of MCH neurons in control, eNpHR3.0 and ArchT animals. Percentage of detected events are represented as mean ± SEM. *, $p<0.05$ **, $p<0.01$, unpaired two-tailed *t* test between control and eNpHR3.0 or ArchT animals. **i, j,** Average spectral distribution of slow and regular theta oscillations during optogenetic silencing of MCH neurons in control (**i**) and eNpHR3.0 (**j**) animals. Note the similar power spectrum profile between control and eNpHR3.0 animals.

2-3-4 MCH neurons release GABA

How do MCH neurons modulate downstream sleep-wake circuits? Subpopulations of MCH neurons express the vesicular GABA transporter, cocaine and amphetamine regulated transcript (CART), Nesfatin-1 and/or MCH-related peptides (Elias et al., 2001; Hanriot et al., 2007; Fort et al., 2008; Sapin et al., 2010; Del Cid-Pellitero and Jones, 2012), however, it remains unclear whether the firing of MCH neurons releases GABA and neuropeptides. First, to determine the role of MCH peptide in sleep, we optogenetically stimulated MCH neurons in mice lacking the MCH receptor 1 [Tg(*Pmch-Cre*) X MCH-R1$^{-/-}$]. Note that MCH-R1 is the only MCH receptor expressed in mice (Tan et al., 2002). We found that optical activation of MCH neurons at 20 Hz extended REM sleep episodes similarly in both Tg(*Pmch-Cre*) : MCH-R1$^{-/-}$ and Tg(*Pmch-Cre*) transduced animals (p = 0.020; Fig. 2-13 a), suggesting that GABA and other neurotransmitters/modulators, rather than MCH peptide, are important for acute extension of REM sleep by MCH cell firing. Using fluorescence *in situ* hybridization (FISH) labeling of GAD-67 mRNA combined with immuno-labeling of MCH (Fig. 2-13 b), we found that 84.86 ± 2.18 % (315/386 cells, N = 9 slices, N = 4 animals) of MCH positive cells were also positive for GAD-67 signal and 17.09 ± 2.06 % of MCH cells that are negative for the GAD-67 signal, suggesting that a large majority of MCH neurons may release GABA.

We then functionally assessed whether MCH neurons release GABA. Anatomical mapping revealed dense ChETA-expressing MCH terminals in the tuberomammillary nucleus (TMN), in close opposition to the wake-promoting histaminergic (HA) neurons (Fig. 2-13 c) (Takahashi et al., 2006). We functionally mapped this LH^{MCH}→TMN circuit using optogenetics in brain slices from control Tg(*Pmch-Cre*) and Tg(*Pmch-Cre*) : MCH-R1$^{-/-}$ animals transduced with ChETA virus. In both animal models, optogenetic stimulation of LH^{MCH}→TMN circuit routinely evoked bicuculline-sensitive IPSCs (p < 0.001; Fig. 2-13 d, e) in postsysynaptic HA and non-HA neurons (Fig. 2-12). IPSCs latencies were typically short, consistent with monosynaptic connections (Atasoy et al., 2008; Schone et al., 2012), and did not differ between Tg(*Pmch-Cre*) and Tg(*Pmch-*

Cre) : MCH-R1$^{-/-}$ slices (Tg(*Pmch-Cre*) ; 2.16 ± 1.61 ms and Tg(*Pmch-Cre*) : MCH-R1$^{-/-}$: 5.36 ± 2.90 ms; $p = 0.58$).

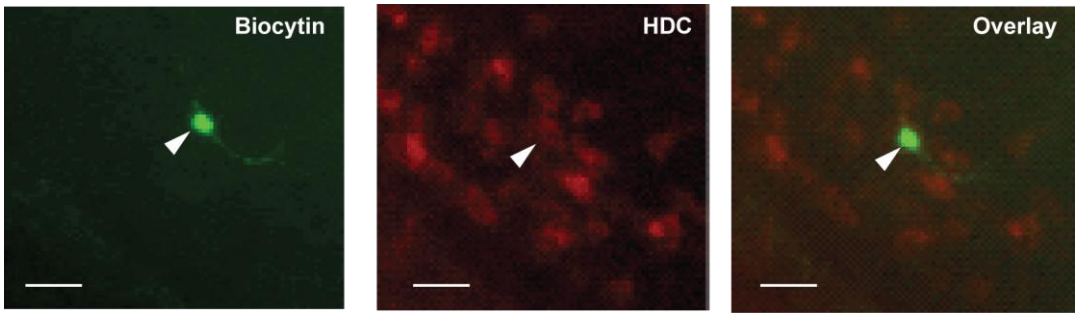

Figure 2-12. Recorded neurons (represented by biocytin labeling in green) co-localized with anti-histidine decarboxylase-positive cells (in red).
Scale bar: 50 µm.

The release of neuropeptides is thought to require the temporal proximity of multiple action potentials (van den Pol, 2012). During optogenetic stimulation, not all light flashes evoked an IPSC, consistent with the low release probability at central synapses(Schone et al., 2012). Nevertheless, in Tg(*Pmch-Cre*) mice, 20 Hz stimulation increased IPSC frequency relative to baseline ($p < 0.05$; Fig. 2-13 f and 2-13 g, *left panel*). However, when repeated in Tg(*Pmch-Cre*) : MCH-R1$^{-/-}$ mice, the effect of 20 Hz stimulation on IPSC frequency was no longer significant ($p = 0.16$; Fig. 2-13 g, *right panel).* Because amplitudes of optically-evoked IPSCs were similar in Tg(*Pmch-Cre*) and Tg(*Pmch-Cre*) : MCH-R1$^{-/-}$ mice ($p > 0.05$; Fig. 2-13 g), the effect of MCH-R1 deletion on IPSC frequency suggests a possible pre-synaptic facilitation of GABAergic transmission by MCH peptide activation of its receptor.

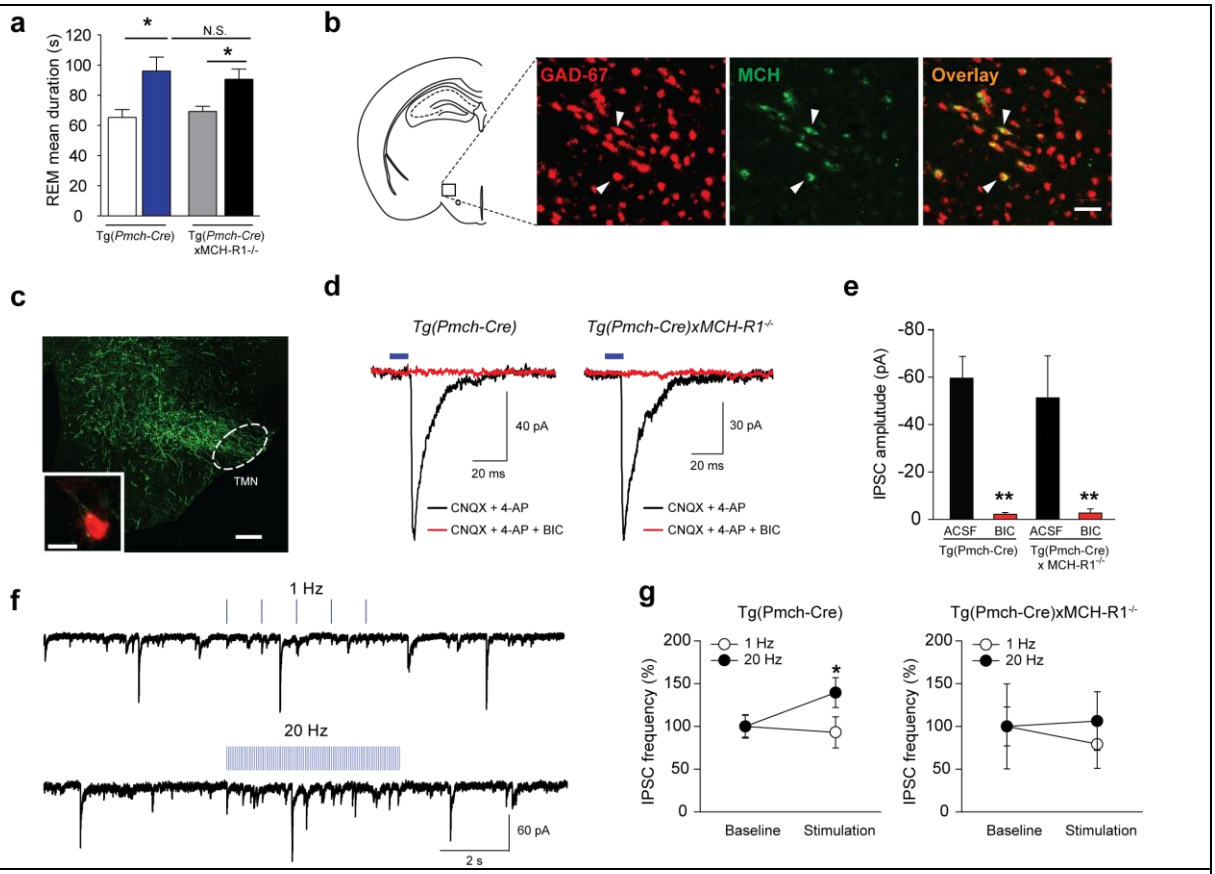

Figure 2-13. MCH neurons release the inhibitory transmitter GABA.

a, Mean duration of REM sleep episodes upon 20 Hz optogenetic stimulation of Tg(*Pmch-Cre*) and Tg(*Pmch-Cre*):MCH-R1$^{-/-}$ animals transduced with YFP or ChETA-EYFP viruses (*n*=6 per group; > 10 stimulations per animal). Data are represented as mean ± SEM. *, *p*<0.05 two-way ANOVA between genotype and viral transduction, followed by Tukey post-hoc test. **b,** Photomicrograph showing colocalization of GAD-67 transcripts (red) with MCH peptide (green). Scale bar: 100 µm. **c**, Photomicrograph showing ChETA-expressing MCH terminals in TMN area. Scale bar: 200 µm. *Inset*, ChETA-EYFP-expressing MCH terminals (green) contacting histamine cell (red). Scale bar: 10 µm. **d,** IPSCs (black) were recorded in TMN histamine and non-histamine neurons (n=3 and 13, respectively) from Tg(*Pmch-Cre*) (left trace) and Tg(*Pmch-Cre*):MCH-R1$^{-/-}$ animals (right trace) transduced with ChETA viruses. Optically-evoked responses (black traces) were blocked by bicuculline (BIC, red traces). **e**, Mean amplitude of evoked IPSCs from Tg(*Pmch-Cre*) (*n* =9 cells in 7 slices, n=6 animals, *left*) and Tg(*Pmch-Cre*): MCH-R1$^{-/-}$ (*n*=5 cells in 4 slices, n=3 animals, *right*) before and after bath application of BIC. **f**, Optogenetic stimulation of ChETA-expressing MCH axons at 20 Hz induced IPSC frequency in histamine neurons in Tg(*Pmch-Cre*), whereas 1-Hz stimulation did not. **g**, Mean IPSC frequency upon 20 Hz stimulation in Tg(*Pmch-Cre*) (left panel, *n*=10 cells in 7 slices, n=7 animals) and Tg(*Pmch-Cre*)xMCH-R1$^{-/-}$ (right panel, *n*= 5 cells in 4 slices, n=3 animals) animals. Mean IPSC amplitudes, latencies, and frequencies are represented as mean ± SEM. *, *p*<0.05, **, *p*<0.01, paired two tailed *t*-test.

2-3-5 MCH neurons modulate multiple targets

What are the downstream targets of MCH neurons for modulation of REM sleep? Results from this study and others suggest that MCH neurons exert an inhibitory action on postsynaptic targets, including the wake-promoting HA neurons located in the TMN (Fig. 2-10), as well as other targets located in the septum and dorsal raphe (Bittencourt et al., 1992; Lagos et al., 2011a). To test this hypothesis, we optogenetically activated ChETA-expressing MCH terminals in the tuberomammillary nuclei (LH^{MCH}→TMN), medial septum (LH^{MCH}→MS) or dorsal raphe (LH^{MCH}→DR) (Fig. 2-15 a, b and Fig. 2-14 for histological verification of optical implant localisation).

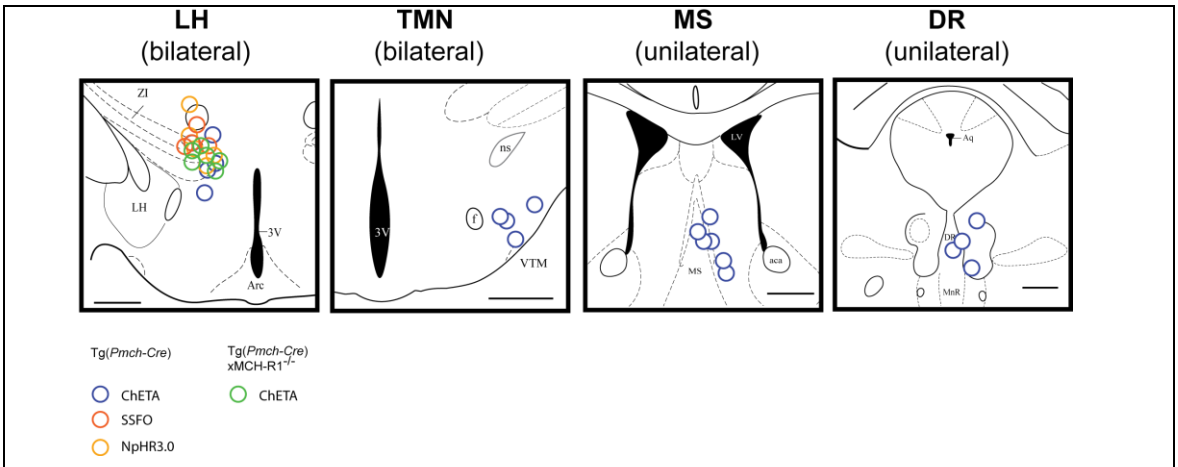

Figure 2-14. Optical fiber positions across experimental conditions over the LH, the TMN, the MS and the DR for Tg(Pmch-Cre) and [Tg(Pmch-Cre) X MCH-R1-/-] animals. Drawings were generated according to the mouse brain atlas. Scale bar: 500 µm. Abbreviations used: ZI: Zona Incerta, LH: lateral hypothalamus, 3V: third ventricle, Arc: Arcuate hypothalamic nucleus, f: fornix, ns: nigrostriatal tract, LV: lateral ventricle, MS: medial septal nucleus, aca: anterior commisure, Aq: Aqueduct, DR: doral raphe nucleus, MnR: Median raphe nucleus, VTM: ventral tuberomammillary nucleus.

We showed that optogenetic stimulation of both LH^{MCH}→TMN and LH^{MCH}→MS circuits significantly increased the duration of REM sleep (TMN: + 48%; p = 0.022, MS: + 38%; p = 0.006, respectively; Fig. 2-15 b) similarly to optical activation of MCH cell bodies in the LH. In contrast, optogenetic stimulation of the LH^{MCH}→DR circuit had no significant effect on REM sleep duration (REM sleep mean duration: 79.14±1 s, *p* = 0.22; Fig. 2-15

b). The EEG power spectrum of REM sleep episodes remained unchanged during these experiments (MS: p = 0.79; TMN: p = 0.27; DR: p = 0.73; Fig. 2-15 c). These results reveal two distinct downstream circuits through which MCH neurons modulate REM sleep episode.

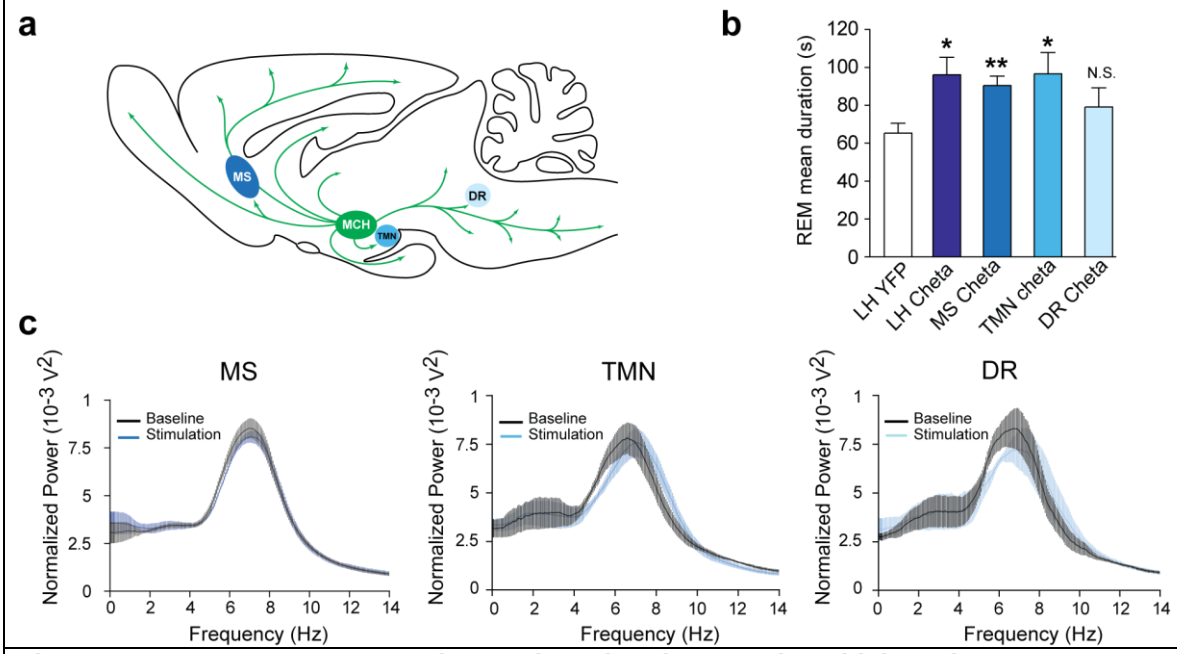

Figure 2-15. MCH neurons control REM sleep duration trough multiple pathways

a, Schematic of the MCH neuron projections in the rodent brain (based on Bittencourt *et al* (1992). Note the presence of MCH projection to the MS, TMN and DR. **b,** Mean REM sleep duration of animals stimulated at 20Hz during REM sleep in the TMN (n = 4 animals; bilateral), the DR (n = 4 animals; unilateral) or the MS (n = 6 animals; unilateral). Results of LH stimulation (LH ChETA) from Fig. 2c were reported for comparison. **c,** Average spectral distribution of relative cortical EEG power density during baseline (black) compared to stimulation (blue) of ChETA-containing MCH terminals within MS (left), TMN (center) and DR (right). Data analysis is based on an average of at least 10 stimulations per frequency for each mouse during REM sleep episodes. Mean duration are represented as mean ± SEM. *, $p<0.05$, **, $p<0.01$, unpaired two-tailed *t* test between YFP animal and ChETA animals in a given target.

2-4 Discussion

In this study, we show that acute activation of MCH neurons selectively modulates REM sleep state, using two distinct activating opsins (ChETA and SSFO) for stimulation of MCH neuron firing rates (Hassani et al., 2009a). Consistent with the neuroanatomical distribution of MCH neurons (see Fig. 2-15 a) (Bittencourt et al., 1992) and the MCH receptor (Bittencourt et al., 1992; Saito et al., 2001a; Chee et al., 2013), our results suggest that LH^{MCH}→TMN and LH^{MCH}→MS circuits represent two distinct pathways mediating MCH neuron control of REM sleep state. None of the optogenetic manipulations affected the duration of NREM sleep episode, which is consistent with the discharge of MCH neuron during spontaneous REM, rather than NREM, sleep (Hassani et al., 2009a).

To functionally map the underlying circuit, we first confirmed that MCH neurons express the glutamic acid decarboxylase (GAD), an enzyme necessary to GABA synthesis (Sapin et al., 2010). Then, we showed that electrical activity in MCH neurons elicits $GABA_A$-mediated IPSCs in post-synaptic neurons *in vitro*, revealing a local inhibitory LH^{MCH}→TMN connection in the hypothalamus. Notably, TMN HA neurons are silent during both NREM and REM sleep but display arousal-specific neuronal activity (Takahashi et al., 2006), in particular during REM sleep to wake transitions. Thus, we could speculate that activation of LH^{MCH} →TMN circuit extends the duration of REM sleep episode by maintaining an inhibitory tone that delays the reactivation of histamine neurons (Takahashi et al., 2006), as well as other hypothalamic (Lee et al., 2005a; Mileykovskiy et al., 2005) and extra-hypothalamic (Hobson et al., 1975; McCarley and Hobson, 1975; McGinty and Harper, 1976; Aston-Jones and Bloom, 1981; Saper et al., 2010) arousal centers shown to be quiescent during REM sleep.

In agreement with previous studies (Wu et al., 2009; Lu et al., 2013), we further found that activation of the LH^{MCH}→MS, but not LH^{MCH}→DR, circuit was sufficient to extend the duration of REM sleep episode, suggesting that MCH neurons provides a functional input to septo-hippocampal circuits where they participate to the stabilization of hippocampus theta rhythm and cortical oscillations (around ~ 7 Hz) during REM sleep

(as shown in Fig. 2-11). This hypothesis is further supported by our silencing experiment showing that rapid and reversible silencing of MCH neurons only during REM sleep episodes reduced the frequency and amplitude of theta oscillations in the hippocampus, which are a hallmark of REM sleep in mammals. Note that the silencing of MCH neurons did not reduced the duration of REM sleep episodes, suggesting that the termination of REM sleep episodes requires additional intra- and/or extra-hypothalamic circuits.

Experimental evidence shows that activation of the MCH-R1 enhances cognitive processing during wakefulness (Adamantidis et al., 2005) and facilitates long-term plasticity at CA1 glutamatergic synapse in the hippocampus (Pachoud et al., 2010), suggesting that MCH neurons may affect sleep-dependent cognitive processing through direct modulation of theta rhythm during REM sleep. However, additional circuits are likely involved and further experiments are now required to identify the mechanisms underlying the regulation of NREM and REM sleep states and their direct relevance in learning and memory.

The persistence of extended REM sleep episodes upon MCH neuron activation in animals lacking known MCH receptors suggests that the MCH peptide may have a minor role in acute regulation of REM sleep, in comparison to long-lasting activation of MCH neurons or MCH-R1. Indeed, previous pharmacological (Verret et al., 2003; Monti et al., 2013) and optogenetic (Konadhode et al., 2013) studies reported an increase of both NREM and REM sleep episode duration after cerebral infusion of MCH peptide or chronic optogenetic activation of MCH neurons. In contrast to our results, Konadhode and collaborators also observed an increase of NREM, as well as delta power, upon optogenetic activation of MCH neurons. This is likely to result from different optogenetic stimulation paradigms or genetic targeting strategies. Based on *in vivo* data showing that MCH neurons are active mainly during REM sleep(Hassani et al., 2009a), we used a temporal and sleep state-specific optogenetic stimulation – optical stimulation (1-20 Hz) were delivered at the onset of NREM or REM sleep and terminated at the following behavioral transition. In contrast, they used a chronic random stimulation paradigm

where optical stimulations were delivered at fixed interval (10 Hz, 1 min every 5 min for 24 h) independently of the behavioral state (i.e., wake, NREM or REM sleep) of the animals. Although we did not observe an extension of NREM sleep episode in our acute experiment, it is possible that chronic activation of MCH neurons results in a long-lasting activation of MCH or $GABA_A$ receptors, as well as receptors for transmitters/modulators produced by MCH neurons (Nesfatin-1, CART)(Elias et al., 2001; Hanriot et al., 2007; Fort et al., 2008), that eventually modulate NREM sleep, as reported in pharmacological experiments (Verret et al., 2003; Monti et al., 2013). Whether this reflects a physiological condition occurring during spontaneous sleep remains to be determined. Furthermore, we generated a new Tg(Pmch-Cre) driver mouse to target the entire MCH neuron population in the LH-ZI area, whereas they used a promoter-specific AAV that limited the transduction of opsin to half of the MCH neuron population (dorsal to the LH). Thus, it is likely that this partial targeting limited their optogenetic modulation of REM sleep, however, we cannot rule out the existence of anatomical subpopulation of MCH neurons with distinct modulatory function on NREM sleep.

Overall, our experiments and the results of others (Verret et al., 2003; Sapin et al., 2010; Clement et al., 2012), together with the neuroanatomical distribution of MCH neuron terminals, suggest that MCH neurons represent a subset of inhibitory cells outside the brainstem that stabilize REM sleep state, possibly through a distributed inhibition of arousal centers located in hypothalamus, forebrain and brainstem structures (Pace-Schott and Hobson, 2002; Luppi et al., 2013). Further functional dissections of NREM and REM sleep circuits in the mammalian brain will undoubtedly support the identification of selective targets that protect the integrity, the structure and the function of sleep in healthy subjects and pathological conditions.

Chapter 3:

Role of MCH peptide *vs* GABA release from MCH neurons in sleep-wake control

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3-1 Introduction

In mammals, melanin-concentrating hormone (MCH) is a conserved cyclic neuropeptide expressed by neurons localized in the lateral hypothalamus (LH) and the zona incerta (ZI) (Kawauchi et al., 1983; Skofitsch et al., 1985; Bittencourt et al., 1992; Saito and Nagasaki, 2008). MCH-expressing neurons project to many brain regions throughout the central nervous system (Bittencourt et al., 1992) including brain structures involved in the sleep-wake cycle such as the thalamus, septum, TMN of the posterior hypothalamus, the preoptic area of the anterior hypothalamus, VTA, PAG, LC, SLD, LDT-PPT nuclei and DR, cortex, hippocampus and nucleus accumbens (Bittencourt et al., 1992; McGregor et al., 2005; Elias et al., 2008; Torterolo et al., 2009). The only functional MCH receptor in rodents is MCH receptor 1 (MCH-R1) which activates Gi, Gq and Go intracellular signaling pathways (Bachner et al., 1999; Chambers et al., 1999; Lembo et al., 1999; Shimomura et al., 1999).

In agreement with the localization of MCH neuron projections, MCH receptors are found in most brain areas and limbic structures as well as areas related to the control of sleep and wakefulness mentioned above (Hervieu et al., 2000; Saito et al., 2001a; Saito and Nagasaki, 2008; Chee et al., 2013) However, there is not always a clear relationship between the density of MCH projections to a given brain region and density of MCH-R1 located within the same brain region. For instance, the LS contains the highest concentration of MCH projections but is not the brain area with the highest density of MCH-R1. Other reported sites of MCH activity, including the nucleus accumbens (Sears et al., 2010) and arcuate nucleus (Davidowa et al., 2002), contained relatively few fibers (Croizier et al., 2010) despite having been shown to express the highest level of MCH-R1 (Hervieu et al., 2000; Chee et al., 2013). In these examples it is possible that MCH was able to act by volume transmission and diffuse through the brain tissue to distal target sites (Tallent, 2008; van den Pol, 2012; Chee et al., 2013). Alternatively, MCH can be absorbed from the CSF by tanycytes and subsequently liberated remotely where it could act upon non classical targets (Torterolo et al., 2008).

In addition to MCH and MCH-derived peptides, MCH neurons also produce other neurotransmitters/modulators (Elias et al., 2001; Fort et al., 2008) including GABA (Elias et al., 2008; Sapin et al., 2010; Jego et al., 2013), and possibly glutamate (Abrahamson et al., 2001; Chee et al., 2015). Interestingly, MCH varicosities in the locus coeruleus (LC) were found to be immuno-positive for VGAT and a similar proportion of abutted puncta were immunostained for gephyrin, the postsynaptic marker for GABAergic synapses (Del Cid-Pellitero and Jones, 2012). Altogether, these results suggest a possible inhibitory role of MCH neurons. Accordingly, studies in lateral hypothalamic neurons *in vitro* have shown a predominantly inhibitory effect of MCH at both pre- and post-synaptic levels (Gao and van den Pol, 2001). MCH reduced the amplitude of glutamate-evoked inward currents and the amplitude of miniature excitatory currents, indicating an inhibitory modulation of postsynaptic glutamate receptors. MCH also reduced the frequency of miniature excitatory currents indicating that MCH reduced the release probability or the number of release sites at pre-synaptic terminals (Gao and van den Pol, 2001). Extra-hypothalamic targets showed an inhibitory response to MCH through MCH-R1-dependent action on K^+ channels and a reduction of the activity of AMPA receptors (Wu et al., 2009; Sears et al., 2010).

Finally, we previously demonstrated that MCH neuron terminals release GABA and exert strong inhibitory actions on wake-promoting histaminergic cells located in the tuberomamillary nucleus (TMN) following optogenetic stimulation at 20Hz (Jego et al., 2013). We further showed that MCH peptide might regulate this process pre-synaptically since the frequency of inhibitory post-synaptic currents (IPSCs) is reduced when stimulating the MCH projections to the TMN in animals without MCH-R1 (Jego et al., 2013) suggesting that MCH peptide can act both pre- and post-synaptically on arousal centers to promote sleep.

We and others have shown that MCH neurons have a strong sleep-promoting effect, although the selectivity of the sleep-promoting effect for NREM and/or REM sleep appears to vary largely depending on the experimental approach used. Random chronic stimulation of the MCH system using optogenetic stimulation of MCH cell bodies for several hours affects both NREM and REM sleep (Verret et al., 2003; Konadhode et al.,

2013; Tsunematsu et al., 2014). In contrast, state-specific acute optogenetic stimulation time-locked to the onset of NREM or REM sleep states and terminated at the next transition promotes and extends REM, but not NREM, sleep episodes (Jego et al., 2013). In addition, intracerebral injection of MCH agonist into specific brain regions induced a non-selective increase of both NREM and REM sleep depending on the structure targeted. Indeed, microinjection of MCH into the preoptic area increases NREM sleep (Benedetto et al., 2013) while injection into the raphe, LC and the basal forebrain increases REM sleep (Lagos et al., 2009; Lagos et al., 2011a; Lagos et al., 2012; Monti et al., 2014).

The discrepancies between these results could be explained by technical limitations including partial genetic targeting of the opsins, the use of different mouse lines, non-physiological administration of MCH peptide, rates of peptide / antagonist degradation and diffusion as well as the interdependence of synaptic transmission modalities and stimulation strengths. Indeed, amino-acid neurotransmitters such as GABA are packaged in clear small synaptic vesicles and activate ionotropic receptors located on the postsynaptic neurons (Leenders et al., 1999; Sudhof, 2012). This type of transmission is fast (< 1 ms) and very efficient compared to neuropeptide transmission (10 ms to several seconds) due to fast vesicle recycling and the short distance to the ionotropic receptors from presynaptic release sites. Peptides such as MCH are packaged in dense core vesicles that have a low recycling rate. Once released, peptides diffuse from the releasing site to act on G-protein coupled receptors (such as MCH-R1) that are mostly located away from the synaptic site (Bruns and Jahn, 1995; van den Pol, 2012). Generally, the release of neuropeptides requires stronger and more prolonged stimulation to occur relative to the release of GABA or other fast neurotransmitters (Bartfai et al., 1988; De Camilli and Jahn, 1990; Tallent, 2008). Amino-acids and neuropeptides are often co-expressed and co-released (Merighi et al., 2011; van den Pol, 2012) but the quantification of the release of one or another is largely unknown. The stimulation paradigm (acute vs. chronic) used in previous MCH studies would probably favor the release of different neurotransmitters and peptides and might explain the different effects on sleep. In this study we further investigated the role

of MCH peptide *vs.* GABA using different protocols of optogenetic stimulation (acute *vs*. semi-chronic) in combination with pharmacological manipulation and genetically-engineered mouse models with disrupted MCH and/or GABAergic transmission within MCH cells.

3-2 Methods

3-2-1 Characterization of Tg(*Pmch-Cre)* transgenic mice

Tg(*Pmch-Cre)* mice use the Pmch gene promoter to restrict cre-recombinase expression to MCH neurons (Kong et al., 2010; Chee et al., 2015). Specificity of cre-expressing MCH neurons was assessed in Tg*(Pmch–cre; tdTomato)* mice (n=4) obtained by crossing Pmch–cre (stock #014099; Jackson laboratories) and tdTomato reporter Rosa-CAG-LSL-td Tomato-WPRE (line Ai9, Jackson Laboratories). Quantification of co-localization was performed on adjacent sections containing the LH. Ectopic expression was found in the septum.

To evaluate the role of MCH peptide in sleep regulation, we optogenetically stimulated MCH neurons in mice lacking the MCH receptor 1, using Tg(*Pmch-Cre)* : *MCH-R1*$^{-/-}$ mice (Adamantidis et al., 2005) by breeding Tg(*Pmch-Cre)* mice with MCH R1$^{-/-}$ mice. To assess the role of GABA expressed in MCH neurons, we optogenetically stimulated MCH neurons in mice lacking the vesicular GABA transporter (Vgat), using Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ mice generated by breeding Tg(*Pmch-Cre)* mice with Vgat $^{flox/flox}$ mice (Slc32a1 $^{tm2(cre)Lowl}$/J, 016962, Jackson laboratories). To test whether Vgat from MCH cells acting on MCH-R1 was the primary sleep promoting mechanism, we generated triple transgenic mice Tg(*Pmch-Cre)* : *MCH-R1*$^{-/-}$*:* Vgat $^{flox/flox}$ by breeding Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ mice with Tg(*Pmch-Cre)* : *MCH-R1*$^{-/-}$ mice. Genotypes of mice from each line were determined by PCR.

Only male mice were used for sleep recordings and optogenetic stimulations. Mice were individually housed in custom-designed polycarbonate cages at constant temperature (22 ± 1 °C), humidity (30–50%), and circadian cycle (12 h light-dark cycle, starting at

8:00 a.m.). Food and water were available *ad libitum*. Animals were treated according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

3-2-2 Plasmid and viral targeting

Mice were anesthetized with isoflurane (5% induction, 1% maintenance) and then head-fixed in a small animal digital stereotaxic frame. 0.8 µl of recombinant AAVdj carrying Ef1α- DIO-ChETA- EYFP or control Ef1α-DIO-EYFP vector were bilaterally injected into the lateral hypothalamus (anteroposterior (AP), -1.50 mm; mediolateral (ML), ± 0.9 mm; dorsoventral (DV) 5.35 mm) at a rate of 50 nl/min.

3-2-3 Surgery

At 9-weeks age Tg(*Pmch-Cre*), Tg(*Pmch-Cre*): MCH-R1$^{-/-}$ and Tg(*Pmch-Cre*): Vgat lox mice were chronically implanted with bilateral fiber implants above the lateral hypothalamus (AP, 1.50; ML, 0.95 mm; DV, 5 mm) and with a custom-made-EEG/EMG implant, as described previously in Chapter 2 (see section 2-2-8) .

3-2-4 Polysomnographic recording & Spectral EEG/EMG analysis

A 24-h baseline recording was completed before starting the optogenetic stimulation on each mouse to ensure a proper sleep-wake cycle. All sleep recordings took place between 12:00 and 19:00. EEG and EMG signals were acquired and analysed as reported in chapter 2 (see section 2-2-9).

For all EYFP and ChETA-EYFP transduced mice used for state-specific stimulations, EEG power spectra were computed for 6 to 10 stimulated events per mouse (each event of at least 20 s duration). Power spectra were calculated in Matlab using the Chronux toolbox (Bokil et al., 2010) and a 5 s sliding window (0.062 s increments). For

each mouse the mean spectral density of stimulated events was sorted into successive 0.062 Hz frequency bands between 0 and 100 Hz, and each frequency band was subsequently normalized relative to the sum of the power over the entire range (0-100 Hz). EEG frequency bands defined as delta (0.5–4 Hz), theta (6–9 Hz), and slow theta (3-5 Hz) were analysed statistically.

3-2-5 Optical stimulation and pharmacology

All optical stimulation experiments in the LH were conducted bilaterally and light pulse trains (5-ms pulses of various frequency and duration) were programmed using a waveform generator (Master 9 - A.M.P.I) that triggered two blue-diode lasers (473 nm, 40 mW continuous light intensity output; LaserGlow Inc.). For acute optical stimulation experiments, we used the same protocol described in chapter 2 (see 2-2-12).

A semi-chronic stimulation protocol (5 ms at 1-20 Hz for 10 s every minute over 1 h) was used to assess the behavioral consequences of sustained stimulation of the MCH network during the second half of the light phase (5 ms at 1-20 Hz for 10 s every minute over 1 h).

To test the effect of MCH peptide during semi-chronic stimulation an MCH-R1 antagonist SNAP 7941 (gift from Dr. Mark Millan, Servier, France) was administered. To evaluate specificity and determine the right dose of SNAP 7941 to use, WT and MCH-R1 $^{-/-}$ mice were intraperitoneally (IP) injected with different concentrations of the MCH-R1 antagonist (1, 5, 10 and 30 mg/kg; all dilutions in sterile water with Tween 80 (1 to 5 %)) at 13:00 and their vigilance states were subsequently recorded and analysed. Then, to test the effect of MCH peptide during the semi-chronic stimulation ChETA and EYFP transduced Tg(*Pmch-Cre*) mice were the intraperitoneally (IP) injected with saline or SNAP 7941 at different concentrations (1 and 10 mg/kg, 0.1 ml/10g of body weight) at 13:00. Semi-chronic stimulation (as described above) was started 20 min after the IP injection.

3-2-6 Immunohistochemistry & microscopy

After completion of experiments, mice were deeply anesthetized and perfused transcardiacally with 4% paraformaldehyde in PBS. The brains were extracted, postfixed overnight in the same fixative at 4 °C, and cryo-protected as described in the section 2-2-13. Each brain was sectioned and collected in PBS with 0.3% Triton X-100 (PBST). For double immunolabeling GFP/MCH, the same protocol reported in the section 2-2-13 was used.

Non-confocal images were collected on an Axio Observer Carl Zeiss fluorescent microscope using fluorescent reflected light. Digital images were minimally processed using Image J to enhance brightness and contrast for optimal representation of the data. All digital images were processed in the same way between experimental conditions to avoid artificial manipulation between different datasets.

3-2-7 Statistical analysis

Statistical analyses of electrophysiological properties and synaptic responses were assessed using ANOVAs or unpaired t-tests, and significant effects were investigated using pairwise multiple comparisons followed by a Tukey post hoc test. Data are presented as mean ± SEM. All data were analyzed using Prism 5.0 (GraphPad Software as described in the text. Data were exported into Adobe Illustrator CS3 (Adobe Systems) for preparation of figures.

3-3 Results

3-3-1 Genetic targeting of MCH cells and Characterization of Tg*(Pmch-Cre)*, Tg*(Pmch-Cre):MCH-R1*$^{-/-}$,Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice.

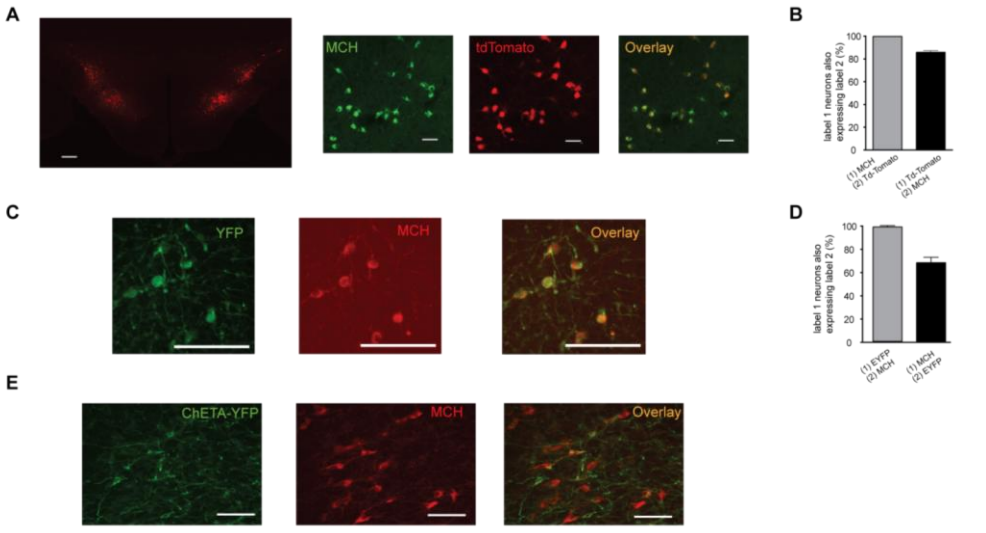

Figure 3-1: Selective targeting of MCH neurons in Tg(Pmch-cre) mice.
a, Left: Photomicrograph of the native fluorescence from the hypothalamus of a Tg*(Pmch-cre)*x R26 tom mouse. Scale bar represents 500 µm. Right: Photograph of a brain section from Tg*(Pmch-cre)*; R26 tom mouse showing colocalization of tdTomato-positive cells (red) with MCH-positive cells (green). Scale bars represent 50 µm. **b,** Quantification of the colocalization of MCH positive cells identified by immunofluorescence (green) and native fluorescence of the tomato (red). **c,** Photograph of a brain section from Tg*(Pmch-cre)* mouse showing colocalization of YFP transfected neurons (green) with MCH-positive cells (red). Scale bars represent 50 µm. **d,** Quantification of the colocalization of MCH positive cells identified by immunofluorescence (red) and YFP fluorescence (green). **e,** Photograph of a brain section from Tg*(Pmch-cre)* mouse showing colocalization of ChETA-YFP transfected neurons (green) with MCH-positive cells (red). Scale bars represent 50 µm.

To optogenetically modulate the activity of MCH cells in the lateral hypothalamus (LH) and the zona incerta (ZI, dorsal to LH), we used Tg(*Pmch-Cre*) mice (Kong et al., 2010). To assess the selectivity of this transgene, Tg(*Pmch-Cre*) mice were crossed with cre-dependent TdTomato reporter (Rosa-CAG-LSL-tdTomato-WPRE). 85.87 ± 1.41 % of MCH-immuno-positive cells were found to express TdTomato in the LH, whereas 99.81 ± 0.09 % of TdTomato cells expressed MCH peptide (2432 cells, n = 3 mice) (Fig. 3-1 a-b).

After stereotactic injection of Cre-inducible adeno-associated virus AAVdj-EF1-DIO-EYFP into the LH of Tg(*Pmch-Cre*) mice, we found that 67.7 ± 4.7% of MCH-immunopositive cells co-expressed EYFP (1271 cells, n=4 mice) (Fig. 3-1 c-e) and MCH immunoreactivity could be detected in 98.4±1.2 % of EYFP expressing cells (955 cells, n = 4 mice). The diffusion of the virus was not restricted to the injection site in the lateral hypothalamus as evidenced by the presence of transfected MCH cells in both the Zona Incerta and the lateral hypothalamus.

Before starting optogenetic activation, Tg*(Pmch-Cre),* Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*, Tg(Pmch-Cre): Vgat* $^{flox/flox}$ and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice were recorded under baseline conditions. During the light phase (rest period) when all optogenetic experiments occurred, we did not see any difference in sleep-wake quantities among Tg*(Pmch-Cre):MCH-R1*$^{-/-}$, Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ compared to Tg*(Pmch-Cre)* mice with the exception that triple transgenic mice exhibited more REM sleep compared to Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ and Tg*(Pmch-Cre): Vgat* $^{flox/flox}$ mice *(*$p < 0.01$ and $p < 0.05$ respectively; Fig. 3-2 a),

During the dark phase (active period), and as reported previously (Smith et al., 2005; Ahnaou et al., 2011; Jego et al., 2013), Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice were hyperactive, as suggested by the increased period of wakefulness compared to Tg*(Pmch-Cre)* mice ($p < 0.05$; Fig. 3-2 b-d). Interestingly, triple transgenic mice and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice exhibited very similar sleep-wake cycles whereas the sleep-wake cycle in Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice was similar to that in Tg*(Pmch-Cre)* mice.

These results confirmed the hyperactivity phenotype observed during the dark phase in mice which had disrupted MCH transmission, however, the disruption of GABA transmission within MCH neurons had no effect on the sleep wake cycle unless it is coupled with the absence of MCH-R1. Indeed, triple transgenic mice showed a marked increase in REM sleep amount during the light phase which might indicate the existence of an interaction between MCH and GABA to control REM sleep.

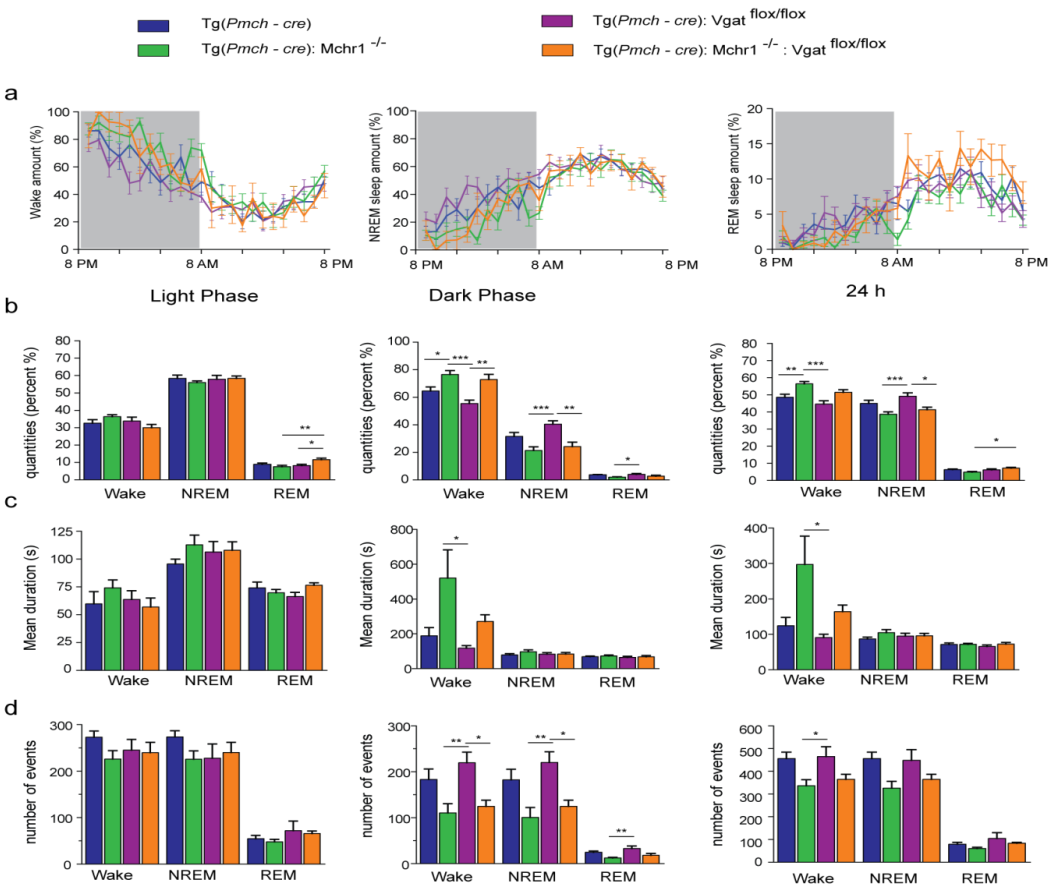

Figure 3-2. Spontaneous sleep-wake cycle of Tg*(Pmch-cre)*, Tg*(Pmch-cre):MCH-R1*$^{-/-}$, Tg*(Pmch-cre): Vgat* $^{flox/flox}$ and Tg*(Pmch-cre): MCH-R1*$^{-/-}$*: Vgat* $^{flox/flox}$ mice.
a, Spontaneous duration of wake, NREM and REM sleep (expressed as a percentage of time) of Tg*(Pmch-Cre)* (blue, n =8),Tg*(Pmch-cre): MCH-R1*$^{-/-}$ (green, n=10), Tg*(Pmch-cre): Vgat* $^{flox/flox}$ (purple, n=9) and Tg*(Pmch-cre): MCH-R1*$^{-/-}$*: Vgat* $^{flox/flox}$ (green, n=6) mice during the light and dark phases, and the full 24 h light/dark cycle. **b-c,** Mean duration of episodes (b) and mean number (c) of Wake, NREM and REM sleep events of each mouse line across the sleep wake–cycle. Mean durations are represented as mean ± SEM. *:p < 0.05,**: p < 0.01, ***: p < 0.001 one-way ANOVA between subject design followed by Tukey post-hoc test.

3-3-2 Removal of both MCH-R1 and VGAT from MCH cells increases basal REM sleep consolidation during acute sleep-specific stimulation

To confirm and extend our previous results illustrated in chapter 2 (Jego et al., 2013), we first delivered 20 Hz bilateral optical stimuli to the LH area during REM sleep using real-time EEG/EMG detection (Fig. 2-2) in Tg*(Pmch-Cre),* Tg*(Pmch-Cre):MCH-R1*$^{-/-}$, Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice. A stimulation

frequency of 20 Hz was previously determined to be both the highest frequency that could be used with 100% fidelity response to light pulses and the frequency that affects sleep most (Jego et al., 2013). Consistent with our previous study (Jego et al., 2013), we confirmed that 20 Hz optical stimulation of MCH neurons during the REM sleep state significantly increased (~86 %) the duration of REM sleep episodes in ChETA Tg*(Pmch-Cre)* mice compared to control mice (99.36 ± 8.7 *vs* 57.65 ± 6.0 s, respectively, $p < 0.01$; Fig. 3-3 a).

To determine whether the observed increase in REM sleep duration was due to MCH receptor 1 (MCH-R1) activation and/or GABA within MCH cells, we optogenetically stimulated MCH cells in Tg*(Pmch-Cre): MCH-R1*$^{-/-}$, Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice during REM sleep. We found that optical activation of MCH neurons at 20 Hz extended REM sleep similarly in both Tg(*Pmch-Cre*) and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice ($p < 0.05$, Fig. 3-3a), demonstrating that other neurotransmitters and modulators co-released with MCH peptide are equally important for acute extension of REM sleep. Most MCH neurons colocalize with *Gad 67* mRNA and release GABA (Sapin et al., 2010; Jego et al., 2013) therefore it is possible that the prolonged REM sleep duration during REM sleep-specific stimulation is due to GABA release by MCH neurons, rather than MCH peptide. To assess this, we optogenetically stimulated MCH cells in Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice during REM sleep. Surprisingly, we found that REM sleep duration remained extended in these mice and was similar to the REM sleep durations observed in Tg(*Pmch-Cre*) and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice ($p < 0.05$, Fig. 3-3 a). Note that this effect did not result from perturbed sleep-wake architecture of Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ or Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice during the light cycle (Fig. 3-2).

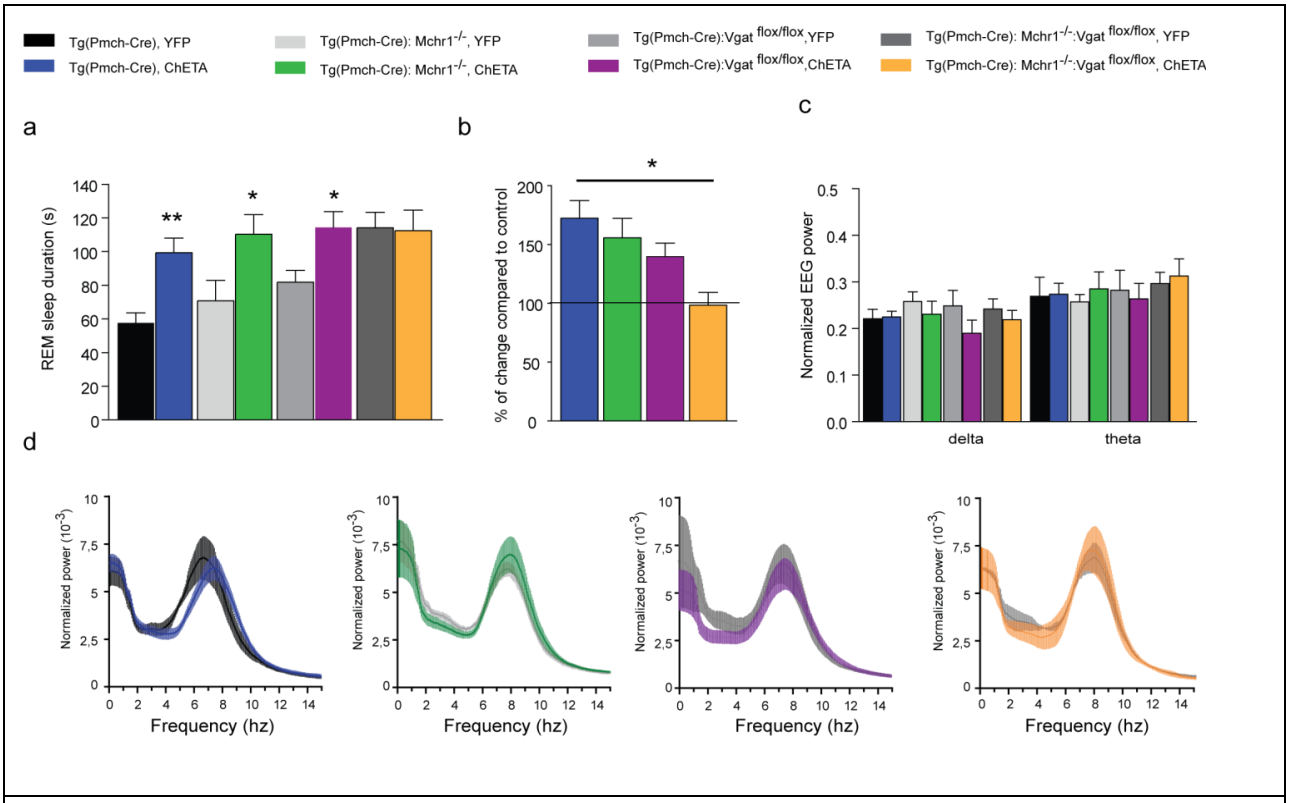

Figure 3-3: Prolonged REM sleep duration during REM sleep stimulation of MCH cells is not mediated by MCH peptide.
a, Mean duration (± s.e.m) of REM sleep following optogenetic stimulation at 20 Hz of EYFP- and ChETA-EYFP-injected mice in Tg*(Pmch-Cre)* (n=5 control,n=8; ChETA >8 stimulations per mouse), Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ (n=6 per group; >8 stimulations per mouse), Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ (n=6 control, n=7 ChETA; >8 stimulations per mouse) and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ *:Vgat* $^{flox/flox}$ (n=2 control, n=4 ChETA; >10 stimulations per mouse) mouse lines. **b,** Percentage of change of REM sleep duration represented in a compared to their respective control. **c,** Power spectrum analysis of the EEG during optogenetic REM sleep stimulation shown in a (delta:0.5-4 Hz and theta : 6-9 Hz). **d,** Profile of the EEG during REM sleep. (delta: 0.5-4Hz and theta : 6-9 Hz) *P<0.05, unpaired t-test within the same mouse line or One way ANOVA between subject designs.

Stimulation of MCH cells in triple transgenic Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice resulted in a similar increase in REM sleep duration (~ 115 s) to that of ChETA-expressing Tg*(Pmch-Cre)* and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$mice ((p < 0.05, Fig. 3-3 a) confirming that neither GABA nor MCH-R1 are necessary for REM sleep extension. However, REM sleep mean duration in control Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice reached the same value as ChETA-expressing Tg(*Pmch-Cre*) mice when stimulated (~ 115 s), which masked the REM sleep promoting effect (p=0.93; Fig. 3-3 b). Consistent with their increased REM sleep amount during the light phase during baseline, it is

possible that the REM sleep amount in mice with the absence of both MCH-R1 and VGAT in MCH cells already reached a ceiling value and their optogenetic activation could not further increase REM sleep duration.

Consistent with our previous reports (Jego et al., 2013), the delta and theta power during optical stimulation remained unchanged (Fig. 3-3 c-d) suggesting that the optogenetically extended REM sleep state was similar to spontaneous REM sleep episodes.

We next investigated the consequence of optogenetic activation of MCH neurons by limiting the optical stimulation to real-time EEG/EMG detection of NREM sleep. Optical stimulation (20 Hz every 10 s; 5 s duration, Fig. 2-2) of MCH neurons at 20hz significantly decreased the duration of NREM sleep episodes (140.0 ± 18.3 *vs* 205.5 ± 25.0 s, respectively, $p = 0.05$; Fig. 3-4 a) and significantly increased the probability of NREM-to-REM sleep transitions in ChETA mice compared to controls ($p< 0.05$, Fig. 3-4 b). Consistent with a NREM-REM sleep transition event, analysis of spectral power revealed that delta power was decreased ($p < 0.05$) while theta activity was increased ($p < 0.01$) during the NREM sleep stimulation (Fig. 3-4 c-d). Interestingly, optogenetic activation of MCH cells during NREM sleep in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ and Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice also induced a decrease in NREM sleep duration ($p < 0.05$) and an increase in the number of NREM-to-REM sleep transitions ($p < 0.05$) with a magnitude of change that is similar to the changes seen in Tg(*Pmch-Cre*) mice (Fig. 3-4 a-b right panels). Power spectrum analysis of ChETA Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice revealed a decrease in slow theta band power (3-5 Hz) ($p < 0.05$; Fig. 3-4 c-d), as previously reported to occur during silencing of MCH neurons (Fig. 2-8 e-g). On the other hand the power spectrum of Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice is similar to that of Tg*(Pmch-Cre)* mice and was consistent with a transition from NREM to REM sleep (decrease in delta power: $p < 0.05$; increase in theta power: $p < 0.05$; Fig. 3-4 c-d). However, ChETA Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice did not show a reduction in NREM sleep duration ($p = 0.64$) compared to YFP controls but showed a marked increase in NREM-to-REM sleep transitions ($p < 0.05$) (Fig. 3-4 b left panel). Power spectrum analysis of

Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice did not revealed any change although more animals are needed to confirm this effect in this particular mouse line. Interestingly, an absence of MCH-R1, VGAT or both MCH-R1 and VGAT in MCH neurons almost doubled the number of transitions from NREM to REM sleep compared to control Tg*(Pmch-Cre)* mice. The fact that ChETA Tg*(Pmch-Cre):MCH-R1*$^{-/-}$, Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ animals were still able to increase the number of transitions with the same magnitude (Fig. 3-4 b right panel) following MCH neural activation during NREM sleep suggests that the «ceiling value» for NREM to REM sleep transitions was increased and neurotransmitters other than GABA and MCH peptide are responsible for this effect. Consistent with a transition to REM sleep, a decrease in delta power concomitant with an increase in theta power was consistently observed.

Altogether, acute sleep-specific stimulation revealed that the presence of both MCH peptide and GABA from MCH neurons is essential for normal REM sleep duration. The absence of both GABA and MCH-R1, but of neither individually, increased the REM sleep mean duration to a ceiling value in control-YFP mice. This could suggest a synergistic inhibitory action on REM sleep promoting structures of both MCH peptide and GABA from MCH neurons. On the other hand, the absence of MCH-R1, GABA or both systematically increased NREM to REM sleep transitions to a similar extent although the number of transitions from NREM to REM sleep in control-YFP animals was elevated suggesting that neurotransmitters other than GABA and MCH peptide are responsible for the REM sleep switch following NREM sleep stimulation.

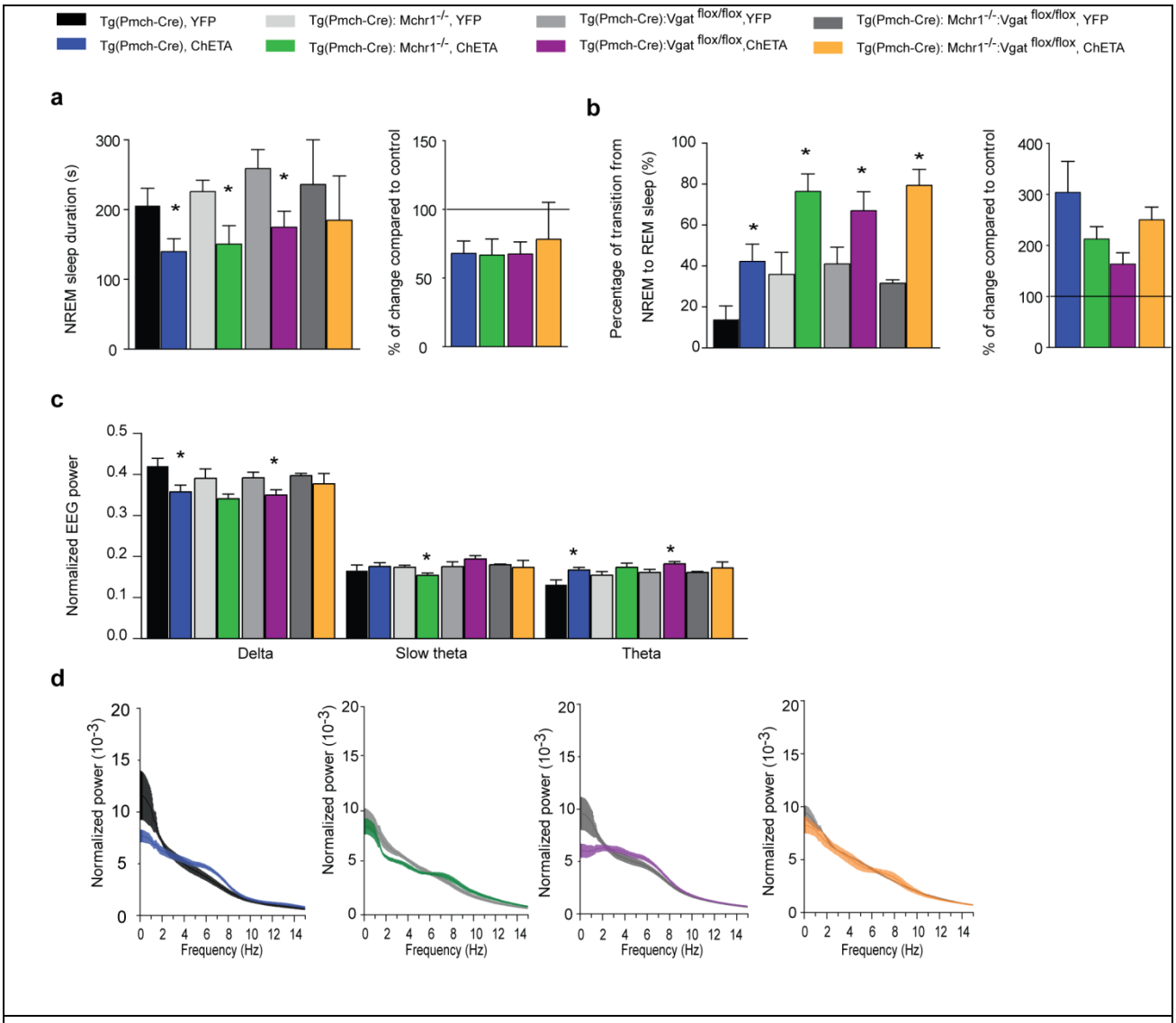

Figure 3-4: NREM to REM sleep transition following the activation of MCH neurons does not require MCH and Vgat transmission.

a, Left: Mean duration (± s.e.m) of NREM sleep following optogenetic stimulation at 20 Hz of Tg*(Pmch-Cre)* mice (n=5 control, n=8 ChETA; >8 stimulations per mouse), Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice (n=8 control, n=6 ChETA; >8 stimulations per mouse), Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ mice (n=9 control, n=8; >10 stimulations per mouse) and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ *: Vgat* $^{flox/flox}$ mice (n=2 control, n=4 ChETA; >10 stimulations per mouse). Right: : Percentage of change of NREM sleep duration compared to their respective control **b,** Left: Percentage of successful NREM-to-REM sleep transitions following optogenetic stimulation during NREM sleep shown in a. Data are shown as a percentage of the total number of NREM-to-REM sleep transitions relative to the total number of stimulations during NREM sleep. Right: Percentage of change of transition from NREM to REM sleep represented compared to their respective control. **c,** Power spectrum analysis of the EEG during optogenetic NREM sleep stimulation shown in a (delta: 0.5-4Hz, slow theta 3-5 Hz and theta : 6-9 Hz). **d,** EEG power spectrum during NREM sleep. *P<0.05, unpaired t-test within the same mouse line or One way ANOVA between subject designs.

3-3-3 MCH peptide promotes NREM sleep during semi-chronic stimulation of MCH neurons and GABA controls basal REM sleep amount.

Semi-chronic *in vivo* optical stimulation at 20 Hz (10 s / 5 ms light pulse trains delivered once per minute over 1 hour) was used to mimic a more sustained activation of MCH cells that would occur following pharmacological administration of the peptide. A stimulation frequency of 20 Hz was chosen because previous data using another Tg*(Pmch-Cre)* mouse line described in Chapter 2 revealed that chronic stimulation of MCH neurons using frequencies ranging from 1 to 10 Hz had no effect on the sleep-wake cycle, whereas 20 Hz had a sleep-promoting effect (NREM sleep: $p<0.001$, REM sleep: $p<0.01$) (Fig. 3-5).

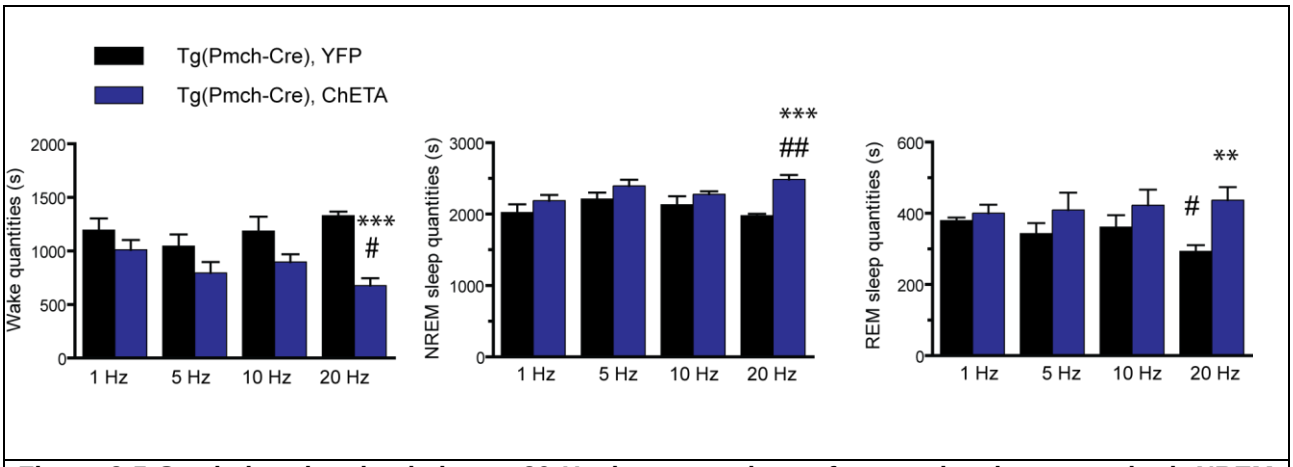

Figure 3-5 Semi-chronic stimulation at 20 Hz, but not at lower frequencies, increases both NREM and REM sleep.

Semi-chronic stimulations (10 s illumination, 5 ms every minute over 1hour) performed on the Tg*(Pmch-Cre)* mouse line from Jego et al., 2013, n=6 per group. **p<0.01 and ***<0.001, ChETA animals compared to YFP at a same frequency condition. # P<0.05, ##<0.01 compared to 1Hz condition for the same mice group (ChETA or YFP animals). Two way-ANOVA mixed design followed by Tukey post-hoc test.

We conducted the same protocol of stimulation in Tg*(Pmch-Cre),* Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice. We found that random semi-chronic activation of the MCH neuronal population throughout the sleep-wake cycle at high frequencies (20 Hz) in ChETA-injected Tg*(Pmch-Cre)* mice

promoted NREM and REM sleep states ($p<0.05$ and $p<0.01$ respectively; Fig. 3-7 a) concomitant with a decrease in wakefulness ($p<0.01$) due to shorter episodes (Fig. 3-8 a). These results are in agreement with pharmacological (Verret et al., 2003; Lagos et al., 2009; Torterolo et al., 2009) and chronic optogenetic (Konadhode et al., 2013) studies. Semi-chronic activation of MCH neurons while MCH transmission was disrupted (Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice*)* resulted in a blockade of the increase in NREM sleep ($p = 0.74$) but not the increase in REM sleep ($p < 0.05$; Fig. 3-7 a, top). This increase was due to an increased number of REM sleep bouts (Fig. 3-8 b), suggesting that other neurotransmitters participate in the regulation of REM sleep. Semi-chronic stimulation of ChETA-expressing Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice had no effect on wake, NREM and REM sleep relative to YFP-injected control mice ($p=0.38$, $p=0.18$, $p=0.65$ Fig. 3-7 a, top). Interestingly, REM sleep quantity in control Tg*(Pmch-Cre):Vgat*$^{flox/flox}$ mice was at the same level (~ 400 s) as in ChETA activated intact MCH neurons. Similarly, semi-chronic stimulation in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice had no effect on wake, NREM and REM sleep compared to YFP-injected control mice ($p=0.10$, $p=0.41$, $p=0.39$ respectively Fig. 3-7 a, top). However, the net effect of the stimulation across mouse lines revealed that the absence of both MCH-R1 and VGAT in MCH neurons reversed the sleep promoting effect observed in Tg*(Pmch-Cre)* mice (Fig. 3-7 a bottom). Indeed, Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice spent significantly more time awake (due to an increase of Wake mean duration, Fig. 3-8 a) and the NREM and REM sleep promoting effect was abolished relative to Tg*(Pmch-Cre)* mice ($p<0.001$, $p<0.05$ and $p<0.05$ respectively Fig. 3-7 a, bottom). In addition, REM sleep quantities in control Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$ mice were almost tripled (due to an increase in NREM to REM sleep transitions, Fig. 3-7a) compared to Tg*(Pmch-Cre)* mice (217 ± 24 vs. 583 ± 10 s), which would make REM sleep amount difficult to further increase, suggesting the existence of a ceiling value for REM sleep amount.

Altogether, these results suggest that the absence of MCH-R1 alone seems to abolish the NREM sleep-promoting effect but not the REM sleep-promoting effect in response to semi-chronic stimulation of MCH neurons. However, the absence of VGAT in MCH neurons of (Tg*(Pmch-Cre): Vgat*$^{flox/flox}$ and Tg*(Pmch-Cre):MCH-R1*$^{-/-}$*:Vgat*$^{flox/flox}$) mice

seems to disinhibit REM sleep-promoting structures resulting in dramatically increased REM sleep, likely to a ceiling value, in control mice.

To further characterize the sleep promoting effect of stimulation of MCH neurons using a semi-chronic protocol in Tg*(Pmch-Cre)* mice, we combined stimulation with the administration of the MCH-R1-specific antagonist SNAP 7941. The purpose of employing pharmacology to block MCH signaling was to prevent of the possible occurrence of compensatory mechanisms that could occur in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice.

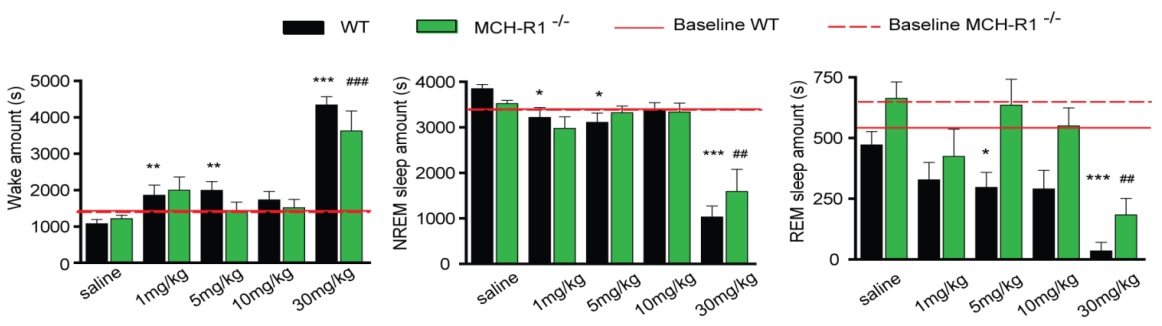

Figure 3-6. Distribution of Wake, NREM sleep and REM sleep in MCH-R1$^{-/-}$ mice and their age matched (10 weeks) control littermates following the administration of the MCH-R1 antagonist, SNAP 7941.

SNAP injection occurs at 1 PM and quantification started 20 min after the administration of the drug (saline, 1mg/kg, 5mg/kg, 10mg/kg and 30mg/kg of SNAP 7941) for a duration of 1 h 30 min.
The red lines represent the amount of wakefulness or sleep during the baseline condition corresponding to the period of the pharmacological testing for both mouse lines. The data are presented as mean ±SEM (n=6 per group). *P<0.05, **<0.01 and ***<0.001 compared to WT saline condition. $^{\#\#}$ P<0.01, $^{\#\#\#}$<0.001 compared to MCH-R1 $^{-/-}$ saline condition. Two way-ANOVA mixed design followed by Tukey post-hoc test.

First, we determined the dose of SNAP 7941 that minimally modified the sleep-wake cycle in order to isolate the effect optogenetic stimulation. 4 doses were tested: 1-5-10 and 30 mg/kg. 1 to 5 mg/kg of SNAP 7941 increased wakefulness (p=0.01 and p=0.01 respectively) and decreased both NREM (p<0.05 and p<0.05 respectively) and REM

sleep ($p<0.05$ and $p<0.05$ respectively) in intact MCH neurons of WT mice but had no effect on MCH-R1 $^{-/-}$ mice (1mg/kg Wake: $p=0.11$, NREM: $p=0.10$ and REM: $p=0.71$. 5 mg/kg Wake : $p=0.38$, NREM: $p=0.16$ and REM: $p=0.85$) demonstrating the specificity of the compound for blocking MCH-R1. The 10 mg/kg dose had no effect on the sleep-wake cycle in both WT and MCH-R1 $^{-/-}$ mice (Wake: $p= 0.08$, NREM: $p= 0.06$ and REM: $p=0.15$; Wake: $p= 0.08$, NREM: $p= 0.46$ and REM: $p=0.22$ Fig. 3-6), making it the ideal dose to use to study the effect of MCH peptide on the optogenetic semi-chronic activation of MCH neurons. Interestingly, the administration of 30 mg/kg of SNAP 7941 induced a non-specific effect since both WT and MCH-R1 $^{-/-}$ mice were significantly more awake (WT; Wake: $p<0.001$, NREM: $p<0.001$ and REM: $p<0.001$. MCH-R1$^{-/-}$; Wake: $p<0.001$, NREM: $p<0.01$ and REM: $p<0.01$) and while demonstrating visible signs of distress (shivering, isolation in their cage).

We found that administration of SNAP 7941 in association with semi-chronic activation of MCH neurons abolished the reduction of wakefulness ($p=0.26$ and $p=0.13$ for 1 and 10 mg/kg of SNAP 7941 respectively, Fig. 3-7 b) and the increase of NREM sleep observed using the lowest doses ($p=0.46$, $p=0.15$ for 1 and 10 mg/kg of SNAP 7941 respectively) but required a higher dose (10 mg/kg) of antagonist to attenuate the increase in REM sleep ($p= 0.22$, Fig. 3-7 b). However, considering the percentage of change of the state amount for each dose of SNAP 7941, there was no significant change in REM sleep amount induced by optogenetic stimulation as a function of SNAP 7941 concentration ($p=0.21$) whereas the effect on Wake and NREM sleep were both clearly abolished following SNAP 7941 application ($p<0.05$ and $p<0.01$).

Collectively, these results as well as those from Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice suggest that the MCH peptide regulates the NREM sleep-promoting effect whereas the regulation of REM sleep is under the control of other transmitters.

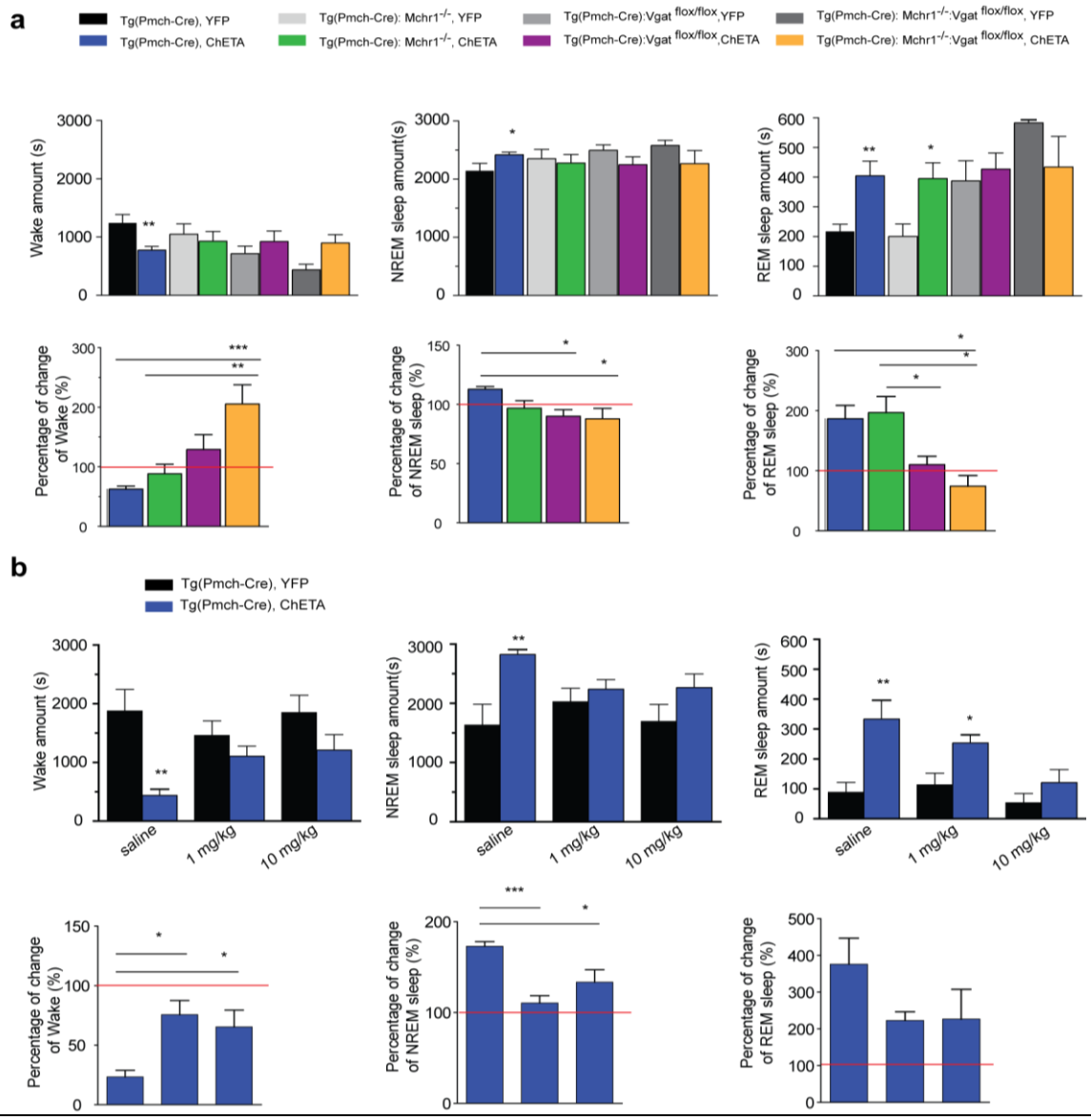

Figure 3-7: Increase of both NREM and REM sleep following semi-chronic stimulation requires functional MCH transmission.

a, Top: Mean duration (± s.e.m) of Wake, NREM and REM sleep during 1 h of optogenetic semi-chronic stimulation at 20 Hz (10 s/5 ms every 60 s) in Tg*(Pmch-Cre)* mice (n=7 control, n=8 ChETA ; triplicate), Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice (n=7 per group ; triplicate), Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ mice (n=6 control, n=7 ChETA; triplicate) and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ *: Vgat* $^{flox/flox}$ mice (n=2 control, n=4 ChETA ; triplicate). Bottom: Percentage of change of state amount compared to their respective control *P<0.05, **<0.01 and ***<0.001 Top: unpaired t-test within the same mouse line. Bottom: One-way ANOVA between subject designs. **b,** Top: Effect of MCH R1 antagonist SNAP 7941 on semi-chronic stimulation in Tg(Pmch-Cre) mice (n=7 per group ; once). Bottom: Percentage of change of state amount represented compared to the same dose of antagonist. *P<0.05, **<0.01 and ***<0.001. Top: 2 way-ANOVA mixed design followed by Tukey post-hoc test. Bottom: One-way ANOVA within subject designs.

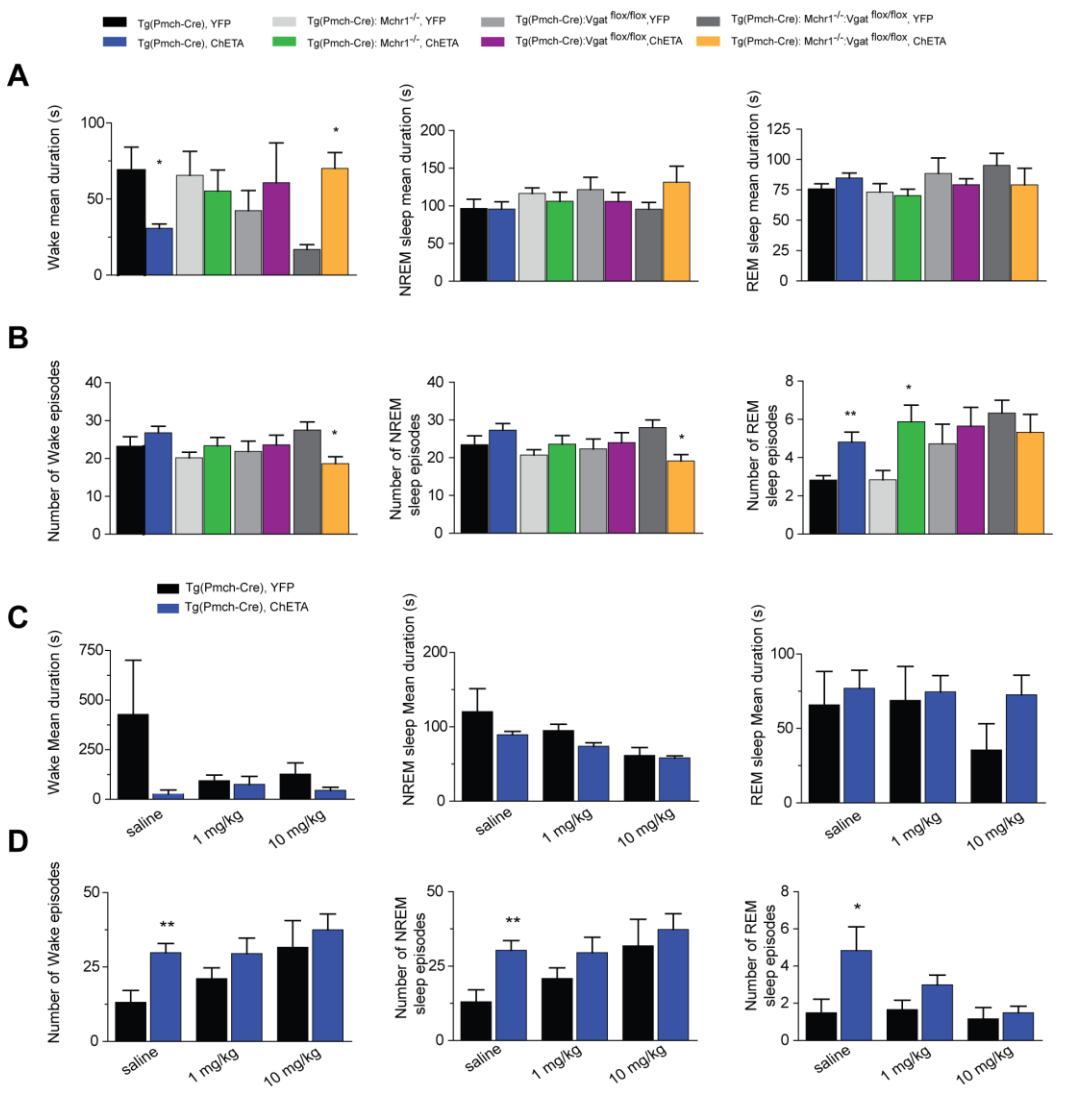

Figure 3-8: Mean duration and number of episodes of vigilance states during semi-chronic stimulation

A-B. Mean duration (A) and number of episodes (B) (± s.e.m) of Wake, NREM and REM sleep episodes during 1h of optogenetic semi-chronic stimulation at 20 Hz (10 s/5 ms every 60 s) illustrated in Fig. 3-7 of Tg*(Pmch-Cre)* mice (n=7 control, n=8 ChETA ; triplicate), Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice (n=7 per group ; triplicate), Tg*(Pmch-Cre):Vgat* $^{flox/flox}$ mice (n=6 control, n=7 ChETA; triplicate) and Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ *: Vgat* $^{flox/flox}$ mice (n=2 control, n=4 ChETA ; triplicate). *P<0.05, **<0.01, Unpaired t-test within the same mouse line. **C-D.** Mean duration (C) and number of episodes (D) (± s.e.m) of Wake, NREM and REM sleep episodes during semi-chronic stimulation in Tg*(Pmch-Cre)* mice (n=7 per group ; once) following application of MCH R1 antagonist SNAP 7941. *P<0.05, **<0.01, 2-way ANOVA mixed design followed by Tukey post-hoc test.

3-4 Discussion

In this study, we showed that acute activation of MCH neurons selectively increased NREM to REM sleep transitions as well as REM sleep duration whereas semi-chronic activation of the same neurons given at random with respect to sleep/wake state favors both NREM and REM sleep. On one hand, the effect of REM sleep-specific stimulation seems to result from the interaction between GABA and MCH peptide which modulates basal REM sleep mean duration. Indeed, the absence of both GABA and MCH dramatically increases REM sleep to a ceiling value in baseline and control condition. On the other hand, NREM sleep-specific stimulation consistently reduced NREM sleep mean duration, increased NREM to REM sleep transitions and showed a decrease of delta associated with an increase of theta. Interestingly, the modulation of the number of transitions from NREM to REM sleep, but not the NREM sleep duration, was controlled by MCH peptide and/or GABA from MCH cells under control conditions. However, optogenetic stimulation of MCH neurons during NREM sleep still generated an increased chance for a switch to REM sleep in the absence of MCH-R1 and GABA suggesting that MCH neurons release other neurotransmitters to mediate this effect. Although CART promotes arousal (Keating et al., 2010), Nesfatin-1, co-expressed in MCH neurons, is a REM sleep promoting factor (Jego et al., 2013; Vas et al., 2013) and might be released from MCH neurons during optogenetic stimulation to act on targets modulating REM sleep.

In addition, semi-chronic activation of MCH neurons revealed that MCH peptide likely mediates the NREM sleep promoting effect whereas the REM sleep promoting effect might involve additional neurotransmitters. Similar to REM sleep-specific stimulation, REM sleep amount during semi-chronic activation is high in control mice relative to animals with disrupted GABA transmission within MCH neurons, probably reaching a ceiling value. Although experiments using triple transgenic mice are not yet fully completed, these results have confirmed that the mode of MCH activation (acute vs. semi-chronic) has a different effect on sleep, probably due to the release of various neurotransmitters and their length of action.

3-4-1 Acute activation of MCH neurons in the literature: comparative analysis among studies

From our previous study, we know that MCH neuron activation during REM sleep was capable of prolonging the REM episode whereas activation during NREM sleep favors NREM-to-REM sleep transitions (Jego et al., 2013). In the present study we confirmed our previous results using a different Tg*(Pmch-Cre)* mouse line. Even though the transfection reported here was less efficient compared to our previous study (67% vs. 88%), the specificity of MCH neuron targeting was improved (98% vs. 89%), demonstrating that the consolidation of REM sleep was not due to non-specific transfection of non-MCH neurons in the previous study. The only difference we found between our two studies is the NREM sleep latency-to-REM sleep transition that is reduced in the present study but not in the previous one (Jego et al., 2013). This could be due to differences in genetic background (C57BL/6 vs. FVB/NJ) or the activation of different MCH subpopulations. Interestingly, in a recent study, Tsunematsu et al. used a chronic stimulation paradigm (10 Hz, 1 min light every 5 min for 20 h) to activate MCH neurons and quantify the number of times the animals entered to REM sleep when the stimulation occurred during NREM sleep or Wake. They found that MCH activation during NREM sleep, but not during wakefulness, favored transition to REM sleep. They also reported that during these stimulated NREM events, the EEG delta power was decreased and the EEG theta power was increased, which is consistent with a transition to REM sleep (Tsunematsu et al., 2014). In our present study, using a NREM sleep specific stimulation protocol in Tg*(Pmch-Cre)* mice , we replicated these results and showed that the delta is indeed reduced while the theta increased. Interestingly, they observed this effect during both the light and the dark phase (Tsunematsu et al., 2014), suggesting that activation of MCH neurons has a sleep promoting effect even against a strong waking drive. Collectively, these results are in good agreement with the response to NREM sleep specific stimulation in the present study and strengthen the idea that MCH neurons promote REM sleep under the acute stimulation conditions.

There are several points that need to be discussed regarding the difference between the studies outlined above. First, the latency of transition from NREM-to-REM sleep

reported in Tsunematsu is less than what we observed in our Tg*(Pmch-Cre)* mice. Indeed, they found that activation of MCH neurons during NREM sleep induced a transition to REM sleep in ~12 s after the light onset, compared to 140 s in the present study (Fig. 3-3-4), which was a very potent effect (Tsunematsu et al., 2014). One possible explanation is that we might not have targeted the entire population of MCH cells. Indeed, the expression rate among individual mice can differ since our technique is dependent upon inter-animal replicability of each virus injection; we reported here that 67% of MCH cells were transfected by the virus. In the Tsunematsu study, biogenic mice, which do not require viral injection, were used and might reduce inter-animal variation. Another possible explanation is related to the different stimulation protocols used in the present study and that of Tsunematsu. In our study, we randomly stimulated NREM or REM sleep epochs for an average of 10 stimulated events collected over 2 or 3 days to avoid over-stimulation or build up of neurotransmitters in between stimulated events. However, the Tsunematsu study used a chronic stimulation protocol (1 min of stimulation every 5 min over 20 h, 10 Hz) to activate MCH cells, treating each stimulated epoch as an acute stimulation to calculate the latency to REM sleep. A problem with this approach is that we cannot rule out the possibility of peptide accumulation in the extracellular space that could differentially modulate the targets of MCH stimulation. Indeed, it has been consistently reported that higher frequencies and/or more prolonged stimulation are required to release peptides as opposed to fast amino-acid transmitters (Hawes et al., 2000). However, under acute stimulation conditions, it is likely that the stimulation is not prolonged enough to activate a significant peptide release (through peptide-activated G protein coupled receptors).

Collectively, it is therefore possible that the shorter latency for transition from NREM to REM sleep might be due to an additional action from other peptides co-released from MCH cells. Accordingly, in the absence of functional MCH transmission, sleep specific acute stimulations still promote REM sleep, suggesting that short stimulation of MCH neurons does not release MCH (or not enough MCH to cause an effect) and that the effect is mediated by another neurotransmitter, most likely the fast amino acid transmitter GABA. Several groups have shown that MCH neurons co-express GAD67 or

65 (Harthoorn et al., 2005; Meister, 2007; Elias et al., 2008; Sapin et al., 2010; Jego et al., 2013) and we previously showed that GABA was released in TMN cells (Jego et al., 2013). Surprisingly Tg*(Pmch-Cre): Vgat* $^{flox/flox}$ mice have the same response to sleep-specific stimulation as Tg*(Pmch-Cre)* mice whereas our preliminary results using triple transgenic Tg*(Pmch-Cre): MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice indicate that REM sleep mean duration is up-regulated in the control condition making it nearly impossible to generate more REM sleep. The existence of a ceiling value for REM sleep mean duration suggests that REM sleep amount is tightly regulated and that intact MCH neurons are necessary to maintain this balance. On the other hand, NREM sleep specific stimulation showed that neurotransmitters other than MCH peptide and GABA are involved in the increase in the number of transitions from NREM to REM sleep since the amplitude of the response across the different mouse lines was unchanged. For instance, Nesfatin-1, a peptide which is present in all MCH cells, has been shown to promote REM sleep specifically (Jego et al., 2012; Vas et al., 2013).

Recently, two studies using constitutive VGAT-EYFP mice revealed that MCH neurons don't express VGAT (Chee et al., 2015; Jennings et al., 2015). Considering this data our results should be discussed carefully although *in situ* hybridization is required to provide a definitive confirmation of these results. Indeed, a study looking at the expression of *Vgat, Vglut2, Gad 67* and *Gad 65* mRNA in hypothalamic POMC neurons reveals that transgene expression may not always faithfully reflect native gene expression. Accordingly, the results of these studies indicate that some transgenic markers are detectable only in a fraction of cells identified using *in situ* hybridization techniques (Jarvie and Hentges, 2012). Conversely, transgenes can be ectopically expressed and genes may have a pattern of expression during development that differs from that observed in the adult. Indeed, GABA plays a critical role in brain ontogeny and the expression of critical components of the GABA system (including GABA, GAD 65, GAD 67, GAT 1-3, and VGAT), present at early developmental stages, changes dynamically during maturation (Dupuy and Houser, 1996; Yan et al., 1997; Minelli et al., 2003; Vitellaro-Zuccarello et al., 2003; Boulland and Chaudhry, 2012). This is of particular importance because one molecule of Cre would allow the expression of the fluorescent

marker. Therefore, neurons which express VGAT during development but not in their mature state would still express the fluorescent marker. Interestingly, the study of Jarvie ((Jarvie and Hentges, 2012)) showed that POMC neurons, similarly to MCH cells, highly colocalize with *Gad 65* and *Gad67* mRNA but not *Vgat*. For this reason, VGAT *in situ* hybridization is required in order to quantify accurately the expression of VGAT in MCH cells. We are currently working on VGAT and VGLUT2 *in situ* hybridization on Tg*(Pmch-Cre) and* Tg*(Pmch-Cre): MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice to confirm these results.

3-4-2 Chronic activation of MCH neurons

Several studies have looked at chronic activation of MCH cells and the resulting effect on sleep by administrating pharmacological compounds (MCH peptide or MCH-R1 antagonist) (Verret et al., 2003; Ahnaou et al., 2011) or, more recently, by using optogenetic techniques (Tsunematsu et al., 2011; Konadhode et al., 2013).

Here, we report that semi-chronic stimulation over one hour increased NREM and REM sleep in both Tg*(Pmch-Cre)* mouse lines, suggesting that the mode of activation of MCH neurons changed either the content of the neurotransmitter released and/or the structures activated. As mentioned earlier, the semi-chronic protocol was designed to favor the release of peptides (including MCH) over the amino-acid transmitter (likely GABA). However, the proportion of peptides and fast amino-acid transmitters released under these conditions is largely unknown and it is unclear whether they are released from the same axon terminals. Interestingly, Tg*(Pmch-Cre): MCH-R1*$^{-/-}$ mice subject to semi-chronic activation of MCH cells showed no increase in NREM sleep amount although REM sleep quantities remained high. Accordingly, a low dose of selective MCH-R1 antagonist administrated in Tg*(Pmch-Cre)* mice before semi-chronic activation of MCH neurons blocked the increase in NREM sleep whereas the REM sleep-promoting effect induced by optogenetic stimulation was not significantly affected by the administration of MCH-R1 antagonist. In light of these results, it is clear that NREM sleep promotion during semi-chronic activation of MCH neurons is mediated by MCH-R1, however the REM sleep-promoting effect is independent of MCH-R1 activation. It is

possible that semi-chronic activation favors the build-up of MCH and other long-acting peptides. We propose that MCH neurons would release different neurotransmitters at targets that modulate the sleep-wake cycle differently. Indeed, a subpopulation of MCH neurons could project and release their content in a structure regulating REM sleep such as the medial septum whereas others can project to structures regulating both NREM and REM sleep or arousal centers. Stimulation of such targets during a semi-chronic stimulation protocol would allow for the identification of subpopulations.

Similar to what was described for REM sleep-specific stimulation, the absence of GABA in MCH neurons raised the amount of REM sleep to what appears to be a ceiling value. Together these results demonstrate that MCH peptide promotes NREM sleep whereas GABA and others modulate the amount of REM sleep, probably by inhibiting directly or indirectly REM sleep promoting centers.

3-4-3 GABA release from MCH neurons

We previously demonstrated that MCH neuron terminals release GABA and exert strong inhibitory actions on wake-promoting histaminergic cells located in the TMN following optogenetic stimulation (Jego et al., 2013). In addition, we demonstrated here that the absence of VGAT in MCH neurons in our Tg*(Pmch-Cre): MCH-R1*$^{-/-}$*:Vgat* $^{flox/flox}$ mice alters the basal REM sleep amount by increasing the REM sleep mean duration to a ceiling value and by increasing the number of transitions from NREM to REM sleep with acute optogenetic stimulation. However, recent studies suggesting the absence of VGAT in MCH cells questioned the ability of MCH neurons to release GABA. If MCH neurons do not use VGAT to release GABA, how could GABA be released?

First, there is the possibility that IPSCs seen following optical stimulations are due to polysynaptic transmission. Similar to what is observed in neighboring histaminergic cells (Williams et al., 2014), Chee et al. commented that MCH neurons can synthesize GABA but might lack the machinery to package it for synaptic release. They then showed that optogenetic stimulation of MCH inputs evoked GABA release in the LS although GABA

is not directly released by MCH terminals. Rather, it results from the activation of glutamatergic MCH projections on an unknown source of GABA innervating the LS (Chee et al., 2015). However, our previous study showing that optogenetic activation of MCH fibers in the TMN induced IPSCs with a short latency (2-3 ms) that were blocked by $GABA_A$ receptor antagonist Bicuculline application suggests a GABAergic monosynaptic response (Jego et al., 2013). These findings along with those of the Chee paper (Jego et al., 2013; Chee et al., 2015) suggest the possibility that MCH neurons release both glutamate and GABA, likely in a region-specific manner.

Another possibility is that GABA release from MCH neurons is independent of VGAT. Intriguing results from Sabatini's group showed that dopaminergic neurons originating from the substantia nigra inhibit striatal neurons through GABA release but in a manner that is independent of VGAT. GABA release instead required functional activity of VMAT2, the vesicular transporter for dopamine (Tritsch et al., 2012). The same group also showed that dopamine neurons projecting to the striatum activated $GABA_A$ receptors despite an absence of detectable GABA synthetic enzymes GAD65 and GAD67 in dopamine cells. Instead, these cells used the membrane GABA transporters mGAT1 (Slc6a1) and mGAT4 (Slc6a11) that are known to rely on GABA uptake from the extracellular space as opposed to the direct synthesis of GABA by dopamine neurons themselves (Tritsch et al., 2014). Future studies might reveal the existence of other atypical mechanisms of GABA release in other neuron types, including MCH cells.

3-4-4 Existence of MCH neuron subpopulations

Using chronic optogenetic activation of MCH neurons, we confirmed a previous pharmacological (Verret et al., 2003) and optogenetic (Konadhode et al., 2013) study showing an increase of REM as well as NREM sleep. However, our stimulations occurred during the second half of the light phase while the recordings of Verret and Konadhode started at the beginning of the light onset. Interestingly, Konadhode *et al* reported that the same protocol of stimulation during the day did not have any effect on sleep (Konadhode et al., 2013), whereas recent work from Tsunematsu showed that 3 h

of 10 Hz stimulation (from 10 AM to 1 PM) increased REM sleep quantities and decreased NREM sleep over the 3 h period (Tsunematsu et al., 2014). In addition, the Konadhode study reported that delta power during NREM sleep was increased, although it is worth mentioning that this increase occurred during the light phase and not during the dark phase when the promotion of sleep is observed. However, theta power during REM sleep remained unchanged. On the other hand, Tsunematsu reported an increase of delta and theta power when MCH neurons were chronically activated (for 8-9 h) during the light phase (Tsunematsu et al., 2014). To clarify this issue further power spectrum analysis is required during semi-chronic activation of MCH cells, especially during NREM sleep.

The difference in the amount of non-REM and REM sleep reported between our present study and those from Konadhode and Tsunematsu might be related to activation of a specific population of MCH neurons. MCH neurons are located densely in the zona incerta (ZI), in the lateral portions of the lateral hypothalamus, along the perifornical area, and ventrally along the dorsal ridge of the ventromedial hypothalamus. In the Konadhode study, transfected MCH neurons were mainly found in the ZI, and less so in the lateral divisions. The other two studies, along with the present study, infected on average about 80% of the MCH neurons located throughout the lateral hypothalamus and the ZI (Jego et al., 2013; Tsunematsu et al., 2014). It is very likely that several subsets of MCH cells exist and might differentially modulate the sleep wake cycle. One can imagine that MCH neurons located in the ZI promote NREM and REM sleep whereas lateral hypothalamic MCH neurons may participate in the regulation of REM sleep only. Retrograde tracing in different sleep structures would help to determine if subpopulations exist.

Interestingly, based on the neurochemical identity of MCH neurons, it has been demonstrated that MCH neurons containing CART send ascending projections to the septum whereas non-CART MCH neurons send descending projections to the lower brainstem and the spinal cord, particularly to the LC and DR (Cvetkovic et al., 2004; Hanriot et al., 2007; Yoon and Lee, 2013). In addition, MCH neurons that were c-Fos activated during the recovery period following 72h REM sleep deprivation were shown

to be part of both subpopulations (Hanriot et al., 2007). This study is a good demonstration of the high heterogeneity existing among MCH positive cells based on neurochemical identity (CART, Nesfatin-1, MCH, GABA, etc.), their localization in the hypothalamic area (ZI vs. LH) and their projection to different targets (ascending vs. descending).

Nevertheless, the optogenetic studies completed on MCH cells, along with pharmacological reports underscore the importance of MCH neurons in both NREM and REM sleep. A better characterization of MCH subpopulations in terms of their pattern of projections with regards to their neurochemical distinction across the posterior hypothalamic area, as well as further study into the role of GABA and glutamate in MCH cells and their action on their respective targets, will help to better understand the influence of MCH neurons on sleep.

Chapter 4

Pharmacogenetic excitation of MCH cells across the sleep-wake cycle

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4-1 Introduction

MCH neurons densely innervate brain areas involved in the regulation of the sleep-wake cycle (Bittencourt et al., 1992; McGregor et al., 2005; Elias et al., 2008; Torterolo et al., 2009) and numerous studies point to a sleep promoting effect of MCH and MCH cells (Verret et al., 2003; Lagos et al., 2009; Lagos et al., 2012; Benedetto et al., 2013; Jego et al., 2013; Konadhode et al., 2013; Tsunematsu et al., 2014). However, depending on the technique used (pharmacology, transgenic animals or optogenetics) and the pattern of the stimulation (acute vs. chronic, constitutive vs. transient), activation of MCH cells can lead to different results. We showed in chapter 2 (Fig. 2-7 and 2-9) that MCH neurons consolidate REM sleep when activated acutely during specific sleep states. However, we found that the activation of MCH neurons for 1 h using a semi-chronic protocol increased both NREM and REM sleep (Chapter 3, Fig. 3-7) suggesting that the mode of activation of MCH cells could lead to differential effects on sleep. In addition, previous reports suggest that the activation of MCH cells at different timepoints during the sleep-wake cycle can affect sleep differently (Verret et al., 2003; Jego et al., 2013; Konadhode et al., 2013; Tsunematsu et al., 2014). However, these studies have been conducted under differing experimental parameters (genetic background of mice, virus transfection, protocol of stimulation), which make the comparison of the effect on sleep difficult.

One common problem with optogenetic tools is that the activation of the population of interest depends on opsin targeting in addition to the delivery of sufficient levels of light into the brain tissue. Given the diffuse distribution of MCH neurons, it is therefore challenging to evaluate what proportion of MCH cells are activated by light in a given experiment. However, Designer Receptors Exclusively Activated by Designer Drugs (DREADD), a pharmacogenetic tool, can target a much broader brain area. Indeed, this method utilizes extrinsic muscarinic receptors ($hM3D_q$ for excitation), that have lost their affinity for endogenous Acetylcholine but can still be activated by a synthetic and pharmacologically inert ligand, clozapine-N-oxide (CNO). Since CNO is capable of

penetrating brain tissue better than light, the proportion of activated MCH cells should be limited only by the efficiency of the virus targeting.

In order to clarify the contribution of substained activation of MCH neurons to REM and/or NREM sleep regulation across the sleep-wake cycle, we studied the effect on sleep of excitatory pharmacogenetic manipulation of MCH cells in Tg*(Pmch-Cre)* mice. The advantage of this study is that the same virus-transfected mice will receive CNO administration at different time points allowing a direct comparison of the effect of MCH activation on sleep during the dark and the light period.

4-2 Methods

4-2-1 Plasmid and viral targeting

To chronically stimulate MCH neurons, we took advantage of the DREADD technique. This method utilizes extrinsic muscarinic receptors, ($hM3D_q$ for excitation), that have lost their affinity for endogenous Acetylcholine but can still be activated by a synthetic and pharmacologically inert ligand, clozapine-N-oxide (CNO). A Cre-dependent version of the hM3Dq receptor was packaged into an AAV virus for injection into the LH of Tg(*Pmch-Cre*) mice. Mice were anesthetized with Isoflurane (5% induction, 1% maintenance) and then head-fixed in a small animal digital stereotaxic frame. 0.8 µl of recombinant AAV2 carrying the hSyn-DIO-hM3D(Gq)-mCherry vector were bilaterally injected into the lateral hypothalamus (anteroposterior (AP), -1.50 mm; mediolateral (ML), ± 0.9 mm; dorsoventral (DV) 5.35 mm) at a rate of 50 nl/min. To ensure that CNO administration did not have any non-specific effect on the sleep-wake cycle, we injected 6 Tg*(Pmch-Cre)* with AAV2-Ef1α-DIO-eYFP and administrated the CNO at the same time as in the hM3D-mCherry transfected mice.

4-2-2 Surgery

9-week old Tg(*Pmch-Cre*) mice were chronically implanted with a custom-made-EEG/EMG implant, as described previously in section 2-2-8.

4-2-3 CNO Administration

IP injection of 1 mg/kg, 0.2mg/kg of CNO (0.1ml/10g of body weight) or saline was completed at different times of day (8 AM, 12 PM, 4 PM, 8 PM, 12 AM) and the sleep-wake cycle was monitored for the six hour post-CNO injection period (light onset: 8 AM). EEG and EMG signals were acquired and analysed as reported in chapter 2 (see section 2-2-9). The same mice received CNO at several time points with at least 6 days between successive CNO administrations. For a given time point, animals were allowed at least one day free of drug administration (each animal received drugs at 2 to 4 time points) before receiving another dose of CNO. The different doses of CNO were given in a random order.

To test whether the effect on sleep induced by chronic activation of MCH neurons is due to MCH peptide, we coupled pharmacogenetic activation of MCH neurons with the administration of SNAP 7941 (MCH-R1 receptor antagonist, 10 mg/kg, 0.1ml/10g of body weight). CNO was administrated at noon and SNAP 7941 was administrated at 2:30 PM. Sleep was quantified for a period of 1 h, 20 min following the administration of SNAP 7941 or saline.

4-2-4 Statistical analysis

Statistical analysis of the effect of CNO on each state was determined using repeated measures ANOVA. Significant effects were investigated using pairwise multiple comparisons with Tukey post hoc test or paired t-tests. Data are presented as mean ± SEM. All data were analyzed using Prism 5.0 (GraphPad Software as described in the

text. Data were exported into Adobe Illustrator CS3 (Adobe Systems) for preparation of figures.

4-3 Results

4-3-1 MCH increases REM sleep specifically during the day

For the 8 AM injection time we found that 1mg/kg of CNO significantly increased REM sleep ($p<0.05$) compared to saline within the 6h following administration without affecting NREM sleep or Wakefulness ($p=0.72$, $p=0.88$ Fig. 4-3 a). A smaller dose showed a moderate increase of REM sleep compare to saline but this was not significant ($p=0.07$, Fig. 4-3 a). No changes in the mean duration or the number of epochs mice spent in each state were found. However, administration of 1mg/kg of CNO at 12 PM dramatically increased REM sleep ($p<0.001$) without affecting NREM sleep or Wakefulness ($p=0.16$, $p=0.54$, respectively, Fig. 4-1 a). The REM sleep-promoting effect lasted for at least 5h (Fig. 4-1 b) and was due to both an increase in the duration of REM sleep episodes ($p<0.05$) and the number of REM episodes ($p<0.01$, Fig. 4-1 c-d). Note that administration of 1 mg/kg of CNO in control animals (EYFP) had no effect on Wake ($p=0.69$), NREM ($p=0.45$) and REM sleep ($p=0.41$), demonstrating that in the absence of the G protein–coupled muscarinic receptor, hM3D (data not shown), the CNO drug had no effect on the sleep-wake cycle.

We next investigated the role of MCH peptide in the promotion of REM sleep at the same time point by combining CNO administration with administration of MCH-R1 antagonist SNAP 7941. The application of SNAP 7941 following CNO administration reduced the REM sleep-promoting effect induced by CNO ($p < 0.05$; CNO-saline vs. CNO-SNAP, Fig. 4-1 e) but did not prevent it ($p < 0.05$; CNO-SNAP; saline-SNAP, Fig. 4-1 e). NREM sleep and Wake states were not affected by the pharmacological treatments ($p = 0.39$ and $p = 0.83$). Note that SNAP 7941 administration by itself reduced REM sleep quantities ($p< 0.01$) whereas CNO administration increased REM sleep ($p< 0.001$) compared to the saline condition.

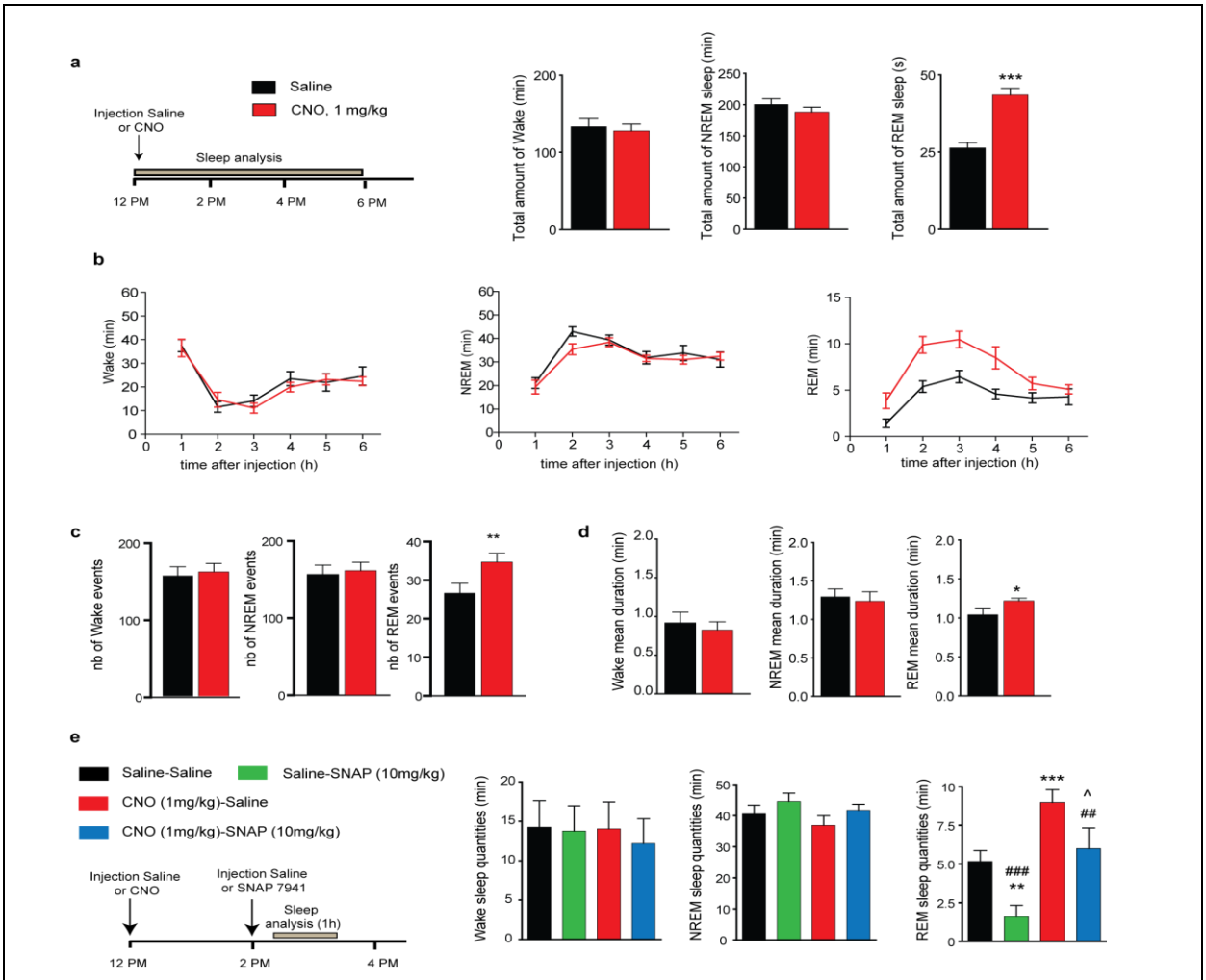

Figure 4-1: Effect of pharmacogenetic activation of MCH neurons during the second half of the day

a. Wake, NREM and REM sleep quantities 6h after CNO or saline injections (injection at 12PM) in Tg*(Pmch-Cre)* mice transfected with hM3D-mCherry (n=11 per group). **b.** Time course of sleep-Wake quantites during the 6h following the injections in MCH Cre mice transfected with hM3D-mCherry. **c-d.** Number of state events (c) and state mean duration (d) during the 6h following the injection. **e.** Wake, NREM and REM sleep quantities on Tg*(Pmch-Cre)* mice transfected with hM3D-mCherry during 1h following saline or SNAP 7941 treatment with a pre-treatment with CNO or Saline (n=6 per group). Paired t test or 1-way ANOVA within subject design followed by Tukey post-hoc test. Compared to saline-saline ** p< 0.01, *** p< 0.001, compared to CNO-saline ## p< 0.01, ### p< 0.001, compared to saline-SNAP ^ p< 0.05.

These results suggest that MCH peptide is involved in the promotion of REM sleep but is not the only mediator of this effect.

To further characterize the role of MCH peptide in REM sleep promotion, we administrated CNO to Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice and found that CNO administration increased REM sleep duration compared to saline (p< 0.05, Fig. 4-2 a-b).

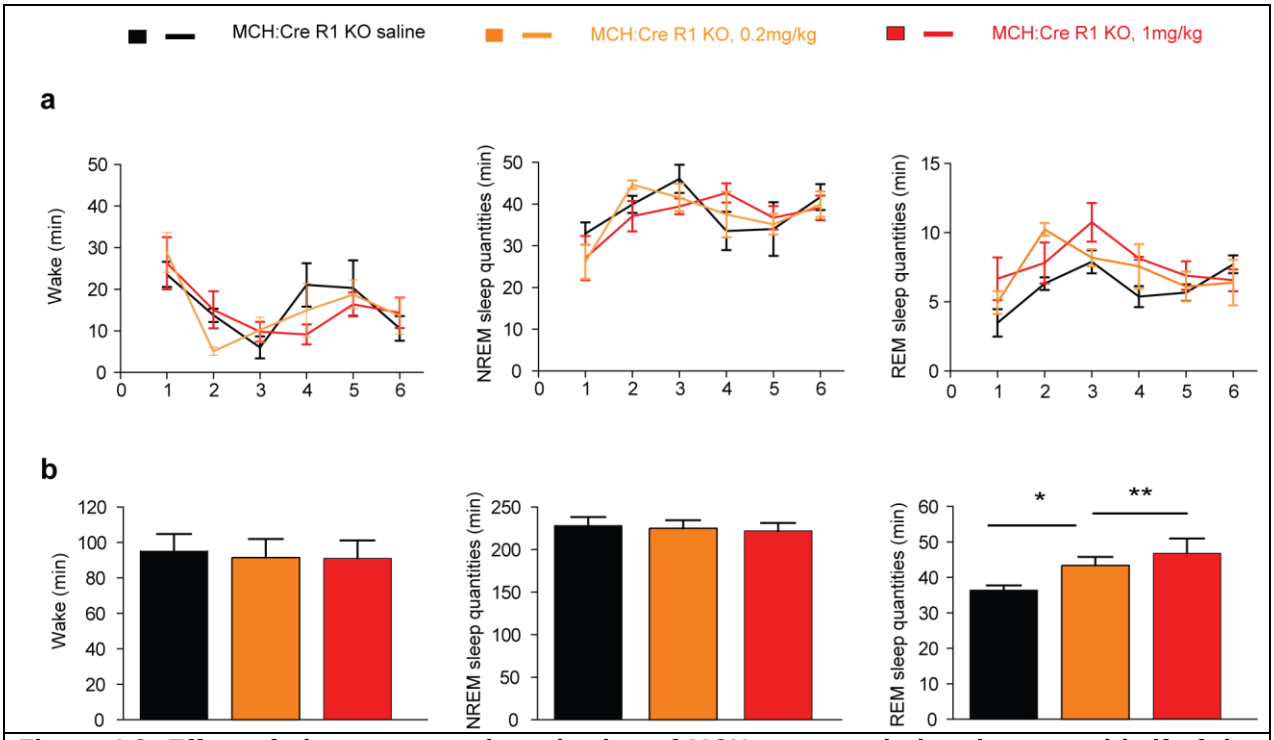

Figure 4-2: Effect of pharmacogenetic activation of MCH neurons during the second half of the day in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice.
a. Time course of sleep-Wake quantities during the 6h following injections in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice transfected with hM3D-mCherry. **b.** Wake, NREM and REM sleep quantities 6h after CNO or saline injections (injection at 12PM) in Tg(Pmch-Cre):MCH-R1$^{-/-}$ mice transfected with hM3D-mCherry (n=4 per group). One way ANOVA, repeated measured followed by Tukey pots-hoc test * p< 0.05, ** p< 0.01.

Interestingly, the increase in REM sleep in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice was less potent than in Tg(*Pmch-Cre*) mice (64% increased in KO (Fig. 4-7 e) vs. 82.7% in Tg(*Pmch-Cre)*(Fig. 4-7 a)*),* however REM sleep quantities in control condition in Tg*(Pmch-Cre):MCH-R1*$^{-/-}$ mice were significantly higher (p< 0.01) compared to Tg(*Pmch-Cre)* but not after treatment with CNO (p=0.46). These results confirmed our previous results obtained with Tg(*Pmch-Cre)* mice, suggesting that MCH peptide is not the only modulator involved in REM sleep promotion.

When administrated at 4PM, the CNO has no effect on Wake, NREM or REM sleep quantities (p=0.35, p=0.15, p= 0.33; Fig. 4-3 b).

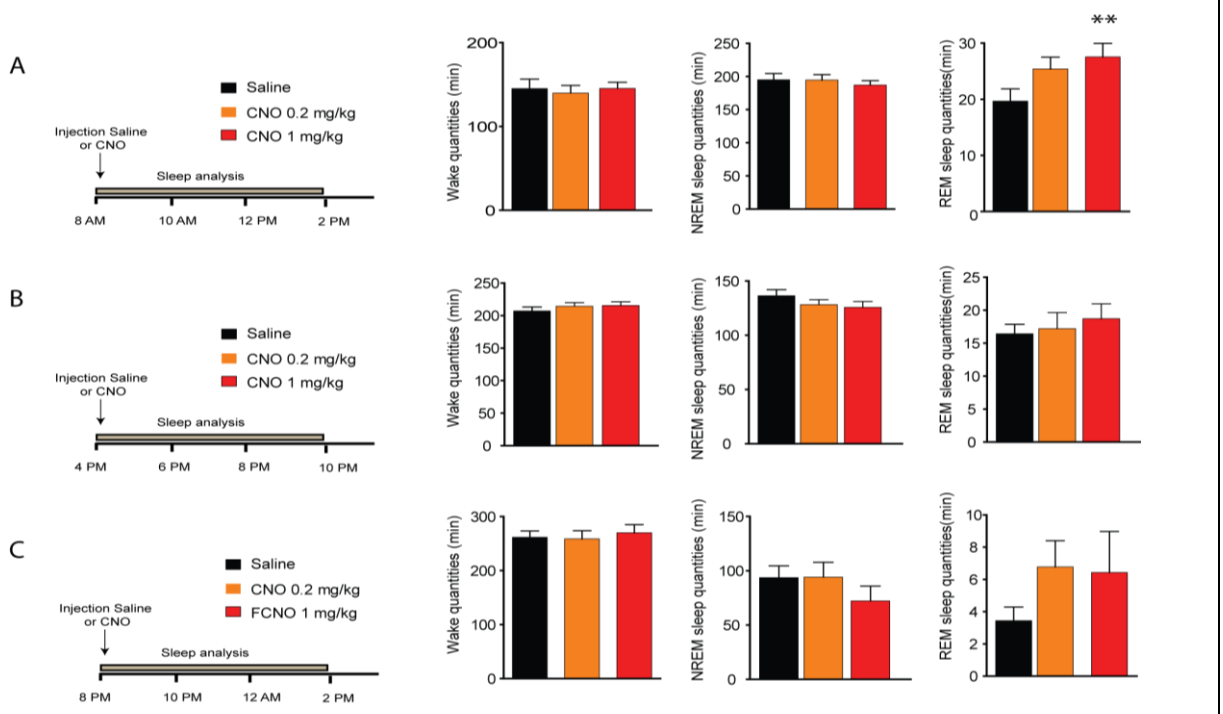

Figure 4-3: Effect of pharmacogenetic activation of MCH neurons at different circadian timepoints.
a, Wake, NREM and REM sleep quantities 6h after CNO or saline injections (injection at 8 AM) in Tg(Pmch-Cre) animals transfected with hM3D-mCherry (n=11 per group). **b,** Wake, NREM and REM sleep quantities 6h after CNO or saline injections (injection at 4 PM, n=11 per group).**c,** Wake, NREM and REM sleep quantities 6h after CNO or saline injections (injection at 8 PM, n=13 per group). One-way ANOVA within subject design followed by Tukey Post-hoc test * p< 0.05.

4-3-2 MCH has an arousal promoting effect during the night

In contrast to previous studies in which MCH neuron activation at the beginning of the dark phase was found to be sleep-promoting using pharmacological, pharmacogenetic or optogenetic approaches (Verret et al., 2003; Konadhode et al., 2013; Tsunematsu et al., 2014), our study did not reveal any hypnogenic effect. Administration of CNO at 8 PM had no effect on either state quantities (Wake, p=0.50; NREM, p=0.37; REM, p=0.30, Fig. 4-3 c) or sleep architecture.

Surprisingly, CNO administration at 12 AM increased wakefulness for at least 6 h ($p<0.05$, Fig. 4-4 a) by prolonging wake events ($p<0.05$, Fig. 4-4 d). The arousal effect was associated with a decrease in NREM sleep quantities ($p<0.05$) for at least 6 h (Fig. 4-4 b) whereas the quantity of REM sleep was decreased only during the first 3 hours following the CNO administration ($p<0.05$).

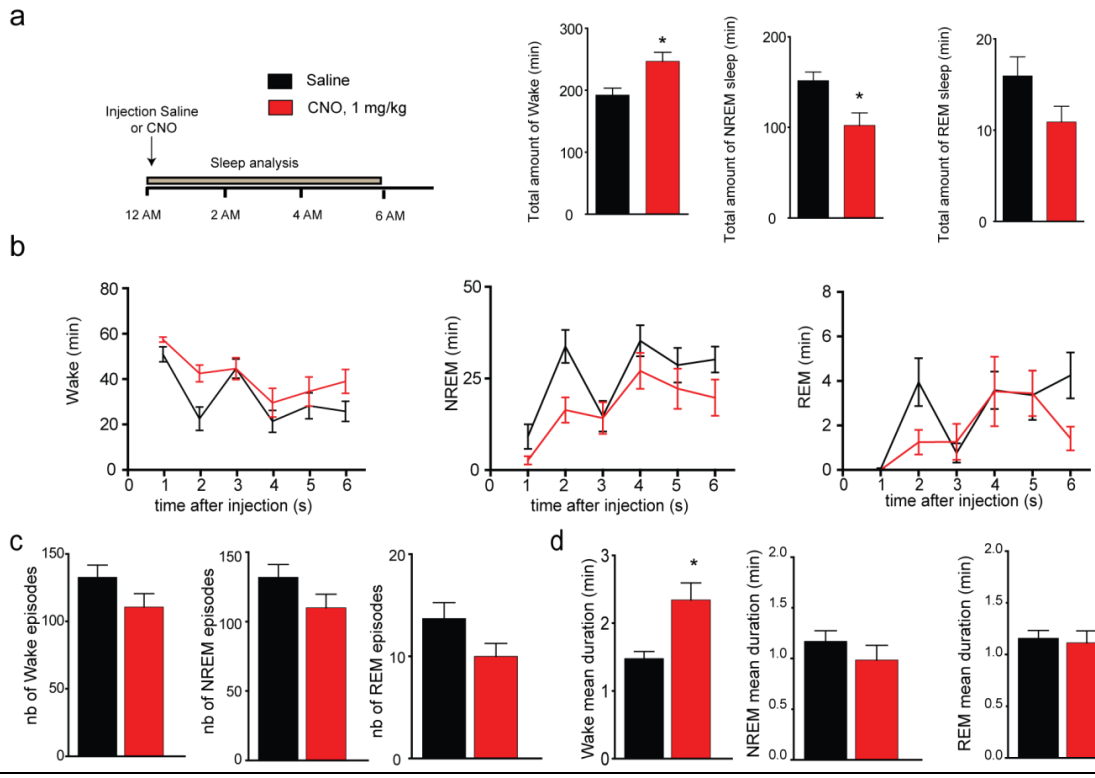

Figure 4-4: Effect of pharmacogenetic activation of MCH neurons during the second half of the night

a. Wake, NREM and REM sleep quantities 6 h after CNO or saline injections (injection at 12 AM) in Tg*(Pmch-Cre)* animals transfected with hM3D-mCherry (n=11 per group). **b.** Time course of sleep-Wake quantities during the 6 h following injections in Tg(Pmch-Cre) animals transfected with hM3D-mCherry. **c-d.** Number of state events (c) and state mean duration (d) during the 6 h following the injection. Paired t-test. * $p< 0.05$.

4-4 Discussion

In the present study, we showed that pharmacogenetic activation of MCH neurons promotes REM sleep, but not NREM sleep during the light phase as was previously reported (Verret et al., 2003; Konadhode et al., 2013; Tsunematsu et al., 2014). This suggests the existence of MCH neuron subpopulations. In contrast, the activation of the same MCH neurons during the dark phase induces arousal suggesting a circadian regulation of MCH neurons.

Results described in Chapter 2 and 3 were obtained when optogenetic stimulation of MCH neurons occurred during the second part of the afternoon. We initially chose this time of the day because we believed that activation of MCH cells during the dark phase would not be sufficient to overcome the powerful drive for arousal occurring during that period. On the other hand, during the first part of the light phase, the sleep pressure is very high and MCH-R1 could already be saturated. In addition, we and others showed that during the second phase of the light period, MCH activation increases REM sleep, and to a lesser extent NREM sleep (Jego et al., 2013; Tsunematsu et al., 2014). However, using different pharmacological or optogenetic stimulation, others found that MCH neuron activation had a sleep promoting effect when it occurred at the beginning of the dark phase (Verret et al., 2003; Konadhode et al., 2013), but not during the light phase (Konadhode et al., 2013). These results raised the questions of both the temporal and spatial resolution of the stimulation paradigms, as discussed previously (Jego and Adamantidis, 2013).

4-4-1 Circadian role of MCH peptide on sleep

Using pharmacogenetic activation, we were able to replicate our results since administration of CNO at light onset (8 AM) or at 12 PM significantly increased REM sleep for at least 5 hours without affecting Wakefulness or NREM sleep (Fig. 4-1 a-c). In addition, we showed that the REM sleep promoting effect was partially mediated by MCH peptide (Fig. 4-1 e and Fig. 4-2). Furthermore, we found that the effect on REM sleep was more pronounced when the injection was made at 12 PM rather than 8 AM,

probably due to MCH-R1 de-sensitization. Consistent with a sleep promoting role, it has been shown that CSF levels of MCH in rats were higher during the light phase compared to the dark phase (Pelluru et al., 2013). It was argued by the authors of this study that the absence of a sleep promoting effect during the day reported by Konadhode was due to a "ceiling effect" because it was impossible to generate more sleep during the resting period (Konadhode et al., 2014). Our results contradict this hypothesis since MCH neuron activation was still able to increase REM sleep even though the CSF level of MCH was supposedly high. A possible explanation is that the MCH system is "prepared" for sleep consolidation during the light phase, while the arousal promoting monoaminergic systems from the brainstem are inhibited and the sleep centers disinhibited which overall may improve MCH signalling efficiency.

4-4-2 The existence of possible MCH subpopulations

As discussed in section 3-4-4, it is likely that functionally distinct subpopulations of MCH neurons do exist. In our study, we found that the virus diffusion of hM3D (Gq)-mCherry was limited to the LH injection site. It seemed that serotype 2 of hM3D(Gq)-mCherry did not diffuse as well as serotype 8 or 9 (personal communication, Jimmy Fraigne); consequently, activated MCH cells in the present study would not be expected to be the same cells that Konadhode were stimulating since they were mostly located in the ZI and rarely in the LH (Konadhode et al., 2013). Hence, one can imagine that MCH cells from the ZI would be responsible for NREM and REM sleep (or NREM sleep only) induction whereas LH-MCH neurons would regulate more specifically REM sleep. Since we only targeted cells in the LH but not in the ZI, this would explain why we did not see any effect on NREM sleep in the present study (Fig. 3-6).

4-4-3 Arousal effects of MCH neurons

Interestingly, pharmacogenetic activation of MCH cells at 4 PM and 8 PM did not modify the quantity of sleep, suggesting that the action of MCH on sleep is under circadian control as previously suggested (Abrahamson et al., 2001). Unexpectedly, activation of

MCH at midnight induced a strong arousal effect. These results are surprising because MCH neurons have been consistently reported as sleep promoting even during the night (Verret et al., 2003; Konadhode et al., 2013; Tsunematsu et al., 2014). However, the arousal-promoting effect observed following CNO administration is strong and the same animals showed a strong REM sleep-promoting effect following CNO administration at noon. Therefore, these results demonstrate that activation of a subpopulation of MCH neurons located in the LH can have opposite effects depending on circadian time. As mentioned in the introduction (section 1-5, 1-6 and 1-7), MCH cells are not only implicated in sleep modulation but also in many other physiological functions such as feeding, energy homeostasis, anxiety/depression-like behaviors and reward. A longstanding challenge has been to reconcile the orexinergic effect of MCH cells with the sleep-promoting effect they are also shown to induce. As suggested for the orexins (Sutcliffe and de Lecea, 2002), MCH might not be a signal for sleep or feeding *per se*, but provide a means by which metabolic and other homeostatic needs can affect sleep states. Then, MCH neurons will respond accordingly to these signals to modulate sleep states and arousal (Sutcliffe and de Lecea, 2002). It is therefore possible that the MCH subpopulation we targeted with hM3D(Gq)-mCherry is responding to circadian and homeostatic factors providing a signal to sleep and decrease energy expenditure during the light phase but to eat and to increase arousal during the dark phase. This would explain why MCH induces sleep during the day and arousal during the night.

Another related possibility could be that activity-dependent neurotransmitter "re-specification" occurs within MCH cells. This phenomenon involve either the induction or elimination of excitatory or inhibitory transmitters or their switch (Spitzer, 2012). The capacity for multidirectional change enables neurotransmitter re-specification to exert a homeostatic effect on levels of activity within neuronal circuits. It is not yet clear which neurons undergo neurotransmitter re-specification, but it seems that not all neurons exhibit these changes (Spitzer, 2012). On one hand, sensory neurons or motoneurons must retain reliable input; consequently it seems unlikely that changes in transmitters would occur. On the other hand, hypothalamic neurons are well known to modulate various physiological functions in response to homeostatic stimuli raising the possibility

that neurotransmitter respecification may actually occur. For instance, neurotransmitter respecification occurs in the mature nervous system of rats; electrically or convulsant-induced seizures promote the expression of the GABAergic phenotype in glutamatergic pyramidal hippocampal neurons counteracting the imposed perturbation in the circuit activity (Gomez-Lira et al., 2005) in order to maintain homeostasis of nervous system excitability.

Another example of an adaptation of a system to homeostatic demands is the change of the response of Hcrt neurons to noradrenaline (NA) after sleep deprivation (Grivel et al., 2005; Uschakov et al., 2011). In control condition, when the animal is not sleep deprived, NA excites Hcrt neurons. However, following a short 2-h sleep deprivation the action of NA switches from excitatory to inhibitory (Grivel et al., 2005) due to α2-ARs availability at the membrane surface following sleep the deprivation (Uschakov et al., 2011). Similarly, MCH-R1 might also respond differently to NA and/or other arousal-promoting neurotransmitters in function of the time of day and the homeostatic needs.

The homeostatic nature of activity-dependent transmitter re-specification raises the possibility that it contributes to sleep (Spitzer, 2012). Inhibition of MCH cells during the active period may change the identity of the transmitters expressed in MCH cells, converting some neurons from inhibitory to excitatory. On the other hand, activation of MCH cells during the resting period could then turn these excitatory neurons back into inhibitory neurons. The fact that MCH neurons would be capable of activity-dependent neurotransmitter re-specification is purely speculative and this hypothesis has to be tested, but it could explain the opposite effect of MCH activation on sleep promotion during the dark and light phase.

More work is needed in order to characterize all MCH subpopulations, the role of MCH co-expressed neurotransmitters and which factors they release to their respective targets in order to regulate the sleep-wake cycle.

Chapter 5:

Concluding remarks: Significance of results and future experiments

During the course of the work completed during this PhD, we provided consistent experimental evidence that demonstrated a central role for MCH neurons in the regulation of REM sleep. Acute optogenetic stimulation of MCH cell somata either during NREM or REM sleep in the light phase strongly promotes REM sleep state. *In vivo* stimulation of MCH axon terminals in distinct anatomical targets demonstrated that MCH neurons modulate REM sleep by acting on multiple structures, suggesting different possible mechanisms. Indeed, we showed that the TMN and the medial septum were important targets whereas the DRN was contributing less to REM sleep promotion. Although this work answered some questions, it also opened new questions that await further investigation in order to better understand the mechanisms by which MCH neurons promote NREM or REM sleep. In particular:

1- The role of neurotransmitters co-released with MCH in sleep control.
2- The homeostatic and circadian inputs to MCH neurons and their integration in MCH control of sleep.
3- The existence of anatomical and/or functional subpopulation of MCH neurons in the LH/ZI area and their actions on distinct topographical targets.

The first problem we sought to solve was to decipher the specific role of MCH neurons, in particular the MCH peptide, in the regulation of vigilance states relative to that of other co-expressed neurotransmitters (i.e., GABA, and other peptides). Indeed, our *in vitro* study revealed that optogenetic activation of MCH neurons induced inhibitory post-synaptic currents in TMN cells within a short latency (2-3 ms). This effect was blocked by the application of Bicuculline, a $GABA_A$ receptor antagonist, demonstrating that GABA could be released from MCH cell terminals. These findings, in conjunction with

the recent reports by Chee et al. (2015) showing evidence of glutamate release from MCH cells, demonstrate that MCH neurons produce and release GABA as well as glutamate (Chee et al., 2015). While it remains unknown if the co-release occurs from the same axon terminal, from different terminals originating from single neurons or from different MCH cells, the fact that MCH neurons could release both excitatory and inhibitory neurotransmitters seems at first glance odd. Interestingly, this has been demonstrated for other cell types in the brain (Root et al., 2014) and it has been proposed that this phenomenon may expand the functions of these cells (Uchida, 2014). One possible advantage would be to modulate the strength of synapse signaling and ultimately the excitability of postsynaptic neurons. Also, having both excitation and inhibition originating from the same source might help to maintain a balanced increase or decrease of excitatory and inhibitory inputs coming from other sources (Uchida, 2014). More work is now needed to characterize the glutamate and GABA co-transmission and to better understand its role in MCH neuron's physiological functions, especially sleep. In Chapter 4 (see section 4-4), the hypothesis that MCH cells could undergo neurotransmitter re-specification across the sleep-wake cycle was also proposed. The circadian rhythm could control MCH cell neurotransmitter expression across the resting/active period to promote sleep or arousal when it is needed, although this hypothesis would need to be validated experimentally.

In addition, our results demonstrated that MCH peptide participates in the regulation of NREM sleep when semi-chronic optogenetic activation of these cells is applied but not when acute stimulations during specific states are used. This result is consistent with the idea that peptide release requires prolonged stimulation (van den Pol, 2012). However, we did not fully address the question of the role of GABA in MCH neurons. Despite the expression of *Gad 67* and *Gad 65* mRNA (Elias et al., 2001; Sapin et al., 2010; Jego et al., 2013) in MCH cells in addition to the fact that MCH neurons can release GABA (Del Cid-Pellitero and Jones, 2012; Jego et al., 2013), it seems unclear whether MCH neurons express VGAT. Until recently, the GABAergic phenotype of MCH cells was generally accepted; however new studies revealing the absence of VGAT protein question this idea (Jarvie and Hentges, 2012; Chee et al., 2015). Current

investigation focus on the detection of *Vgat* mRNA and the presence of *vGLUT2* mRNA in MCH cells using *in situ* hybridization, which is absolutely needed to clarify this issue as discussed in Chapter 3 (see section 3-4-1). Nonetheless, these results have raised many other questions regarding the mode of GABA release within MCH cells. Several studies showed that GABA can be released independently of a VGAT-mediated vesicular loading (as discussed in the section 3-4-3) and more studies are needed to unravel the role of GABA on sleep and others functions of MCH cells. We and others have confirmed the presence of GAD 67 in MCH cells (Sapin et al., 2010; Jego et al., 2013), which would make transgenic GAD $67^{flox/flox}$ mice an ideal tool to address the question of GABA release within MCH cells. However, at the present time, and to the best of our knowledge, no such mouse line is available yet.

Another important question that was raised by our studies, along with those of others, is the existence of distinct subpopulations of MCH neurons. This would explain the variation of the effect of MCH neurons activation on sleep across some of the studies. This question is very challenging considering the heterogeneity of the hypothalamus and the MCH cells themselves. Anatomical, neurochemical and functional evidence has shown diversity among MCH neurons. One example of the heterogeneity of MCH neurons was reported in the first study demonstrating a sleep promoting effect of MCH on sleep. Even though ~60% of all the MCH cells are c-Fos activated during REM sleep hypersomnia, they account for only 25% of the total cells in the same area activated in that particular condition (Verret et al., 2003). The other 75% of the remaining cells activated are still unknown, suggesting that some MCH neurons are part of a bigger population of cells that might control sleep and particularly REM sleep. Finding a common marker of all cells regulating REM sleep is extremely challenging.

At the present time, few MCH neurons have been recorded across the sleep-wake cycle (Hassani et al., 2009a). Although they showed the same profile of discharged across the sleep-wake cycle (discharging maximally during REM sleep)(Hassani et al., 2009a), it is possible that other MCH neurons might have a different pattern of firing or are active during other behaviors including negative energy balance, anxiety or depression-

like behaviors (Pissios et al., 2006). Development of new technology such as deep brain calcium imaging in freely behaving animals will unquestionably help to decipher the role of MCH cells and other hypothalamic cells on NREM versus REM sleep as well as other hypothalamic functions.

Last but not least, wide projections throughout the brain originating from MCH neurons strongly suggest that these neurons are capable of influencing many brain regions regulating wakefulness, NREM and REM sleep. Studies by many groups, including ours, tried to identify these targets and their functions on the regulation of sleep; I will try to summarize the main results in order to identify what would be the next step for future investigations.

MCH neurons send anatomical/functional projections to:

1- Structures that are active maximally during wakefulness such as the Dorsal Raphe (DR), locus coeruleus (LC), hypocretin neurons and the periaqueductal gray (VLPAG) and the adjacent dorsal deep mesencephalic nucleus (dDpMe).

2- Structures that are active during both Wakefulness and REM sleep such as the medial septum (MS) and;

3- Neurons that are active during NREM sleep such as neurons in the ventro-lateral preoptic area (VLPO) and the POA and BF regions.

Using various experimental approaches such as electrophysiology, immunohistochemistry, pharmacology or optogenetics, experimental evidence consistently pointed out a sleep-promoting role of MCH neuron projections to different targets.

1- <u>Activation of MCH neurons induces sleep by inhibiting arousal centers.</u>

<u>Hcrt neurons:</u> Slice patch-clamp study revealed that MCH exhibited an indirect inhibitory action on excitatory inputs to hypocretin neurons (Rao et al., 2008). Due to the proximity of the two cell populations, it is very difficult to specifically manipulate MCH projections to Hcrt neurons, however, the coupling of optogenetic and calcium imaging (Apergis-Schoute et al., 2015) would certainly help to determine the mechanism by which MCH neurons inhibit Hcrt neurons.

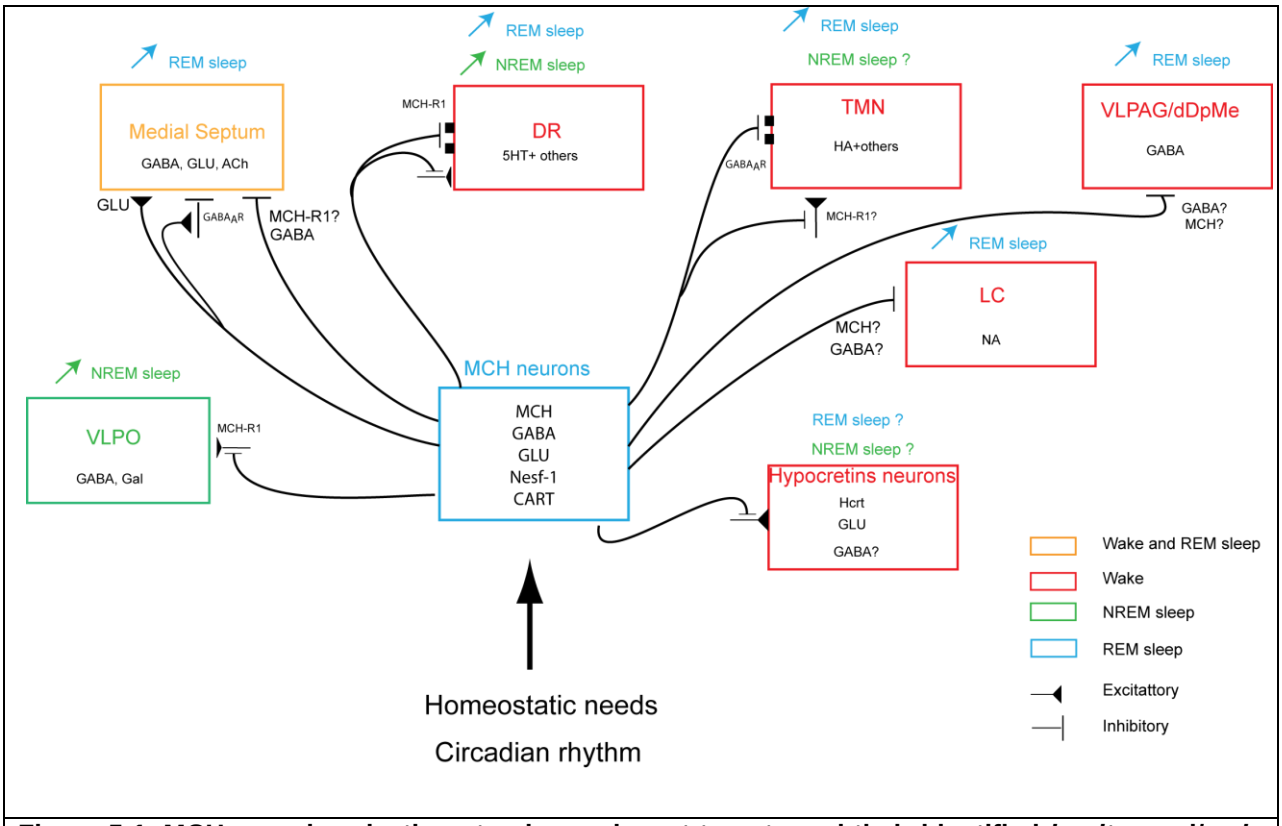

Figure 5-1. MCH neural projections to sleep-relevant targets and their identified *in vitro* and/or *in vivo* action on sleep. Note that the afferences to MCH neurons are not represented for clarity purpose. Dorsal Raphe (DR), locus coeruleus (LC), hypocretin neurons and the periaqueductal gray (VLPAG) and the adjacent dorsal deep mesencephalic nucleus (dDpMe), medial septum (MS), ventro-lateral preoptic area (VLPO).

LC: Immunohistochemical evidence suggest that MCH neurons would inhibit the LC through the release of GABA and MCH (Del Cid-Pellitero and Jones, 2012) however this anatomical data still requires functional investigation using, for instance, optogenetic stimulations of MCH fibers to this structure.

DR: Pharmacological studies, along with unit recordings in anesthetized rats, suggest that MCH peptide infusion inhibits DR neurons, including serotonergic neurons (Lagos et al., 2009; Lagos et al., 2011a; Devera et al., 2014). In addition,

these pharmacological studies showed that MCH infusion into DR increases both NREM and REM sleep (Lagos et al., 2009; Lagos et al., 2011a). In our study, we stimulated specifically the MCH projection to DR and we did not see an increase of REM sleep duration (NREM sleep specific stimulation awaits further investigation) (Jego et al., 2013), however, it is possible that DR modulation by MCH neurons alone is not sufficient to increase REM sleep or that the sleep promoting effect requires chronic activation of MCH neurons or concomitant inhibition of other wake promoting nuclei.

TMN: We showed that MCH projections to the TMN enhance REM sleep *in vivo* during acute optogenetic stimulation. In addition, we showed that the stimulation of these fibers induced GABA release in the TMN (Jego et al., 2013). The origin of GABA is likely from MCH neurons themselves but remains to be confirmed considering recent study suggesting the involvement of local GABAergic innervations in the MS (Chee et al., 2015).It remains possible that MCH neurons release GABA since the presence of GAD 67 indicates their ability to synthesise it, however, the accumulation of GABA into synaptic vesicles via VGAT might not be possible (Chee et al., 2015; Jennings et al., 2015). Either GABA is loaded into the vesicles via a transporter other than VGAT or GABA release is limited due to low efficiency refilling of recycling vesicles. More investigation is needed to unravel GABA transmission in MCH neurons and their targets.

VLPAG/dDpMe: MCH neurons project to the VLPAG/dDpMe (Clement et al., 2012). GABA neurons from VLPAG/dDpMe are thought to be responsible for the inhibition during wakefulness and NREM sleep of the pontine sublaterodorsal tegmental nucleus (SLD), a structure active during REM sleep (Boissard et al., 2002). Inactivation of the LH by muscimol (a GABA agonist) increases the c-Fos activity of VLPAG/dDpMe neurons and induces an reduction of REM sleep amount (Clement et al., 2012). Optogenetic stimulations of MCH fibers to this structure are necessary to understand the functional implication of this connection.

2- MCH neurons can induce sleep by inhibiting and/or exciting the medial septum.

There is strong evidence showing that the septo-hippocampal pathway is important for theta rhythm generation *in vivo* (Petsche et al., 1962; Stumpf et al., 1962; Gogolak et al., 1968; Alonso et al., 1987). The MS contains wake/REM-active neurons which may be activated by wake- or REM-promoting neurons in the hypothalamus and brainstem. We showed that *in vivo* stimulation of MCH neural projections to this structure prolonged REM sleep episodes during acute optogenetic stimulation (Jego et al., 2013). Moreover, a recent study revealed that MCH neurons both stimulate the MS through glutamatergic receptor and inhibit MS cells through feedforward inhibition (Chee et al., 2015). Therefore, one can imagine that the action of MCH neurons on the MS would regulate specifically the REM sleep state in which theta rhythm is predominant. Interestingly, infusion of MCH peptide in this area increased REM sleep and reduced wakefulness (Lagos et al., 2012).

3- MCH neurons can induce NREM sleep by disinhibiting/activating the VLPO

MCH neurons project to the preoptic area, a region though to be responsible for the promotion of NREM sleep. A study reported that infusion of MCH in this area induced NREM sleep without any effect on REM sleep (Benedetto et al., 2013). Optogenetic stimulation of these projections is needed to confirm the NREM sleep-promoting effect.

In conclusion, MCH neurons send projections to many brain regions involved in sleep regulation. Optogenetic stimulation of these different targets is a valuable approach to determine their functional relevance in sleep control. Combining imaging of MCH cellular activity and combinatorial optogenetic techniques (Carter et al., 2012) will undoubtedly unravel the precise network mechanisms underlying MCH cell control of NREM and REM sleep.

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