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The role of MS-PV GABAergic neurons and theta rhythm in the regulation of REM sleep

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Abstract

Rapid-eye-movement (REM) is a distinct phase of sleep and the mechanisms responsible for its regulation remain to be clarified. Theta (5-10 Hz) is the dominant brain rhythm present in the EEG and in all hippocampal regions during REM sleep. The medial septum-diagonal band of Broca (MS-DBB) neurons are considered as the fundamental theta pacemakers. Crucial for pacing of theta are the MS parvalbumin-positive (PV) GABAergic cells which synapse onto interneurons in hippocampus CA1 and rhythmically release pyramidal cells from inhibition. In this pilot study, we examined the involvement of MS-PV GABAergic neurons and hippocampal theta oscillations in REM sleep regulation. To address this question, we combined *in vivo* electrophysiological recordings in behaving mice with optogenetic stimulation of the MS-PV GABAergic neuronal projections to hippocampus (HC) CA1 region during different vigilance states. Our results indicated that optogenetic stimulation of MS-hippocampal projections during REM sleep at theta 8 Hz frequency reduced the time intervals between consequent REM episodes by nearly 4 minutes compared to the equivalent control light experiments and disrupted the relationship between REM sleep episode duration and the preceding REM sleep interval, without disrupting vigilance state architecture in general. Moreover it was demonstrated that theta oscillation entrainment fidelity around transitions from non REM (NREM) sleep to active wakefulness (AW) was higher than around transitions from quiet wakefulness (QW) to NREM sleep. These preliminary results could indicate that MS-PV GABAergic neurons and the theta rhythm which they pace, are involved in the circuit that regulates REM sleep.

1. Introduction

Insufficient sleep has been recognized as a public health problem and according to the Center for Disease Control (CDC) sleep insufficiency is strongly associated with motor vehicle crashes, industrial accidents and medical errors (http://www.cdc.gov/features/dssleep/). Moreover, disturbances in sleep have also been linked to a wide spectrum of psychiatric and chronic medical conditions like depression, bipolar disorder, diabetes and cardiovascular diseases. Therefore, studies on brain mechanisms controlling sleep are of great importance for the society. Even though the purpose of sleep remains one of the most interesting but yet unsolved mysteries in the fields of biology and neuroscience, a lot of progress has been made in understanding the brain mechanisms underlying sleep and wakefulness control. Sleep is not anymore considered as a period of relative inactivity and rest. According to the current view, sleep is recognized as a highly organized state, generated and controlled by the attuned activity of multiple neural and behavioral components (reviewed in Brown et al., 2012).

Rapid eye movement (REM) sleep, also called paradoxical sleep (PS), is a unique phase of sleep. It was first discovered in 1953 in humans by Eugene Aserinsky and Nathaniel Kleitman (Aserinsky and Kleitman, 1953) and it is characterized by the low muscle tone throughout the body (muscle atonia), the high frequency and low amplitude waveform of the electroencephalogram (EEG) signal and the random ocular movements.

The endeavor to reveal the center of REM sleep in the brain began in 1959 with Jouvet and Michel who discovered a sleep phase, in cats, resembling REM sleep as described in humans (Jouvet and Michelle, 1959). However, most studies conducted ever since indicate that REM sleep is rather controlled by a sparse brain network (Peever and Fuller, 2016) in which many brain regions and different types of neurons are involved. Still not all the components of this network have been identified and the exact mechanisms in which they interact need to be more clarified.

Local and global interactions between different brain structures and persistent activity constitute two of the most fundamental characteristics of cerebral cortex, which contribute in computation processes (Dehaene and Changeux, 2011; Buzsaki and Watson, 2012), and they can be maintained by interactive systems of brain oscillations (Buzsaki and Draguhn, 2004).

According to Buzsaki and Watson (2012), sleep could be considered as an extreme example of persistent activity, since activity is maintained without the interference of external inputs. Consequently, it is possible for brain oscillations to contribute to the mechanisms of sleep regulation.

The most prominent brain rhythm during REM sleep in the EEG of mammals is theta (5-10 Hz). Importantly, theta oscillations are also present in all hippocampal regions during REM sleep (Jouvet, 1969; Buzsaki, 2002), and although theta rhythm is one of the most well studied rhythms of the brain in the context of memory consolidation, even during REM sleep (Miller, 1989; Lisman and Idiatar, 1995; Raghavachari et al., 2001; Boyce et al., 2016), however its role in REM sleep regulation remains poorly understood. In this pilot study we employed a combination of optogenetic stimulation and electrophysiological local field potential (LFP) recordings in freely behaving mice in order to study the effect of hippocampal theta oscillations on REM sleep regulation.

This introductory section provides a brief overview of the mechanisms and functions of theta rhythm, followed by a brief literature review on current knowledge regarding the brain circuits underlying REM sleep, and finally explains how the availability of transgenic mouse lines in combination with the optogenetic toolkit enables the entrainment of brain rhythms and the identification of different types of neurons involved in innate behaviors.

1.1 Hippocampal theta oscillations: Mechanisms and functions

Theta rhythm was first identified in rabbits (Jung and Kornmuller, 1938) and ever since in other species as well, including cats, rats, mice, monkeys and humans (Vanderwolf, 1969; Green and Arduini, 1954; Grastyan et al., 1959; Ekstrom, 2005; Ulanovsky and Moss, 2007; Jutras et al., 2013). Theta oscillations are sinusoidal waves and have a frequency of 5-10 Hz. They are present in all hippocampal regions during REM sleep (Jouvet, 1969; Buzsaki 2002) and during exploratory and voluntary locomotion activity (Vanderwolf., 1969; Buzsaki, 2002; Bender et al., 2016). However, theta can be recorded in most regular frequency and highest amplitude in CA1 stratum lacunosum moleculare, as both the amplitude and the phase of theta alter as function of depth in the different hippocampal layers (Bullock et al., 1990; Buzsaki 2002). Besides hippocampus, theta oscillations and neurons that discharge in a phase locked

manner to theta waves, have been observed in several brain regions counting the subicular complex, entorhinal cortex, amygdala and medial prefrontal cortex (Steriade, 2000; Buzsaki 2002; Colgin, 2011; Hutchison and Rathore, 2015). Nonetheless, the occurrence of theta patterns in cortical structures in different behavioral states is not always consistent with theta waves in hippocampus, as indicated by human intracranial recordings (Kahana et al., 1999; Raghavachart et al., 2001; Buzsaki, 2002).

1.1.1Mechanisms

The extracellular currents contributing in theta rhythm are generated by the perforant path inputs (entorhinal input), CA3 Schaffer collaterals and the voltage dependent Ca^{2+} currents in the dendrites of pyramidal cells (Buzsaki, 2002). Even though assorted subcortical nuclei have been suggested to be implied in theta rhythm generation, the medial septum-diagonal band of Broca (MS-DBB) neurons are considered as the fundamental theta pacemakers that supply phasic modulation to hippocampus (Petsche et al., 1962; Buzsaki, 2002; Wulff et al., 2009; Bender et al., 2015) since MS lesions or genetic disruption of MS projections to hippocampus nullify hippocampal theta (Green and Arduini, 1954; Wulff et al., 2009).

In detail, the MS parvalbumin- positive (PV) GABAergic cells are considered to carry the "theta- pacemaker" properties and the fact that they express HCN channels (Varga et al., 2008; Hangya et al., 2009) further supports this hypothesis. MS-PV GABAergic cells are synaptically connected to interneurons in hippocampal CA1, CA3 and the dentate gyrus regions (Freund and Antal, 1988), and lift the inhibition of hippocampal pyramidal cells in a rhythmic manner that promotes their firing at theta frequency (Colgin, 2016).

Even though the minimal conditions for theta generation are the intact connections between hippocampus and MS- DBB, the exact mechanisms underlying theta generation are more complicated than can be described in this brief overview. MS-PV GABAergic neurons terminate in different types of hippocampal interneurons (Freund and Antal, 1988) and the distinct classes of hippocampal interneurons are, in turn, phase locked in their firing to different phases of the theta cycle (Somogyi et al., 2014). Interestingly, it was demonstrated that the silencing of MS-PV GABAergic interneurons increased place cells spiking in the early part of the cell place field which corresponds to the late phase of theta (Skaggs et al., 1996). This

implies MS-PV GABAergic neuron involvement in the determination of the place cells' firing associated with theta oscillations (Colgin, 2016).

Compatible with these findings, it has also been evident that different types of interneuron mechanisms are involved in theta generation (Korotkova et al., 2010; Royer et al., 2012) and one of them is the rhythmic suppression of pyramidal cell activity (Royer et al., 2012). Furthermore, non GABAergic mechanisms are involved in theta generation as well. One of the most characteristic studies on this topic was conducted by Kamondi et al. (1988), where it was shown that excitatory inputs are necessary to activate specific place cells within a theta cycle, as the MS-PV neuron silencing had no effect on place cells outside their place field. Moreover, cholinergic inputs from MS also contribute to theta rhythm entrainment, as cholinergic projections from MS are required for the atropine-sensitive theta, the theta oscillations occurring when the animal engages in passive behaviors. During these quiet states, the cholinergic stimulation may provide the necessary excitation to hippocampus (Nakajiima et al., 1986), since movement-related inputs are absent. Another potent role of cholinergic inputs in theta entrainment could also involve the suppression of sharp wave ripples, which function is antagonistic to theta (Kubota et al., 2003; Vandecasteele et al., 2014). Finally, studies employing *in vitro* hippocampal preparations showed that theta oscillations occur even without the MS connections, suggesting that local circuit interactions are capable to induce theta rhythms (Goutagny et al., 2009). Nonetheless, it is clarified that the MS inputs are necessary for theta entrainment in behaving animals.

Last, it is worth to mention that even if theta oscillations appear to have a similar frequency range during wakefulness and REM sleep, there is some evidence supporting that wakefulness and REM theta differ in their generation or regulation mechanism (reviewed in Hutchison and Rathore 2015), and therefore, serve different functions.

1.1.2 Functions

Regarding the functions of theta rhythm, it is considered that theta oscillations represent the "online" state of hippocampus and have a key role in the temporal coding and decoding of functional neuronal networks (Buzsaki, 2002). Moreover, it has been postulated that they are carriers of mnemonic processes (Miller, 1989; Lisman and Idiatar, 1955) and may facilitate

hippocampal inputs to the medial prefrontal cortex during mnemonic tasks (Colgin, 2011). In fact, theta rhythm involvement in memory has received considerable attention. Several studies provide indirect evidence for theta oscillation involvement in synaptic plasticity (Larson and Lynch, 1988) and have also shown that long term potentiation is facilitated during theta (Fox et al., 1983; Buzsaki et al., 1983; Buzsaki, 2002).

However, causal relationship between theta and memory consolidation was yet to be proven until a recent study, conducted by Boyce et al. (2016), on the role of theta oscillations during REM sleep in contextual memory consolidation. Specifically, they were able to "erase" object place recognition and fear memories by inhibiting the hippocampus theta-pacemaker neurons, the MS PV GABAergic interneurons, during REM sleep. Furthermore, a growing body of research provides evidence for REM sleep theta activity implication in the processing of emotional memories. Specifically, in one of their recently published literature reviews on this topic, Hutchison and Rathore proposed that REM sleep mediates for the prioritized processing of emotional memories, the integration of memory traces that were previously consolidated within the neocortex and the disengagement of neocortical memory traces from the hippocampus. According to them in this mediation process, theta activity in hippocampus, amygdala and neocortex have a key role, as the timing of hippocampal activity in coherence with theta phase is crucial for the determination of neural activity potentiation and because of theta correlation to the reduced levels of acetylcholine, observed in REM sleep, which is believed to decrease neocortex inputs from hippocampus (Hutchison and Rathore, 2015).

1.2 Brain circuits involved in REM sleep

As already mentioned, Jouvet and Michel efforts to discover the center of REM sleep inside the brain were fruitful; they proved that the brainstem is necessary and sufficient not only to trigger, but also to maintain REM sleep in cats. Specifically, by employing electrolytic and chemical lesions they demonstrated that the neurons responsible for REM sleep onset were located in the dorsal part of pontisoralis (PnO) and caudalis (PnC) (Jouvet, 1962; Carli et al., 1965; Sastre et al., 1981; Webster et al., 1988). Later this control area was further defined as a region immediately ventral to the main cluster of noradrenergic neurons in the locus coeruelus (LC) and dorsal to the gigantocellular tegmental field (FTG) and coined the term subcoeruleus nucleus (SubC). The discovery of a SubC-subset of neurons which exhibit tonic firing selectively

during REM sleep (termed REM-on neurons) (Sakai et al., 1981; Sakai,1985; Sakai et al., 1996; Sakai et al., 2001; Fort et al, 2009) is a considerable milestone in this field of study.
These findings provided a background for the "Jouvet hypothesis" on REM sleep, according to which the brainstem is necessary and sufficient to generate a state characterized by REM activity and muscle atonia (reviewed in Fort et al., 2016). However, REM sleep is not induced, maintained and controlled exclusively by the brainstem. Not only different brain regions are involved, but also different types of neurons located and projecting to these regions contribute to these processes. Some of the most important brain regions participating in REM sleep are reviewed below:

Sublaterodorsal nucleus (SLD)/ Subcoeruleus nucleus (SubC)

The terminology used to describe the responsible for REM-sleep control reticular formation regions is different between brain atlases, between species, and between different investigators. In the cat the REM control area was defined as a region immediately ventral to the main cluster of noradrenergic neurons in the LC and dorsal to the FTG. This region was termed SubC. However, the functionally equivalent region in the mouse and rat has been termed as sublaterodorsal nucleus (SLD) and corresponds to the rostral part of the SubC area as defined in the rat brain atlas of Paxinos and Watson (Brown et al., 2006).

This region consists of neurons that are predominantly active during REM sleep episodes (REM-on neurons) (Boissard et al., 2002; Lu et al., 2006; Siegel et al., 1991; Maloney et al., 1999; Boissard et al., 2003) and even though the vast majority of them is glutamatergic, GABAergic SubC cells are implicated in the control of REM sleep as well (Lu et al., 2006). It is believed that these cells induce REM sleep muscle paralysis by recruiting GABAergic/glycine neurons in the mentromedial medulla (VMM) and spinal cord, which produce motor atonia by inhibiting skeletal motor neurons (Lu et al., 2006; Lai and Siegel, 1988; Schenkel and Siegel, 1989; Holmes et al., 1994; Vetrivelan et al., 2009). Moreover, cholinergic REM-on neurons have a key role in REM sleep initiation and muscle inhibition control (Hobson et al., 1975). It was demonstrated that acetylcholine activated spinally projecting SubC (Weng et al., 2014) and these cholinergic inputs mediate muscle atonia by enhancing the glutamate-driven postsynaptic excitation, and strengthen transitions by facilitating presynaptic glutamate release (Grace et al., 2014).

Dorsal paragigantocellular reticular nucleus (DPGi)

DPGi is localized in the medulla, it consists of GABAergic neurons that are also REM-on neurons. These neurons are hypothesized to inhibit LC, dorsal raphe (DR) and a part of the ventrolateral periaqueductal gray (vIPAG) (Luppi et al., 2006), and therefore facilitate REM sleep by inhibiting these wake-promoting areas (Gervasoni et al., 2000).

Ventral medulla (VM)

The GABAergic REM-on neurons of VM play a causal role in the generation of REM sleep. Precisely, in a recent study by Weber et al. it was demonstrated that the activation of rostral GABAergic projections was sufficient to induce and maintain REM sleep. It was proposed that this effect is probably mediated by the inhibition of vIPAG REM-off neurons (Weber et al., 2015). VM is also a critical component of the core network generating muscle atonia and cortical activation associated with REM sleep (Burges and Peever, 2013; Soya et al., 1995; Grace et al., 2013). GABAergic VM projection neurons to LC are likely to provide an inhibition to noradrenergic neurons whose activity enhances wakefulness (Lai et al., 2001) and excitability of spinal motor neurons.

Ventrolateral periaqueductal gray (vIPAG)

The GABAergic neurons of vIPAG are divided into two subpopulations: the REM active (REM-on) neurons and the REM inhibiting (REM-off) neurons. vIPAG REM-on neurons send projections to LC and DR, which are wake-active regions (Gervasoni et al., 2000; Luppi et al., 2006,Verret et al., 2006). On the other hand, the vIPAG REM-off neurons send inhibitory inputs to SLD and may prevent the activation of the REM generating circuit (Lu et al., 2006; Boissard et al., 2003; Sapin et al., 2009).

Extended ventrolateral preoptic area (eVLPO)

REM-on neurons of eVLPO send GABAergic projections to the REM inhibiting neurons of vIPAG and free the SLD from its silenced state (Lu et al., 2002).

Lateral Hypothalamic Area (LHA)

GABAergic interneurons, located in the posterior part of hypothalamus, are considered one of the most important regulators of REM sleep; specifically, melanin concentrating hormone (MCH) neurons of lateral hypothalamus (LH) which are REM-on neurons and fire exclusively during REM sleep (Hassani et al., 2009; Jego et al., 2013). MCH neurons send dense projections to wake-active serotoninergic neurons of the DR and experiments employing pharmacological application of MHC on DR resulted in an increase of the number of transitions to REM (reviewed in Fort et al., 2009). Interestingly, in a recent study by Jego et al. it was shown that the optogenetic activation of MCH neurons prolongs the duration of REM sleep, providing causal evidence for the involvement of LH networks in REM sleep regulation (Jego et al., 2013). They also suggested that MCH neurons provide a functional input to septohippocampal circuits in which they participate in theta rhythm stabilization in hippocampus. On the other hand, when they silenced MCH neurons optogenetically, REM sleep duration was not reduced, and therefore it was proposed that additional circuits are required for the regulation of REM sleep duration. In our study we investigate the role of theta-pacemaker MS-PV GABAergic neurons as a potential part of these additional regulatory circuits.

Network model of the mechanisms controlling REM sleep onset and maintenance (by Fort et al., 2009)

The activation of SLD glutamatergic REM-on neurons, causes REM sleep onset. These neurons remain silent during wakefulness and NREM sleep due to a tonic inhibitory signal from REM-off GABAergic neurons originating from the vIPAG and dorsal deep mesencephalic reticular nuclei (dDpMe). In turn, these REM off neurons are activated during wakefulness by the hypocretin and monoaminergic neurons.

In the beginning, an intrinsic mechanism activates REM-on MCH-GABAergic neurons in hypothalamus, as well as the REM on neurons of DPGi and vIPAG. These neurons would also be able to inhibit the REM-off monoaminergic and hypocretin neurons and so, release SLD REM on neurons from inhibition. The activated SLD REM-on neurons would stimulate cortex via their projections to intralaminar thalamic relay neurons, in synchrony with the Wakefulness-on and REM-on cholinergic and glutamatergic neurons fromlaterodorsal tegmental nucleus (LDT) andtuberomammillary nucleus (PPT), mesencephalic and pontine reticular nuclei and the basal

forebrain. Following, the descending REM-on SLD neurons would induce muscle atonia and inhibit the sensory neurons via their excitatory projections to glycinergic premotor neurons in the alpha and gigantocellular reticular nuclei and the nucleus raphe magnus.

Regarding the exit from REM sleep, it would be triggered by the activation of wakefulness circuits, because REM sleep episodes are terminated by an arousal in most of the times. Taking this into account, the wakefulness circuit would inhibit MCH-GABAergic neurons and the GABAergic REM-on neurons of DPGi and vIPAG and therefore ensure the exit from REM sleep in order to restore competing physiological parameters.

1.3 Optogenetics and transgenic mouse lines

The term optogenetics refers to the technique in which optics and genetics are combined aiming to achieve gain or loss of function of well defined events taking place in specific cell types (Deisseroth et al., 2006; Yzhar et al., 2011) and involve the use of light in order to activate or inhibit specific types of neurons that have been genetically modified to express light-sensitive ion channels. Additionally, the generation of conditional knockout mice employing the Cre/lox system enables cell type-specific expression of optogenetic molecules and allows temporally precise manipulation of targeted neuronal activity (Madisen et al., 2012). The use of optogenetics in combination with conditional knockout mice lines provides researches with the handles to examine the involvement of specific neuronal types and oscillatory patterns in different innate behaviors including locomotion (Bender et al., 2015), food intake (Carus-Cadavieco et al., in revision) and sleep (Jego et al., 2013) to establish causal relationships between them.

In our study, we employed PV::Cre transgenic mice in order to selectively target Cre recombinase expression to PV-positive GABAergic neurons and a Cre-dependent channelrhodopsin-2(ChR2) viral construct, which was injected in the MS. This combination enabled the expression of ChR2 specifically in MS PV GABAergic interneurons and therefore, their optogenetic manipulation (Bender et al., 2015).

1.4. Aim of the study

The aim of this pilot study was to examine the involvement of MS-PV GABAergic neurons as a potent compartment of REM sleep circuit and to explore the role of hippocampal theta oscillations in REM sleep regulation, in mice.

2. Methods

2.1. Animals

In this study 8 PV::Cre transgenic mice (The Jackson Laboratory, Bar Harbor, Maine, USA), 10- 20 weeks of age were used in order to selectively target Cre recombinase expression to PV-positive GABAergic neurons. However, only 5 survived enough time to complete the experiments and were used in our analysis. All animals were housed individually after their implantation, in a 12h:12h light:dark cycle (light on at 6.00 a.m.) at 22^{o}C with access to standard rodent chow and water *ad libitum*. All procedures were performed according to the national and international guidelines and were approved by the animal care committee and local health authority (Landesamt fur Gesundheit und Soziales, Berlin).

2.2. Viral injections

All PV::Cre transgenic mice were injected in MS (AP: 0.98, L: 0.0, V: -5.0 and -4.5 mm, all coordinates relative to bregma) with a total of 900 μl (450μl per injection site) of Cre-dependent ChR2 (*AAV2/1.CAGGS.flex.ChR2.tdTomato*, Penn Vector Core) approximately 6 weeks before the initiation of the experiments.

For the viral injections, all animals were anaesthetized with inhalant isoflurane (1.5 –3% isoflurane in oxygen) (Gas Vaporizer) and then placed in a stereotactic head frame using non-traumatic earholders (David Kopf Instruments, Tujunga, CA, USA). The animal's eyes were protected from drying by applying an artificial lipid solution gel (Artelac Lipids EDO) and 0.1 ml of lidocaine were injected intradermaly under the skin of the head. All hair right above the skull was cut and an incision of the skin above the midline was done, so that bregma and lambda could be visible. A small hole on the skull was drilled according to the stereotactic coordinates above MS and the virus was infused in MS via a 5ul Nanofil Hamilton syringe connected to a microsyringe pump (PHD Ultra, Harvard Apparatus, Holliston, MA, USA) at a rate of 100nl per

minute. Following the infusion the needle was kept in each injection site and after 10 min was slowly withdrawn. Finally, the incision was sutured and lidocaine was applied locally on the skin. Additionally, 0.3ml of Erythromycin (0.1 ml Erythromycin diluted in 0.4 ml 0.9% NaCl) was administered intraperitoneally (i.p.). All animals were left to recover for at least 5 weeks before their implantation in order to ensure the virus expression in MS-PV GABAergic neuronal terminals.

2.3. Stereotactic implantations

Two EEG miniature stainless steel screws, each one attached to a copper wire, were placed in the skull bilaterally, above PFC connected with 2 EMG electrode wires which were embedded to dorsal neck muscles.

For the optogenetic entrainment of theta oscillations in the MS PV-GABAergic projections to the HC during REM sleep and the simultaneous CA1 LFP signal recording, all mice were implanted with one optic fiber attached to a wire array (Fig. 2.1), in CA1 stratum pyramidale (AP: -1.94, L: 1.4, V: -1.4) . All optic fiber implants were prepared using 100 μm diameter fiber (0.22 NA, Thorlabs, Newton, NJ, USA) combined with a ceramic ferrule stick (Precision Fiber Products, Milpitas, CA, USA) and the intensity of light transmitted was at least the 50% of the light intensity delivered by the laser patch cord. The wire arrays used for the EEG and EMG signal acquisition as well as for the hippocampal LFP recordings were custom made. In detail, 8 copper wires were soldered to the pins of a nanoconnector (Omnetics Connector Corporation, Minneapolis, USA) and 4 of them were subsequently soldered to one separate row of 4 parallel assembled tungsten wires (40μm, California Fine Wire Company) with an impedance approximately 100kΩ each. The 4 remaining wires were soldered to the EMG and EEG electrode wires, respectively. Bone screws above the cerebellum were used as reference and ground electrodes.

The entire implant and the bone screws were secured to the skull using dental acrylic cement and the entire instrument was built as light as possible in order to minimize the impact on the animal's natural sleep behavior. After the surgery, all mice were allowed to recover in their home cages for 1 week and 0.3ml of Erythromycin was administered intraperitoneally (i.p.) to them for 3 consecutive days after their implantation to prevent infection.

2.4. Sleep recordings

After the 1 week recovery period, the mice were recorded inside a sleep-promoting set-up, consisting of a small clay flowerpot with soft paper tissues as bedding, some of which were placed inside the home cage of each mouse a few days before the experiment. For the sleep recordings the mice were habituated inside the flowerpot for 1.5 to 2h before the initiation of the experiments in order to acquire more REM sleep episodes as indicated in 6h preliminary baseline recordings of 2 mice (Fig. S1). All recordings took place during the dark cycle between 1 0a.m and 4 p.m. and the duration of each sleep session was 1h. No more than 2 sessions were recorded within one day, except for the preliminary 6h baseline recordings. 2-4 baseline sleep sessions were recorded for all animals.

2.5. Data acquisition

The chronically implanted electrode arrays were connected to operational amplifiers (HS-8, Neuralynx, Bozeman, Montana USA) to eliminate artifacts caused by the cable movements. Additionally, the EEG, EMG and CA1 LFP electrophysiological signals were differentially amplified, band pass filtered (1 Hz-10kHz, Digital Lynx, Neuralynx) continuously acquired at 32kHz and the timestamps of the laser pulses were recorded simultaneously.

2.6. Optogenetic stimulation

In the optical experiments a 473nm-wavelength diode-pumped solid-state laser (R471005FX, Laserglow Technologies, Toronto, ON, Canada) connected to a 3m long fiberoptic patch cord (Thorlabs), was used in order to deliver blue light pulses inside the brain of the freely moving mice. Before the initiation of each experiment the patch cord was coupled with the chronically implanted optic fiber using a ferrule sleeve. The laser output was controlled using a stimulus generator and MC_Stimulus software (Multichannel Systems, Reutlingen, Germany) and the light power output from the tip of the patch cord was at 21 mW as measured with a power meter before each experiment (PM100D, Throlabs), ensuring that the light power output from the tip of the implanted optic fiber would be approximately 10 to 12 mW.

In order to entrain theta oscillations in HC, the EEG, EMG and CA1 LFP signals an open loop without feedback system was used, continuously throughout each session and the real time vigilance state determination was based on the same criteria used for the sleep scoring and the

analysis of the vigilance state. Whenever the mice entered REM sleep during each session the laser was manually activated and 30ms-blue light pulses were delivered to MS-PV GABAergic projections to the HC at 8Hz (typical dominant theta frequency), via the patch cord-optic fiber system, until a transition from REM sleep occurred. Then, the laser was immediately turned off until the next REM sleep episode detection. Exactly same protocol was applied for the control light experiments in these mice, where the patch cord was connected to a dummy ferrule attached to the headset instead of the implanted optic fiber. These sessions were performed in order to control for any changes in sleep architecture due to the blue light emission from the patch cord during the stimulation. Additionally, one other group of ChR2-expressing animals was subjected to one 1h and 3min automated random-state-stimulation protocol, according to which the animals received rectangular blue light pulses to MS-PV GABAergic projections to HC at 8Hz for 2 minutes followed by a 5 minutes break regardless the vigilance state.

2.7 Sleep scoring and vigilance state analysis

The EEG, EMG and CA1 LFP signals recorded from each sleep session were processed in Neurophysiological Data Manager (NDManager, http://neurosuite.sourceforge.net/), plotted (sampling rate: 1250) and visually scored in 5s epochs in Spike 2 software (Cambridge Electronic Design, CED). Scoring of each epoch was based on the visual characteristics of EEG, EMG and CA1 LFP traces according to a standardized set of scoring rules (Fig. 2.2, Louis et al., 2004), the spectral analysis of the EEG signal (Hamming window, fast Fourier transformation (FFT) size = 2048) and the video monitoring of each mouse during each session. Quiet wakefulness (QW) was defined by de-synchronized cortical activity with a low-amplitude waveform EEG consisted of different frequencies (Delta-δ: 1-4.88Hz; Theta-θ: 4.88-10 Hz; Alpha-α: 10-14.65 Hz and Beta-β: 14.65-20.14 Hz) and tonic EMG activity with periods of movement-associated bursts of EMG activity (Fig. 2.2.A). In cases where these movement-associated bursts of EMG activity lasted for more than half of the epoch duration and the video showed that the mouse was actively moving, these epochs were characterized as active wakefulness (AW; Fig. 2.2.B). NREM sleep epochs were characterized by synchronized cortical activity, high amplitude - low frequency EEG waves with a predominant delta power (δ-power in bands > 45%) and reduced EMG activity (Fig. 2.2.C). REM sleep was defined by the desynchronized, saw-tooth like EEG waveform, the absence of muscle activity in EMG and the

characteristic theta prominent activity observed in CA1 LFP (Fig. 2.2.D). Hypnogram analysis was performed using custom-written MATLAB (Mathworks, Natic, MA, USA) scripts. The fidelity of theta oscillation entrainment for the stimulation epochs of all recordings was quantified as the ratio of cumulative power spectral density (PSD) around the optogenetic stimulation frequency (±0.5 Hz) to the cumulative PSD in the 5–12 Hz band (calculated for the hippocampus LFP signal).

2.8 Statistical analysis

Sleep behavior parameters were analyzed using GraphPad Prism (GraphPad Software v.7.00, La Jolla, California, USA). All values are expressed as mean ± SEM, unless it is otherwise stated. All samples were tested for normality, using D'Agostino and Pearson omnibus normality test. The level of significance was pre-determined at $\alpha < 0.05$. In cases where sample distribution proofed to be normal, the data were analyzed by using two-tailed unpaired t-tests and for correlation analysis Pearson's r was calculated. In cases where sample distribution proofed to be non-normal or the sample sizes were too small to test for normality, the data were analyzed using Kruskal-Wallis or Mann-Whitney non parametric tests and for correlation analysis Spearman's r was calculated. In cases where P-values were less than 0.05 in non-parametric analysis, the median is also reported in the text and legends.

2.9 Histology

Histological analysis was performed after the completion of experiments. ChR2 expression was determined in MS PV-positive GABAergic neuronal cell bodies and their projections in HC, as well as the recording electrode tracks. The implantation sites of selected electrodes were confirmed by performing electrolytic lesions in order to visualize the recording electrode tracks. All mice were perfused intracardially using 4% paraformardehyde (PFA) solution (Fisher Reagents, Schwerte DL), decapitated and their brains were fixed in 4% PFA for 24h and kept in phosphate buffer saline (PBS) 1X until slicing. All brains were cut in 50μm slices using a vibratome (EMS 4500, Electron Microscopy Slicer, Hatfield, PA, USA) and mounted (Fluoromount Aqueous Mounting Medium, Sigma- Aldrich) for visual inspection of the viral construct fluorophore *tdTomato* distribution. Images were obtained using a fluorescence

microscope (Olympus BX6 61, Hamburg, DL) combined to a QimagingExi mono fluorescence camera (Qimaging, Surrey, BC).

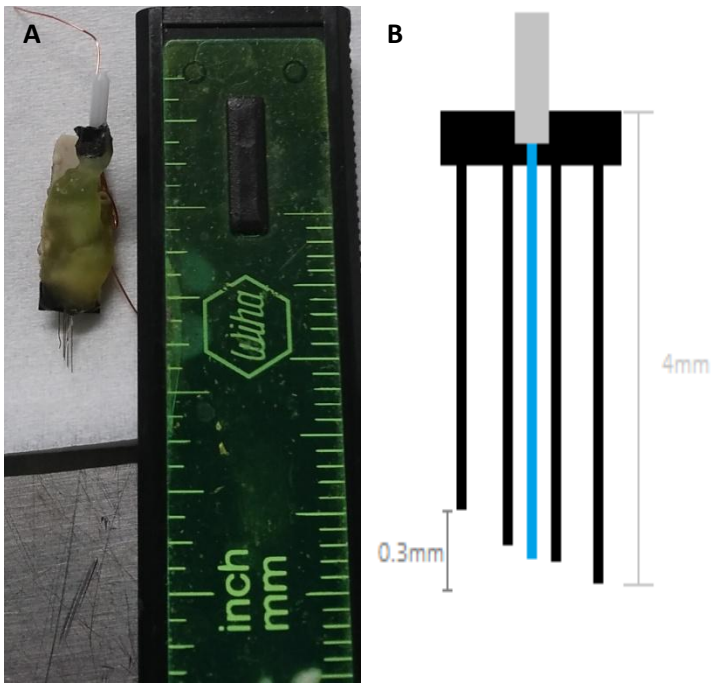

Figure 2.1 Electrode array-optic fiber implant. A. Implant preparation **B.** Scheme.

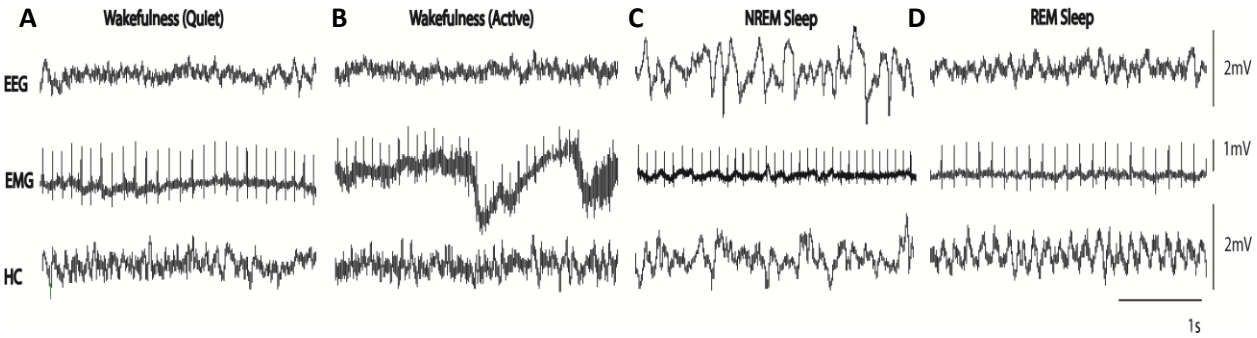

Figure 2.2 Representative traces of EEG, EMG and Hippocampus LFP recordings during the different behavioral states. A. Quiet wakefulness, **B.** Active wakefulness, **C.** NREM sleep and **D.** REM sleep.

3. Results

3.1 ChR2 was expressed in MS-PV GABAergic neuronal cell bodies in MS and in their projections to HC.

To study the role of hippocampal theta oscillations in REM sleep, we used the preparation developed by Bender et al., 2015 which enabled the temporal control of theta oscillations in freely moving animals. Precisely, we targeted MS PV-GABAergic neurons by injecting a Cre dependent ChR2 viral construct into the MS of PV::Cre mice (Fig. 3.1A). ChR2 expression was confirmed in MS PV-GABAergic neurons (Fig. 3.1B and C) and in their projections to HC (Fig. 3.2C, D, E, F) before the statistical analysis for all animals. The electrode array tracks were also verified for all animals (Fig. 3.2B)

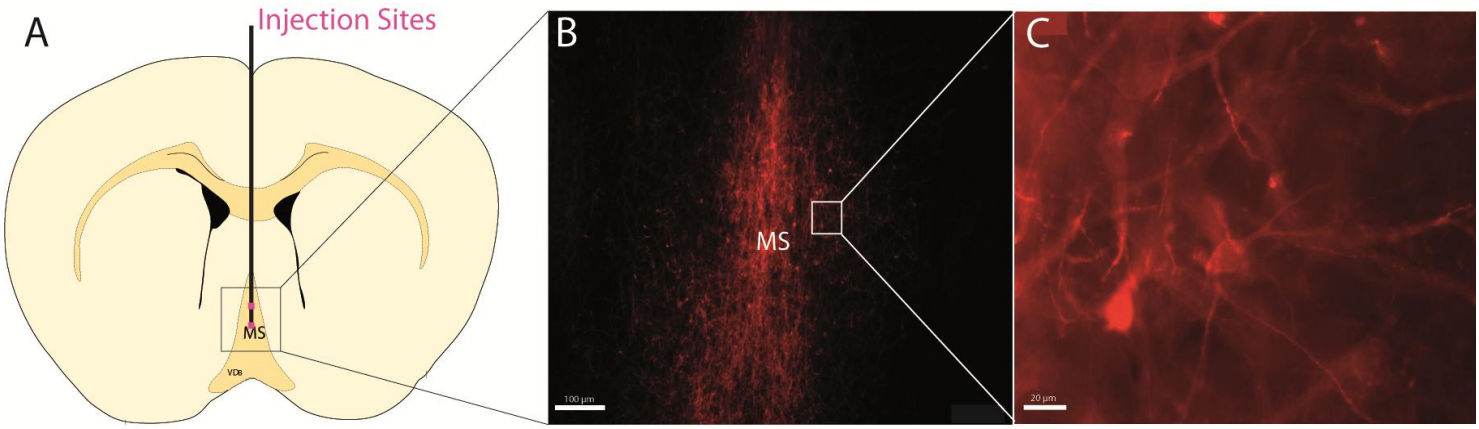

Figure 3.1 ChR2 expression in MS PV-GABAergic neurons. A. Injection sites of Cre-dependent ChR2 in the MS of PV::Cre mice. **B.** and **C.** Representative histological image of AAV2/1.CAGGS.flex.ChR2.tdTomato expression in the neuronal cell bodies of PV-GABAergic neurons in MS (in red). Scale bars: 100 µm (B), 20 µm (C).

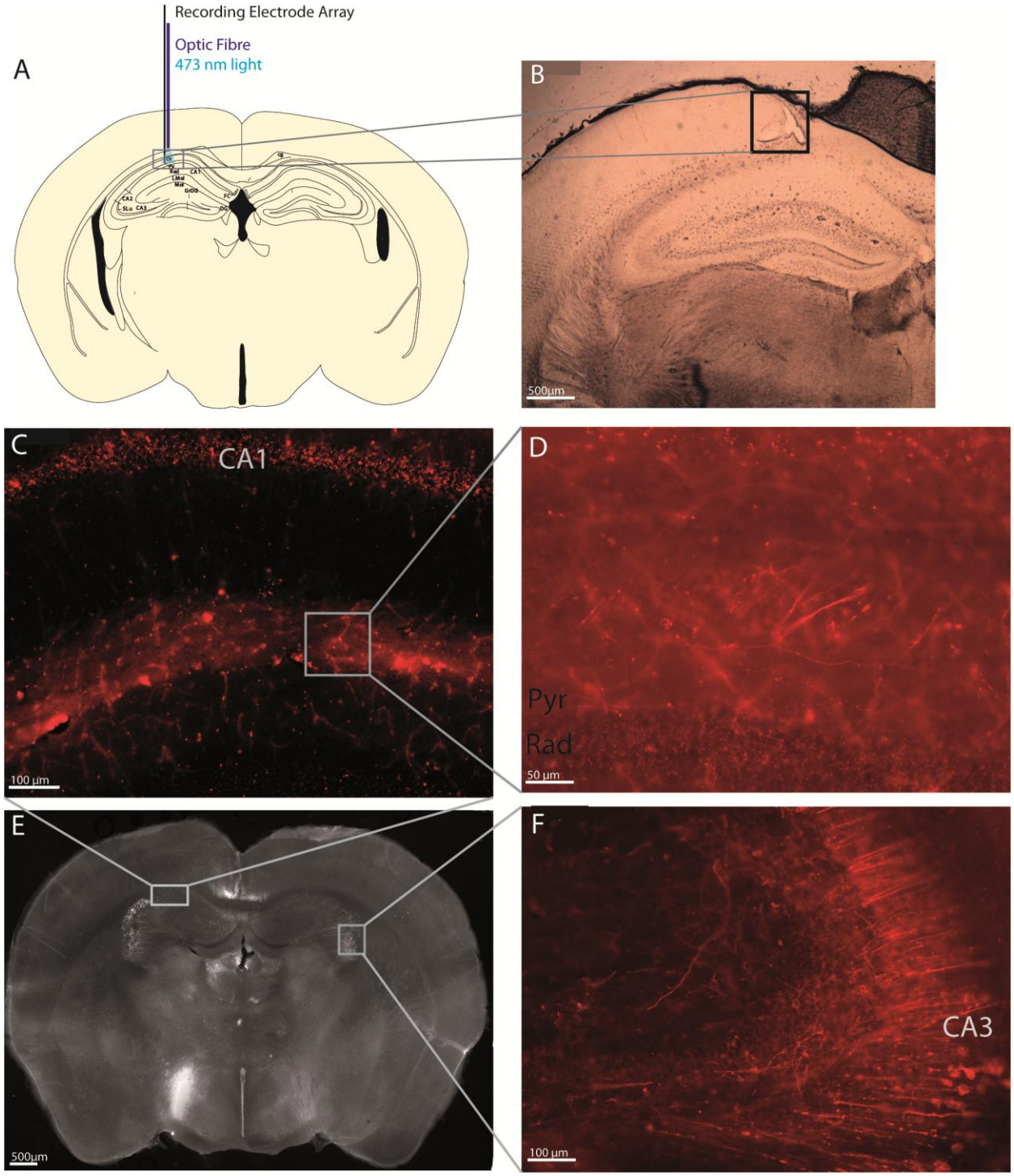

Figure 3.2 ChR2 expression in MS PV-GABAergic neuronal projections to HC. A. Scheme of optic fiber and electrode array position in HC. Representative histological images of **B.** wire array track **C.** *AAV2/1.CAGGS.flex.ChR2.tdTomato* expression in MS PV-GABAergic neuronal projections to hippocampal CA1 region **D.** stratum pyramidale (Pyr) and stratum radiatum (Rad) **E.** Overall overview of ChR2 expression in MS PV-GABAergic neuronal projections to HC and **F**. ChR2 expression in CA3 region. Scale bars 500 µm (B), 100 µm (D, F), 20 µm (E).

3.2 Targeting of ChR2 to MS hippocampal projections did not alter vigilance state homeostasis.

In order to examine the effect of theta oscillations in the vigilance state homeostasis of mice, we developed two different protocols; one automated random-state stimulation protocol and one manual REM-specific stimulation protocol. According to the automated random-state stimulation protocol, 473 nm blue light pulses at 8Hz were delivered at MS projections to HC for 2 minutes followed by a 5 minutes break, for one hour regardless the vigilance state. With the manual REM stimulation protocol we targeted specifically the effect of REM sleep theta oscillations on vigilance state homeostasis. Therefore, according to our manual REM stimulation protocol 473 nm blue light pulses at 8Hz were delivered at the MS projections to HC upon the detection of REM sleep until a transition to another state occurred.

Subsequently we compared the following vigilance state parameters between baseline control light and light stimulation sleep recordings for both protocols: cumulative duration of each state, mean episode duration of each state and number of episodes of each state.

3.2.1. Optogenetic stimulation of MS-PV GABAergic neuronal projections to HC did not alter the cumulative duration of individual vigilance states.

Random-state theta (8Hz) stimulation of MS-PV GABAergic projections to HC did not alter the cumulative duration of all vigilance states (Fig. 3.2.1 A-D). A slight increase in the cumulative duration of AW (light stimulation: 38.0 ± 8.4% versus control light: 27.4 ± 9.3% of total recording time) and REM sleep (light stimulation: 9.3 ± 4.0% versus control light: 4.6 ± 3.5% of total recording time) was observed upon light stimulation, which was not statistically significant (QW: $p = 0.13$; AW: $p = 0.13$; NREM: $p = 0.86$; REM: $p = 0.78$; Kruskal-Wallis test; $n = 6$, $N = 3$ for light stimulation recordings, $n = 4$, $N = 2$ for control light recordings; n = number of recordings; N = number of animals). Similarly, no statistically significant alterations regarding the cumulative duration of all vigilance states were observed when employing the REM-stimulation protocol (Fig. 3.2.2 A-D, QW: $p = 0.10$; AW: $p = 0.83$; NREM: $p = 0.23$; REM: $p = 0.13$; Kruskal Wallis test; $n = 12$, $N = 3$ for light stimulation recordings; $n = 11$, $N = 3$ for control light recordings).

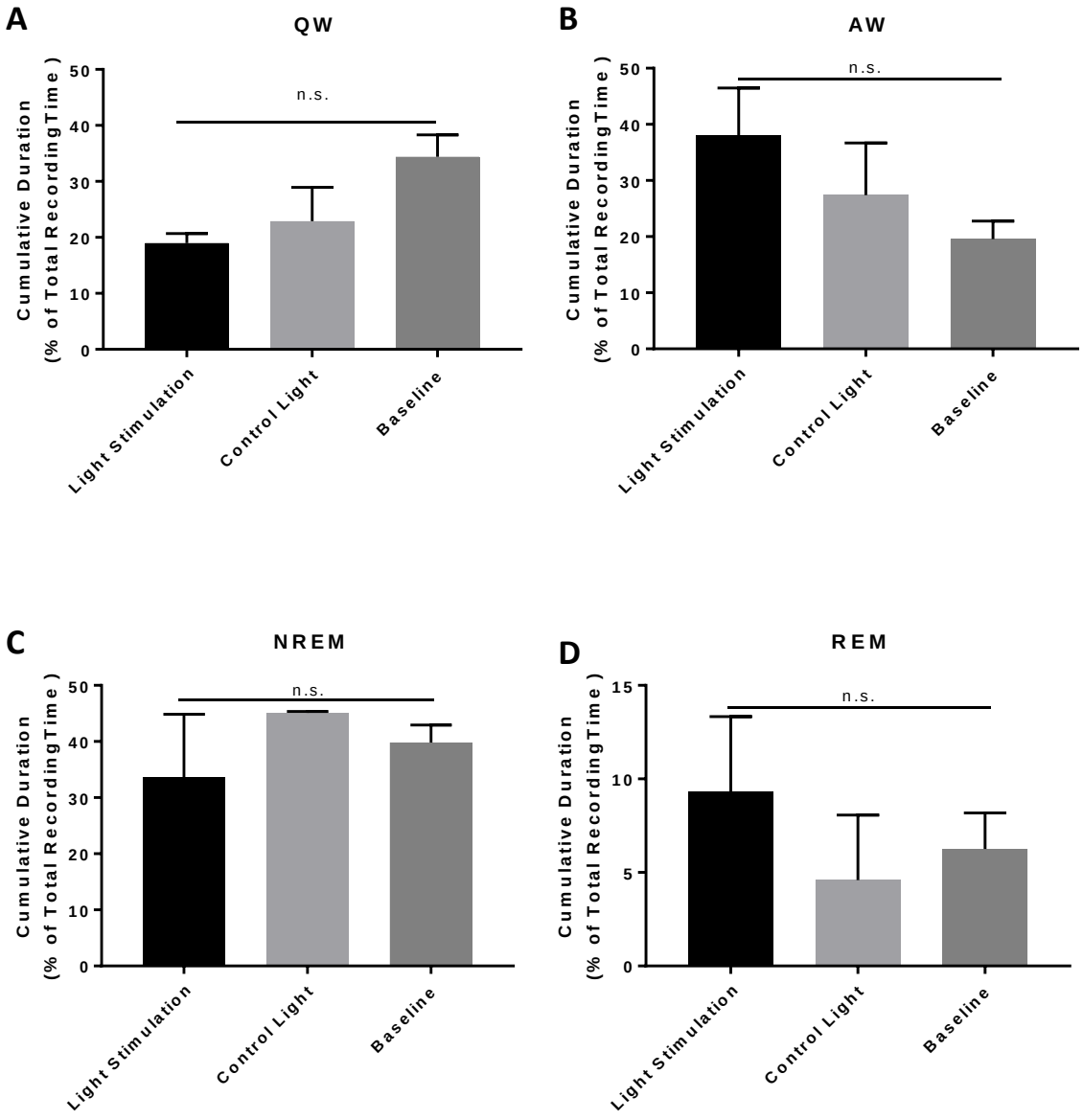

Figure 3.2.1 Cumulative duration (mean ± SEM) **as a percentage of total recording time** of **A.** Quiet wakefulness (QW) **B.** Active wakefulness (AW) **C.** NREM and **D.** REM sleep during random-state theta (8Hz) optogenetic stimulation, control light stimulation and baseline experimental sessions. No statistically significant differences between the different experimental conditions were observed (QW p= 0.13; AW p = 0.13 NREM p = 0.86; REM p = 0.78; Kruskal Wallis test; n= 6, N = 3 for light stimulation recordings; n = 4, N = 2 for control light recordings; n = 7, N = 3 for baseline recordings).

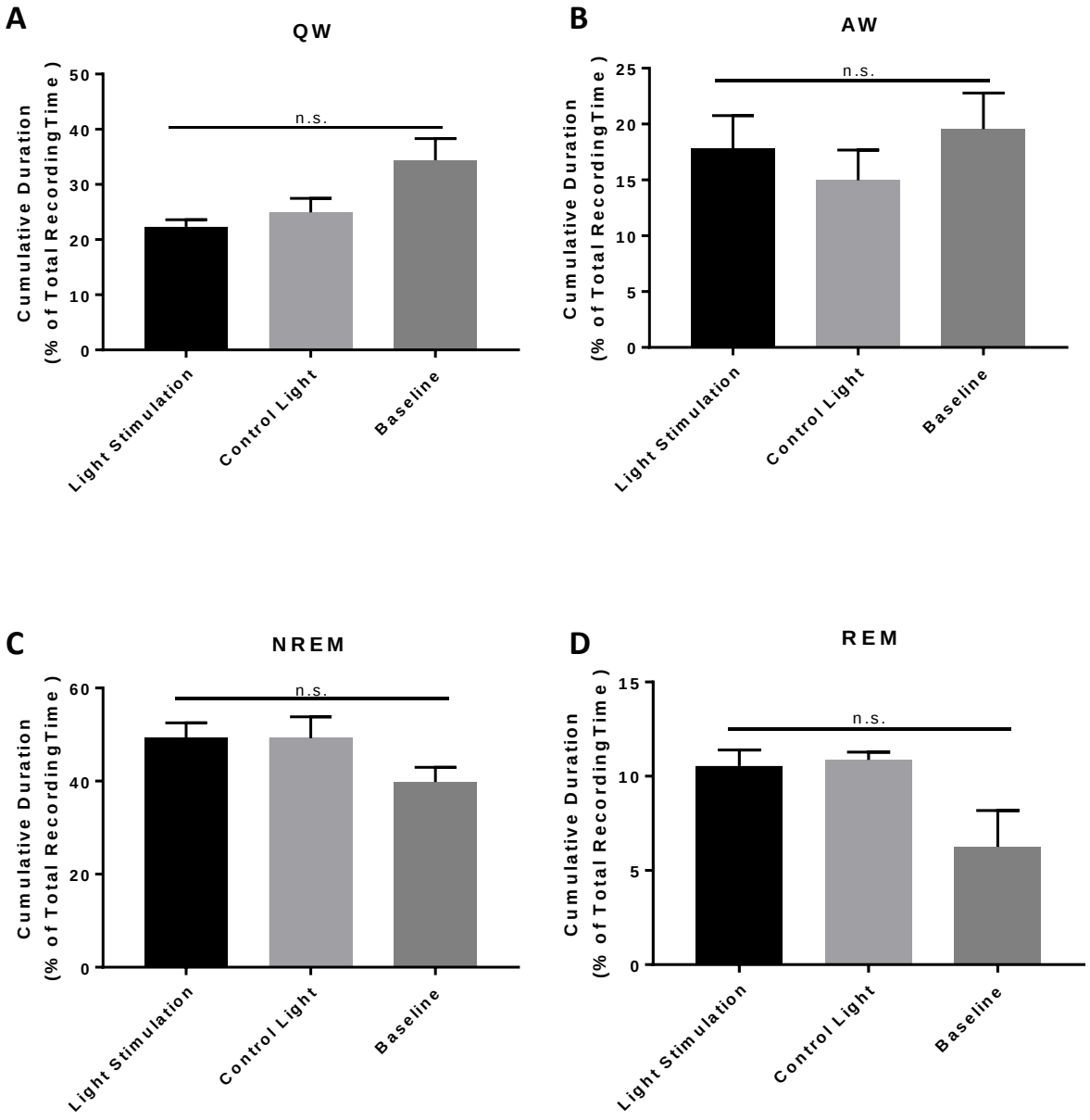

Figure 3.2.2 Cumulative duration (mean ± SEM) **as a percentage of total recording time** of **A.** quiet wakefulness **B.** Active wakefulness **C.** NREM and **D.** REM sleep during REM theta (8Hz) optogenetic stimulation, control light stimulation and baseline experimental sessions. No significant differences between the different protocols were found (QW p = 0.10; AW p = 0.83; NREM p = 0.23; REM p = 0.13, Kruskal Wallis test; n = 12, N = 3 for light stimulation recordings; n = 11, N = 3 for control light recordings; n = 7, N = 3 for baseline recordings).

3.2.2 Optogenetic stimulation of MS-PV GABAergic neuronal projections to HC did not affect fragmentation of vigilance states.

The uninterrupted period of time an animal spends in one vigilance state is defined as an episode of this state. In our vigilance state analysis we also examined the mean episode duration of each vigilance state for each protocol. The random-state theta stimulation of MS hippocampal projections did not alter the mean episode duration of QW, NREM and REM sleep (Fig. 3.2.3A, C, D; QW: $p = 0.23$; NREM: $p= 0.43$; REM: $p = 0.2$). However, a statistically significant decrease in the mean episode duration of active wakefulness (Fig. 3.2.3B; AW: $p = 0.01$) was observed in baseline, compared to the light stimulation and control light sessions (medians: 16.41; 30.48 and 27.40 s, respectively). Nevertheless, the differences between light stimulation and control light recordings ($p = 0.2$, Mann- Whitney test). In accordance with these observations, theta stimulation during REM sleep exclusively did not affect the mean episode duration of all states (Fig. 3.2.4A-D; QW: $p = 0.83$; AW: $p = 0.3$; NREM: $p = 0.82$; REM: $p = 0.72$).

Further we focused on REM sleep episode duration only during the stimulation epochs of all light sessions from all animals, in order to specifically examine the effect of theta oscillations during REM sleep on REM sleep mean episode duration. There were no differences between the light stimulation and the control light sessions (Fig. 3.2.5; 41 ± 6.4 s and 41.38 ± 3.8 s respectively; $p = 0.66$ unpaired t-test).

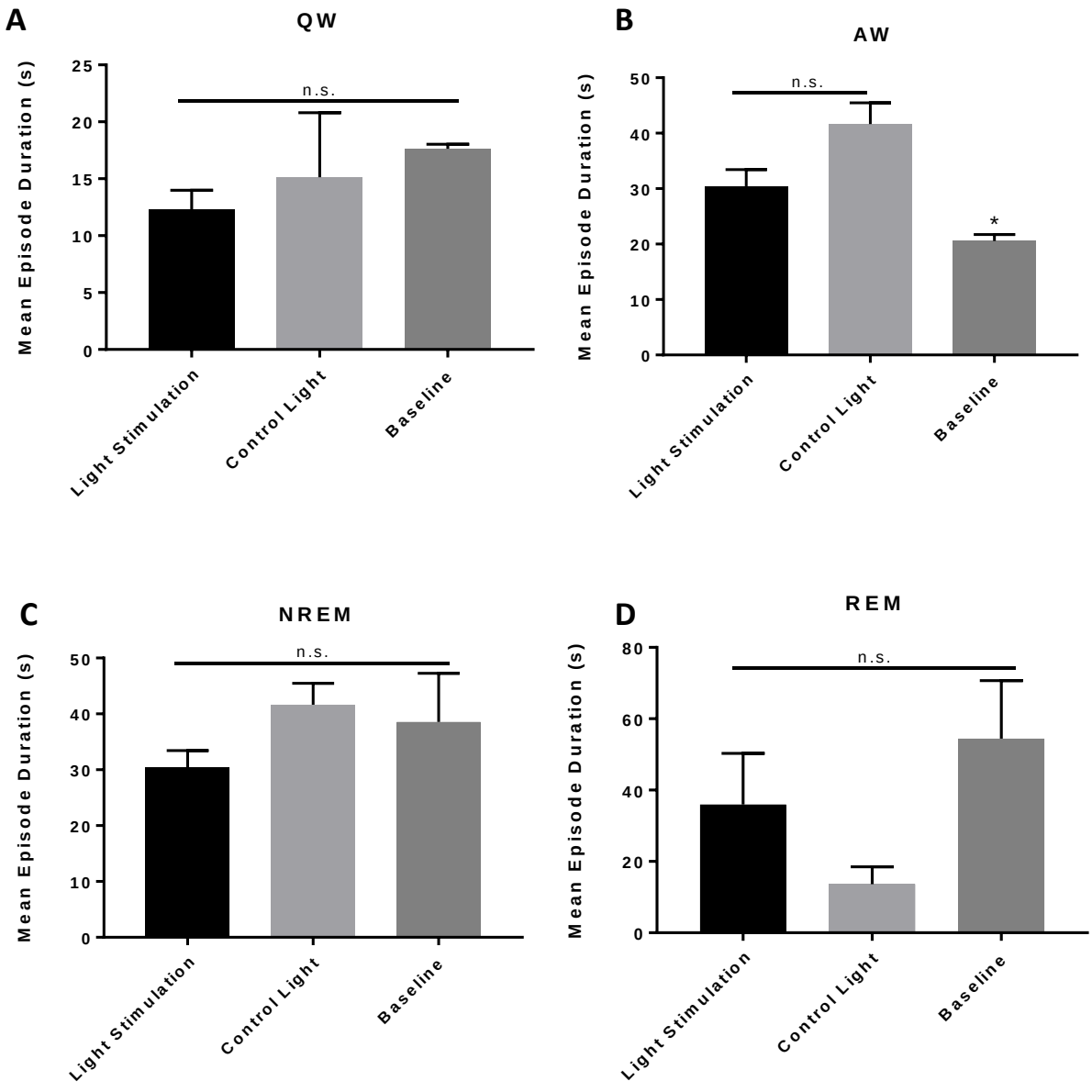

Figure 3.2.3 Mean episode duration (mean ± SEM) of **A.** Quiet wakefulness (QW) **B.** Active wakefulness (AW) **C.** NREM and **D.** REM sleep during random-state theta optogenetic stimulation, control light and baseline experimental sessions. No significant differences between the different experimental conditions except for AW p = 0.01 (*see text*) were found (QW p = 0.29; NREM p = 0.43; REM p = 0.20 Kruskal Wallis test; n = 6, N = 3 for light stimulation recordings; n = 4, N = 2 for control light recordings; n = 7, N = 3 for baseline recordings).

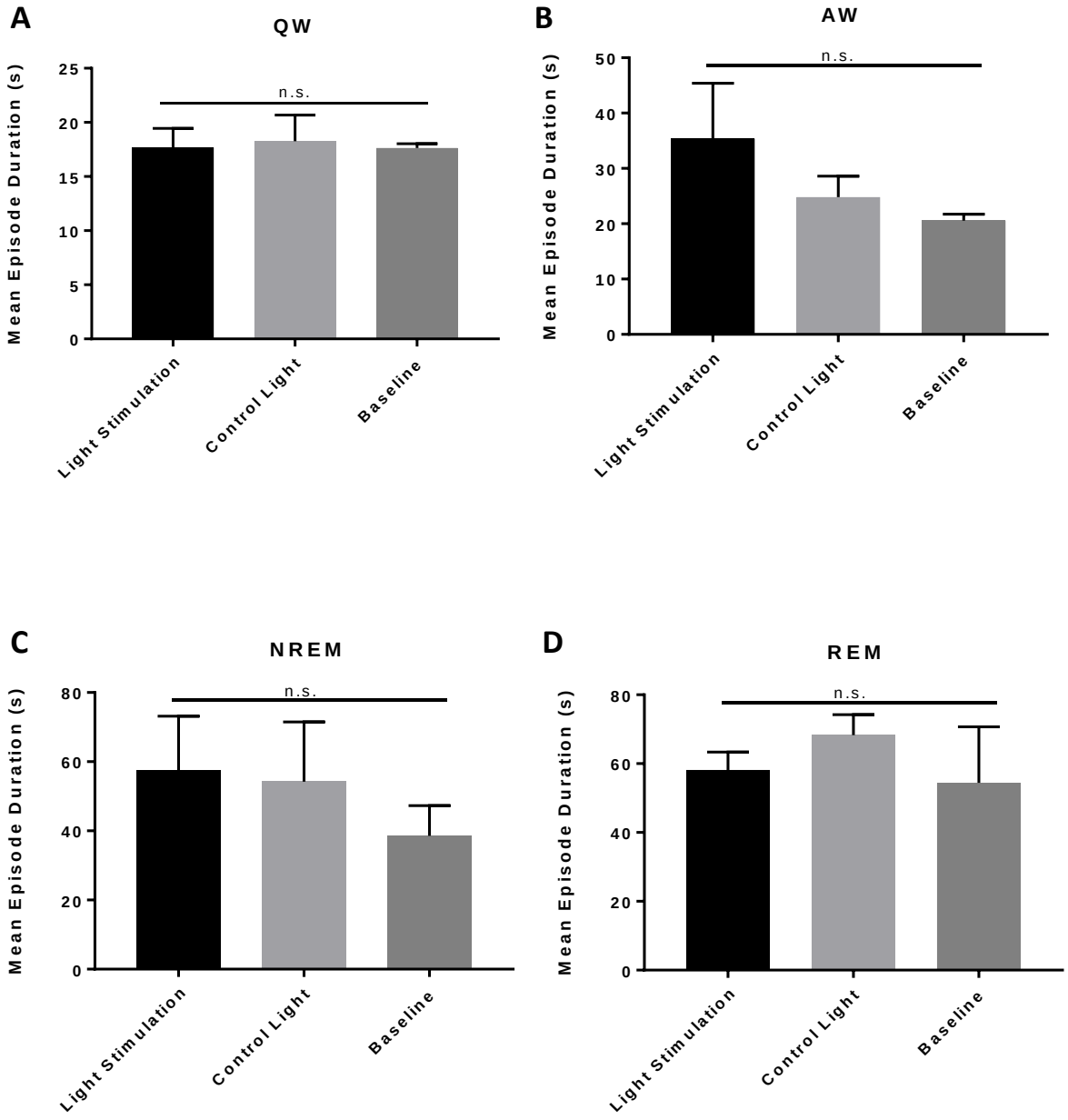

Figure 3.2.4 Mean episode duration (mean ± SEM) of **A.** Quiet wakefulness **B.** Active wakefulness **C.** NREM **D.** REM sleep during REM theta optogenetic stimulation, control light stimulation and baseline experimental sessions. No statistically significant differences between the different protocols were detected (QW p = 0.83; AW p = 0.30; NREM p = 0.83; REM p = 0.73 Kruskal Wallis test; n = 12, N = 3 for light stimulation recordings; n = 11, N = 3 for control light recordings; n = 7, N = 3 for baseline recordings).

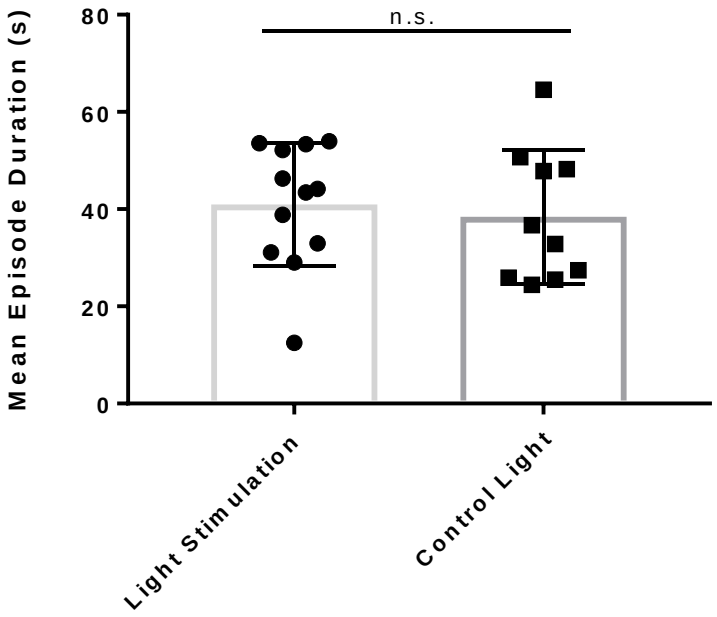

Figure 3.2.5 Mean REM episode duration within stimulation epochs (mean ± SEM) during REM theta optogenetic stimulation (light gray borders; circles) and control light (dark gray borders; squares) experimental sessions. Each symbol represents the mean REM episode duration during stimulation epochs in one experiment. No statistically significant differences between light stimulation (40.95 ± 6.4 s) and control light (41.38 ± 3.8 s) sessions were observed ($p = 0.66$ unpaired t-test; $n = 12$, $N = 3$ for light stimulation recordings; $n = 11$, $N = 3$ for control light recordings; $n = 7$, $N = 3$ for baseline recordings).

3.2.3 Optogenetic stimulation of MS-PV GABAergic neuronal projections to HC did not alter the number of episodes of individual vigilance states.

The last parameter examined within the vigilance state analysis was the number of episodes of each state for each stimulation protocol. In agreement with the cumulative duration and mean episode duration findings, the number of episodes of all vigilance states was not modified upon random state (Fig. 3.2.6; QW: $p = 0.20$; AW: $p = 0.24$; NREM: $p = 0.20$; REM: $p = 0.6$) or specifically during REM theta stimulation of MS hippocampal projections (Fig. 3.2.7; QW: $p = 0.10$; AW: $p = 0.34$; NREM: $p = 0.44$; REM: $p = 0.13$).

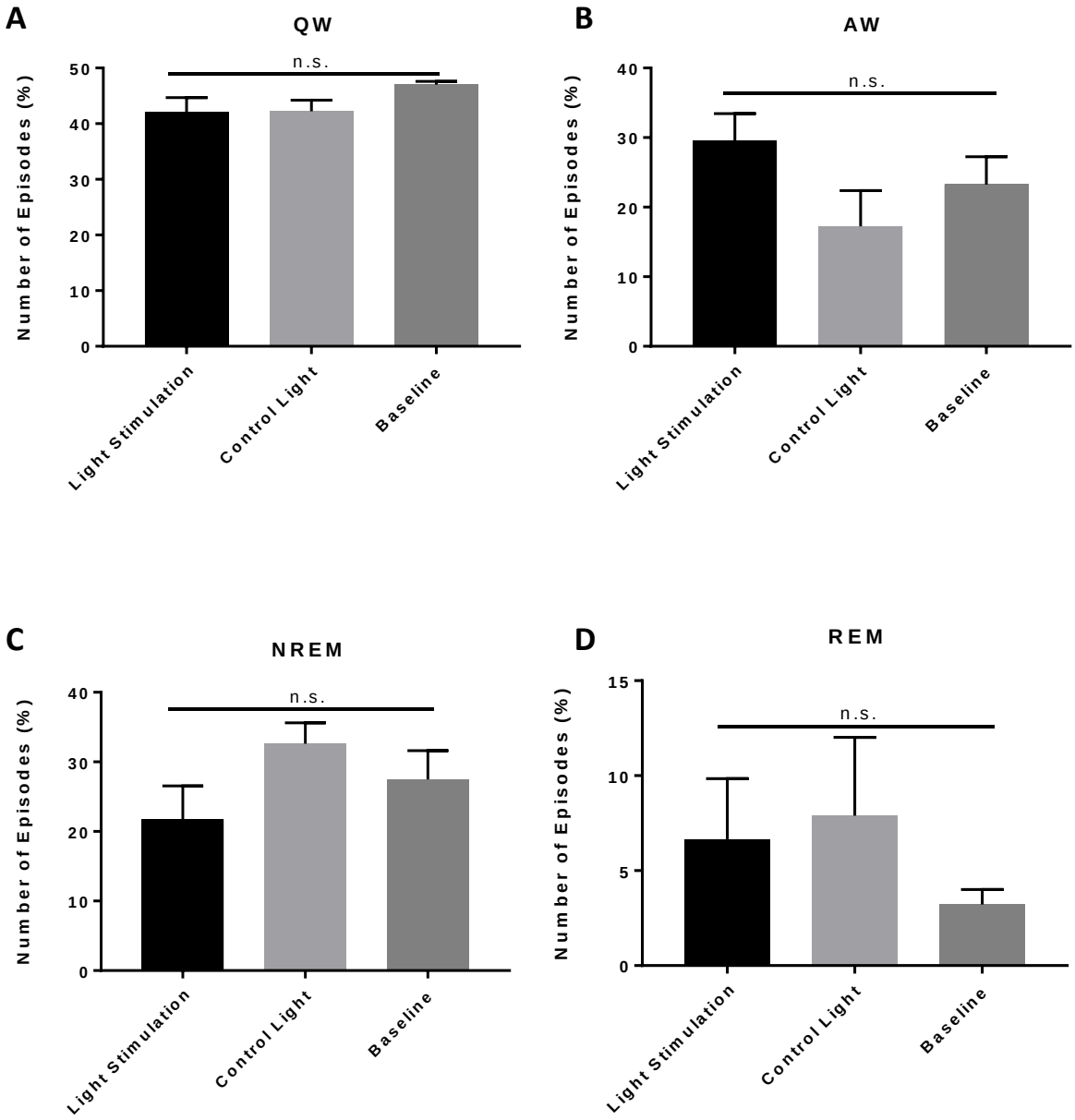

Figure 3.2.6 Number of episodes (mean ± SEM), as a percentage of total number of all vigilance state episodes (per recording). **A.** Quiet wakefulness **B.** Active wakefulness **C.** NREM and **D.** REM sleep number of episodes during random-state theta optogenetic stimulation, control light and baseline experimental sessions. No significant differences between the different experimental conditions were identified (QW: $p = 0.20$; AW: $p = 0.24$; NREM: $p= 0.20$; REM: $p = 0.60$ Kruskal Wallis test; ($n = 6$, $N = 3$ for light stimulation recordings; $n = 4$, $N = 2$ for control light recordings; $n = 7$, $N = 3$ for baseline recordings).

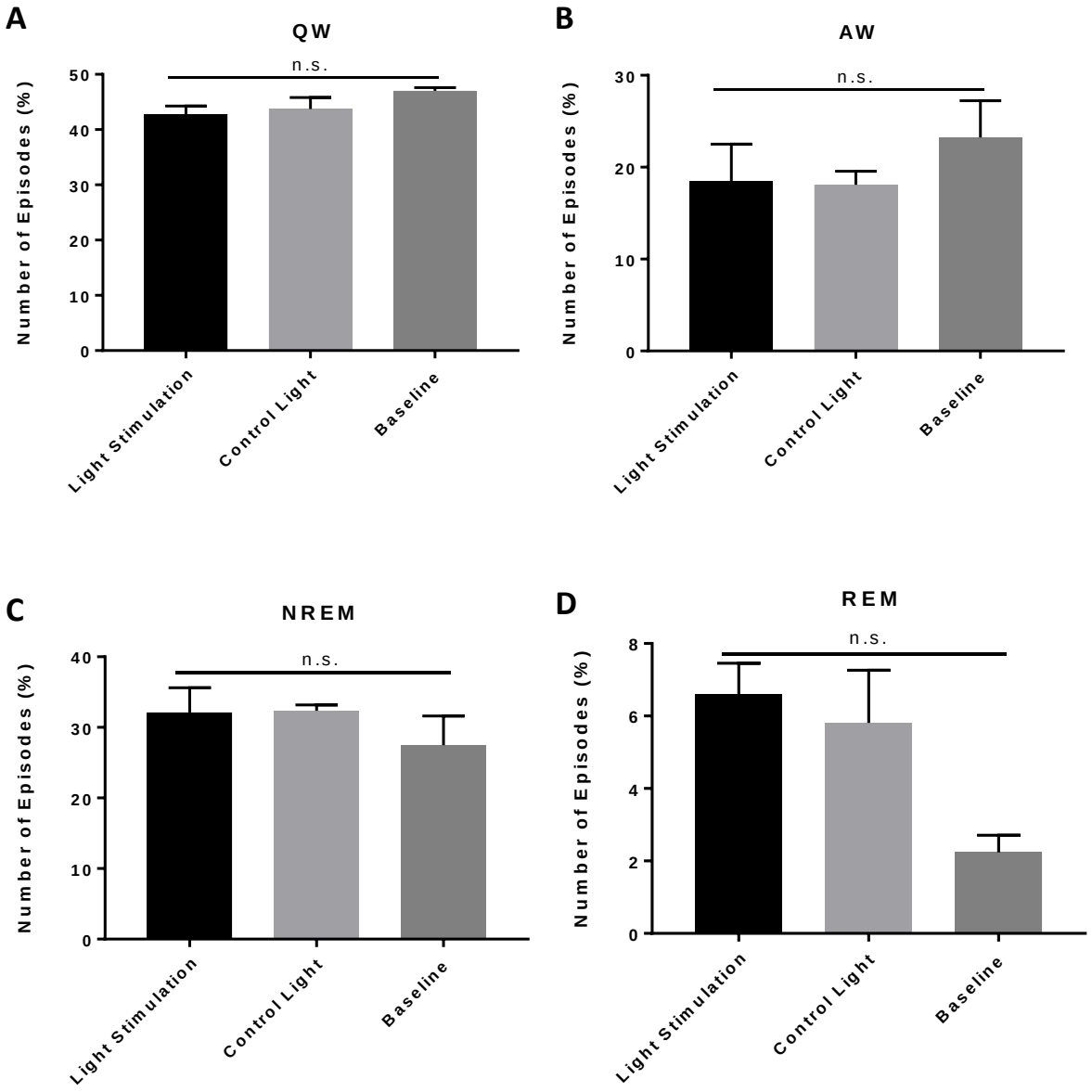

Figure 3.2.7 Number of episodes (mean ± SEM), as a percentage of total number of all vigilance state episodes (per recording). **A.** Quiet wakefulness **B.** Active wakefulness **C.** NREM **D.** REM sleep number of episodes during REM theta optogenetic stimulation, control light stimulation and baseline experimental sessions. No statistically significant differences between the different protocols were identified (QW: $p = 0.10$; AW: $p = 0.34$; NREM: $p = 0.44$; REM: $p = 0.13$ Kruskal Wallis test; $n = 12$, $N = 3$ for light stimulation recordings; $n = 11$, $N = 3$ for control light recordings; $n = 7$, $N = 3$ for baseline recordings).

3.2.4 The relationship between the number of REM episodes and the mean REM episode duration.

Aiming to gain a deeper insight in REM sleep homeostasis we examined whether the number of REM episodes recorded in our animals was correlated to the mean REM episode duration. However, no statistically significant correlations between the number of REM episodes and the mean REM episode duration were proven either for the light stimulation sessions (Fig. 3.2.8A; r = -0.33; p = 0.16) or for the control light sessions (Fig. 3.2.8B; r = -0.36; p = 0.30).

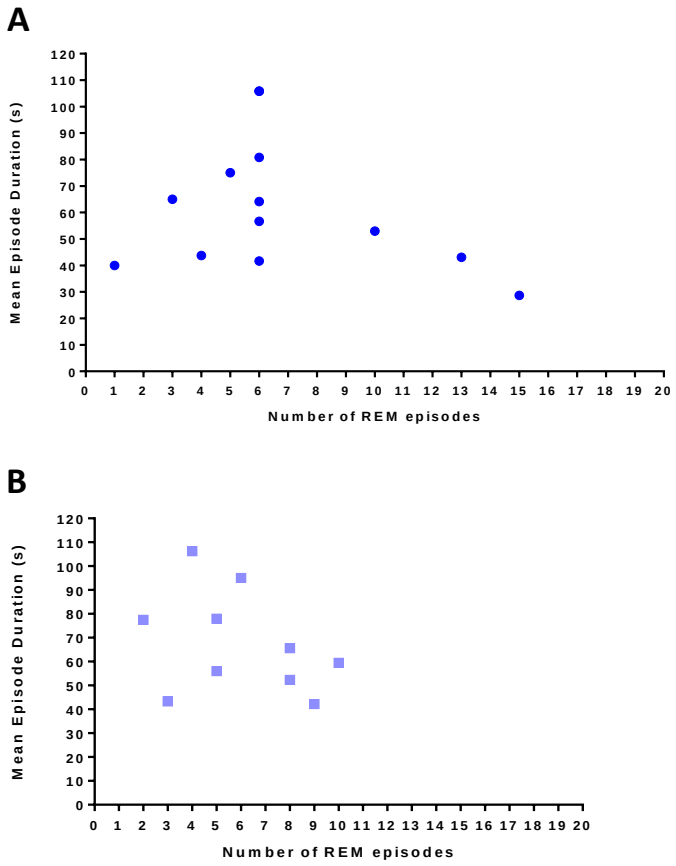

Figure 3.2.8 Relationship between mean REM episode duration and number of REM episodes for theta optogenetic stimulation during REM sleep (blue dots) and control light (light blue squares) experimental sessions. Each point represents the mean REM episode duration of one experiment in correlation with respect to the corresponding number of episodes of the same experiment. **A**. No correlation between the mean REM episode duration and the number of REM episodes upon REM theta stimulation (Pearson's r = -0.3293; p = 0.16) or **B**. upon control light (Pearson's r = -0.36; p= 0.30) conditions was identified.

3.3 Targeting of ChR2 to MS hippocampal projections during REM sleep reduced the REM intervals

The time intervals between REM sleep episodes represent a biological trait which is believed to be under specific regulation (reviewed in Parmeggiani and Velluti, 2005) and it is an important parameter when studying REM sleep regulatory aspects. Consequently, we examined the effect of optogenetically entrained hippocampal theta oscillations during REM sleep on the intervals between consequent REM sleep episodes. Therefore, the latency to the next REM sleep episode was significantly reduced in the light stimulation experiments compared to control light experiments (Fig. 3.3. 1; 362.2 ± 55.4 and 613.5 ± 63.1 s for light stimulation and control light experiments respectively; p = 0.04; unpaired t-test).

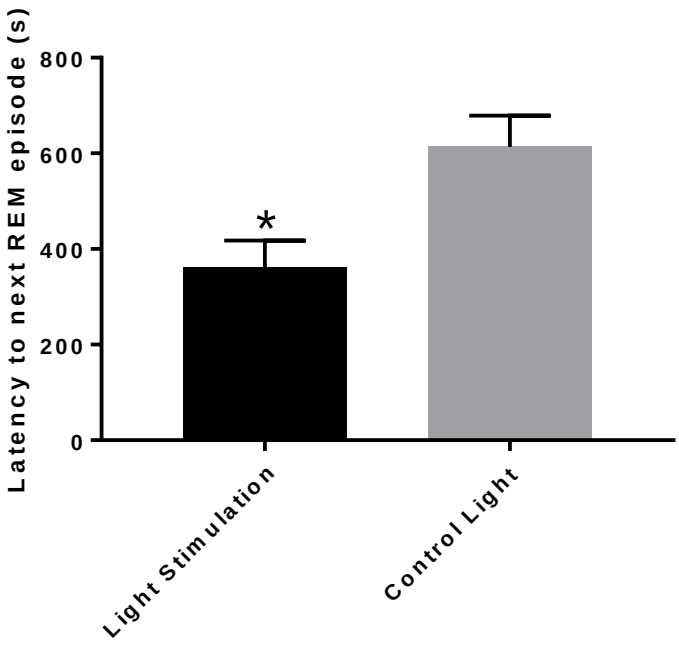

Figure 3.3.1 Latency to the next REM episode initiation (mean ± SEM). Stimulation reduced the latency the next REM episode initiation (362.2 ± 55.35 s) during REM theta optogenetic stimulation compared to the control light sessions (613.5 ± 63.12 s; p = 0.0429; unpaired t-test; n = 12, N = 3 for light stimulation recordings; n = 11, N = 3 for control light recordings).

Furthermore we examined the relationship between REM sleep intervals and the duration of the following REM episode. Even though the relationship between the duration of a REM sleep episode and the previous REM sleep interval remains to be further determined according to the literature (Parmeggiani and Velluti, 2005), in our study the duration of REM sleep episodes appeared to be positive correlated to the previous REM sleep interval in control light sessions (Fig. 3.3.3A Spearman's r = 0.2529; p = 0.0375). Interestingly, in light stimulation sessions this relationship was disrupted and the duration of REM sleep episodes showed a tendency to be negatively correlated to the previous REM sleep interval, which was not statistically significant however (Fig. 3.3.3B Spearman's r = - 0.068; p = 0.52).

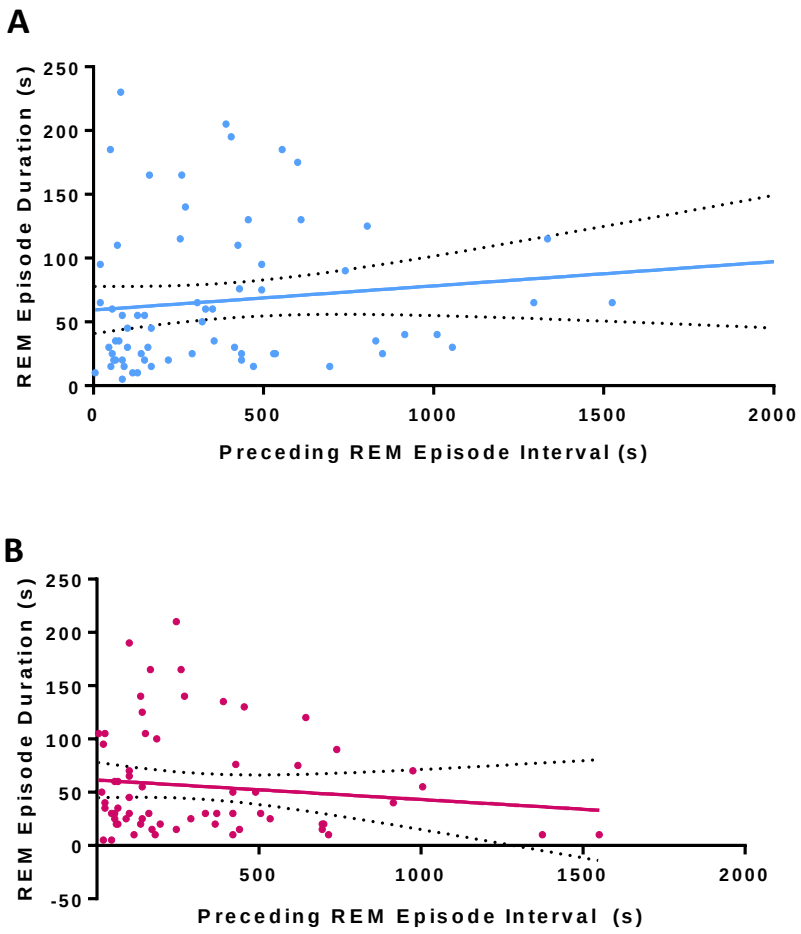

Figure 3.3.3 REM episode duration with respect to the preceding interval. A. During control light sessions (light blue) the duration of REM sleep episodes was proved to be positive correlated to the previous REM sleep interval (Spearman's r = 0.25; p = 0.04; slope: 0.02 ± 0.02). **B.** Theta optogenetic stimulation during REM sleep (magenta) disrupted the positive correlation between the duration of REM sleep episodes and the previous REM sleep interval (Spearman's r = - 0.07; p = 0.52). Dotted lines define the 95% confidence intervals.

3.4. Theta entrainment fidelity: implications in vigilance state transitions and REM episode duration

Besides the examination of sleep homeostasis upon theta stimulation of MS-PV GABAergic neurons, it was crucial to validate how efficient was our preparation in studying the role of theta oscillations in REM sleep regulation. The first step in the validation process of theta rhythm entrainment at 8Hz during REM sleep, was to examine the local field potential (LFP) signal from the electrodes implanted in HC and the corresponding power spectra. In the absence of optogenetic stimulation or during control light sessions, theta oscillations during REM sleep appeared to have a fluctuating frequency from 10 to 12 Hz (Fig. 3.4.1C-F and B-E, respectively) However, during the optogenetic stimulation of MS hippocampal projections the power spectra of HC LFP demonstrated a peak at 8Hz, in agreement with stimulation frequency (Fig. 3.4.1A-D).

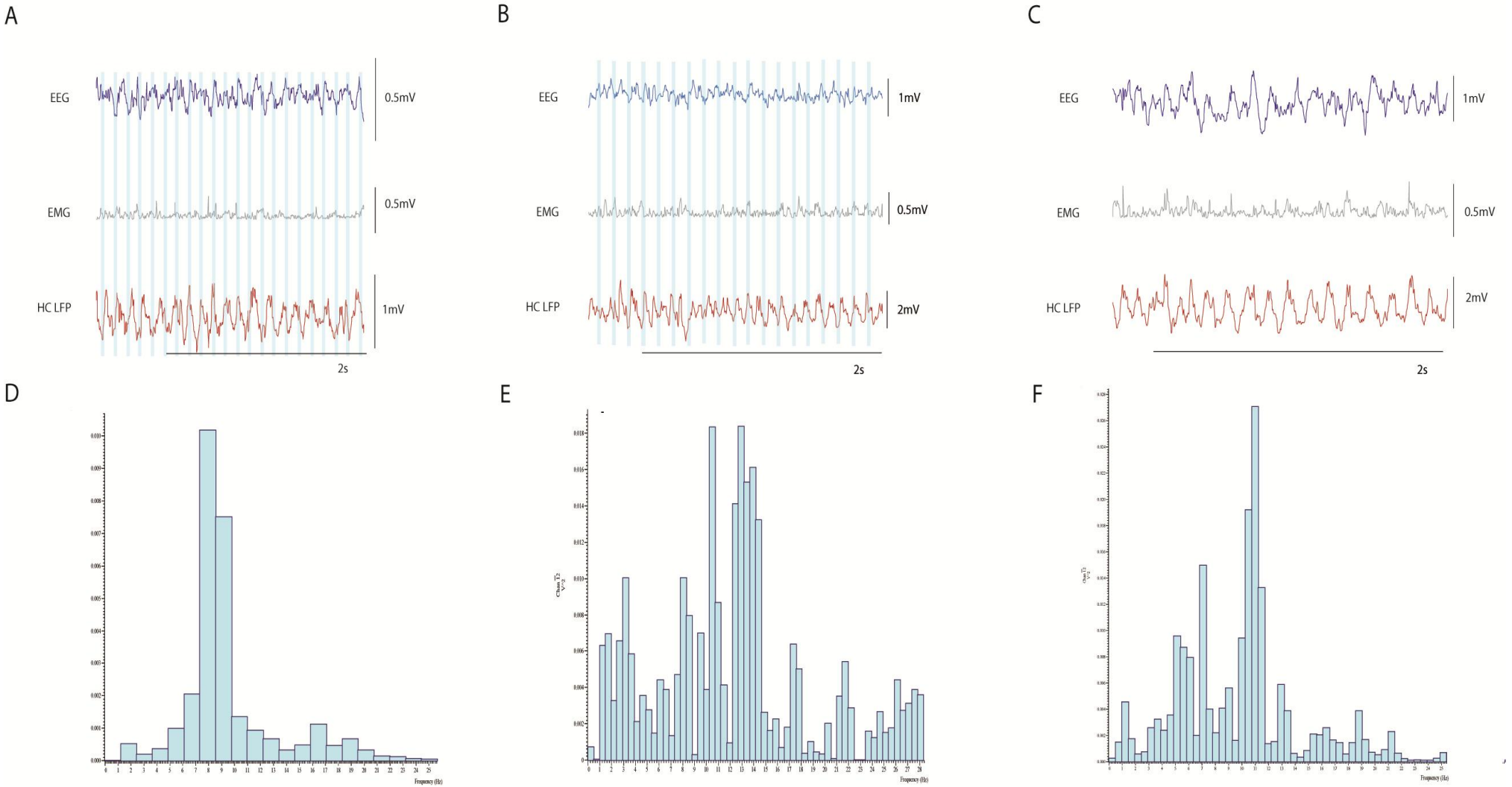

Figure 3.4.1 Entrainment of theta oscillations during REM sleep. A. Representative LFP signal traces from HC during REM sleep theta (8Hz) optogenetic stimulation (red) **B.** Representative LFP signal traces from HC during REM sleep 8Hz control light **C.** Representative LFP signal traces from HC during REM sleep during baseline recording **D. E.** and **F.** Corresponding power spectra of these HC LFP signals for light stimulation, control light and baseline, respectively. All selected traces were derived from recordings of the same animal. Power spectra were calculated using Hamming window and 1024 FFT size for all traces. EEG corresponds to electroencephalogram trace (blue) and EMG to electromyogram (grey) traces during these REM sleep epochs. Light pulses are indicated with light blue lines.

Following this rough estimation we proceeded to a more accurate method in order to evaluate the efficacy of theta oscillation control and correlate it with our findings. In order to achieve this goal we calculated the fidelity of the entrainment for all recordings as described in Bender et al. (2015). First, we assessed whether the fidelity of theta entrainment is dependent on the behavioral state during which stimulation takes place. The results showed that the fidelity of theta entrainment was not dependent on the state during which stimulation takes place (Fig. 3.4.2A $p = 0.95$; Kruskal Wallis test). However, the fidelity values, for experiments with high fidelity (>0.3), around the transition points from QW to NREM sleep were lower compared to the fidelity values around NREM to AW transitions (Fig. 3.4.3, $p = 0.0373$; Kruskal Wallis test and $p = 0.015$ between the two groups; Mann- Whitney test; $n = 3$, $N = 1$).

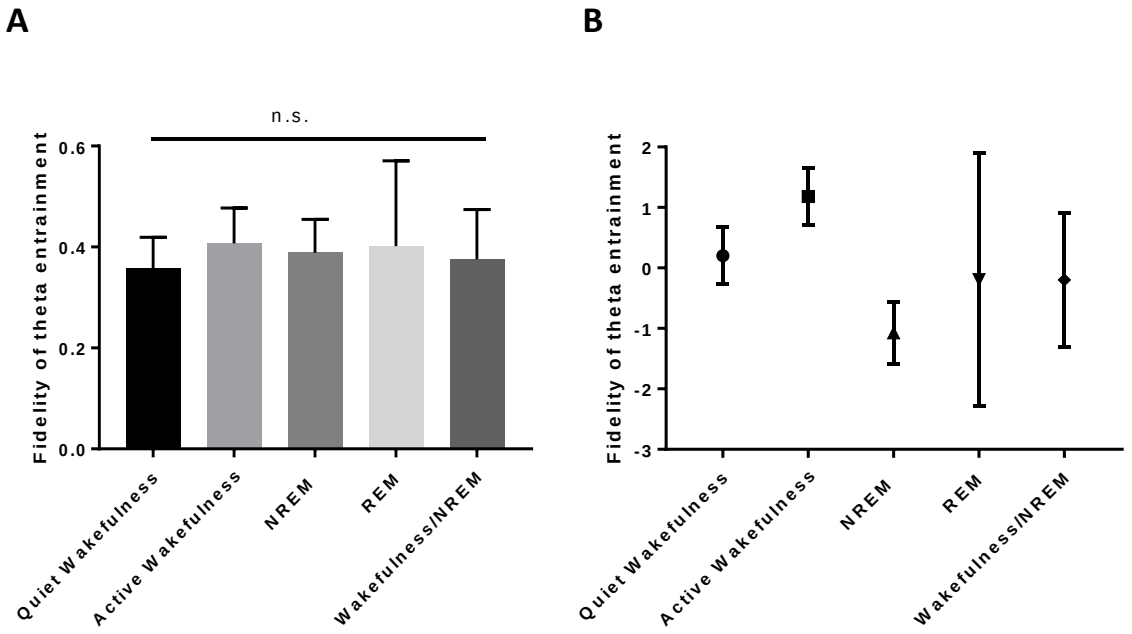

Figure 3.4.2 Fidelity of theta oscillation (mean ± SEM) **entrainment during the different vigilance states. A.** There were no statistically significant differences in the fidelity of theta oscillation entrainment between the different vigilance states ($p = 0.95$; Kruskal Wallis test; $n = 6$, $N = 3$,). **B.** Z-transformation of A data set ($p = 0.2$; Kruskal Wallis test).

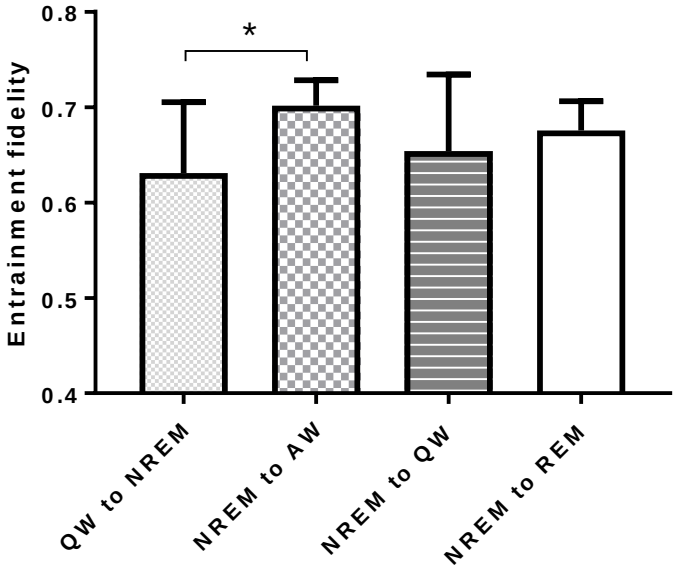

Figure 3.4.3 Fidelity of theta oscillation entrainment (mean ± SEM) **around transitions.** Fidelity entrainment was calculated for 5s before and 5s after the transition. An increase in fidelity of theta entrainment was detected around NREM to AW transitions (0.70 ± 0.02, median= 0.71) compared to QW to NREM transitions (0.63 ± 0.01, median = 0.66; p = 0.015 Mann Whitney test between the two and p = 0.037 Kruskal Wallis test between all groups n = 3, N = 1).

Finally, we examined whether theta entrainment fidelity of the entire recording was correlated with the mean REM episode duration in both protocols. The fidelity of theta oscillation entrainment during REM sleep stimulation and the mean REM episode duration were not correlated (Fig. 3.4.4 Pearson's r = -0.47; p = 0.14). Similar results were noted for the automated random state theta stimulation protocol recordings (Fig. 3.4.3 Pearson's r = -0.49; p = 0.32). However, a slight tendency for a negative correlation between mean REM episode duration and the fidelity of the entrainment can be observed.

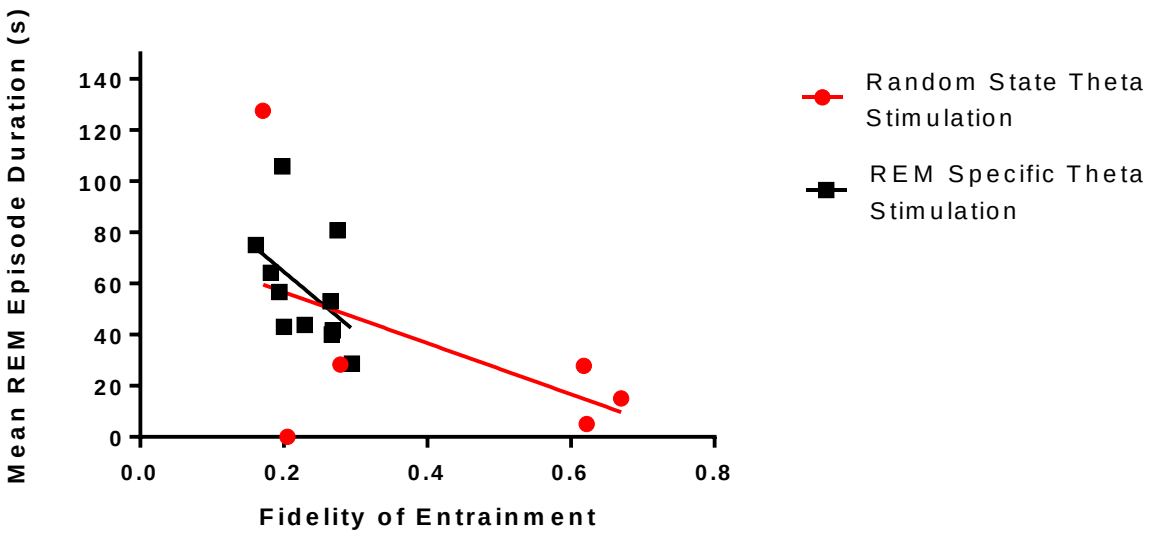

Figure 3.4.4 Mean REM episode duration is independent from theta entrainment fidelity. Mean REM episode duration with a respect to the fidelity of theta entrainment during random state (red dots) and during REM optogenetic theta stimulation (black squares). No statistically significant relationship between the two parameters was proven under random state or REM optogenetic theta stimulation (Pearson's r = -0.47, p = 0.14 and Pearson's r = -0.49, p = 0.32, respectively).

4. Discussion

Pilot studies are one of the fundamental parts in research process and represent one of the best ways to examine not only the feasibility of an approach but also provide clues about the direction of the large scale experiments that will follow. In this pilot study, we completed the initial steps in exploring the role of MS-PV GABAergic neurons and hippocampal theta oscillations in REM sleep regulation in mice.

We combined EEG, EMG and electrophysiological recordings in behaving mice with optogenetic stimulation of the MS-PV GABAergic neuronal projections to hippocampal CA1 region in order to examine whether these neurons constitute a part of REM sleep regulatory circuit. Specifically, since MS-DBB neurons are considered to be the fundamental theta pacemakers, supplying phase modulation to hippocampus (Petsche et al., 1962; Buzsaki, 2002), this approach provided us with the handles to investigate the involvement of theta oscillations in REM sleep regulation. By employing a modified version of the mouse preparation for controlling theta oscillations during locomotion, previously developed in our lab (Bender et al.,

2015), and two different approaches regarding the state specificity of theta oscillation entrainment, our results suggest that MS-DBB neurons and theta rhythm during REM sleep could have a role in the regulation of REM sleep intervals, rather than in the actual duration of REM sleep episodes.

4.1 Impact of MS hippocampal projections on sleep structure

In our study, we examined the time intervals between REM sleep episodes as they represent a biological trait which is believed to be under specific regulation (Zamboni et al., 1999). The results showed that theta-8Hz stimulation of MS hippocampal projections during REM sleep reduced the time interval between consequent REM episodes by nearly 4 minutes compared to the control light stimulation experiments. Moreover, optogenetic stimulation of MS hippocampal projections during REM sleep disrupted the positive correlation between REM episode duration and the previous REM sleep interval which was observed in control light sessions. These findings indicate that these neurons and theta rhythm may play a role in the REM propensity that is discharged during the episode as in the homeostatic model of REM sleep-timing, the interval between consequent REM sleep episodes is affected by the REM propensity discharged in the preceding REM episode (Hartman 1966; Beninghton and Heller, 1994). Additional analysis at the scale of REM sleep cycle in each recording could reveal more information regarding the balance in REM sleep timing between the cycles and possible alterations in wakefulness and NREM sleep episode duration within these intervals.

Moreover, it is worth noting that specific attention should also be paid at the "intermediate" state between NREM to REM sleep. This intermediate state is characterized by the appearance of short bouts of high amplitude and high frequency spindle-like waves in the EEG and is accompanied by the initiation of theta rhythm in hippocampus (Gottesman, 1996). In rats this state does not always result in REM sleep consolidation and according to previous studies conducted by Mandille et al. and Franken, such episodes were followed by wakefulness at 50% of the times (Mandille et al., 1996; Franken, 2002). In our experiments, this intermediate state was apparent in nearly all our baseline, control light and stimulation sessions however, since our primary goal at this stage was to assess the effect of theta oscillations in the "big picture" of REM sleep architecture, we included it in NREM sleep episodes. This small time period of transition may represent the time at which autonomic structures control for the

possibility to leave a behavioral state with fully operating homeostatic regulation like NREM sleep, before proceeding to a state where the same homeostatic regulatory mechanisms are inhibited such as in REM sleep (Parmeggiani and Velluti, 2005). MS-PV GABAergic neurons and theta rhythm could be a part of a circuit that "decides" whether to leave NREM and enter REM sleep since they are wired with numerous cortical (i.e. entorhinal cortex) and subcortical regions including midbrain and hypothalamus, the principal regulator of autonomic processes, temperature regulation and circadian rhythms. Finally, it is worth noting that these neurons receive functional input from MCH neurons of LHA and the activation of MCH neurons prolongs the duration of REM sleep, providing causal evidence for the involvement of LH networks in REM sleep regulation (Jego et al., 2013). However, this circuit is not the only one participating in REM sleep duration regulation. During our study we investigated the role of MS-PV GABAergic neurons as a potential part of these additional regulatory circuits responsible for REM sleep duration.

Aiming to address the role of MS -PV GABAergic neurons and specifically theta rhythm in the regulation of REM sleep duration, we examined the effect of theta rhythm entrainment during random states and during REM exclusively, in vigilance state architecture parameters. Theta rhythm entrainment during different vigilance states or during REM sleep exclusively did not change the vigilance state architecture in a statistically significant manner, regarding the cumulative duration, the mean episode duration and the number of episodes of each vigilance state. These results agree with the findings of a recent study, where optogenetic inhibition of the same neurons and theta rhythm reduction during REM sleep did not alter either cumulative duration or the mean episode duration of all vigilance states (Boyce et al., 2016). However, a tendency for an increase in REM sleep total duration was noted only in the random automated protocol recordings, which was also accompanied by high theta entrainment fidelity during the preceding NREM episode in most of the cases. These observations suggest that theta rhythm could be involved in the regulatory mechanisms, providing supporting evidence to the hypothesis that it is operating in the transition period from NREM to REM sleep, and affect homeostatic regulatory circuits. Additional experiments focusing on theta entrainment and inhibition during NREM sleep exclusively as well as during the "intermediate" state between NREM to REM sleep could provide reliable evidence to build the background for this hypothesis.

Furthermore, it should be noted that the experiments of theta rhythm entrainment exclusively during REM sleep were accompanied with a relatively low fidelity (~0.2) value compared to the fidelity values extracted from Bender et al. behavioral experiments during locomotion (Bender et al., 2015). Even though our data from the random state stimulation protocol suggest that the fidelity of the entrainment is not state dependent, it should be taken into account that differences in the fidelity of entrainment were noted around NREM to AW transitions and the fact that the number of REM episodes accompanied by optogenetic stimulation during these recordings was very low. This low fidelity could result from methodological reasons including the low number of animals and the variability in ChR2 expression in each animal. Furthermore, the emergent nature of theta oscillations should also be taken into account as a possible explanation for these low fidelity values (Sohal, 2009; Goutagny et al., 2009) and finally the possibility of a different mechanism in theta generation during REM to be involved (Hutchison and Rathore 2015) since higher fidelity values of theta entrainment (>0.3) were noted during active wakefulness stimulation experiments (Bender et al., 2015). Nonetheless, longer in duration sleep recordings employing a random state stimulation protocol, or even recordings employing state specific stimulation protocols for all vigilance states could provide further evidence regarding the relationship between theta entrainment fidelity and the vigilance state, or even establishing distinct fidelity threshold values for each state.

4.2 Conclusion

Sleep disorders constitute an important public health problem and can interfere with normal physical mental social and emotional functioning of people who suffer from them. The crucial role of gamma-amino butyric acid (GABA) in sleep induction and maintenance is well established. Still nowadays, most insomnia treatments including barbiturates, benzodiazepines (Visser et al., 2003) even the third generation Z-drugs (Mohler, 2006), target GABAergic transmission, by binding to specific sites of GABAa receptors. Multiple populations of GABAergic neurons are involved in sleep regulation. Consequently, the delineation of all GABAergic neuronal populations that mediate the effects of GABA on sleep is critical in order to design specific treatments for the different sleep pathologies (Luppi et al., 2016). Combining these suggestions with Buzsaki and Watson view on sleep as an example of persistent activity (Buzsaki and Watson, 2012) and the potential role of brain oscillations in the mechanism that

controls sleep regulation, we could add that not only the characterization of neuronal populations that mediate for specific effects in sleep regulation is essential, but also their oscillatory/firing profile should be determined. If so, then the design of even more specific treatments with the incorporation of deep brain stimulation could provide a more effective way to treat sleep disorders.

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7. Supplementary Material

7.1 List of abbreviations

AW:	active wakefulness
ChR2:	channelrhodopsin-2
dDpMe:	dorsal deep mesencephalic reticular nuclei
DPGi:	dorsal paragigantocellular reticular nucleus
DR:	dorsal raphe
EEG:	electroencephalogram
eVLPO:	extended ventrolateral preoptic area
FFT:	fast Fourier transformation
FTG:	gigantocellular tegmental field
HC:	hippocampus
i.p.:	intraperitoneally
LC:	locus coeruelus
LDT:	laterodorsal tegmental nucleus
LH:	lateral hypothalamus
MCH:	melanin concentrating hormone
MS-DBB:	medial septum-diagonal band of Broca
NREM:	non REM
PnC:	pontiscaudalis
PnO:	pontisoralis
PS:	paradoxical sleep
PSD:	power spectral density

PV: parvalbumin

QW: quiet wakefulness

REM: Rapid- eye- movement

SLD: sublaterodorsal nucleus

SubC: subcoeruleus nucleus

TMN: tuberomammillary nucleus

vIPAG: ventrolateral periaqueductal gray

vIPAG: ventrolateral periaqueductal gray

VM: ventral medulla

VMM: mentromedial medulla

7.2 Figures

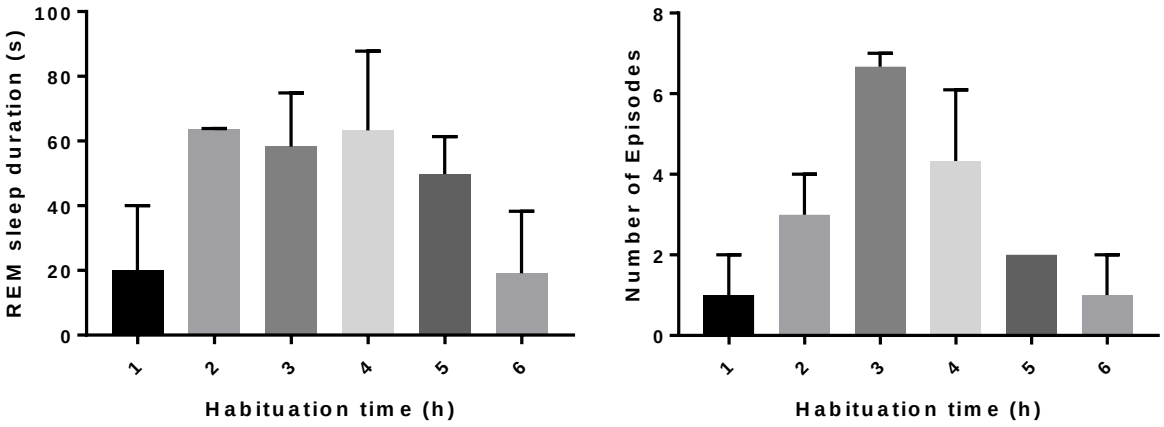

Figure S1. Increase in REM sleep total duration and number of REM sleep episodes after 2 hour habituation in the sleep promoting environment. Data obtained from preliminary 6-hour baseline recordings (n = 2).

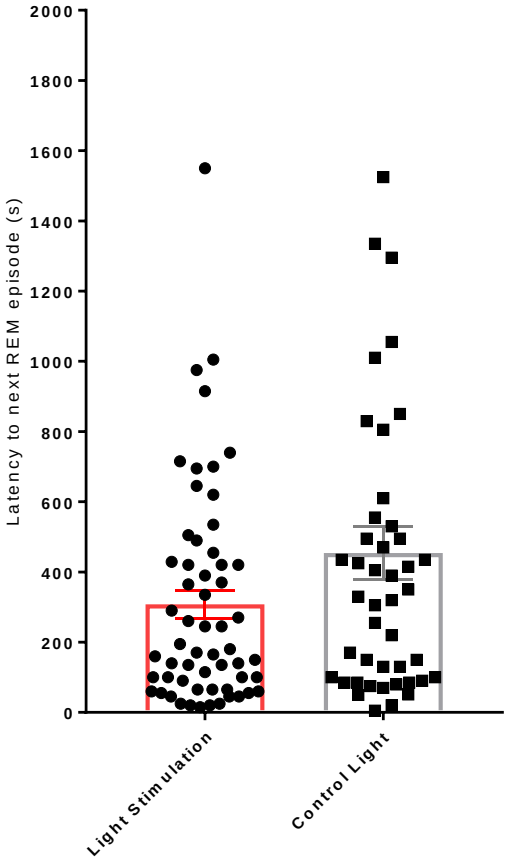

Figure S2. Analytical representation of raw data (represented by the symbols) of Fig. 3.3.1 (Latency to the next REM episode initiation) for theta (8Hz) optogenetic stimulation during REM sleep (red border bar-black circles) and control light (light gray border bar-black squares) experimental sessions. In this graph, each symbol corresponds to one REM episode interval and REM episode intervals are pooled from all stimulation or control light recordings respectively.

Περίληψη

Ο ύπνος REM (rapid-eye-movement) αποτελεί ξεχωριστό στάδιο του ύπνου και οι υπεύθυνοι για την ρύθμισή του μηχανισμοί δεν έχουν αποσαφηνιστεί πλήρως. Ο κυριότερος εγκεφαλικός ρυθμός στο ηλεκτροεγκεφαλογράφημα (EEG) αλλά και στην περιοχή του ιππόκαμπου κατά τη διάρκεια του ύπνου REM είναι ο θήτα (5-10 Hz). Οι νευρώνες της περιοχής του μέσου διαφράγματος και της διαγώνια ζώνης του Broca (medial septum- diagonal band of Broca, MS-DBB) θεωρούνται οι θεμελιώδεις βηματοδότες του ρυθμού θήτα. Πιο συγκεκριμένα, σημαντικοί στον καθορισμό του ρυθμού θήτα είναι οι MS- GABAεργικοί νευρώνες που εκφράζουν την παρβαλβουμίνη (parvalbumin positive, PV) και σχηματίζουν συνάψεις με ενδονευρώνες της περιοχής CA1 του ιππόκαμπου και αίρουν την αναστολή που ασκούν στους πυραμιδικούς νευρώνες με ρυθμικό τρόπο. Στην παρούσα πιλοτική μελέτη εξετάσαμε τον πιθανό ρόλο των MS-PV GABAεργικών νευρώνων και των θήτα ταλαντώσεων στη ρύθμιση του ύπνου REM. Προκειμένου να διερευνήσουμε το συγκεκριμένο ερώτημα συνδυάσαμε *in vivo* ηλεκτροφυσιολογικές καταγραφές σε ελεύθερα κινούμενα ποντίκια με οπτογενετική ενεργοποίηση (optogenetic stimulation) των προβολών των MS-PV GABAεργικών νευρώνων στην περιοχή CA1του ιππόκαμπου, κατά τη διάρκεια διαφορετικών σταδίων εγρήγορσης. Τα αποτελέσματά μας έδειξαν πως η οπτογενετική ενεργοποίηση με συχνότητα 8 Hz των προβολών των MS-PV GABAεργικών νευρώνων στον ιππόκαμπο κατά τη διάρκεια του ύπνου REM, μείωσε τα ενδιάμεσα διαστήματα μεταξύ διαδοχικών επεισοδίων ύπνου REM κατά περίπου 4 λεπτά σε σχέση με τα αντίστοιχα πειράματα έλεγχου για το φώς (control light) και διατάραξε τη σχέση μεταξύ της μέσης διάρκειας επεισοδίων ύπνου REM και του προηγούμενου ενδιάμεσου διαστήματος μεταξύ επεισοδίων REM, χωρίς να επηρεάσει την αρχιτεκτονική του ύπνου και των υπόλοιπων σταδίων εγρήγορσης. Επιπλέον, δείχθηκε ότι η πιστότητα στην επαγωγή των θήτα ταλαντώσεων στα μεταβατικά διαστήματα από ύπνο non REM (NREM) σε εγρήγορση ήταν υψηλότερη σε σχέση με εκείνη στα μεταβατικά διαστήματα από εγρήγορση σε NREM. Τα πρωταρχικά αυτά αποτελέσματα υποδεικνύουν πως οι MS-PV GABAεργικοί νευρώνες και ο ρυθμός θήτα εμπλέκονται στο δίκτυο του εγκεφάλου που ρυθμίζει τον ύπνο REM.