Diversity of simultaneous sleep in the motor cortex and hippocampus in rats

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Summary

We investigated the homogeneity/heterogeneity of spontaneous sleep, simultaneously recorded in the motor cortex and the hippocampus of control rats, and particularly analyzed simultaneous and non-simultaneous motor cortical and hippocampal non-rapid eye movement (NREM)/rapid eye movement (REM) sleep.

We demonstrate that the sleep architectures of motor cortex and hippocampus are different in control rats. There was an increase of NREM duration and a decrease of REM duration in the hippocampus versus the motor cortex. In terms of duration, NREM state is the most heterogeneous in the hippocampus, while the REM state is the most heterogeneous in the motor cortex. Whereas the hippocampal NREM duration was increased due to the prolongation of NREM episodes, the hippocampal REM duration decreased due to the decreased number of REM episodes.

The heterogeneity of sleep in the motor cortex and hippocampus in control rats was particularly expressed through the inverse alteration of: sigma amplitude during NREM sleep and beta/gamma amplitudes during REM sleep in the hippocampus along with the delta, sigma, beta and gamma amplitudes only during non-simultaneous NREM/REM sleep in the hippocampus.

We demonstrated the brain structure-related and NREM/REM state-related heterogeneity of the motor cortical and hippocampal local sleep in control rats. The distinctly altered local NREM/REM state, alongside their episode dynamics and electroencephalographic (EEG) microstructures, suggest the importance of both the local neuronal network substrate and the NREM/REM neurochemical substrate in the control mechanisms of sleep.

Keywords: local sleep, simultaneous sleep, EEG microstructure, NREM/REM stage dynamics, local NREM/REM episode dynamics, rat

1. Introduction

Sleep is classically considered to be a global, complex, reversible behavioral state common to all mammals that is homeostatically regulated, and whose control mechanisms are manifested at every level of biological organization – from genes and intracellular mechanisms to networks of neuronal populations within the central nervous system (Hobson and Pace-Schott, 2002; Pace-Schott and Hobson, 2002; Siegel, 2008). Sleep is not a homogenous state but rather a continuum of a number of mixed states (Hobson and Pace-Schott, 2002; Pace-Schott and Hobson, 2002).

Sleep is traditionally constituted of two global behavioral states, NREM and REM, characterized by quiescence and reduced responses to sensory stimuli (Cirrelli and Tononi, 2008). Whereas the hallmarks of NREM sleep are slow waves and spindles throughout the cerebral cortex, REM sleep is characterized by an 'activated', low-voltage fast EEG activity, paradoxically similar to that of wake, along with the rapid eye movements and muscle atonia (Funk et al., 2016). In addition, there are evidence for the intermediate state preceding the REM sleep, characterized by large amplitude spindles interspersed with large amplitude slow waves in the cerebral cortex together with low frequency theta activity in the dorsal hippocampus that correspond to the functional isolated forebrain in cats, rats and mice (Glin et al., 1991). Furthermore, beside the fact that REM sleep can be subdivided into 'tonic' (continuing without an interruption throughout the active sleep episode) and 'phasic' phenomenon (occurring briefly many times, accompanied with the twitches of somatic muscles, ponto-geniculo-occipital waves, and presynaptic inhibition of the sensory Ia afferents during the active sleep episode), and that the low voltage fast neocortical electrical activity and the hippocampal theta activity were classically considered as tonic phenomenon, there are evidence that electrical activity during REM sleep can be also subdivided into 'tonic' and 'phasic' component (Robinson et al., 1977).

Although sleep is classically considered to be a global phenomenon, orchestrated by the specialized neuronal networks that modulate whole-brain activity, it is also a local phenomenon or rather a fundamental propriety of small neuronal groups (Krueger et al., 2008; Vyazovskiy et al., 2011; Nobili et al., 2012; Funk et al., 2016; Soltani et al., 2019). Moreover, in the sleep-deprived brain of freely behaving rats (after a long period in awake state) the local neuronal subpopulation of one cortical area can go briefly offline as in sleep, accompanied by slow waves in the EEG (local sleep), but not in another cortical area (local awake), and even within the same cortical area, some neurons may be off while others remain on during awake state (Vyazovskiy et al., 2011). In addition, recent study in freely moving mice evidenced that cortical activity during REM sleep is not homogeneously wake-like, and that slow waves occur regularly only in the superficial layers of primary cortical areas during REM sleep (Funk et al., 2016).

Furthermore, experimental evidence in animals and humans suggest that sleep and wakefulness might be simultaneously present in different cerebral regions without strictly defined boundaries and that the brain-sleep state may be spatially non-uniform (Nobili et al., 2012). Tiredness after prolonged sleep deprivation can be manifested as “microsleeps” during an awake state: brief episodes of 3-15 s during which a person appears suddenly asleep with closed

eyes, without response to stimuli, and with sleep-like EEG activity (Tirunahari et al., 2003).

Moreover, recent animal studies have demonstrated topographically distinct changes to the EEG microstructures in the sensorimotor and motor cortex during NREM and REM sleep following severely impaired thalamo-cortical cholinergic innervation (Petrovic et al., 2013a, 2013b) and the emergence of two distinct REM states in the motor cortex with distinct EEG microstructures, different electromyographic (EMG) power and cortical drives (Petrovic et al., 2014). In addition, the intracranial EEG recordings in humans has provided evidence for the dissociation of activity in different brain regions during sleep walking and lucid dreaming (Bassetti et al., 2000; Voss et al., 2009), and evidence that the hippocampus and cortex in humans display features of different sleep states – notably REM and NREM at the same time (Moroni et al., 2012). Therefore, it is suggested that normal sleep is much more of a complex and local process than it is a global phenomenon (Genzel and Dresler, 2012).

In addition, hippocampal studies in both humans and animals have reported a dichotomy in the EEG states between the hippocampus and the neocortex, with the hippocampus showing slow wave activity typical of NREM sleep, while the neocortex shows the low-voltage, fast EEG signals which are commonly associated with waking (Vanderwolf et al., 1977; Sarasso et al., 2014). Simultaneous sleep

recording from the hippocampus and motor cortex in freely moving rats demonstrated that REM sleep occurred long before it occurred in the neocortex and the sleep state heterogeneity between the hippocampus and the neocortex (Emrick et al., 2016).

Based on all aforementioned studies, in this study we investigated the homogeneity/heterogeneity of spontaneous sleep simultaneously recorded in the motor cortex and hippocampus of control rats in order to further explain the simultaneous/non-simultaneous local sleep differences at the level of sleep architecture, Wake/NREM/REM stage and episode dynamics alongside their EEG microstructures in physiological condition.

2. Methods

2.1. Experimental design

The experiments were performed on 19 adult male Wistar rats, which were 2-and-a-half month old at the beginning of the study.

We followed their sleep bi-weekly from 14 to 42 days following the surgical procedure for the implantation of EEG and EMG electrodes for chronic sleep recording. All the sleep recording sessions, at each time point, were done during the same circadian phase (during the inactive circadian phase for rats, from 9 a.m. to 3 p.m.).

This study was carried out in accordance with the recommendations of EEC Directive (2010/63/EU) on the Protection of Animals Used for Experimental and Other Scientific Purposes, and the protocol was approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Sinisa Stankovic”- National Institute of Republic of Serbia, University of Belgrade (Approval N^{o} 2-21/10).

Prior to surgery and throughout the experimental protocol, the animals were maintained on a 12 hour light-dark cycle (7 a.m. lights on, 7 p.m. lights off), and were housed at 25^{o}C with free access to food and water.

2.2. Surgical procedure

The surgical procedures employed for the implantation of the EEG and EMG electrodes for chronic sleep recording have been described previously (Ciric et al., 2015, 2018, 2019) and are outlined below.

We implanted under ketamine/diazepam anesthesia (Zoletil 50, VIRBAC, France, 50 mg/kg; i.p.), in 2-and-a-half month old rats two epidural stainless-steel screw electrodes for EEG cortical activity from the motor cortex (MCx; A/P: +1.0 mm from bregma; R/L: 2.0 mm from the sagittal suture; D/V: 1.0 mm from the skull), and two stainless-steel teflon-coated wires (Medwire, NY, USA) into the CA1 hippocampal regions (Hipp; A/P: -3.6 mm from bregma; R/L: 2.5 mm from

the sagittal suture; D/V: 2.5 mm from the brain surface, following Paxinos and Watson, 2005).

For the most accurate stereotaxically guided implantation of the hippocampal EEG electrodes we always used the rats weighing between 250 to 290 g, according to Paxinos and Watson (2005), and a Digital Lab Standard Stereotaxic Instrument (Stoelting Co., Europe). Typical example of the histological identification of the positions of bilaterally implanted hippocampal electrodes into the CA1 is depicted in **Supplemental Fig. 1**.

In addition, we implanted two stainless-steel teflon-coated wires into the dorsal nuchal musculature, for the assessment of skeletal muscle activity (EMG), and a stainless-steel screw electrode in the nasal bone as a ground.

All the electrode leads were soldered to a miniature connector plug (39F1401, Newark Electronics, Schaumburg, IL, USA), and the assembly was fixed to the screw electrodes and skull using acrylic dental cement (Biocryl-RN, Galenika a.d. Beograd, Serbia) (Ciric et al., 2015, 2018, 2019).

2.3. Recording procedure

At the end of surgical procedure, the scalp wounds were sutured and the rats were allowed to recover for 13 days before their adaptation to the recording cable and plexiglass chamber (30 cm x 30 cm x 30 cm) for one day (Petrovic et al., 2013a, 2013b). The EEG and EMG activities were carried from the connector plug

on the rat head by a cable, passed through a sealed port on the recording box, and differentially recorded (Ciric et al., 2015, 2016, 2018, 2019). The differential mode consisted of 6 inputs (left MCx, right MCx, left Hipp, right Hipp, left EMG, right EMG), each with a (+) on the left and a (-) on the right, and all with the same ground (a screw electrode implanted in the nasal bone) (Ciric et al., 2015, 2016, 2017, 2018, 2019).

The EEG and EMG activities were displayed on a computer monitor and stored on a disk for further off-line analysis. After conventional amplification and filtering (0.3-100 Hz band pass; A-M System Inc. Model 3600, Calrborg, WA, USA), the analog data were digitized (at a sampling frequency of 256/s) and recorded for 6 hours, during the normal inactive circadian phase for rats (from 9 a.m. to 3 p.m.) using DataWave SciWorks Experimenter Version 8.0 (DataWave Technologies, Longmont, CO, USA) (Ciric et al., 2015, 2016, 2018, 2019).

In this study, at each time point of our follow-up period (14, 28, 42 days after surgical procedure for the implantation of the EEG and EMG electrodes for chronic sleep recording), the sleep recordings were done in all rats simultaneously for both the motor cortex and the hippocampus. We repeated sleep recording sessions at three time points because only sleep alterations that repeated consecutively at least two times were considered as the consistent results.

2.4. Data Analysis

The analysis of the sleep recorded signals was conducted using software developed in MATLAB 6.5 (Petrovic et al., 2013a, 2013b, 2014; Ciric et al., 2015, 2016, Lazic et al., 2015, 2017), and upgraded to MATLAB R2011a (Ciric et al., 2017, 2018, 2019).

2.4.1. Differentiation of the sleep/wake states

We applied Fourier analysis to the signals acquired throughout each 6 hour recording (a total of 2160 10 seconds Fourier epochs), and each 10 second epoch was differentiated as a Wake, NREM or REM state for further sleep analysis (sleep architecture, Wake/NREM/REM stage dynamics, Wake/NREM/REM episode dynamics, and state-related EEG microstructures) (Petrovic et al., 2013a, 2013b, 2014; Ciric et al., 2015, 2016, 2019; Lazic et al., 2015, 2017; Saponjic et al., 2016).

First, by using the motor cortical or hippocampal EEG, we extracted all the 10 second Wake epochs from each 6 hour recording, based on the product of sigma and theta frequency power on the y-axis, and the EMG power on the x-axis (Petrovic et al., 2013a, 2013b, 2014). Furthermore, the differentiation of the NREM and REM 10 second epochs was done using the EMG power on the y-axis, and the delta/theta power ratio on the x-axis (Petrovic et al., 2013a, 2013b, 2014). The differentiation of all Wake/NREM/REM epochs was improved by using the logarithmic values of the quantities on both the EEG and EMG axes, and was

finally achieved using the two clusters K means algorithm (Petrovic et al., 2013a, 2013b, 2014; Ciric et al., 2015, 2016; Lazic et al., 2015, 2017; Saponjic et al., 2016).

In this study, we particularly extracted the simultaneous and non-simultaneous Wake/NREM/REM 10 second epochs in the motor cortex and the hippocampus for the further analyses of the local sleep architectures, as well as local Wake/NREM/REM stage dynamics along with their state-related EEG microstructures (Ciric et al., 2018, 2019).

When we differentiated the NREM/REM sleep 10 second epochs at the same time we named them simultaneous NREM/REM sleep of the motor cortex and hippocampus. When we differentiated the NREM sleep in the motor cortex but REM sleep in the hippocampus or vice versa, we named them non-simultaneous NREM/REM sleep.

Fig. 1A depicts the individual examples of the motor cortical and hippocampal hypnograms and sleep recordings during 1 hour, with extracted 20 seconds of analog signals, and the simultaneous and non-simultaneous NREM/REM 10 second epochs, at the beginning (Day 14) and at the end (Day 42) of our follow-up period. Typical examples of the final scatter-grams for the differentiation of simultaneous and non-simultaneous Wake/NREM/REM states in the motor cortex and the hippocampus are depicted in **Fig. 1B**, while typical

scatter-grams of brain structure related Wake/NREM/REM states homogeneity/heterogeneity are depicted in **Supplemental Fig. 2.**

Furthermore, to determine the diversity (homogeneity/heterogeneity) of the sleep states between the motor cortex and the hippocampus, we calculated the individual percentage of simultaneous and non-simultaneous Wake/NREM/REM states for each rat and brain structure at each time point with respect to their total duration over 6 hours in the motor cortex, taken as 100%, to further calculate the means for the overall follow-up period.

In addition, we analyzed the motor cortical versus hippocampal Wake/NREM/REM episode dynamics. The average "episode" duration of each state was calculated by concatenating all bouts of each state at each time point and by dividing the total duration obtained by the number of all bouts (10 seconds, 20 seconds, 30 seconds, and so on). All the values were expressed for the overall follow-up period as means ± SE in minutes. To be specific, we consider as an episode every single 10 seconds of Wake/NREM/REM (a 10 second "episode") or a number of the consecutive 10 second epochs of the same state of Wake/NREM/REM (20 second or 30 second "episodes", and so on). Although the classical term "episode" is not entirely appropriate to our form of data analysis (Ciric et al., 2015, 2016, 2019; Lazic et al., 2017) we have retained this term as it is conventional.

2.4.2. Sleep state related EEG analysis

To analyze the sleep/wake state related EEG amplitude changes we calculated the group probability density distributions of all the Wake, NREM and REM conventional EEG frequency bands (δ = 0.3-4 Hz; θ = 4.1-8 Hz; σ = 10.1-15 Hz; β = 15.1-30 Hz; γ = 30.1-50 Hz) relative amplitudes over 6 hours, from each brain structure and at each time point, using the Probability Density Estimate (PDE) routine supplied with MATLAB R2011a. In this study, we particularly analyzed the EEG microstructure of simultaneous and non-simultaneous motor cortical and hippocampal NREM/REM sleep. In order to eliminate any influence from absolute signal amplitude variations on the recordings, we computed the relative Fourier amplitudes (Petrovic et al., 2013a, 2013b, 2014):

$$(RA)_b = \frac{\sum_{b} \text{Amp}}{\sum_{tot} \text{Amp}},\ b=\{\ \delta,\ \theta,\ \sigma,\ \beta,\ \gamma\}.$$

In addition, for each sleep/wake state and each frequency band, PDE analysis was performed on the assembles of relative amplitudes (Petrovic et al., 2013a, 2013b; Lazic et al., 2015, 2017) by pooling the measured values from all the animals at each time point of the overall follow-up period (14, 28 and 42 days after the surgical procedure for the implantation of the EEG and EMG electrodes for chronic sleep recording). For the statistical analysis of the PDE/6 hours of each EEG frequency-specific relative amplitude $(RA)_b$ distribution, and in each state, we

calculated the relative amplitude means for Wake and REM during each 30 minutes, while for NREM we calculated it during each 60 minutes (Ciric et al., 2018, 2019).

2.5. Statistical analysis

All statistical analyses were performed using a Kruskal-Wallis ANOVA (X^2 values) with the Mann Whitney U (z values) two-tailed post-hoc test. The accepted level of significance was $p \leq 0.05$.

3. Results

3.1. The diversity of the sleep architecture and Wake/NREM/REM stage and episodes dynamics in the motor cortex versus the hippocampus

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In our study, the control rats, during 6 hours of sleep, spent in the motor cortex (mean ± SE) 66.01 ± 7.01 minutes awake; 183.82 ± 10.11 minutes in NREM sleep, and 109.83 ± 7.75 minutes in REM sleep (**Table 1**). On the other hand, in the hippocampus they spent (mean ± SE) 56.59 ± 3.90 minutes awake, 228.29 ± 6.72 minutes in NREM sleep and 74.80 ± 6.83 minutes in REM sleep (**Table 1**).

While there was no difference between the motor cortical and hippocampal Wake duration (z = - 0.78; p = 0.44), NREM duration was longer (z = - 3.19; p =

10^{-3}) and REM duration was shorter ($z = -3.10$; $p = 10^{-3}$) in the hippocampus versus the motor cortex during 6 hours of sleep (**Table 1**). Moreover, the mean numbers of Wake/NREM/REM episodes were lower in the hippocampus versus the motor cortex (**Table 1**; $z \geq -4.52$; $p \leq 0.02$), but there mean durations were longer in the hippocampus versus the motor cortex (**Table 1**; $z \geq -4.52$; $p \leq 0.02$).

In addition, the difference in the simultaneous sleep episode dynamics were expressed as a decreased number of short NREM/REM episodes and an increased number of long NREM episodes in the hippocampus versus the motor cortex (**Fig. 2**).

The Wake state was determined as simultaneous between the motor cortex and the hippocampus (mean ± SE) in 76.24 ± 3.87%; as non-simultaneous in the motor cortex in 21.65 ± 2.37%; and as non-simultaneous in the hippocampus in 15.54 ± 1.97% (**Table 2**).

The NREM state was determined as simultaneous between the motor cortex and the hippocampus (mean ± SE) in 84.87 ± 2.22%; as non-simultaneous in the motor cortex in 15.50 ± 2.42%; and as non-simultaneous in the hippocampus in 34.40 ± 4.80% (**Table 2**).

The REM state was determined as simultaneous between the motor cortex and the hippocampus (mean ± SE) in 45.57 ± 4.42%; as non-simultaneous in the

motor cortex in 57.04 ± 6.19%; and as non-simultaneous in the hippocampus in 11.09 ± 1.23% (**Table 2**).

In addition, we demonstrate that while the hippocampal non-simultaneous NREM state is longer than the motor cortical non-simultaneous NREM state (**Table 2**; $z = -2.95$; $p = 10^{-3}$), the hippocampal non-simultaneous REM state is shorter than the motor cortical REM state (**Table 2;** $z = -4.16$; $p = 10^{-4}$).

3.2. The homogeneity/heterogeneity of local total, simultaneous and non-simultaneous NREM/REM sleep EEG microstructures in the motor cortex versus hippocampus

The most consistent EEG microstructure differences in the local NREM sleep in the motor cortex versus the hippocampus were (**Fig. 3**): the higher delta and theta amplitudes ($z \geq -8.80$; $p \leq 10^{-3}$) versus the lower beta and gamma amplitudes ($z \geq -7.48$; $p \leq 10^{-4}$) during total, simultaneous and non-simultaneous NREM sleep in the hippocampus. In contrast to the higher sigma amplitude during non-simultaneous NREM ($z = -3.32$; $p = 10^{-3}$) there was a lower sigma amplitude ($z \geq -4.40$; $p \leq 10^{-4}$) during simultaneous and total NREM in the hippocampus versus the motor cortex.

The most consistent difference in the local EEG microstructures was a higher theta amplitude ($z = -9.19$; $p = 10^{-4}$) alongside a lower sigma amplitude ($z = -5.05$; $p \leq 10^{-4}$) in the hippocampus versus the motor cortex during total REM

sleep (**Fig. 4**). During simultaneous REM sleep the theta amplitude in the hippocampus was consistently higher ($z = -6.52$; $p = 10^{-4}$), whereas the beta and gamma amplitudes were consistently lower ($z \geq -6.02$; $p = 10^{-4}$) versus the motor cortex (**Fig. 4**). During non-simultaneous REM sleep there were consistently increased theta ($z = -6.37$; $p = 10^{-4}$), beta and gamma ($z \geq -3.53$; $p \leq 0.01$) amplitudes, and consistently decreased delta and sigma amplitudes ($z \geq -5.07$; $p \leq 10^{-4}$) in the hippocampus versus the motor cortex (**Fig. 4**).

Although this augmented theta amplitude was a common feature of the hippocampal simultaneous and non-simultaneous REM sleep versus the motor cortical simultaneous and non-simultaneous REM sleep, the attenuated delta and sigma amplitudes together with the augmented beta and gamma amplitudes were the hallmarks of hippocampal non-simultaneous REM sleep (**Fig. 4**). Moreover, our results show the heterogeneity of hippocampal REM sleep through the inverse alteration of beta and gamma amplitudes during hippocampal simultaneous and non-simultaneous REM sleep (**Fig. 4**).

When we compared the simultaneous versus non-simultaneous NREM of the motor cortex we evidenced: no difference in delta amplitudes ($z = -1.88$; $p = 0.06$), lower theta and sigma ($z \geq -3.67$; $p \leq 10^{-3}$), but higher beta and gamma amplitudes ($z \geq -4.62$; $p \leq 10^{-4}$) during non-simultaneous NREM sleep. Conversely, there were the higher delta and theta ($z \geq -6.49$; $p \leq 10^{-4}$) and lower beta and gamma (z

$\geq$ - 6.86; $p \leq 10^{-4}$) amplitudes during non-simultaneous versus simultaneous motor cortical REM sleep (**Supplemental Fig. 3**).

On the other hand the only difference between the simultaneous and non-simultaneous hippocampal NREM/REM sleep were higher theta amplitude during non-simultaneous NREM sleep ($z = - 3.72$; $p = 10^{-4}$) and higher delta amplitude ($z = -4.22$; $p = 10^{-4}$) during non-simultaneous REM sleep (**Supplemental Fig. 3**).

4. Discussion

Our present study demonstrates the diversity of simultaneous sleep in the motor cortex and the hippocampus in control rats at the level of local Wake/NREM/REM stage and episode dynamics, as well as at the level of local NREM/REM related EEG microstructures.

We have shown that the sleep architectures of the motor cortex and hippocampus (the mean duration of Wake/NREM/REM sleep/6 hours or the total number of Wake/NREM/REM 10 second epochs/6 hours) in the control rats were different. There was an increase of NREM duration and a decrease of REM duration in the hippocampus versus the motor cortex. Whereas the hippocampal NREM duration was increased mainly due to the prolongation of NREM episodes,

the hippocampal REM duration decreased due to the decreased number of REM episodes (**Table 1**).

However, the extraction of the simultaneous and non-simultaneous 10 second Wake/NREM/REM epochs from the total Wake/NREM/REM epochs, which were differentiated based on the EEG from the motor cortex or the hippocampus and the EMG of the dorsal nuchal muscles, enabled us to show that the duration of the non-simultaneous NREM stage was increased in the hippocampus versus the motor cortex (**Table 2**). By contrast, the durations of non-simultaneous REM stage of the hippocampus was decreased versus the motor cortex (**Table 2**), indicating that, in terms of duration, NREM state is the most heterogeneous (the longest duration of non-simultanoues NREM state) in the hippocampus, while the REM state is the most heterogeneous (the longest duration of non-simultaneous REM state) in the motor cortex.

In addition, we have shown the consistent difference in the NREM/REM episode dynamics during simultaneous sleep in the motor cortex and the hippocampus, particularly in the lower number of short NREM episodes/the higher number of long NREM episodes in the hippocampus (**Fig. 2**, upper panel), alongside the lower number of short REM episodes in the hippocampus (**Fig. 2**, bottom panel) versus the motor cortex.

The heterogeneity of the spontaneous sleep of the motor cortex and the hippocampus in control rats was particularly expressed through the inverse alteration of the sigma amplitude during total, simultaneous and non-simultaneous hippocampal NREM sleep (**Fig. 3**), alongside the complex alterations to the EEG microstructure of the hippocampal REM state (**Fig. 4**). Specifically, whereas the theta amplitude during the total, simultaneous, and non-simultaneous REM states was generally higher in the hippocampus versus the motor cortex, the delta and sigma amplitudes were lower during the non-simultaneous REM state in the hippocampus (**Fig. 4**). In addition, in contrast to the decreased beta and gamma amplitudes during the simultaneous hippocampal versus the motor cortical REM state, there were increased beta and gamma amplitudes during the non-simultaneous hippocampal versus the motor cortical REM state (**Fig. 4**). Alongside the higher hippocampal theta amplitude during total, simultaneous and non-simultaneous NREM and REM sleep, the state-related local sleep heterogeneity of non-simultaneous NREM/REM sleep was expressed through the inverse alterations of the delta, sigma, beta and gamma amplitudes in the hippocampus versus the motor cortex (**Figs. 3, 4**).

In addition to our present results that demonstrated the physiological brain structure-related and NREM/REM state-related heterogeneity of the motor cortical

and hippocampal local simultaneous/non-simultaneous sleep, at the level of NREM/REM stage and episode dynamics

alongside their local EEG microstructures, recent studies in rats have demonstrated also the distinct motor cortical/hippocampal simultaneous sleep alterations as an expression of the impaired cholinergic (Ciric et al., 2017) or dopaminergic (Ciric et al., 2019) control. The long-lasting alteration of the hippocampal EEG microstructure (delta amplitude augmentation/beta amplitude attenuation) during simultaneous NREM sleep with the motor cortex, and the long-lasting alteration of high voltage sleep spindle dynamics in the hippocampus and the motor cortex during their simultaneous REM sleep were the first biomarkers of Parkinson's disease cholinopathy in rats (Ciric et al., 2017). Moreover, in the hemiparkinsonian rats, there was the augmented NREM/REM theta amplitude and a distinct NREM/REM related alteration to the sleep spindles and high voltage sleep spindle dynamics in the hippocampus and the motor cortex, as a locally functionally distinctly expressed dopaminergic neuronal loss of the substantia nigra pars compacta (Ciric et al., 2019). Although dissociated sleep states have been presented as an explanation for parasomnias such as sleepwalking and nightmares in humans (Bassetti et al., 2000; Nielsen 2000; Voss et al., 2009), there is evidence for the long-lasting heterogeneity of simultaneous sleep states between the

neocortex and the hippocampus in rats as a normal sleep asynchrony (Emrick et al., 2016).

In this study we demonstrate, beside the physiological inter-structure sleep heterogeneity, the heterogeneity of the NREM/REM state related EEG microstructures of non-simultaneous versus simultaneous motor cortical or hippocampal sleep. Whereas the delta amplitude did not change, there were lower theta and sigma versus higher beta and gamma amplitudes during non-simultaneous versus simultaneous NREM sleep in the motor cortex (**Supplemental Fig. 3**). Conversely, there were higher delta and theta amplitudes versus lower gamma and beta amplitudes during non-simultaneous versus simultaneous REM sleep in the motor cortex (**Supplemental Fig. 3**). But, simultaneous and non-simultaneous NREM and REM sleep in the hippocampus were less different: only theta amplitude was higher during non-simultaneous NREM and delta amplitude was higher during non-simultaneous REM sleep in the hippocampus (**Supplemental Fig. 3**).

Although the local non-simultaneous and simultaneous NREM/REM sleep was different, and non-simultaneous REM sleep was much “slower” than simultaneous REM sleep in the motor cortex, still the non-simultaneous REM sleep in both structures was with common feature - the augmented delta amplitude (**Supplemental Fig. 3**). This delta amplitude augmentation during non-

simultaneous REM sleep was higher in the motor cortex versus hippocampus (**Fig. 4**), and could be a consequence of the higher impact of artefacts in the motor cortex. But, beside the fact that for the analyzes in this study we included only the good quality simultaneous sleep recordings, this delta amplitude augmentation, related only to non-simultaneous REM sleep in both brain structures, does not speak in favor of the impact of artefacts.

Our present study is in accordance to the recent study (Emrick et al., 2016) that suggests if sleep is often independently expressed in two different brain areas, it may also be independently homeostatically regulated in these areas, and thus it is critical to characterize sleep within the brain region in order to understand its function.

It is well known that the hippocampus and neocortical areas are connected independently to areas of the brain contributing to sleep state generation (Gvilia, 2010; Saper et al., 2010), and these independent functional inputs could be the underlying mechanisms for sleep state asynchrony. There is evidence for the different control of local NREM sleep by the thalamic reticular nucleus, based on its anatomical, morphological and neurochemical diversity (Vantomme et al., 2019). Moreover, a recent study of sleep stage dynamics in the neocortex and the hippocampus during their simultaneous sleep (Duran et al., 2018) indicated a region-specific regulation of REM sleep with implications for our understanding of

sleep organization and REM related functions such as memory formation. Regarding the functional importance of physiological local sleep diversity, since in our study we did not follow any other behavior except sleep, the diversity of local sleep, particularly the alterations of hippocampal sleep versus the motor cortical sleep, could be related to behavior, such as memory, in our future study.

We have to note here, that beside the fact that we have differentiated and analyzed only three main sleep states (Wake/NREM/REM) in the motor cortex and hippocampus, we did not follow the peripheral components of REM sleep (rapid eye movements (EOG), alterations in thermoregulation, alterations in autonomic functions), except the simultaneous motor cortical and hippocampal EEG activities and the dorsal nuchal muscle tone from 14 to 42 days after the surgical procedure for the EEG/EMG electrodes implantation for chronic sleep recording. Therefore, the main limitation of our present study is that we are unable to give the behavioral (functional) implication of demonstrated homogeneity/heterogeneity of simultaneous and non-simultaneous hippocampal or motor cortical sleep architecture, NREM/REM sleep episode dynamics and NREM/REM EEG microstructures. Still, this remains an open question for our future studies.

Our present study demonstrates for the first time the brain structure-related and state-related heterogeneity of simultaneous sleep in the motor cortex and hippocampus in control rats at the level of the local NREM/REM state and episode

dynamics, and the local NREM/REM EEG microstructures, suggesting, besides the local nature of sleep, the importance of both the local neuronal network substrate and the NREM/REM neurochemical substrate in the control mechanisms of sleep.

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Table 1. Wake/NREM/REM episode dynamics in the motor cortex (MCx) versus the hippocampus (Hipp).

	Mean duration/6h ± SE (min)			**Mean number of episodes/6h ± SE**			**Mean duration of episodes/6h ± SE (min)**		
	Wake	NREM	REM	Wake	NREM	REM	Wake	NREM	REM
MCx	66.01 ± 7.01	183.82 ± 10.11	109.83 ± 7.75	161.18 ± 19.40	359.94 ± 13.16	295.76 ± 17.01	0.46 ± 0.03	0.51 ± 0.04	0.37 ± 0.02
Hipp	56.59 ± 3.90	**228.29 ± 6.72**	**74.80 ± 6.83**	**115.08 ± 8.09**	**161.92 ± 6.18**	**83.25 ± 7.31**	**0.53 ± 0.09**	**1.53 ± 0.06**	**0.69 ± 0.05**

Bold numbers indicate the statistically significant different mean values between the MCx and Hipp at $p \leq 0.05$.

Table 2. Wake/NREM/REM stage dynamics in the motor cortex (MCx) versus the hippocampus (Hipp). Duration (%) of simultaneous and non-simultaneous Wake/NREM/REM states of the motor cortex (MCx) and the hippocampus (Hipp) in control rats, during simultaneous sleep recording and throughout the overall follow-up period, calculated with respect to the total Wake/NREM/REM duration/6 hours (minutes) in the motor cortex, taken as 100%.

	Total MCx (min)	***Simultaneous***		***Non-simultaneous MCx***		***Non-simultaneous Hipp***	
		min	%	min	%	min	%
Wake	66.01 ± 7.01	47.34 ± 3.19	76.24 ± 3.87	25.28 ± 9.62	21.65 ± 2.37	11.70 ± 1.84	15.54 ± 1.97
NREM	183.82 ± 10.11	157.90 ± 11.25	84.87 ± 2.22	29.48 ± 3.61	15.50 ± 2.42	62.93 ± 6.57	**34.40 ± 4.80**
REM	109.83 ± 7.75	45.12 ± 2.73	45.57 ± 4.42	71.04 ± 13.27	57.04 ± 6.19	12.74 ± 2.06	**11.09 ± 1.23**

Bold numbers indicate the statistically significant different mean values between the MCx and Hipp at $p \leq 0.05$.

Legends

Fig. 1. Typical individual examples of the motor cortical and hippocampal hypnograms and sleep recordings during 1 hour, with extracted 20 seconds of analog signals and the simultaneous and non-simultaneous NREM/REM 10 seconds epochs at the beginning (Day 14) and at the end (Day 42) of our follow-up period (**A**) and the final scatter-grams for the differentiation of simultaneous and non-simultaneous Wake/NREM/REM states in the motor cortex and the hippocampus (**B**). MCx – motor cortex, Hipp – hippocampus; W – total numbers of Wake 10 second epochs, NR – total number of NREM 10 second epochs, R – total number of REM 10 second epochs; Ws/Wns – simultaneous/non-simultaneous Wake 10 second epochs, NRs/NRns – simultaneous/non-simultaneous NREM 10 second epochs, Rs/Rns – simultaneous/non-simultaneous REM 10 second epochs.

Fig. 2. NREM/REM episodes dynamics of the motor cortex versus the hippocampus. The group distributions of the mean number/6 hours of NREM/REM episodes over their durations (minutes) simultaneously recorded from the motor cortex (MCx) and the hippocampus (Hipp).

In these log-log distributions each “horizontal stair” is placed above its corresponding duration, depicted on the x-axis in minutes. The first three episode durations are indicated below the “stair lines” as 10, 20 and 30, presenting the group mean number/6 hours of the NREM/REM 10 second, 20 second and 30 second episodes in the motor cortex versus the hippocampus. The missing lines in the distributions depict the zero group mean number of episodes that causes the logarithms to be identified as “not a number”.

Fig. 3. EEG microstructure differences in the total, simultaneous and non-simultaneous NREM sleep of the motor cortex (MCx) versus the hippocampus (Hipp). The group probability density distributions/6 hours of the data pooled from all rats and from each time point of the follow-up period (14, 28 and 42 days after the surgical procedure for the implantation of the EEG and EMG electrodes for chronic sleep recording). Arrows indicate the statistically significant increase (arrows up) or decrease (arrows down) in the certain EEG frequency range amplitude in the hippocampus versus the motor cortex at $p \leq 0.05$.

Fig. 4. EEG microstructure differences in the total, simultaneous and non-simultaneous REM sleep of the motor cortex (MCx) versus the hippocampus (Hipp). The group probability density distributions/6 hours of the data pooled from all rats and from each time point of the follow-up period (14, 28 and 42 days after the surgical procedure for the implantation of the EEG and EMG electrodes for chronic sleep recording). Arrows indicate the statistically significant increase (arrows up) or decrease (arrows down) in the certain EEG frequency range amplitude in the hippocampus versus the motor cortex at $p \leq 0.05$.

Supplemental Fig. 1. Typical example of histological identification of the position of bilaterally implanted hippocampal electrodes into the CA1, stained by NADPH-diaphorase histochemistry .

The scale bar is not defined, since this image was obtained by the acquisition of the images by using the 10x objective lens of the Olympus BX-51 microscope, equipped with a motorized stage and CCD video camera (Pixelink, Ottawa, ON, Canada), and by using a superimage acquisition option within the newCAST stereological software package (VIS-Visiopharm Integrator System, version 5.3.1.1640; Visiopharm; Denmark).

Supplemental Fig. 2. Typical scatter-grams of the motor cortical/hippocampal Wake/NREM/REM states homogeneity/heterogeneity. MCx – motor cortex, Hipp – hippocampus.

Supplemental Fig. 3. Distinct simultaneous/non-simultaneous NREM/REM EEG microstructure within the motor cortex and the hippocampus of control rats.

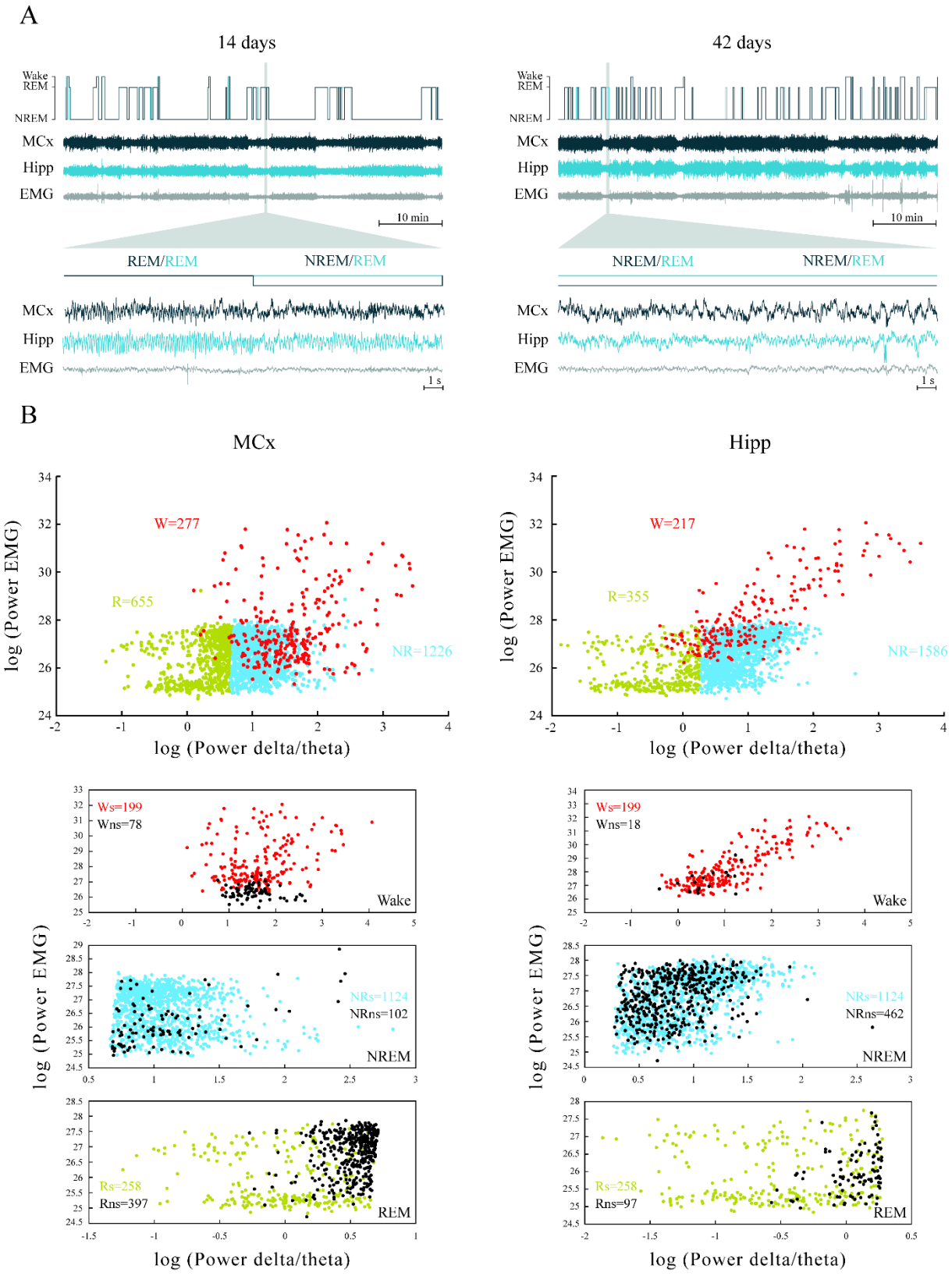

A
14 days
42 days
Wake
REM
NREM
MCx
Hipp
EMG
10 min
REM/REM
NREM/REM
NREM/REM
1 s
B
MCx
Hipp
W=277
R=655
NR=1226
W=217
R=355
NR=1586
log (Power EMG)
log (Power delta/theta)
Ws=199
Wns=78
Wake
NRs=1124
NRns=102
NREM
Rs=258
Rns=397
REM
Ws=199
Wns=18
NRs=1124
NRns=462
Rs=258
Rns=97

Fig. 1.

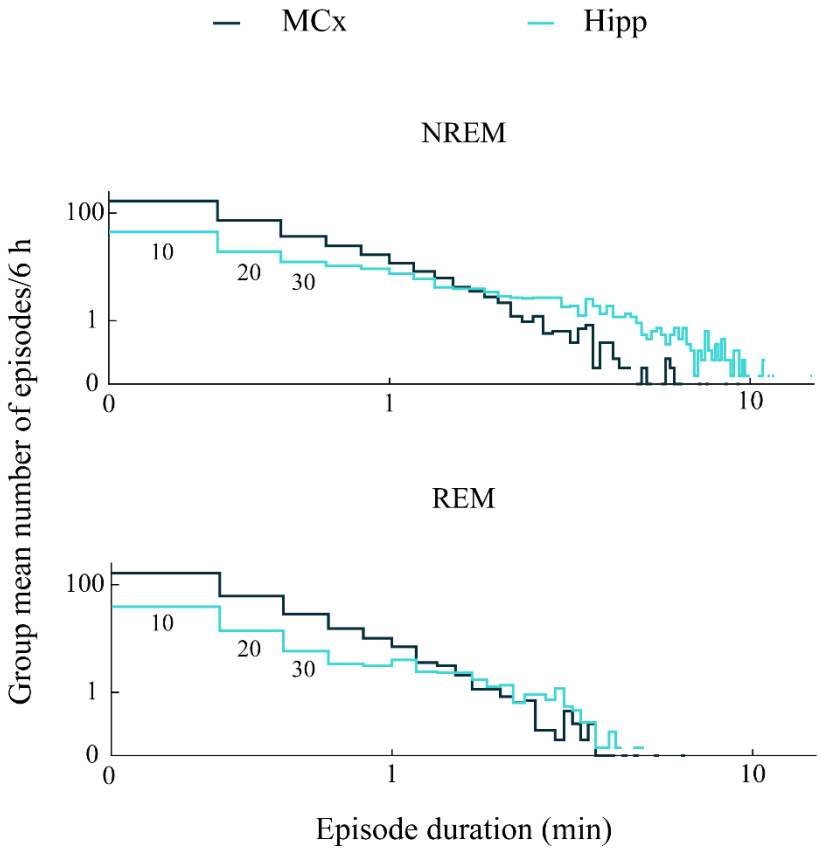

MCx
Hipp
NREM
100
1
0
10
20
30
0
1
10
REM
100
1
0
10
20
30
0
1
10
Group mean number of episodes/6 h
Episode duration (min)

Fig. 2.

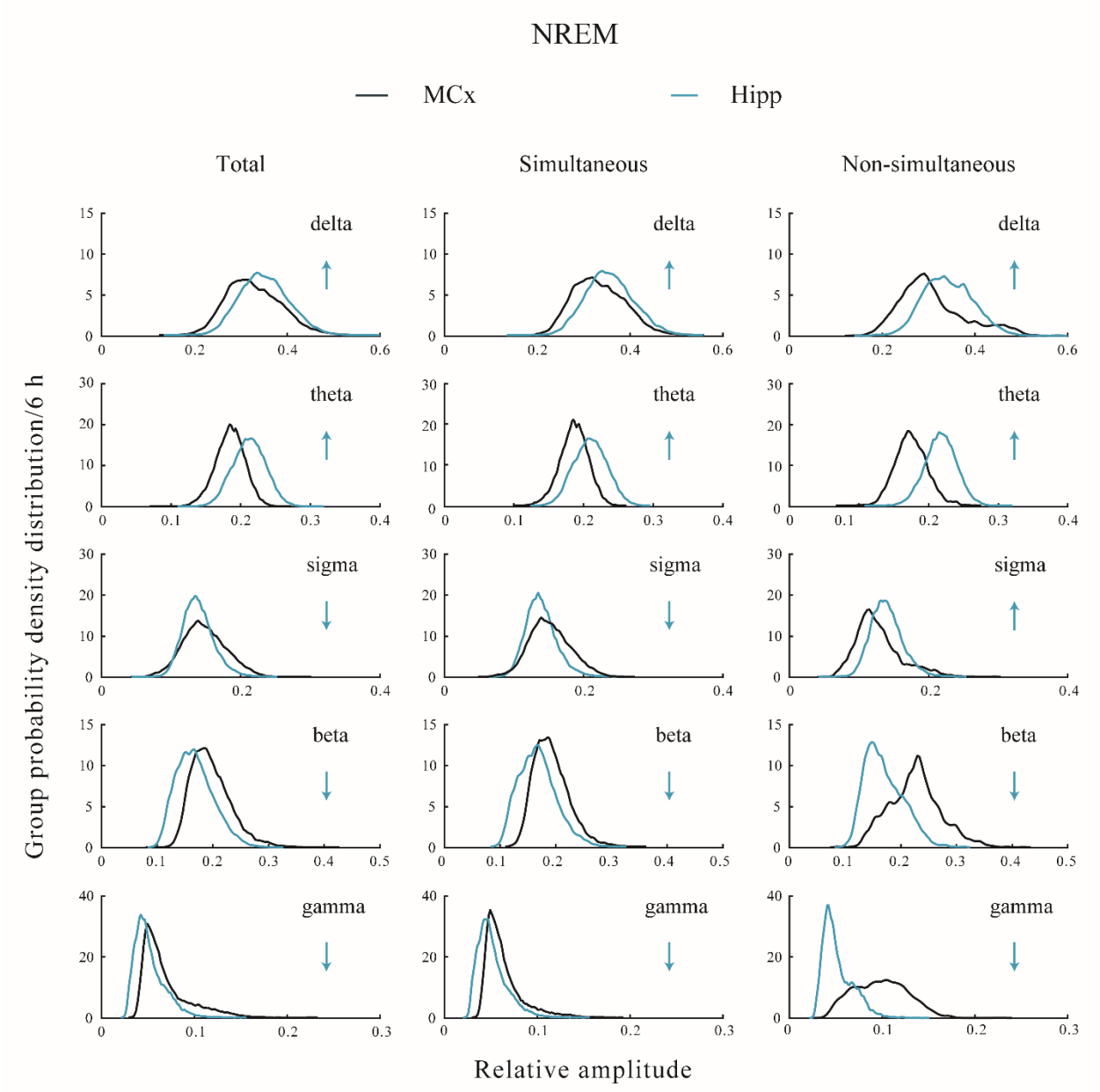

NREM
MCx
Hipp
Total
Simultaneous
Non-simultaneous
delta
theta
sigma
beta
gamma
Group probability density distribution/6 h
Relative amplitude

Fig. 3.

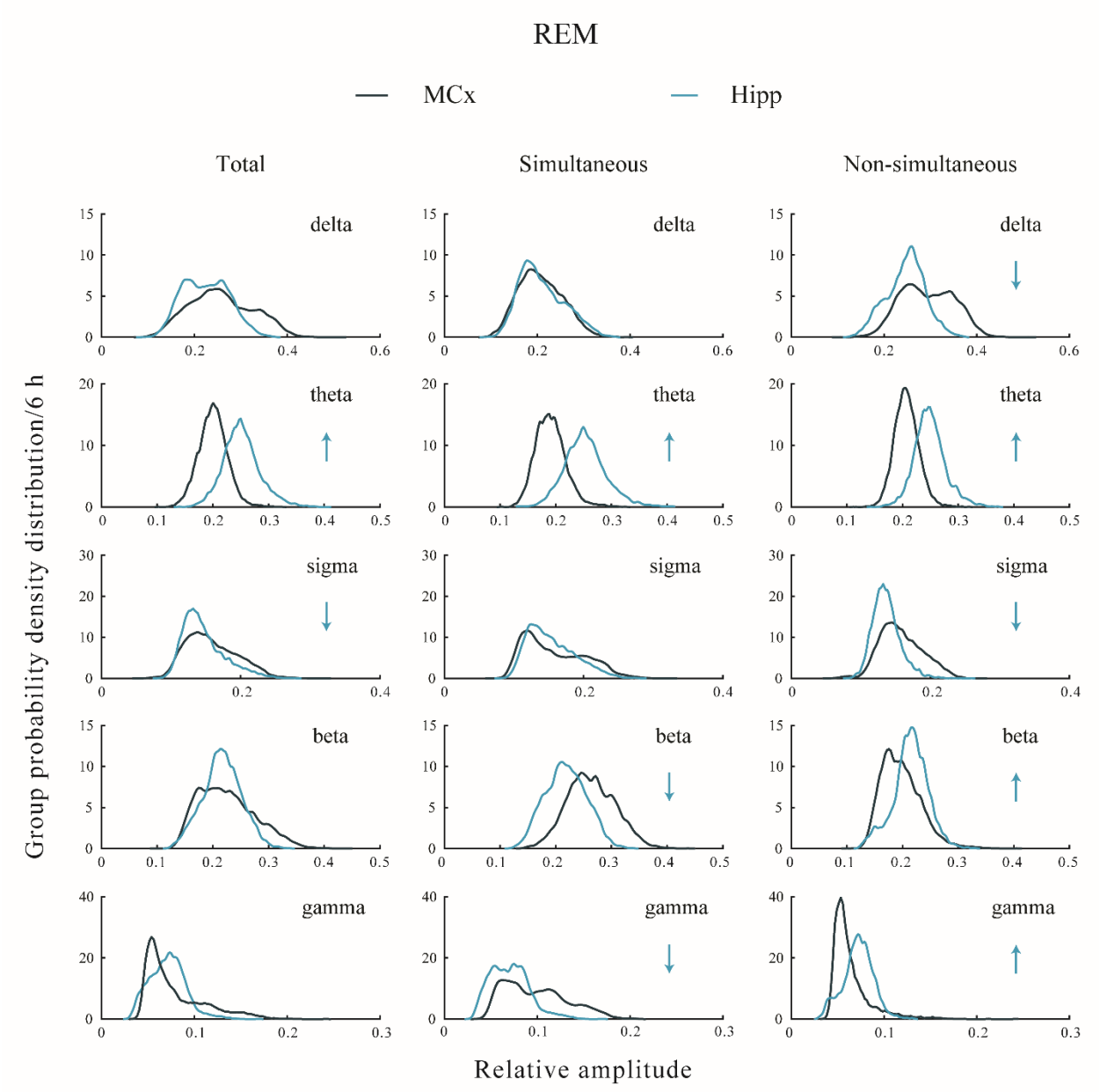
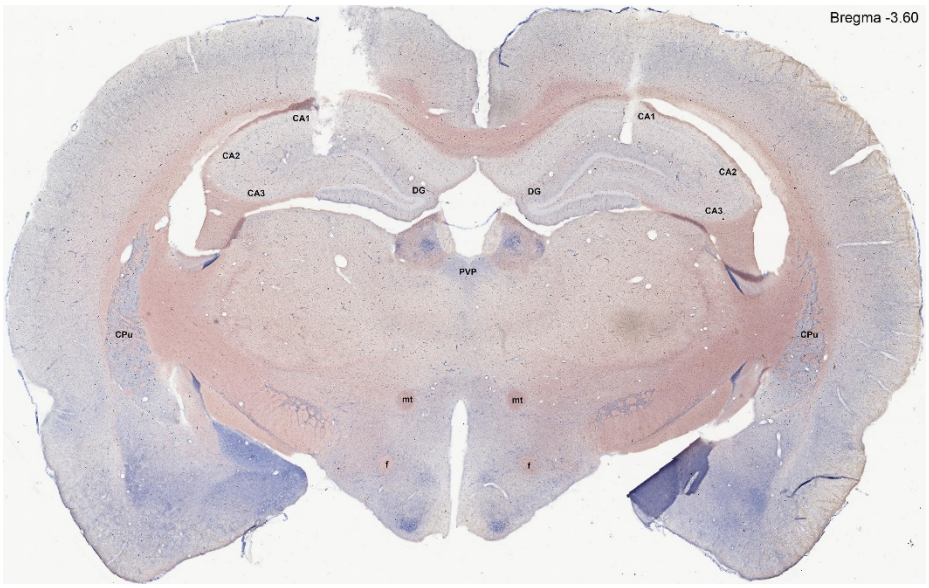
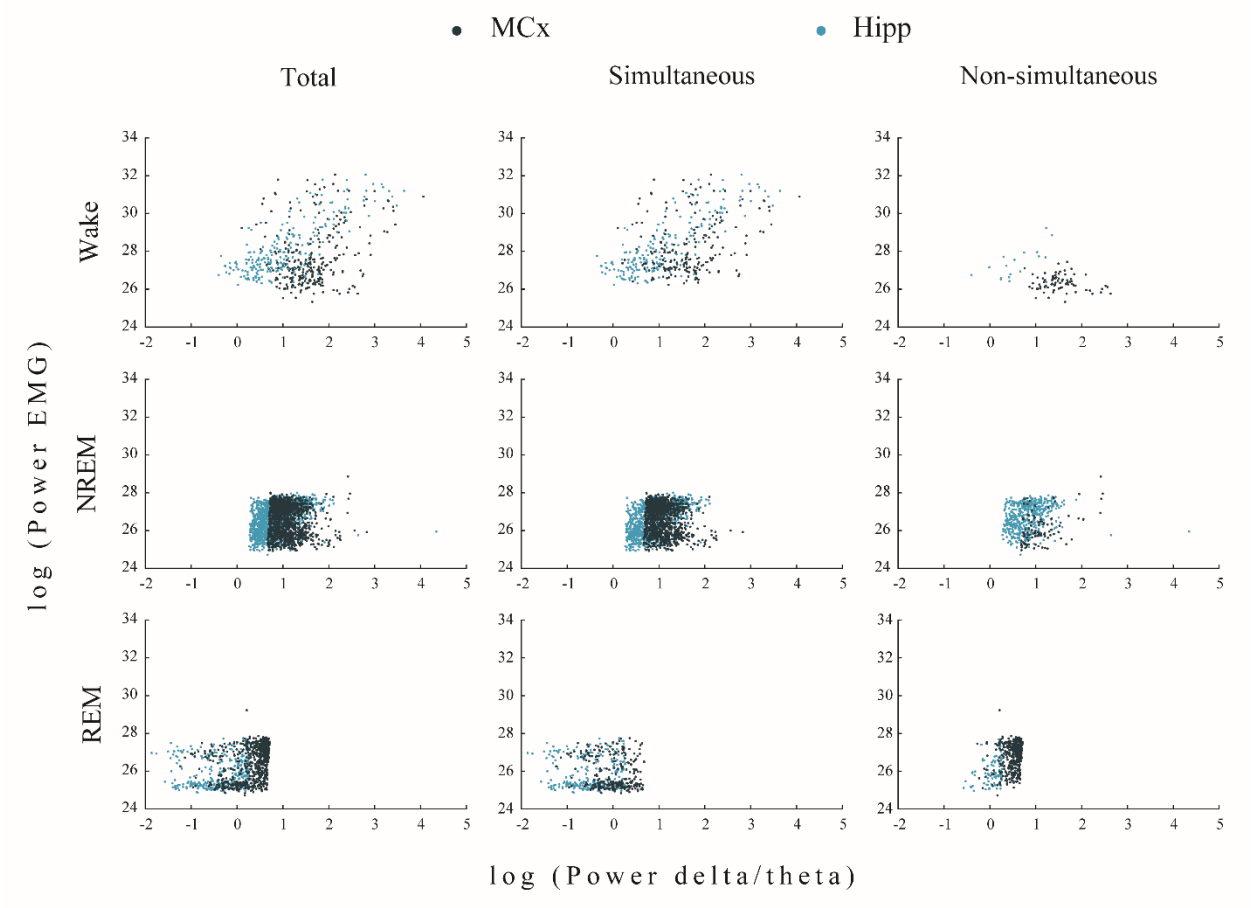

REM
MCx
Hipp
Total
Simultaneous
Non-simultaneous
delta
theta
sigma
beta
gamma
Group probability density distribution/6 h
Relative amplitude

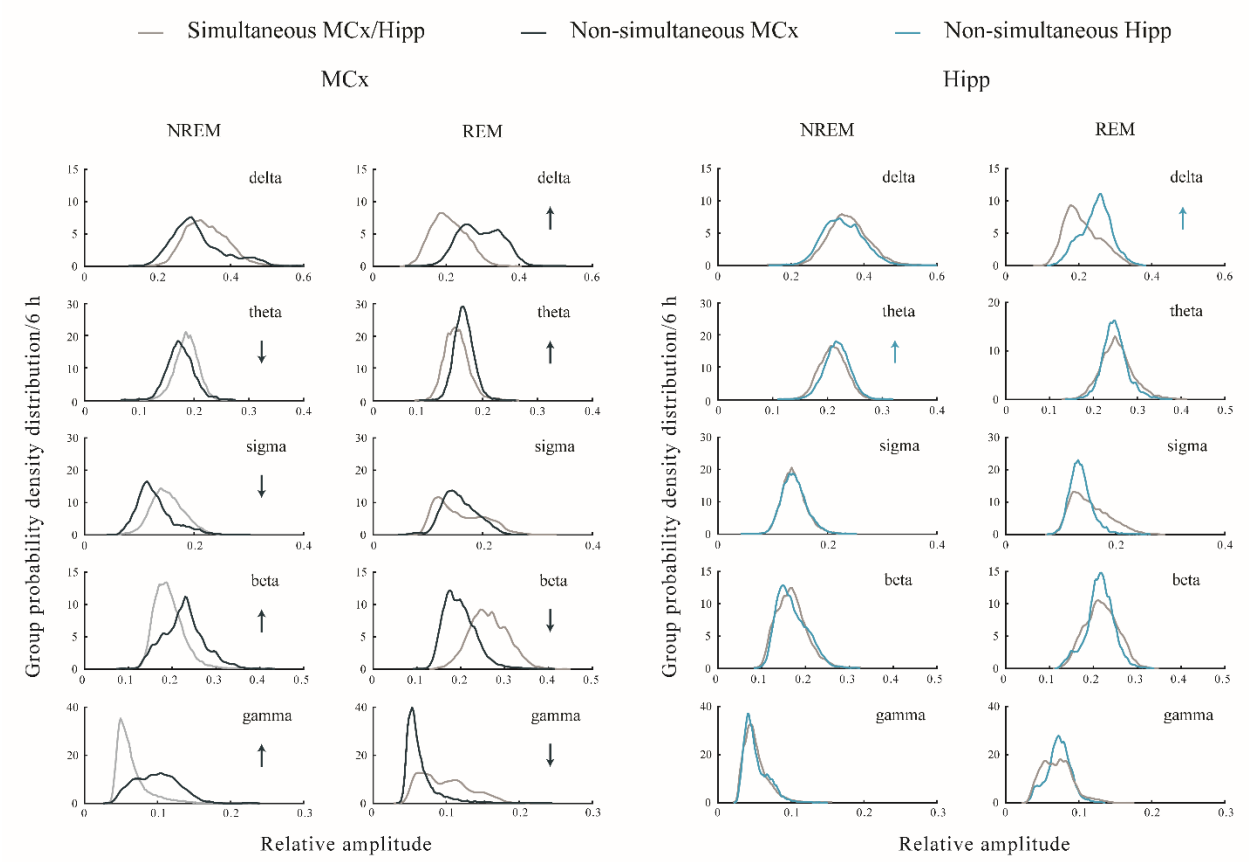
Fig. 4.



Suppl. Fig. 1.


MCx
Hipp
Total
Simultaneous
Non-simultaneous
Wake
NREM
REM
log (Power EMG)
log (Power delta/theta)

Suppl. Fig. 2.


Simultaneous MCx/Hipp
Non-simultaneous MCx
Non-simultaneous Hipp
MCx
Hipp
NREM
REM
NREM
REM
delta
theta
sigma
beta
gamma
Group probability density distribution/6 h
Relative amplitude

Suppl. Fig. 3.