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CIRCADIAN MODULATION OF HUMAN CORTICAL EXCITABILITY

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SUMMARY

It is now well established that human cognitive brain function is set by the interaction between sleep homeostasis (sleep-wake history) and the circadian timing system (biological clock). However, the underlying neuronal mechanisms remain elusive.

Cortical excitability, defined as the electrical reactivity of cortical neurons to a perturbation, is a core neurophysiological parameter of brain function. To date, dynamics in human cortical excitability is considered to be mainly driven by sleep homeostasis which directly depends on time spent awake. However, no study has been properly designed to investigate a putative influence of the circadian timing system on human cortical excitability.

Since cortical excitability assessment has long required invasive tools (i.e intracranial electrodes), it has long been restrained to animal research. Transcranial magnetic stimulation combined to simultaneous electroencephalography recording (TMS/EEG) recently emerged as a gold approach to non-invasively gauge *in vivo* cortical excitability in humans *: "Pertube and measure"*.

In our experiment, 22 healthy young men underwent 8 TMS/EEG recordings during 29 hours of continuous wakefulness under strictly controlled environmental conditions. Cortical excitability was inferred from the amplitude and slope of the first early EEG potential evoked by TMS over the frontal cortex. Our data reveal a circadian modulation of cortical excitability over the 24h day-night cycle. This modulation appears strongest in those individuals with the strongest circadian drive and is associated with changes in macroscopic EEG synchronization and behavior (i.e. subjective sleepiness, affect and performance). Our results suggest that the sleep-wake dependent dynamics in cortical excitability is strongly influenced by the

circadian machinery. This dynamics can arguably sustain the well-known changes in behavior associated with wakefulness extension and time-of-day. Our results also suggest that sleep and sleep homeostasis are not the only processes that regulate and restore brain function. The role of sleep could be therefore reformulated in a more general framework where the crucial factor would be the balance between circadian rhythmicity and the homeostatic sleep pressure**.** The full characterization of the temporal profile of cortical excitation in various healthy and clinical populations brings new insights for time-adapted lifestyles and may pave the way for development of chronotherapy strategies.

RESUME

Il est à présent bien établi que la fonction cognitive humaine est régulée par l'interaction entre l'homéostasie du sommeil (historique du temps passé éveillé et à dormir) et le système circadien (horloge biologique).

L'excitabilité corticale, définie comme la réactivité électrique de neurones corticaux à une perturbation, est un paramètre neurophysiologique fondamental de la fonction cérébrale. Actuellement, la dynamique temporelle de l'excitabilité corticale est considérée comme principalement entraînée par l'homéostasie du sommeil qui dépend elle-même directement du temps passé éveillé. Toutefois, aucune étude n'a encore été proprement dessinée pour investiguer le rôle éventuel du système circadien dans le décours temporel de l'excitabilité corticale chez l'Homme.

La mesure de l'excitabilité corticale a longtemps requis l'emploi d'outils invasifs (électrodes intracrâniennes) et pour cette raison s'est longtemps cantonnée aux expérimentations animales. La stimulation magnétique transcrânienne combinée à l'enregistrement électroencéphalographique (TMS/EEG) simultané a récemment émergé comme une approche de choix pour la mesure *in vivo* de l'excitabilité corticale humaine : « perturber puis mesurer ».

Dans notre étude, vingt-deux jeunes participants de sexe masculin et en bonne santé ont réalisé 8 enregistrements TMS/EEG. L'excitabilité corticale a été inférée de l'amplitude et de la pente du premier potentiel EEG évoqué par la TMS délivrée au cortex frontal. Nos données révèlent une modulation circadienne de l'excitabilité corticale à travers le cycle jour-nuit de 24h. Cette modulation apparait plus importante chez les individus dont le signal circadien est le plus fort et est associée à des changements de la synchronisation de l'EEG spontané et du comportement (somnolence et affects subjectifs, performance). Nos résultats suggèrent que la

dynamique de l'excitabilité corticale dépendante du cycle veille-sommeil est fortement influencée par la machinerie circadienne. Cette dynamique pourrait sous-tendre les changements comportementaux classiquement associés à l'extension du temps d'éveil et l'heure du jour. Nos résultats suggèrent aussi que le sommeil et l'homéostasie du sommeil ne sont pas les seuls processus qui régulent et restaurent la fonction cérébrale. Le rôle du sommeil pourrait ainsi être reformulé dans un contexte plus général où le facteur crucial serait la balance entre la rythmicité circadienne et la pression homéostatique de sommeil. Une caractérisation plus complète du profil temporel de l'excitabilité corticale en conditions physiologiques et pathologiques apporterait de nouveaux éléments pour optimiser nos horaires de vie et le développement de stratégies de chronothérapie.

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CONTENTS

INTRODUCTION

In 350 BCE, Aristotle pointed out "*There is no animal which is always awake or always asleep, such that all sleep is susceptible of awakening and all wake time beyond the natural time limit is susceptible to sleep*". Indeed, animal life is a succession of sleep-wake cycles, which in humans is tightly synchronized to the earth's 24h light-dark cycle.

Most human behavioral activities occur during the day, while light certainly provides, according to our visual system, the best conditions to perform. Although considerable interindividual and intercultural differences in the timing of wakefulness exist, there is no human ethnic group or culture which, by nature, lives exclusively at night.

In our modern societies, our rhythm of living is constrained by growing social, familial or professional obligations sometimes imposing us extended working hours in the night. Furthermore, we are more and more exposed to artificial light (including at night) that may disturb temporal landmarks normally provided by the natural light-dark cycle. Yet the human preference to stay awake during the day and to sleep at night still strongly persists. Our sleep-wake preferred timing is well echoed by the dynamics of our cognition. During a normal waking day, human cognitive performance remains indeed rather stable. However, when wakefulness is extended into the night, it deteriorates abruptly (Cajochen et al., 1999a; Dijk et al., 1992).

How are human beings able to sustain cognitive performance during ~16 continuous hours of wakefulness despite the wears and tears of waking activity? Why is it only when wakefulness is extended into the night that performance sharply decreases? These remain open questions. Conceptually, this phenomenon has been explained through a combined influence of a process tracking the duration of wakefulness (sleep homeostasis) and a ~24h oscillating signal, tracking internal biological time

(the circadian timing system). However, the cortical mesoscopic phenomena sustaining behavior during continuous waking remain largely unknown.

Cortical excitability, defined as the electrical reactivity of cortical neurons to a perturbation, is a core neurophysiological parameter of human brain function and cognition. Its measurement has long been restricted to animal research because only invasive approaches were available (e.g. intracranial electrodes). However, the recent development of transcranial magnetic stimulation combined to simultaneous EEG recording (TMS/EEG) offered the great opportunity to non-invasively gauge *in vivo* cortical excitability in humans.

To date, dynamics in cortical excitability has been considered to be mainly driven by sleep homeostasis, increasing with time spent awake and decreasing after sleep (Huber et al., 2013; Vyazovskiy et al., 2008). However, no study has been properly designed to investigate a putative influence of the circadian master clock on human cortical excitability.

Our objective in this thesis is to explore how the circadian timing system impacts on human cortical excitability and how this influence underpins the circadian fluctuations in cortical EEG dynamics and behavior.

This manuscript will classically start with a **theoretical background** divided in 3 chapters. In Chapter 1, we will address the 2 main regulatory and interacting processes setting the sleep-wake cycle: the circadian timing system and sleep homeostasis. In Chapter 2, we will focus on the development of transcranial magnetic stimulation (TMS) and the possibilities this non-invasive technique provide for a better comprehension of human brain function. Finally, Chapter 3 will discuss

the cortical plastic processes that may underpin cortical excitability changes during wake and sleep.

The stage set up, we will carry on with the core of this work: the **experimental section** (Chapter 4). Using TMS/EEG recordings during a challenging strictly controlled sleep deprivation paradigm, we highlight a novel circadian modulation on human cortical excitability that appears to sustain circadian change on system-level EEG synchrony and cognitive performance.

We hope you will enjoy your read!

THEORETICAL BACKGROUND

CHAPTER I

SLEEP AND WAKE CYCLE REGULATION

It is well established that both human sleep-wake timing and cognitive performance dynamics reflect the influence of two separate but interacting endogenous oscillatory processes: the circadian timing system and the hourglass sleep homeostat (Borbély, 1982; Dijk and von Schantz, 2005).

In this first chapter, we address the bases of these two fundamental processes. The two first sections discuss them separately in terms of neuroanatomy structures, mechanisms, markers and outputs. The third and last section will focus on the interaction between circadian and homeostatic processes in the control of sleep, brain function and cognitive performance.

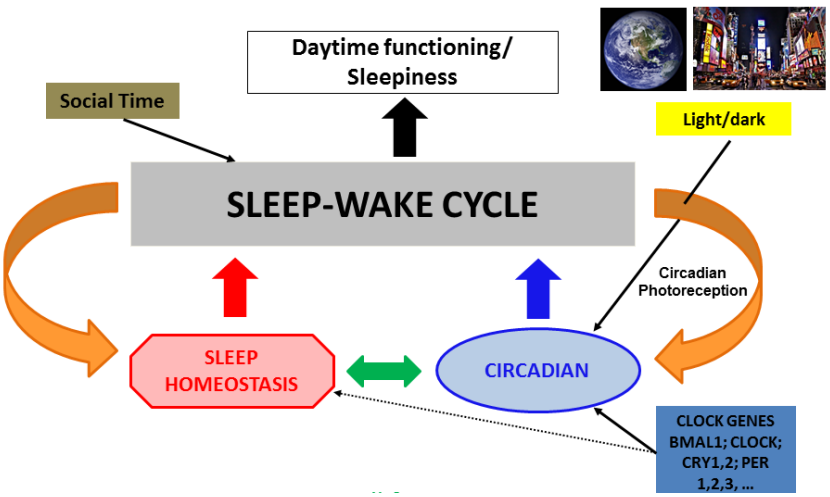


Fig. I.1 Sleep–wake cycle regulation - Sleep wake cycles are regulated by two oscillatory interacting processes: sleep homeostatis (sleep wake history - process H) and the circadian timing system (biological clock - process C). Light input is mediated by the circadian photoreception system. The sleep-wake cycle is a major determinant of this light input. Social time and clock genes also underlie this process (see following text). The major output of this regulation is the daytime functioning and sleepiness level. *Figure modified from (Dijk and Archer, 2010)*

1 THE CIRCADIAN TIMING SYSTEM

The circadian timing system is an internal clock which determines the rhythm of many physiological, biological and behavioral variables in the center of which stands the timing of wake and sleep. This section is dedicated to the circadian process in its fundamental neuroanatomical bases, core molecular components and main biological outputs.

1.1 Neuroanatomical bases of circadian rhythmicity

Circadian oscillators disseminated in the body are orchestrated by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Moore, 1983; Reppert and Weaver, 2002). The intrinsic period of the human circadian pacemaker averages 24.18 hour (Czeisler et al., 1999). However, to adapt to the 24h changes of our environment, the SCN is daily reset by various circadian synchronizers (Zeitgebers), including social time and light (Czeisler et al., 1999).

The most powerful synchronizer of circadian rhythmicity is the external light-dark cycle which entrains the SCN through retinal light inputs. The photoreceptive system responsible for the circadian resetting is distinct from the system mediating vision which mostly involves rods and cones. Indeed light synchronizes the SCN throughout projections from the intrinsically photosensitive retinal ganglion cells (ipRGCs) which express melanopsin, a photopigment maximally sensitive to short wavelength (blue ~ 480 nm)[1] (Hattar et al. 2003; Bailes & Lucas 2013; Chellappa & Ly et al. 2014).

The SCN projects, via multi-synaptic pathways, to the pineal gland, responsible for melatonin secretion (Moore, 1996). It also widely innervates the thalamus and the

[1] By contrast rods and cones are maximally sensitive at ~ 505 (blue-green) and ~ 550 nm (green), respectively (Hattar et al., 2003).

hypothalamus in order to achieve modulation of many physiological and endocrinal rhythms such as core body temperature (CBT) (Eastman et al., 1984), cortisol (Buijs, 1999) or orexin/hypocretin (Moore et al., 2001) secretions (see below). Finally, the SCN has indirect projections via the dorsomedial hypothalamus (DMH) to the sleep promoting ventrolateral preoptic hypothalamus (VLPO) and to the arousing-promoting basal forebrain cholinergic and monoaminergic nuclei. Monoaminergic nuclei include histaminergic tuberomammilary neurons (TMN), noradrenergic locus coeruleus (LC), dopaminergic A10 cell group and serotoninergic median and dorsal raphe nuclei (Cajochen et al., 2010; Saper et al., 2005a) **[Figure I.2].**

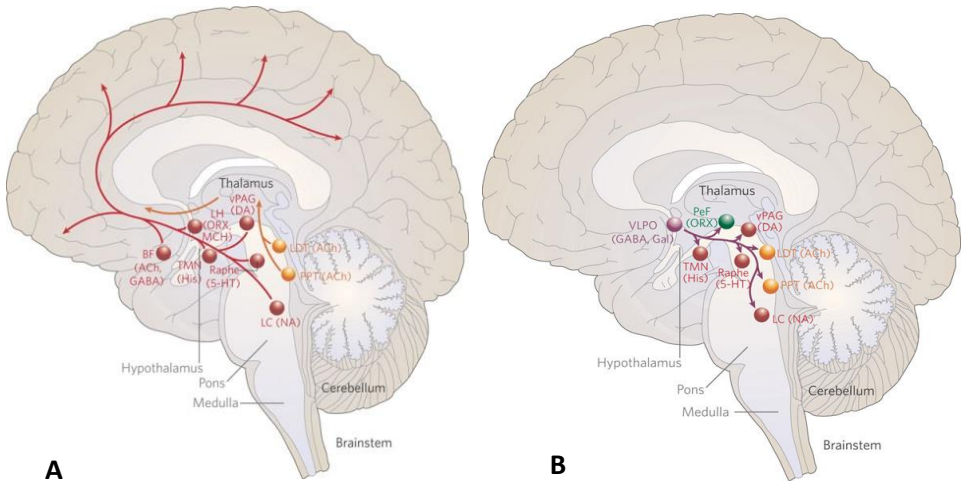


Fig. I.2 : (A) Key components of the ascending reticular arousal system. Cholinergic nuclei (yellow pathway) in the upper pons are major input to the relay and reticular nuclei of the thalamus. They facilitate thalamocortical transmission. Monoaminergic nuclei (red pathway) activate the cortex to facilitate the processing of inputs from the thalamus. (**B) – Key projections of the ventrolateral preoptic nucleus (VLPO) to the main components of the ascending arousal system.** VLPO promotes sleep by sending inhibitory projections on arousing monoaminergic (red), cholinergic (yellow) and orexin (green) neurons.

Abbreviations: Nuclei : LC, locus coeruleus; LDT, laterodorsal tegmental nuclei; PeF, perifornical neurons (lateral hypothalamus), PPT, pedunculopontine tegmental nuclei; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic nucleus. Neurotransmission : Ach, acetylcholine; DA, dopamine; GABA, γ-aminobutyric acid; Gal, galanin; Hist, histamine; 5-HT, serotonin; MCH, melanin-concentrating hormone; NA, noradrenalin; ORX, orexin**.** *Figures from (Saper et al., 2005a)*

1.2 Circadian control of wake and sleep: how does it work?

1.2.1 The "switch flip flop" theory

The "switch flip flop" theory (Saper et al., 2001) constitutes one of the most popular hypotheses to explain how SCN participates to the regulation of the sleep-wake cycle. Although it has been put in doubt in those recent years (see next section), this model may help to schematically understand some of the role(s) of the main subcortical structures implicated in wake and sleep promotion.

During the biological day (time of habitual wake episode), the SCN activates the hypocretin/orexin neurons of the lateral hypothalamus, and brainstem cholinergic and monoaminergic nuclei. Orexin/hypocretin neurons activate monoaminergic nuclei themselves and the cerebral cortex (Saper et al., 2001) [2]. The monoaminergic nuclei promote in turn wakefulness by direct excitation of the cortex and by inhibition of VLPO sleep promoting neurons (Saper et al., 2001).

In the later part of the waking period, the circadian drive for arousal progressively decreases, thus leading to an increase in the firing rate of VLPO GABAergic and galaninergic neurons (Saper et al., 2001). These inhibitory neurons project to the monoaminergic wake-promoting nuclei and excitatory orexin hypothalamic neurons leading to their "flip flop" switch inactivation when falling asleep. These mechanisms allow the progressive synchronization of the thalamo-cortical network enhancing generation of spindles and deeper non rapid eye movement (NREM) stages (Mc Carley, 2007; Saper et al., 2001). Rapid eye movement (REM) sleep mainly occurs later in the second half of the biological night and may be the "sleep output" of the SCN-circadian control (Mc Carley, 2011). During REM sleep, cholinergic neurons

[2] low levels of orexin/hypocretin can lead to the very well-known sleep attacks that occur in narcolepsy (Brisbare-Roch et al., 2007)

within the basal forebrain increase their activation level. When the biological night ends, the SCN stops to promote sleep and restarts to promote wakefulness through reversed switched “flip flop” activation of arousal nuclei and reciprocal deactivation of VLPO (Saper et al., 2001).

To date, the best in vivo representation of this model can be ascribed to sleep attacks seen in narcolepsy both in animal and humans (Baumann and Bassetti, 2005; Mignot, 2014; Taheri et al., 2002). The dynamics of the “flip flop” switch are fairly well established between different behavioral states (i.e. sleep and wakefulness) (Saper et al., 2005b). The circadian timing system is also known to play a key role onto those dynamics (Saper et al., 2005a). However the nature of the circadian sleep and wake promoting signal, in the context of the flip flop model, remains elusive.

1.2.2 The SCN: master clock of multiple peripheral oscillators.

As previously mentioned, the classical view of a unique circadian pacemaker orchestrating all physiological and behavioral rhythms has been challenged in those recent years. Indeed current change in opinion assumes the existence of numerous peripheral oscillators which phase and period length may be adjusted to the rhythm imposed by the SCN through neural and endocrine pathways. In addition, these peripheral clocks may be connected among themselves and may regulate the SCN activity by means of auto-regulatory feedback loops (Buijs et al., 2013; Schibler and Sassone-Corsi, 2002; Yu et al., 2014).

1.3 What are circadian core molecular components?

Human circadian oscillators rely on interacting positive and negative transcriptional feedback loops in clock genes expression which entrain the activity of the SCN to a

near-24-hour intrinsic oscillation. The exact period length is fine-tuned by post-translational mechanisms and synchronized by light to alternation of days and nights.

Clock[3] and *Bmal1*[4] are two fundamental core clock genes which encode for a complexed protein that binds to DNA as a transcriptional factor. The CLOCK-BMAL1 complex translocates to the nucleus whereby it induces transcription of *Cryptochrome* (Cry 1 and 2), *Period* (per 1, 2 and 3), *Rorα/β* (ROR), *Rev-erbα/β* (REV-ERB) and controlled clock genes (CCG) **[Figure I.3]**.

- Cry1 and cry2 inhibit CLOCK and/or BMAL1 transcriptional activity, thus leading to inhibition of their own transcription. Per3 forms heterodimers with Per1/2 and Cry1/2, which enter into the nucleus and inhibit *Clock-Bmal1*-mediated transcription (Hastings and Herzog, 2004; Reppert and Weaver, 2002).
- ROR and REVERB proteins respectively activate or inhibit Bmal1 transcription and thus constitute a second circadian feedback loop reinforcing first loop accuracy and robustness (Liu et al., 2008; Sato et al., 2004).
- Finally, the controlled clock genes (CCG) generate circadian signals responsible of physiological and behavioral circadian outputs (Liu et al., 2008).

The role of these core clock genes in circadian rhythms generation has notably been demonstrated by lack of circadian behavioral rhythms in mice carrying suppressive mutation of Bmal1, Clock, Cry1, Cry2, Per1 and Per2 (Lowrey and Takahashi, 2004). Importantly, this molecular circadian circuitry is not only operative in the SCN but is also expressed and active in most peripheral tissues (Yoo et al., 2004). This supports the view of multiple peripheral oscillators which the SCN, as a

[3] CLOCK = Circadian Locomotor Outputs Cycles Kaput
[4] BMAL1 = Brain and Muscle ARNT-like 1

"master oscillator", may adjust to achieve temporally synchronized physiology (Buijs et al., 2013).

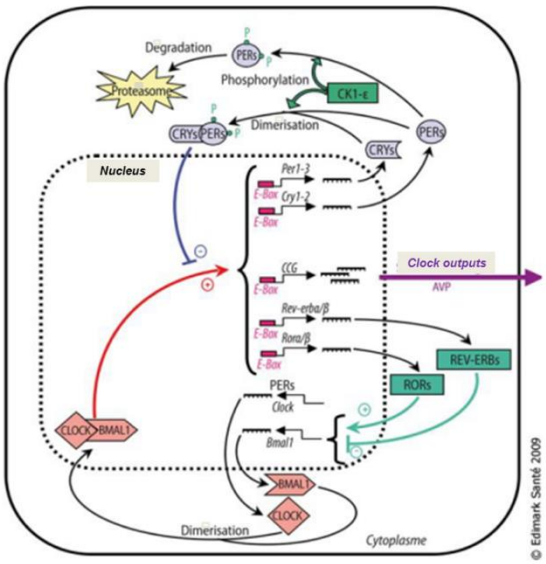


Fig. I.3 : Schematic view of the main clock genes contributing to cellular circadian oscillations. CLOCK-BMAL1 activation constitutes the main positive feedback loop. The main negative feedback loop is formed by indirect inhibition of Per and Cry own transcription**.** See upper main text for details and references. *Figure modified from (Challet et al., 2009)*

1.4 Circadian biological markers

The circadian timing system entrains many physiological and endocrine rhythms such as blood pressure, heart rate, core body temperature, secretion of cortisol, melatonin, prolactin or growth hormone (Czeisler and Gooley, 2007; Morris et al., 2012a, 2012b). In this short subsection, we will discuss the rhythm of the three main established markers of circadian phase in humans: core body temperature, melatonin and cortisol **[Figure I.4]**.

1.4.1 Core body temperature

Core body temperature (CBT) is the most reliable circadian vital parameter. Its variation is strongly tied to the circadian phase and depends on SCN activity (Eastman et al., 1984). CBT slowly increases during the day to reach its peak in the evening. Then it starts to fall to reach its minimum in the early morning (around the middle of sleep episode in normal conditions) when the circadian sleep promoting signal is strongest. The following morning rising portion of CBT coincides with habitual sleep termination (Cajochen et al., 1999a) **[Figure I.4]**.

1.4.2 Melatonin

Melatonin is a sleep promoting neuropeptide which favors sleep initiation and continuity (Cajochen et al., 2003). It is synthesized in a robust circadian fashion by the pineal gland of the epithalamus (Moore, 1996). Melatonin plasma concentrations is low and rather stable during the biological day. In the late evening, a few hours before habitual sleep time, it starts to rise and reaches its peak in the middle of the biological night (~2h before CBT minimum, time of circadian maximal sleep propensity). It then progressively decreases to reach its nadir in the early morning hours (time of minimal circadian sleep propensity) (Cajochen et al., 1999a) **[Figure I.4]**. The on and offsets of melatonin secretion respectively mark the beginning and ending of the biological night (Pandi-Perumal et al., 2007; Wehr et al., 2001). Determination of melatonin secretion onset under dim light conditions, (DLMO, dim light melatonin onset) is the most accurate marker for circadian phase assessment (Pandi-Perumal et al., 2007). Melatonin secretion is under the control of the SCN which synchronizes itself through non-visual retinal light inputs from the melanopsin containing ipRGC. Blue light, typically met in the sky during the day, maximally suppresses melatonin secretion (Brainard et al., 2001). Postures can also

affect melatonin levels (Deacon and Arendt, 1994). The intake of exogenous melatonin can improve sleep efficiency when administered during the biological day before the endogenous melatonin onset but not after, during the biological night (Wyatt et al., 2006).

1.4.3 Cortisol

Cortisol is a steroid hormone produced by the cortico-surrenal glands mostly associated with wakefulness (Czeisler et al., 1989). Its secretion depends on a hormonal and a neuronal pathway both among SCN projections (Buijs, 1999; Kalsbeek et al., 2011). Cortisol shows a robust circadian rhythmicity with nadir in the early biological night, close to the habitual bedtime; rapid rising in the middle of the night (when sleep would normally occur); and peak in the morning, close to habitual wake time (Morris et al., 2012b) **[Figure I.4]**. Interestingly sleep can suppress cortisol levels during its early part when slow wave sleep (SWS) is prominent (Follenius et al., 1992). Conversely, intravenous infusion of corticosteroids increases SWS and decreases REM sleep (Born et al., 1991).

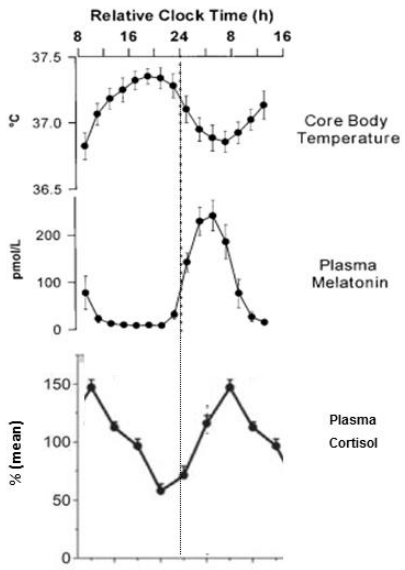


Fig. I.4: Circadian oscillation of core body temperature (CBT), plasma melatonin and plasma cortisol. Vertical reference line indicates transition of habitual wake- and bedtime

Figures combined and modified from (CBT and melatonin : Cajochen et al., 1999a; cortisol : Morris et al., 2012b)

2 THE SLEEP HOMEOSTAT

Sleep homeostat is an hourglass oscillator process which tracks the history of wake and sleep in order to regulate the average level of sleep debt. During wakefulness, sleep debt (also referred as sleep pressure) accumulates. Conversely, it dissipates during sleep. The term homeostasis relies on the observation that the longer and the more active is the waking episode, the longer and the deeper is sleep (Borbély and Achermann, 1999).

In contrast to the circadian timing system, neuroanatomical underpinnings of the sleep homeostat remain unclear. Sleep homeostasis process is likely to be a diffuse system, which, to date, has been more characterized in terms of molecular and electrophysiological aspects

2.1 What are the molecular mechanisms of sleep homeostasis?

Within the molecular framework, adenosine system has certainly been the most implicated in the function of sleep homeostasis. Adenosine is by-product of cellular energy metabolism (derived from adenosine triphosphate, ATP). During wakefulness, extracellular adenosine levels rise due to increased neural activity, whereas they decline during sleep. Those changes appear to be more prominent in the basal forebrain – an area which promotes wakefulness (Saper et al., 2001; see section 1.1). Thus, it has been hypothesized that adenosine is the molecular regulator of the sleep-wake cycle which operates by inhibiting the neural activity during sleep (Porkka-Heiskanen and Kalinchuk, 2011). Experimental evidences support this theory. The intracerebroventricular injection of adenosine or adenosine agonist has been shown to promote sleep (Benington et al., 1995; Virus et al., 1983), whereas intake of adenosine antagonists, such as caffeine, promotes wakefulness (Wyatt et al., 2004).

Other local net increases of molecules have been suggested to play a role in the wake-related progressive build-up in homeostatic sleep pressure. These substances notably include molecular markers of glutamatergic neurotransmission, the main excitatory pathway of the central nervous system (Dash et al., 2009). They also comprise endocrine (Growth Hormone Release Hormone (GHRH)) and pro-inflammatory agents (nitric oxide (NO), prostaglandin D2, tumor necrosis factor (TNF) or interleukin-1 (IL1)), which may mediate parts of deleterious effect of sleep deprivation on health (Krueger et al., 2008; Obal and Krueger, 2003; Wright et al., 2015). Those molecules promote sleep through inhibition of subcortical centers of arousal and interact in a complex synergic way (Krueger et al., 2008).

2.2 What are the electrophysiological markers of sleep pressure?

The accumulation of sleep pressure with time spent awake increases EEG activities in low frequencies (0.75-8 Hz) both in wake and sleep. Indeed the classical and most obvious electrophysiological markers of sleep pressure are slow wave activity (SWA, 0.75 – 4.5 Hz) and slow wave sleep (SWS) during NREM sleep. Their amount increases with the duration of prior wakefulness and they progressively dissipate with NREM sleep independently of circadian phase at which wake and sleep occur (Dijk et al., 1987). Importantly, the homeostatic increase in EEG low frequencies component with wakefulness can be attenuated by scheduling multiple short nap episodes (Dijk et al., 1987). The topographical distribution of this process is not homogeneous but is the most pronounced in frontal cortical areas **[Figure I.5]** suggesting that sleep, as global process, may also be regulated locally (Cajochen et al., 1999b).

Theta activity (4.5-8 Hz) during wake is considered as a marker of sleep need because it increases during prolonged wakefulness (Cajochen et al., 1995; Vyazovskiy and Tobler, 2005). Like SWA, this relative increase shows frontal predominance during

sleep (Cajochen et al., 1999b). However theta activity also displays a marked circadian influence (Cajochen et al., 2002).

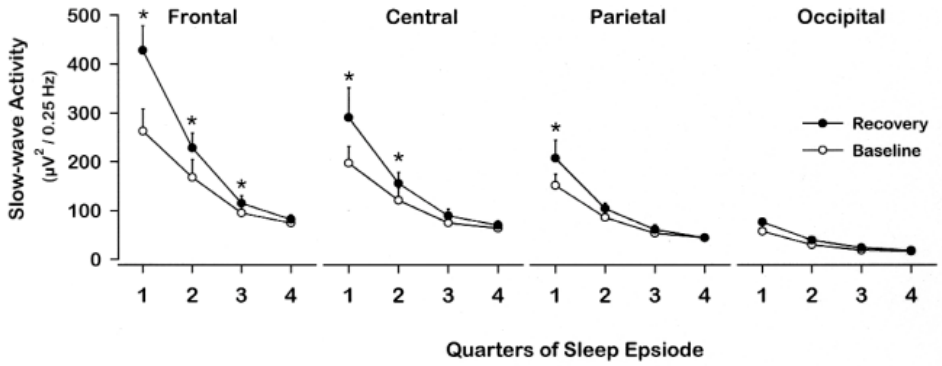


Fig. I.5 : Topographical dynamics of slow-wave activity (SWA, 0.75-4.5 Hz band) during NREM sleep across quarters (2h-intervals) of baseline and recovery (following 40h of total sleep deprivation) sleep episode in the frontal (Fz), central (Cz), parietal (Pz) and occipital (Oz) derivation (n=6; +1 SEM). The increase in SWA is most prominent in the frontal derivation. Asterisks indicate significant differences ($p<0.05$, baseline vs. recovery, Duncan's multiple range test). *Figure From (Cajochen et al., 1999b)*

2.3 Do clock genes influence sleep homeostasis?

There is evidence for a role of clock genes in the homeostatic regulation of sleep. For instance, mice lacking both Cry 1 and Cry 2 (Cry 1,2 $^{-/-}$), which are behaviorally arrhythmic under constant conditions (Vitaterna et al., 1999), also spend more time in NREM sleep, which is more consolidated (longer uninterrupted NREM sleep episodes) and characterized by higher EEG delta power (Wisor et al., 2008). On the other hand, mice knocked-out for per1 or per2 have diminished frontal SWA during NREM sleep, a proxy of sleep pressure level (Kopp et al., 2002). Furthermore, per1 or per2 expression respectively increases and decreases in mice forebrain during sleep deprivation and sleep recovery, which also supports their role in the homeostatic sleep regulation.

A non-circadian role of clock genes has also been demonstrated in humans. The coding region of the PER3 gene has a variable-number tandem-repeat polymorphism encoding for a repetition of 18 amino acids either four (PER3 ^{4}) or five times (PER3 ^{5}). Thus one individual can either be homozygous for PER3 ^{4} (PER3 $^{4/4}$; ~45% ^{*5}) or PER3 ^{5} (PER3 $^{5/5}$; ~10% ^{*5}) alleles or heterozygous (PER3 $^{4/5}$; ~45% ^{*5}) (Archer et al., 2003; Lázár et al., 2012). Homozygosity for the longer allele (PER3 $^{5/5}$) has been shown to predict alterations in sleep structure and slow oscillations, indicative of accelerated increase of homeostatic sleep pressure (Viola et al., 2007). Relative to PER3 $^{4/4}$ individuals, PER3 $^{5/5}$ show increased SWS and SWA during NREM sleep and increased theta and alpha activity during REM sleep **[Figure I.6]**; differences which are amplified after 40h of total sleep loss. (Viola et al., 2007). Deterioration of performance to a prolonged multiple-task test batteries is also more prominent in the PER3 $^{5/5}$ individuals during the circadian night following sleep-loss (Groeger et al., 2008). Interestingly, circadian rhythms of melatonin and cortisol seem to be not affected by PER3 polymorphism (Viola et al., 2007). Altogether, these results suggest that PER 3 $^{5/5}$ individuals are likely to live under higher sleep pressure and are more susceptible to the effects of sleep loss (Viola et al., 2007).

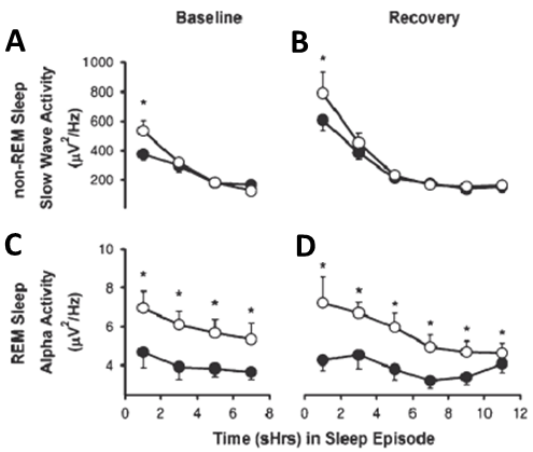


Fig. I.6: PER 3 polymorphism and sleep structure

A-B Dynamics of SWA (0.75-4.5 Hz) in NREM sleep during baseline **(A)** and recovery night **(B)** following 40 h of total sleep deprivation under constant routine conditions (see section 3.2.1) . **C-D** Dynamics of alpha activity (8-12 Hz) in REM sleep during baseline (C) and recovery night (D) following 40 h of total sleep deprivation under constant routine conditions. Open symbols = PER3 $^{5/5}$; filled symbols = PER3 $^{4/4}$; asterisks indicate significant differences between the 2 genotypes : *p<.05*). *Figure modified from (Viola et al., 2007)*

^{*5} Among Caucasian population (Lázár et al., 2012)

3 THE TWO PROCESS MODEL

It is now well established that both circadian pacemaker and sleep homeostat contribute about equally to sleep and wake regulation. Their interacting contribution has been integrated in the "two process model"(Borbély, 1982).

3.1 Through the interaction of sleep homeostasis and circadian oscillators

The two process model has been conceptualized in the eighties (Borbély, 1982). To date, it stands as the most relevant model to understand the timing and the architecture of sleep. According to this model **[Figure I.7]**, the homeostatic sleep pressure (*process H*)[6] accumulates with each waking hour and is dissipated by sleep on an exponential manner. The increase in sleep pressure may be nearly linear during a normal day but actually follows a saturation curve which appears evident under extended sleep deprivation (> 24h) (Achermann and Borbély, 2003). By contrast, the circadian signal *(process C)* oscillates on a sine-wave manner with an intrinsic near 24h period which is synchronized itself, in normal conditions, to the external light-dark cycle. During the biological day, a circadian drive for wakefulness counteracts the progressive sleep pressure build-up until the end of the so-called evening "wake maintenance zone" (WMZ; Dijk and Czeisler, 1995; Strogatz et al., 1987). When entering into the biological night, the circadian timing system switches to a sleep promoting signal, which favors sleep until the end of the early morning "sleep promoting zone" (SPZ; Dijk and Czeisler, 1995). This circadian drive for sleep opposes the progressive tendency to wake-up due to sleep pressure dissipation during sleep and allows reaching REM stage which mostly occurs in the latest part of the night (Mc Carley, 2011).

[6] In the original version of Borbély, sleep homeostasis was referred as process S. Later in this manuscript, we will use a sine-wave function to model the circadian signal which will be referred as S. Thus avoid any confusion, we will use process H instead process S for sleep homeostasis.

The circadian drive for wakefulness peaks just before habitual bedtime for humans entrained to 24h-day. A couple of hours before habitual bedtime, the sleep promoting hormone melatonin is released by the pineal gland. It fixes to melatonin SCN receptors resulting in neuronal SCN firing suppression which quiets the circadian waking promoting signal and thereby facilitates sleep. The circadian sleep promoting signal reaches its maximum just before the habitual wake-up time close to the nadir of core body temperature.

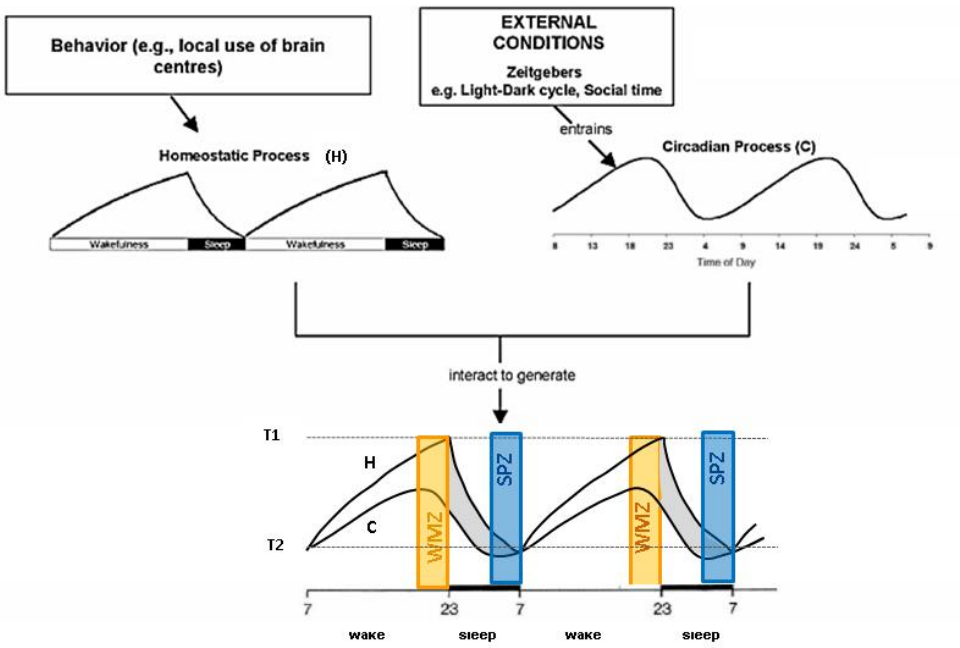


Fig. I.7 : The two process model for sleep regulation

Sleep homeostatic pressure builds up during wakefulness and declines during sleep on an exponential manner (*Process H*)**.** Behavior and more specifically the use of brain regions (e.g. during a specific task) may impact on the local sleep homeostatic drive. The circadian pacemaker oscillates on a sine-wave manner with an intrinsic near 24h period (*Process C*) which is synchronized by external Zeitgebers (e.g. light-dark cycle, social time). The circadian process modulates the two thresholds delimiting *H*. During the biological day, the circadian pacemaker promotes wake and counteracts the build-up of sleep homeostatic pressure build-up. This circadian wake promoting signal is maximal in the evening, a period so-called wake maintenance zone (WMZ, orange rectangle). During the biological night, the circadian pacemaker promotes sleep, favors sleep continuity and prevents from progressive tendency to arouse during sleep because of sleep pressure dissipation. This circadian sleep promoting signal is maximal in the early morning, a period so-called sleep promoting zone (SPZ, blue rectangle). *Figure modified from (Schmidt et al., 2007)*

According to the two process model, the time-of-day modulation of brain function results from a balanced interaction between sleep homeostasis and the circadian signal. To determine the relative contribution of these two processes on cerebral outputs (i.e. neurophysiological or cognitive parameters) remains however challenging. The following section addresses the key study paradigms mostly used in chronobiology research to explore the influence of the sleep homeostasis and circadian processes.

3.2 The exploration paradigms in chronobiology

3.2.1 The constant routine paradigm

The constant routine (CR) protocol has been applied to unmask the endogenous circadian rhythms (Duffy and Dijk, 2002). The intrinsic circadian rhythmicity is indeed embedded in the sleep-wake cycle and can be veiled by any external (e.g. light, food, body posture, social interactions, time cues) or internal (e.g. digestion, stress, motivation) factors with potential to modify physiological and behavioral responses (Duffy and Dijk, 2002). In a CR, these factors are controlled as much as possible in order to attenuate their masking effect. Participants are kept awake for more than 24 hours, staying in a semi-recumbent posture, with no time-of-day information. Instead of main meals, they receive regular isocaloric snacks and water to cover their energy metabolic and hydration demands. Social interaction and physical activity are kept at minimum. Ambient temperature and humidity are maintained unvarying. Light, the most powerful circadian synchronizer, is also kept constant at dim level (~ 5 lux). Under those strictly controlled conditions, physiological (CBT, plasma or saliva melatonin and cortisol, EEG recordings) and behavioral measures are assessed at regular fixed time intervals. Thus, the constant routine allows to determinate the endogenous circadian phase and amplitude, as well as to evaluate the circadian and

sleep homeostatic influence on physiological and/or neurobehavioral variables. For instance, the CR makes possible to compare EEG spontaneous activity and responses or cognitive performance picked at different circadian phases or either at same circadian phase but with large differences in sleep pressure (Dijk et al., 1992; Groeger et al., 2008; Münch et al., 2004; Viola et al., 2012a) **[Figure I.8b]**.

The CR has however some limitations. Since sleep pressure continuously builds up with sustained wakefulness, this protocol does not allow to segregate the two processes. The contribution of the sleep pressure can be investigated by comparing one condition in which participants are totally sleep deprived with one condition in which multiple naps are scheduled in order to dissipate sleep pressure (Schmidt et al., 2007).

3.2.2 The forced desynchrony paradigm

In a forced desynchrony (FD) protocol, volunteers are isolated for several days or weeks in a constant dim light environment which is free from external usual times-cues (Cajochen et al., 2002; Dijk and Czeisler, 1995; Lazar et al., 2015; Wyatt et al., 2006). During this period, they followed a superimposed artificial sleep-wake cycle which is significantly either longer (e.g. 28h **[Figure I.8c]**) or shorter (e.g. 19h) than the normal 24h-day cycle. The imposed period in FD is out of the range the circadian pacemaker can adapt to. Thus, it leads participants to progressive desynchronization of the imposed artificial sleep-wake cycle and their endogenous circadian cycle. In those conditions, the circadian oscillator follows its own intrinsic rhythm which is slightly different from 24h (Dijk and Czeisler, 1995; Schmidt et al., 2007).

FD offers the unique advantage to truly separate the influence of the circadian and the sleep homeostatic processes. In this protocol, sleep can virtually occur at all

circadian phases allowing to unmask sleep-dependent changes in sleep consolidation at all circadian phases (Schmidt et al., 2007).

FD has also drawback. As one can imagine, the technique is extremely time, energy and money consuming. It is also very difficult to conduct considering its long duration, the strict environmental conditions required and unusual schedules imposed to both subjects and experimenters.

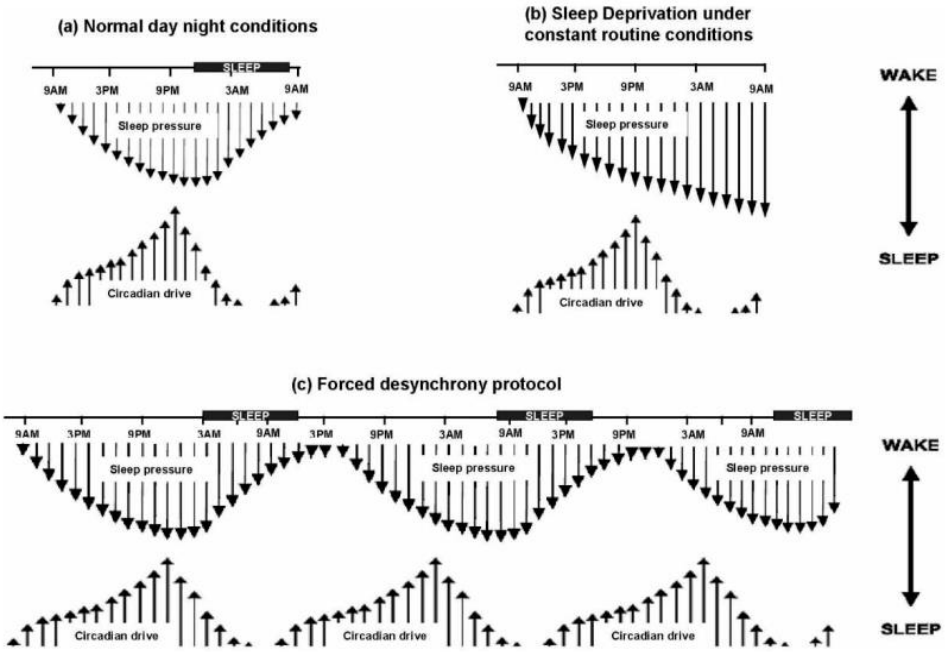


Fig. I.8 : Interaction of sleep homeostatic and circadian drives during (a) normal day-night conditions (NC), (b) during sleep deprivation under constant routine (CR) conditions and (c) during a forced desynchrony (FD) protocol. In NC (a), the sleep episode and the two processes are scheduled according to the individual's preferred sleep-wake schedule. This figure presents a "neutral" chronotype with habitual sleep time at 11 pm. During CR (b), sleep deprivation leads to sleep pressure increase thus allowing the assessment of the circadian alerting signal under different sleep pressure conditions. In FD paradigm (c), participants followed a superimposed artificial sleep-wake cycle which is significantly either shorter or longer (presented here) than 24h. This allows the observation of scheduled sleep and wakefulness episodes at virtually all circadian phases. *Figure and legend modified from (Schmidt et al., 2007)*

We presented the general principles of the interaction between sleep homeostasis and the circadian timing system. We also addressed the main methods used in chronobiology to explore those two processes in or out of their interplay. The two last subsections of this chapter will be dedicated to the cerebral electrophysiological and cognitive outputs of this interplay.

3.3 EEG activities shared between homeostatic and circadian influences

The homeostatic and circadian processes modulate both sleep and waking EEG activities with pronounced frequency specificity. In other words, some frequencies ranges EEG activities seem to be mostly dependent on sleep homeostat whereas others appear to be mostly under circadian influence.

3.3.1 Circadian and homeostatic modulation of sleep EEG

NREM sleep

As previously mentioned in the second section of this chapter, wakefulness duration increases SWA and SWS during NREM sleep (Dijk et al., 1987) which dissipate with time asleep. However, the slowing down in EEG activity during sleep related to sleep pressure does not only concern delta range (0.75 – 4.5 Hz) but also implies the sigma/spindle range (12-15 Hz). Indeed, prolonged wakefulness increases low spindle frequency activity (LSFA ; <13.5 Hz) and decreases high spindle frequency activity (HSFA >13.5 Hz)[7] (Knoblauch et al., 2002). Nonetheless, spindle activity during NREM sleep also exhibits a significant circadian influence with LSFA and HSFA being respectively maximal and minimal during the circadian phase of melatonin secretion. Sleep spindles have been suggested to play a key role in sleep

[7] Spindles are not distributed homogeneously over the brain : ~12 Hz spindles exhibit an anterior dominance whereas ~14 Hz spindles are the most prominent in posterior derivations (Gibbs, 1950)

maintenance, basically by inhibiting the sensory information that reaches the cerebral cortex (Dang-Vu et al., 2010; Siapas and Wilson, 1998). Whether this sleep maintenance effect is underscored by the slow or the fast spindles remains unclear[8]. Nonetheless, it is remarkable that greatest changes in spindle range activity occur during periods of enhanced homeostatic or circadian drive for sleep.

REM sleep

During REM sleep, alpha activity range (8.25 – 10.5 Hz) shows the maximum circadian variance with nadir coinciding with period when melatonin is high, close to the crest of REM sleep rhythm, in the early morning hours (Dijk et al., 1997). Alpha activity has also been proposed as a marker of sleep homeostasis and more specifically of REM sleep homeostasis. Indeed, similarly to SWA in NREM sleep, alpha activity declines during the course of REM (Roth et al., 1999; Viola et al., 2007; see fig. I.5). As compared to PER3 $^{4/4}$, NREM alpha activity is increased in PER3 $^{5/5}$ individual assumed to live under higher sleep pressure (Viola et al., 2007; see fig I.5). However, selective REM sleep deprivation reduces alpha power during subsequent REM sleep (Roth et al., 1999) , which is contradictory with the principle of homeostasis.

3.3.2 Circadian and homeostatic modulation of waking EEG

Alpha activity, recorded at both fronto-central and parieto-occipital derivations, exhibits a pronounced circadian modulation with nadir in the late biological night and peak in the middle of the biological day (Cajochen et al., 2002). This frequency range is also affected by sleep homeostasis since it is reduced with increasing duration of wakefulness (Cajochen et al., 2002).

[8] Due to differences in the ranges used for discriminate LSFA and HSFA

Spectral power in theta range (4.5 – 8 Hz) on waking EEG is usually considered as a marker of homeostatic sleep pressure (Finelli et al., 2000; Vyazovskiy and Tobler, 2005). It increases during sleep deprivation with largest effect on frontal areas (Finelli et al., 2000). Furthermore, this increase predicts the increase of SWA during subsequent sleep (Finelli et al., 2000). Waking theta activity is however not purely homeostatic. In a forced desynchrony study, waking theta and beta (20-32 Hz) activities measured on fronto-central derivation showed a circadian rhythmicity with nadir close to the onset of melatonin secretion, i.e. during the wake maintenance zone (Cajochen et al., 2002) .

3.4 How is cognition influenced by the 2 processes?

3.4.1 Global overview

It is now well established that daily changes in human cognition are set by the interplay between sleep homeostasis and the circadian timing system. This dual influence appears particularly evident under sleep deprivation conditions.

Human cognition is maintained rather stable during the first ~16 hours of a normal waking day, but deteriorates abruptly when wakefulness is extended into the biological night (Cajochen et al., 1999a) **[Figure I.9]**. The overnight impairment is typically followed by a morning partial recovery before undergoing subsequent deterioration if wakefulness is extended into a second day. Finally, after a recovery sleep episode, cognitive function may be restored. This non-linear dynamics of human cognition is well predicted by the two process model. During the biological day, the circadian wake promoting signal opposes the negative impact of homeostatic sleep pressure accumulation. During the biological night, under sleep deprivation (and entrained conditions), the circadian signal and build-up in sleep

pressure promote sleep in synergy. The transient and partial cognitive improvement in the morning corresponds to the "switch" circadian signal back to wake promotion. However, this circadian "rescue" is rapidly overpassed by the ongoing sleep pressure accumulation. To summarize, the two process-model of sleep-wake regulation entails the daily changes in neurobehavioral efficiency because of increasing homeostatic sleep pressure combined with the influence of the circadian timing system which supports more or less optimal performance efficiency.

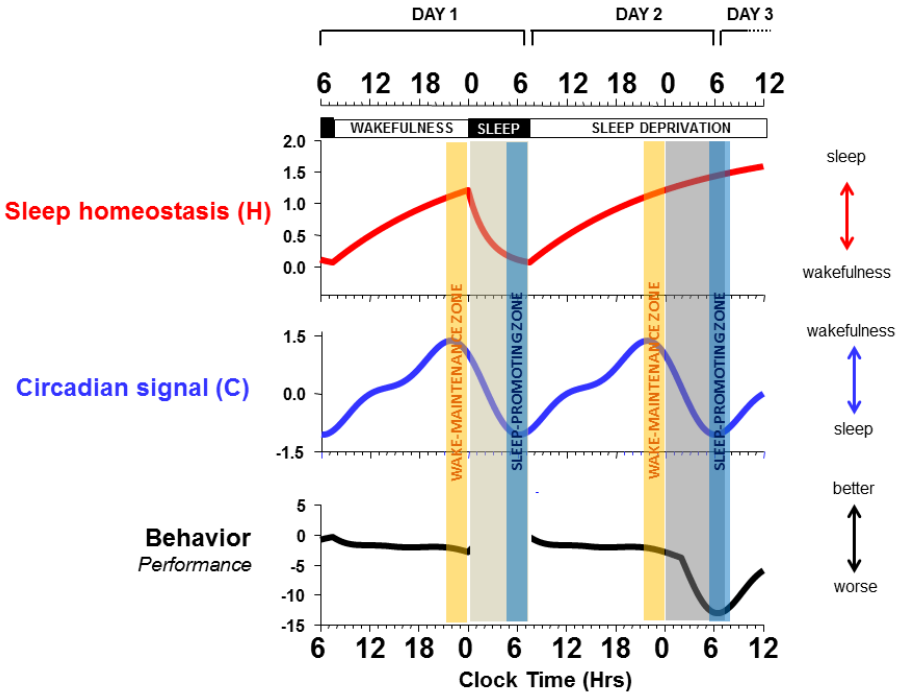


Fig. I.9 : Schematic representation of the sleep homeostatic process (H, red curve) and the circadian drive (C, blue curve) and their impact on behavior (black curve). The putative circadian alerting signal maintains cognitive performance rather stable during the ~16 hour of a normal waking day (day 1). However, when wakefulness is extended into the biological night (day 2), it deteriorates abruptly before partially recovering in the morning following the night without sleep (day 3). *Figure modified from (Dijk and Archer, 2010)*

3.4.2 Specific cognitive aspects

We provided a schematic view of how human cognition is modulated by the two interacting sleep and wake regulatory processes. However, cognition encompasses a variety of processes that may be served by different networks of brain areas. This includes attention, memory, executive or affective domains which themselves comprise subdomains (e.g. sleepiness, sustained attention, …; working memory, declarative memory, …; inhibition, planning, … ;stress, mood, …) that can be tested by more or less specific tasks (for review see: Schmidt et al., 2007) **[see appendix 1 for classification of cognitive processes]**. For instance, sustained attention can be assessed by the psychomotor vigilance task (PVT; Dinges and Powell, 1985), where participants press a button as fast as a digital counter starts with a random interval. Self-reported affect and subjective sleepiness are usually assessed by visual analog scales (VAS; Monk, 1989) and Likert-type rating scales, such as the Karolinska Sleepiness Scale (KSS; Akerstedt and Gillberg, 1990) and the Stanford Sleepiness Scale (SSS; Hoddes et al., 1972). All those tests showed robust circadian rhythmicity in combination with the classical deterioration associated with increasing homeostatic sleep pressure (Cajochen et al., 1999a; Dijk et al., 1992; Monk et al., 1997) **[Figure I.10].**

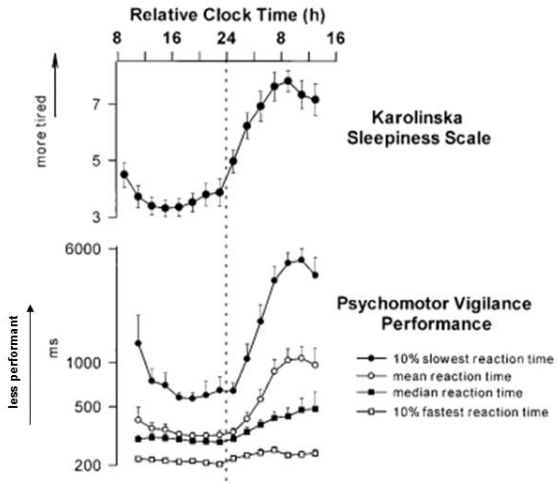


Fig. I.10 : Time course of subjective sleepiness and sustained attention level during 32h of sustained wakefulness under constant routine conditions. Subjective sleepiness and sustained attention are respectively indexed by Karolinska Sleepiness Scale (KSS) and Psychomotor Vigilance Task (PVT) performance. *Figure from (Cajochen et al., 1999a)*

Nonetheless, the time-of-day at which a cognitive test is optimally completed largely depends on the specific parameters of the task. This includes the cognitive domain it belongs to, its duration, its difficulty/complexity, the administration method and the measured variable (Bonnet, 2000; Rutenfranz and Colquhoun, 1979). While performance on simple repetitive (Colquhoun, 1981) and serial search tasks (Monk, 1982) peaks with temperature levels in the evening, performance on more complex cognitive tasks, such as logical reasoning tasks, seems to peak in the late morning (Folkard, 1975), and short-term memory retention performance appears to be optimal in the early to mid-morning (Laird, 1925). Those observations underlie the debate that the circadian drive differentially affects performance subtended by different cognitive processes and networks of brain areas (Maire et al., 2014). However, caution is warranted as those observations were obtained without controlling the influence of primary factors, such as motivation (Minors and Waterhouse, 1983), stress (Orr et al., 1976), food intake (Paz and Berry, 1997), ambient temperature (Mavjee and Horne, 1994), time cues, posture (Kräuchi et al., 1997), caffeine consumption (Ryan et al., 2002), physical activity (Bugg et al.) or light exposure (Leproult et al., 1997); all parameters that can mask the circadian profile of neurobehavioral function (Duffy and Dijk, 2002). Indeed, the rhythm of short-term memory performance was shown to be parallel (and not inversed) to CBT variations when testing was extended into an unmasking constant routine (CR) protocol with 40 hours of continuous wakefulness (Cajochen et al., 1999a).

Those results do not preclude that the impact of the circadian pacemaker and/or the sleep homeostatic drive could vary among different cognitive processes. Indeed another constant routine study (with 38h of continuous wakefulness) showed that sensitivity to the circadian signal and sleep pressure accumulation varies according to the attentional domain investigated (Horowitz et al., 2003). Whereas sustained (vigilant) attention, as indexed by PVT reaction time, worsened with time awake and adverse circadian phase, selective attention, tested by a search task, remained

constant over the protocol (Horowitz et al., 2003). Another study investigated the impact of repeated partial sleep deprivation and acute total sleep deprivation on different cognitive domains (Lo et al., 2012). Partial and total sleep deprivation showed primary detrimental effects on subjective sleepiness and sustained attention whereas their effect on working memory (a form of executive function) was much smaller. Furthermore, while prior partial sleep deprivation led to poorer cognitive performance in a subsequent total sleep deprivation episode, its effect was virtually absent in the evening wake maintenance zone and most prominent in early morning hours indicating a modulation by circadian phase.

To sum, it appears clear that both sleep homeostasis and the circadian timing system interact to set human cognitive function. However, the modulatory effect of this interplay may vary with the cognitive domain and task characteristics and may be altered by external of internal factors. This highlights the importance to select unmasking paradigms (e.g. constant routine protocol) when studying circadian contribution on cognition. A better comprehension of the mechanisms that underpins temporal changes in cognitive performance has significant implications. It must foster the need to individually adapt our work shifts or at least promote countermeasures (e.g. naps, light exposure, physical activity) aimed to limit cognitive deterioration under challenging circumstances (Czeisler and Gooley, 2007). Ultimately, it could improve work efficiency, optimize school time-tables (Laird, 1925), and reduce professional accidents (Barger et al., 2005; Lockley et al., 2004).

CHAPTER II

Transcranial Magnetic Stimulation

1 HISTORY OF CORTICAL STIMULATION

In 1980, Merton and Morton showed it was possible to stimulate human brain motor areas through the intact scalp by means of transcranial electrical stimulation (TES) (Merton and Morton, 1980). They produced motor evoked potentials (MEP) by delivering brief, high voltage electric shocks through external scalp electrodes placed above the motor cortex. However, their technique, although promising, rapidly met limitations because TES also activates pain fibers in the scalp provoking significant discomfort. Thus, five years later (1985), Barker and colleagues presented external magnetic stimulation as a painless non-invasive way to stimulate both brain and nerves (Barker et al., 1985) **[Figure II.1]**. They used a magnetic stimulating coil making the conventional electrode placement unnecessary. Applying external time-varying magnetic fields to stimulate the brain was actually not a new concept since it had been first reported by d'Arsonval in 1896 (D'Arsonval, 1896). However, Barker and colleagues were the first to bring a transcranial magnetic stimulation (TMS) device which was sufficiently compact and easy to handle to be used in a clinical environment.

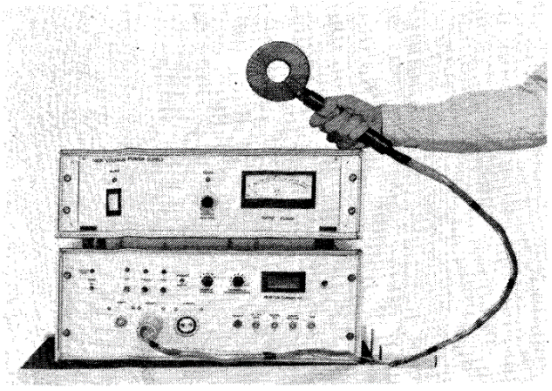


Fig. II.1 Magnetic stimulator and coil *from (Barker et al., 1985)*

2 BASIC PRINCIPLES OF TMS

Transcranial magnetic stimulation is based on the law of electromagnetic induction described by Michael Faraday in 1831. Using a nearby conductor, Faraday showed that currents could only be induced by time-varying magnetic fields but not by static ones. For TMS **[Figure II.2]**, a brief high-current pulse passes through a coil of wire placed above the scalp. The pulse of current induces a rapidly changing magnetic field with lines of flux passing perpendicularly to the plane of the coil and which penetrate the scalp and the skull to reach the cortex with negligible attenuation. The rapid magnetic variation induces a second electric field perpendicularly to the magnetic field but with opposite direction to the original electric field. The resulting secondary ionic current in the brain penetrates neurons membranes, flows through the axons and triggers a post-synaptic excitatory (or inhibitory) action potential (Hallett, 2000; Rosanova et al., 2012a). From the site of direct cortical stimulation, the neuronal excitation propagates to other cortical areas, subcortical structures and spinal cord via associating and projecting fibers (Rosanova et al., 2012a).

The capacity of TMS to depolarize neurons depends on the "activating function" which causes a transmembrane current sufficient to flow and depolarize the rest of the neuron membrane (Roth et al., 1991). Mathematically, the activating function can be represented as the spatial derivative of the induced electric field along the nerve. In such that way, stimulation takes place at the point where this spatial derivative is maximal (Roth et al., 1991). In the case of a neural fiber bends across the induced electric field, the current passes out straightaway across its membrane resulting in a local depolarization that increases this derivative. This phenomena makes an axon bend to be a preferential site of stimulation (Abdeen and Stuchly, 1994). Taking account of the general disposition of axons, for a maximal stimulation efficiency, the coil should be ideally orientated to induce a secondary electric field perpendicular to the targeted gyrus (Rosanova et al., 2012a).

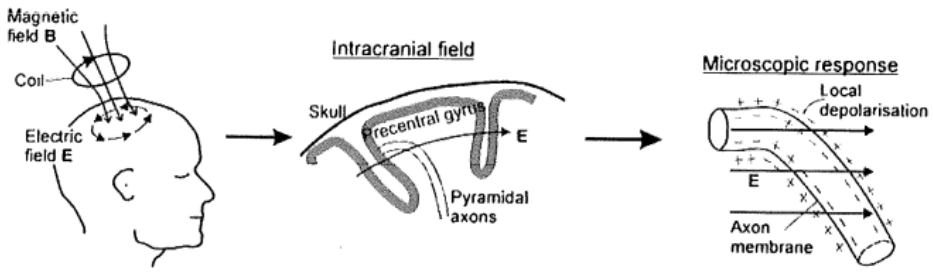


Fig. II.2 : Neural targets of TMS. From left to right: schematic representation of the electric filed (E) induced into subject's head by the magnetic field (B) after a brief pulse of current is passed through the TMS coil.; the macroscopic (cortical gyri) and microscopic targets (bent axons) of the electric field induced intracranially by the TMS coil . *Figure and legend from (Rosanova et al., 2012a)*

3 TMS TECHNICAL ASPECTS

The effects of TMS on brain rely on stimulation parameters which include TMS pulse intensity, focalization, stimulation target and frequency setting. In this section, we depict the influence of all these variables in TMS causal effect.

3.1 TMS intensity

The magnetic field intensity depends on the current pulse intensity which level can be adjusted by the operator. Stimulators and coils currently produced about 1.5-2 Tesla at the face of the coil resulting in currents changes at rates up to 170 A/s (Thielscher and Kammer, 2002) and induced electrical fields voltage in the cortex up to 150 V/m. The stimulation intensity is always maximum at the cortical surface and attenuates rapidly with distance from the coil (Roth et al., 1991). With standard coils, only superficial neurons at a depth of 1.5-3.0 cm beneath the scalp are considered to be directly activated by TMS. Thus, TMS cannot reach deep brain regions (such as hippocampus, amygdala, striatum, thalamus and brainstem).

3.2 TMS focalization

The focus of the magnetic field depends on the shape of the stimulation coil. Most commonly used magnetic coils are the circular and figure-eight shaped coils **[Figure II.3]**. Circular coils induced relatively wide electric fields. This latter property allows bihemispheric stimulation which can be useful, for example, in the study of the central motor conduction (Rossini and Rossi, 1998). The figure-eight shaped coil is less powerful but provides more focal stimulation with maximal current induced at the intersection of the two round components (Hallett, 2000; Kobayashi and Pascual-Leone, 2003). With the figure-eight coil used in the present work experiment (Eximia; Nexstim, Helsinki, Finland), the hotspot (surface of the cortex which is efficiently stimulated) is about 0.68 cm².

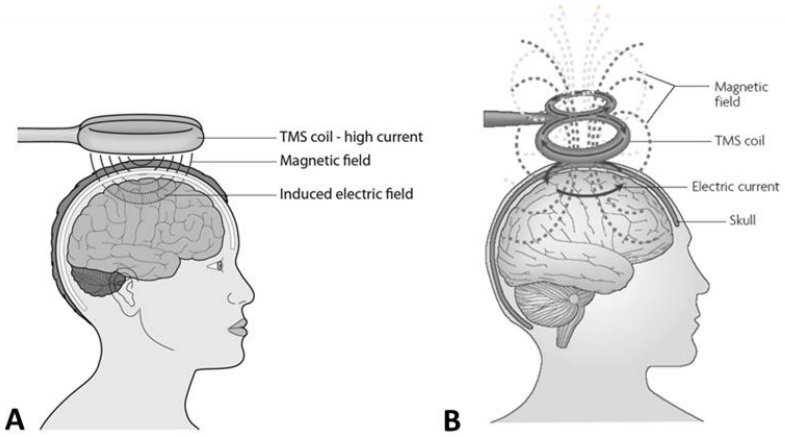


Fig. II.3 : Schematic representation of TMS circular (A) **and figure-eight shaped coils** (B) **and their induced electric field.** *Figures from http://nuffieldbioethics.org (A) and (Ridding and Rothwell, 2007) (B)*

Other shapes of coils exist, including the double cone coil formed of two large adjacent circular wings at an angle of 95". Less focal but stronger than the figure-eight coil (Lontis et al., 2006), it allows direct stimulation of deeper brain regions like lower limb motor area situated at the interhemispheric fissure.

3.3 TMS targeting

TMS allows a stimulation location easy setting by freely moving the coil all above the scalp, whereas, in TES, the operator is constrained by placement of stimulation electrodes. A suprathreshold stimulus delivered over the motor cortex provokes a contraction of a muscle in the contralateral face, upper or lower limb muscle (Barker et al., 1985). A stimulation applied over the occipital cortex can elicit phosphenes and visual perception disruption (Kammer et al., 2005). However, choosing the site of stimulation according to a sensor or a motor response is inaccurate and limits exploration to a few "speaking" brain areas. Two methods were initially employed to reproduce TMS targeting within and across individuals. The first method consists of the estimation of the resting motor threshold (RMT). The latter is defined as the lowest output necessary to obtain a visible twitch of a contralateral peripheral muscle (most commonly the first dorsal interosseous (FDI) of the thumb) for 50 percents of a set of stimulations. If other areas than the motor cortex are stimulated, the coil is moved taking the location used for the estimation of the MT as the spatial reference. The second method is based a 10-20 EEG channel system which electrodes locations are assumed to match to cortical regions. Limitations of this method are obvious since it does not take into account of interindividual variability of the skull shape which can lead to errors up to 20 mm in different directions (Herwig et al., 2003; Rosanova et al., 2012a).

TMS neuronavigated systems have been developed in order to provide precise and reproducible TMS targeting **[Figure II.4]**. Those systems made possible the accurate location of the subject's head and TMS coil, by means of an optic or magnetic tracking system which allows to coregistrate the subject's head with his individual CT scan or MRI 3D reconstructed brain, skull and scalp. Most of available neuronavigation systems input coordinates of the stimulator to a virtual aiming device of the neuronavigation software. In such that way, the experimenter can

ensure coil position stability, direction, angle as well as the intensity of stimulation. Furthermore, using a 3-sphere model taking into account the head's shape, the coil position (and orientation), the distance scalp-to-cortex and the delivered intensity, neuronavigation systems are now able to calculate an estimation of the TMS induced electric field on the cortex surface. Finally, when TMS is combined to an EEG recording (see section 4), the position of EEG scalp electrodes can be digitalized (and stored) by means of a pen visible by the optic tracker (Rosanova et al., 2012a).

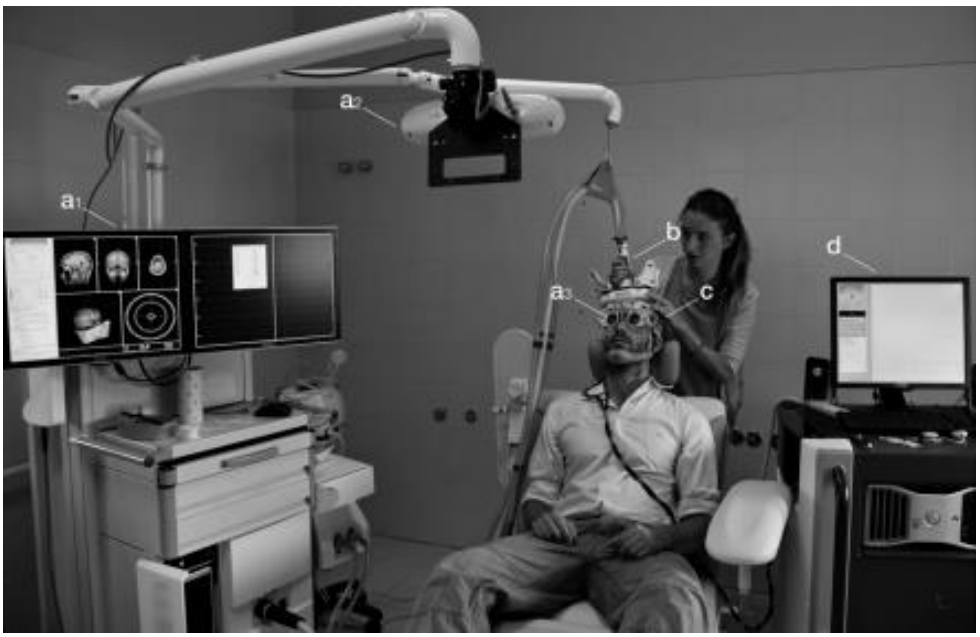


Fig II.4 : - Possible set-up, combining a neuronavigation system (a), the stimulation coil with tracking elements (b), high-density EEG net (c) and EEG amplifier (d). The neuronavigation system is composed of 3D brain reconstruction (a1), an infrared tracking camera (a2) and tracking googles (a3). *Figure and legend from (Napolitani et al., 2014)*

3.4 TMS frequency protocols

In addition to intensity, orientation, location and focalization, stimulation frequency is also critical because it determines the effects of TMS on the targeted cortical region.

Single pulse TMS refers to the application of stimuli one by one at low frequency (< 1 Hz) and with no regular interval. Single pulses are very safe and assumed to have no short term or long lasting after effects on neuronal activity. Single pulses are used, for instance, to map cortical outputs, to study central motor conduction time or causal chronometry in brain-behavior relations (Rossi et al., 2009).

Paired-pulse TMS can provide measures of intracortical facilitation (ICF) or inhibition (ICI) (Rossi et al., 2009). In this method, two TMS pulses are successively delivered to a single cortical target using the same coil or to two different brain regions using two different coils. The first pulse consists of a subthreshold conditioning stimulus whereas the second is a suprathreshold test stimulus. The effects of the conditioning TMS pulse on the size of a test TMS response (e.g. motor evoked potential) depend on the stimulus intensity and the interstimulus interval (Rossi et al., 2009). Study of the motor cortex showed that shorter interstimulus intervals (1-4 msec) and high intensity (60-80% of the resting motor threshold) corresponds to maximum inhibition (Kujirai et al., 1993; Schäfer et al., 1997). Conversely, longer intervals (7-20 msec) and lower intensity brings maximum facilitation (Kujirai et al., 1993; Ziemann et al., 1996). Using this principle, an Italian group showed, for instance, that a cortisol bolus injection transiently reduces the inhibitory effect of a shorter interval (3 msec) conditioning stimulus delivered before a test TMS pulse over the primary motor cortex (Milani et al., 2010).

In ***paired associative stimulation*** (PAS), a single TMS pulse is paired with a peripheral stimulus. The latter could be a motor (Stefan et al., 2000), sensory (Litvak et al., 2007a), auditory (Sowman et al., 2014) or visual (Suppa et al., 2015) stimulus. PAS is based on the idea that if two stimuli arrive at the same time (synchronous stimulation) at the cortex, an excitation will be induced. By contrast, asynchronous stimulation leads to inhibition (Stefan et al., 2000). Repetitive synchronous stimulation results in long term potentiation (LTP) –like effects whereas asynchronous stimulation leads to long term depression (LTD) -like effects (Stefan et al., 2000; Wessel et al., 2015). However, a substantial interindividual variability in PAS effects (which reasons remain unclear) exists (Ziemann et al., 2008).

Conventional (or regular) *repetitive TMS* *(rTMS)* consists of delivering multiple TMS with constant intensity in trains at a given frequency to one single brain area **[Figure II.5]**. High frequency (or rapid) and low-frequency (or slow) rTMS refer to rTMS with stimulus rate of more and less than 1 Hz respectively. Frequency of 5 Hz and higher have been shown to transiently enhance excitability in the motor cortex (Pascual-Leone et al., 1994), whereas frequency at 1 Hz will transiently depress excitability (Chen et al., 1997). Repetitive TMS has been used in clinics. In stroke, for instance, several days of high frequency (excitatory) rTMS applied to the ill hemisphere leads to clinical improvement (Khedr et al., 2005). Slow (inhibitory) rTMS on the unaffected hemisphere brings similar benefits because it may reduce possible interference with the recovery of the stroke hemisphere (Fregni et al., 2006). Induction of transient virtual focal lesions by slow rTMS can be used to determine the temporal organization of specific cortical networks. For example, the delay to deliver slow rTMS after an auditory stimulus that impairs the localization of this latter sound is shorter for extrastriate occipital cortex relative to posterior parietal cortex. This suggests that occipital cortex is involved earlier in the processing of auditory spatial perception than parietal cortex (Collignon et al., 2008).

Patterned repetitive TMS refers to repetitive application of short rTMS bursts at high inner frequency interleaved by short pauses of no stimulation **[Figure II.5]**. Theta burst (TBS) protocols are the most used patterned rTMS. They consist of repetition of short bursts of 50 Hz rTMS at rate in the theta range (5 Hz) as a continuous (cTBS, mostly inhibitory), or intermittent (iTBS, mostly excitatory) train (Huang et al., 2005; Di Lazzaro et al., 2008). Theta burst protocols have been applied, for instance to stroke patients. A single session of iTBS to the stroke hemisphere was shown to decrease reaction times of the paretic hand (Talelli et al., 2007). A similar effect is obtained after cTBS to the unaffected hemisphere (Di Pino et al., 2014). Quadripulse stimulation (QPS) is another patterned rTMS procedures able to induce long-term changes in cortical excitability (Hamada et al., 2008). This technique consists of repeating trains of four monophasic pulses separated by intervals of 1.5-1250 msec. Short intervals produce facilitation whereas longer intervals lead to inhibition (Hamada et al., 2008) .

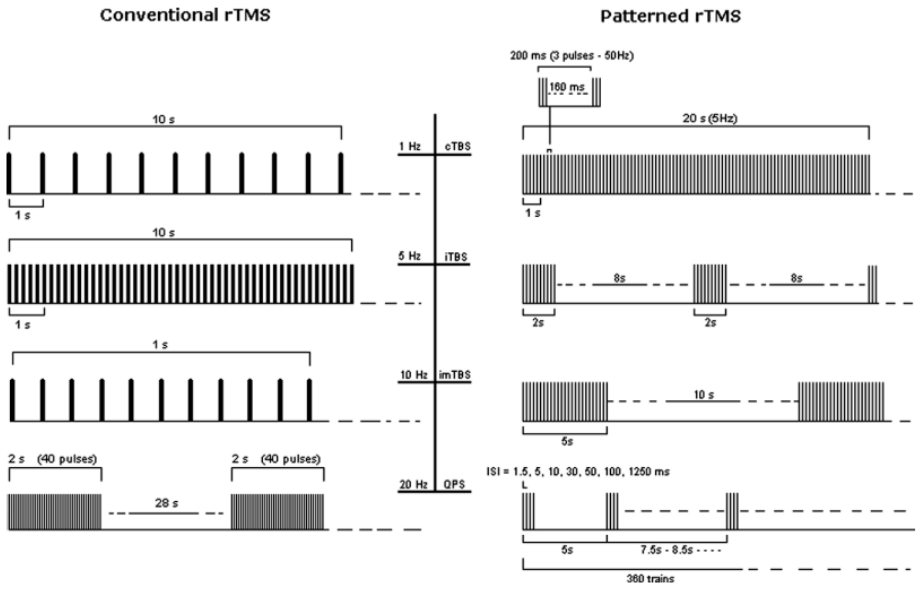


Fig. II.5 Conventional and rpatterned repetitive TMS (rTMS): Left panel (Conventional rTMS). From the top: examples of 10 s of rTMS at 1 Hz (first trace) and at 5 Hz (second trace); 1 s of rTMS at 10 Hz and a typical example of 20 Hz application for therapeutic purposes (trains of 2 s interleaved by a pause of 28 s). Right panel (Patterned rTMS). From the top: 20 s of continuous theta burst (first trace); intermittent theta burst (second trace) and intermediate theta burst (third trace). The fourth trace represents protocols of quadripulse stimulations (QPS). *Figure and legend from (Rossi et al., 2009)*

4 TMS COMBINED TO EEG RECORDING

TMS can evoke, potentiate or inhibit primary motor or sensory responses but is also able to modulate the activity of superior brain regions. For instance, the inhibition driven by slow rTMS is used to map language-related cortical areas before neurosurgery (Pascual-Leone et al., 1991). Rapid and slow rTMS delivered respectively to the left and right prefrontal dorsolateral cortex have been applied in clinical Psychiatry because they may improve depression symptoms (George et al., 1995; Klein et al., 1999). TMS brought great advances in research, clinimetry and therapeutic but, used "alone", its exploration field is restricted to only few "speaking" brain areas. Indeed, in this case, TMS effects can only be assessed by means of indirect outputs; those latter relying themselves on the integrity of a whole pathway (i.e. cortex, spine, peripheral nerves and sensors, neuromuscular junction and muscle for a motor evoked response). The assessment of TMS direct causal effect requires to record cortical activity in the order of the millisecond, a time resolution that only EEG provides.

4.1 TMS/EEG: brief history

Cracco and colleagues were pioneers in the history of TMS/EEG. In 1989, they compared transcallosal responses evoked by magnetic coil and electrical stimulation. Transcallosal responses were recorded with a latency of 8.8 -12.2 msec from the pulse (Cracco et al., 1989). Three years later, the same group recorded evoked response on the interaural line after magnetic stimulation of the human brain cerebellum (Amassian et al., 1992). The combination of TMS with EEG stayed however challenging because it remained large artifact introduced by the TMS coil discharge in the EEG leads. Indeed, a typical pulse intensity of about 1 Tesla with a rise time of 0.1 msec induces a voltage reaching 10 V at scalp electrodes. This largely surpasses the magnitude of brain responses (which are in the range of tens of

microvolts) resulting in large artifacts and saturation of an ordinary EEG amplifier for a few seconds. The latter problem was solved in the late 1990s. In 1999, Virtanen and colleagues presented a 60 channel TMS compatible EEG system equipped with gain control and sample-and-hold circuits able to block the TMS induced artifact **[Figure II.6]**. The system keeps the acquired signal at a constant level for a couple of msec around the TMS pulse, thus preventing EEG amplifier to saturate and allowing to further record TMS evoked EEG potentials free from artifacts (Virtanen et al., 1999).

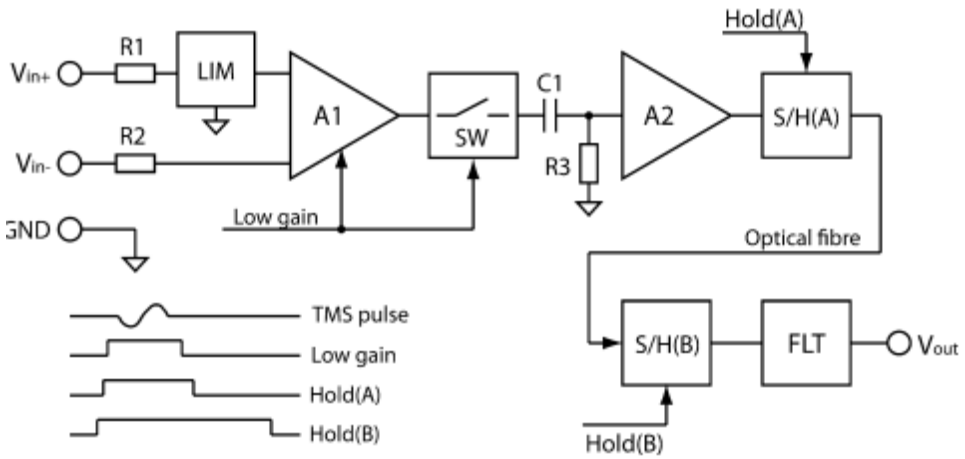


Fig II.6 : Block diagram of the TMS-compatible EEG amplifier by (Virtanen et al., 1999) capable of recording the EEG responses to single TMS pulses after just a few milliseconds

The gain of the first amplifier stage A1 is reduced during the TMS pulse. Simultaneously, the semiconductor switch SW, following A1, opens the signal path during the TMS pulse: the input voltage of the second amplifier stage A2 drops to zero and the voltage over capacitor C1 remains constant. To block large voltage peaks before the optical isolator, the sample-and-hold circuit S/H(A) latches the signal from A2 prior to the TMS pulse and keeps the output at this level during the pulse. S/H (B), located in the non-isolated section of the amplifier, prevents any residual from the stimulus artifact from being stored in the subsequent filters (FLT). To keep the differential input voltage of the preamplifier A1 in the linear operating range, the signal in the positive input terminal V in + is limited to ±9 V (LIM), and the voltage between the negative terminal V in - and the amplifier ground is kept smaller than ±1 V by attaching the reference and ground electrodes close to each other. If the voltage exceeds these values, the 20-kX resistors R1 andR2 limit the current to a safe level in accordance with standards. The sample-and-hold circuit S/H(B) is controlled by the Hold (B) signal, which is activated about 50 ls before the TMS pulse and is released after the pulse (e.g., 2.5 msec later). *Figure and text from (Ilmoniemi and Kičić, 2010)*

Other techniques have been developed to deal with the TMS-induced high-voltage magnetic artifact. These methods include use of slew-rate limited preamplifiers preventing saturation resulting in a short lasting artifact that decays within ~30 msec (Thut et al., 2005). Use of an MRI- compatible DC amplifier with wide dynamic range allows to record TMS evoked EEG responses with a short artifact lasting between 10 and 20 msec (Bonato et al., 2006). Finally, offline methods also exist to effectively remove TMS-induced artifact (Litvak et al., 2007b). In contrast to Virtanen's system, used in the present work, those methods present the drawback for not being able providing a reliable online very early (8-30 msec post stimulus) TMS/EEG response.

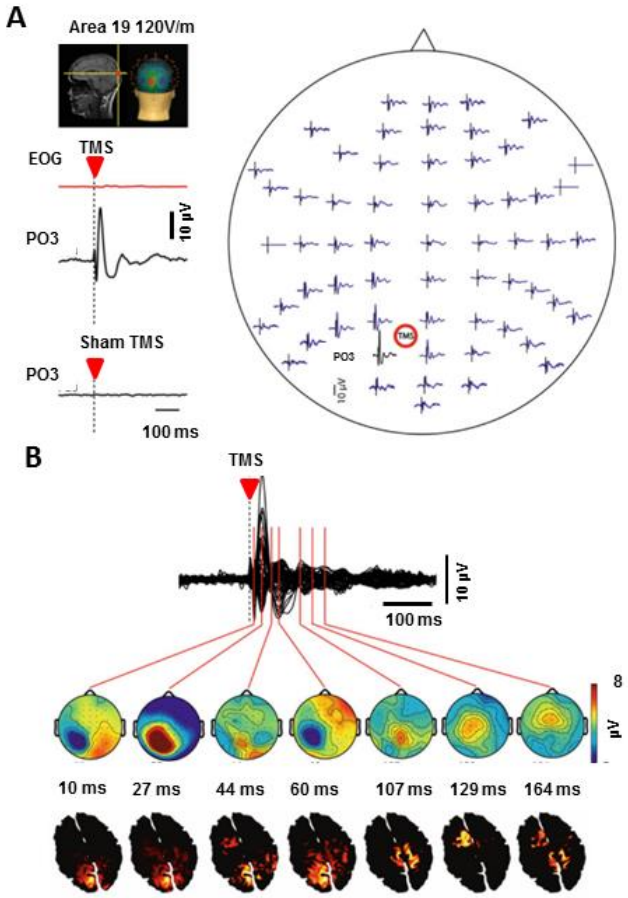


Fig. II.7: EEG responses and cortical activations evoked by TMS of area 19 . The left side of A shows the EOG trace (red trace) and the TMS evoked potential (black trace) recorded from the electrode under the stimulator (PO3) when Brodmann's area 19 is stimulated at 120 V/m (the inset shows the location of the maximal electric field induced by TMS on the cortical surface). On the right side of A, the evoked potentials recorded from all the 60 EEG channels are shown. The upper part of B shows the butterfly plot of the 60 EEG channels. In the lower part of B the voltage scalp maps and the corresponding cortical source reconstructions are represented at different time latencies from the TMS. *Figures and legend from* (Rosanova et al., 2012a)

4.2 EEG artifacts induced by TMS and how to deal with

4.2.1 TMS magnetic artifact

TMS induced magnetic artifacts appear as large spikes on the recording, which are immediately following TMS pulses and are visible on many channels even if the coil is placed far from scalp muscles (e.g. vertex). Despite the use of EEG amplifier compatible with TMS (see previous section), they may still contaminate the recording. Their occurrence may suggest to improve EEG electrodes impedances (especially the ground and the reference), to diminish TMS intensity and/or changing coil orientation to deliver the most efficient stimulation at the lowest intensity (Rosanova et al., 2012a).

4.2.2 Auditory evoked potentials

The TMS pulse elicits a loud "click" (up to 120 decibel) due to a rapid mechanical deformation of the coil when it is energized (Ilmoniemi and Kičić, 2010; Rossi et al., 2009). This noise activates the subject's auditory system and gives rise to an evoked EEG potential (AEP, Nikouline et al., 1999) which may cover the TMS response. AEP typically occur between 100 and 200 msec after the pulse and appear larger at the central derivation (Cz) (Rosanova et al., 2012a) **[Figure II.8 B]**. Both air and bone conducted sounds play a role in the generation of this AEP (Nikouline et al., 1999). The former can be avoided by exposing the subjects to a noise masking obtained by shuffling the time-varying spectral component of the "click" through earphones and set at a sufficiently loud volume level (Rosanova et al., 2012a). The latter can be blocked by interposing a small cube of plexiglass or a thin layer of foam between the coil and the scalp (Rosanova et al., 2012a) **[Figure II.8 A]**.

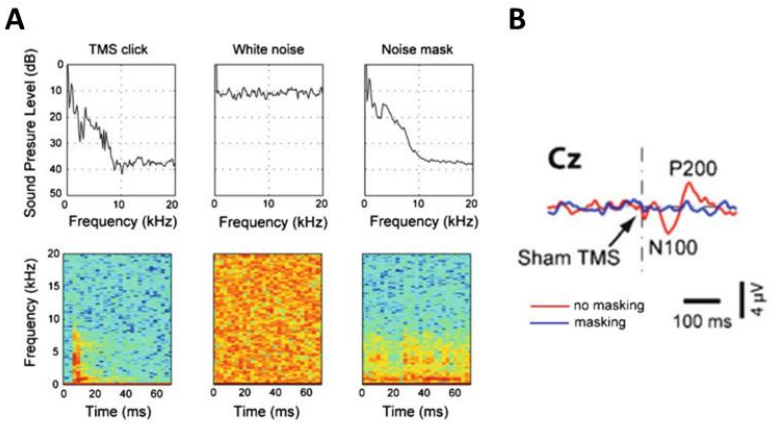


Fig. II.8 : Abolition of the auditory evoked potential (AEP) through noise masking by the TMS "click". A - Comparison of the spectral profile and time frequency characteristics of the TMS induced "click" sound (left with white noise and synthetic noise masking shuffling the time-varying spectral component of the "click". B –evoked potentials on Cz (vertex) obtained with (blue trace) and without noise masking while the coil , connected to the scalp by a plexiglass cube (to preserve bone conduction) was placed 5 cm above the vertex (sham TMS/EEG session). N100 and P200 components of the AEP clearly abolished with noise masking and plexiglass. *Figure and legend modified from* (Rosanova et al., 2012a)

4.2.3 Scalp muscle artifacts

TMS delivered at high intensity over lateral and/or anterior aspects of the scalp may directly activate temporal and/or frontal muscles. Those activations appear on EEG as "M-waves", of hundreds to thousands microvolts, time-locked to the TMS pulse at the more frontal and lateral electrodes (Rosanova et al., 2012a). EEG scalp muscle artifacts can last up to 50 msec and can be prevented by reducing the intensity and stimulating closer to the cerebral midline (Rosanova et al., 2012a).

4.2.4 Eye movements and blinks

Because there is a steady potential of several millivolts across each eyeball (cornea positive as compared to the opposite side of the eye), eye movements and blinks may evoke transient potential at the vertex which are larger as compared to the EEG

signal (Ilmoniemi and Kičić, 2010). TMS may startle the subject and trigger eyes blinks or eyes muscle activations (Corthout et al., 2000). This can be prevented by employing a masking noise and adjusting the coil location and the stimulation intensity. The electrooculogram (EOG) must be also constantly checked during TMS/EEG recordings (Rosanova et al., 2012a).

4.3 What information does TMS/EEG provide?

TMS/EEG is able to record the immediate response of the cortex to a direct perturbation. It represents therefore a straightforward way to non-invasively probe excitability and connectivity of the human cortex. Cortical excitability commonly refers to the amplitude of the immediate neural response to this perturbation whereas cortical connectivity is reflected by the spread of this response to distant interconnected areas (Casali et al., 2010). TMS/EEG studies showed that those aspects of the human brain function may be affected by changes in vigilance states in physiological as well as in pharmacological and pathological conditions.

4.3.1 TMS/EEG changes in physiological conditions

Human cortical excitability, as indexed by the amplitude (and slope) of the early (~ 0-25 msec) EEG potential evoked by TMS, was shown to progressively increase during 40h of *prolonged wakefulness* and to return to baseline levels after one night of recovery sleep (Huber et al., 2013) **[Figure II.9]**. TMS/EEG also showed that transitions from wake to sleep and between sleep stages are accompanied by changes in cortical connectivity. Indeed *during quiet wakefulness*, an initial TMS/EEG response at the stimulation site (~ 15 msec) is followed by a sequence of waves that move to connect surrounding brain areas (Massimini et al., 2005, 2010) **[Figure II.10-11]**. *During NREM sleep*, the same stimulation evokes a stronger initial response but

that rapidly extinguishes and does not propagate (Massimini et al., 2005, 2010) **[Figure II.10-11]**. The authors assume that this breakdown of cortical effective connectivity would explain why consciousness fades during NREM sleep (Massimini et al., 2005). *During REM sleep*, a sleep stage associated with a regain of consciousness, the pattern of cortical activation was found quite similar to the one observed during wakefulness (Massimini et al., 2010) **[Figure II.10-11]**.

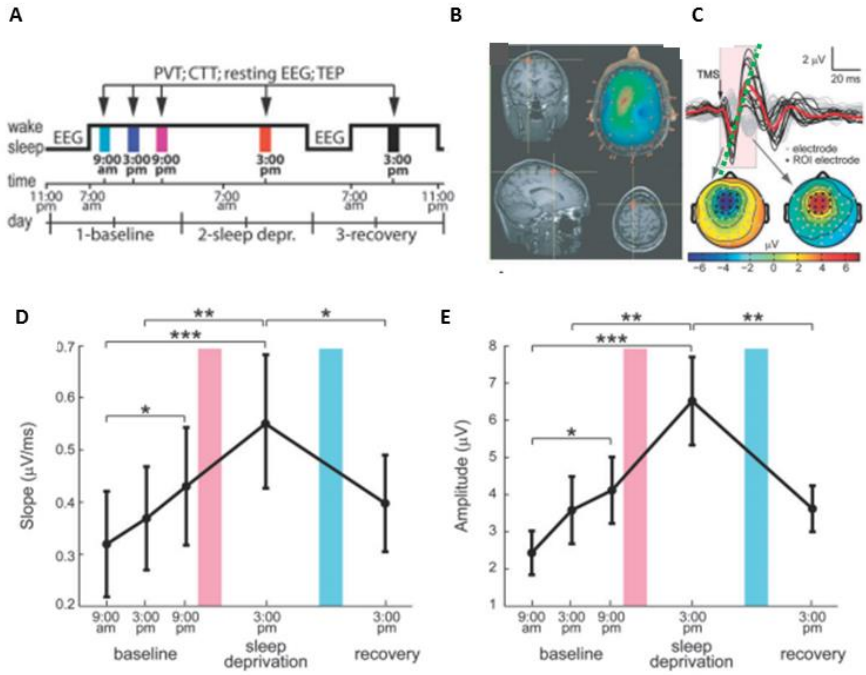


Fig. II.9 : Modulation of human cortical excitability with time spent awake as indexed by slope and amplitude of the early TMS evoked EEG potential (TEP) *from (Huber et al., 2013).*
(A) After an 8 hour baseline night (11 pm – 7 am), 6 healthy volunteers (25 – 41 y.o; 1 female) spent 40h of total sleep deprivation (SD, 7 am – 11 pm) followed by an 8 hour recovery night of sleep (11 pm – 7am). During this protocol, they underwent 5 TMS/EEG recordings while performing a visuomotor vigilance task (Compensatory tracking Task, see our experimental section for details). TMS/EEG sessions were scheduled at 9 am, 3 pm, 9 pm (day 1 : baseline), 3 pm (day 2: sleep deprivation), 3 pm (day 3 : recovery). **(B)** Left supplementary motor area (SMA) was set as stimulation target (hotspot) based on individual MRI. **(C)** Slope (green dot line) and amplitude were extracted at each session from the average TEP across 14 electrodes in the region of interest (hotspot). TEP slope **(D)** and amplitude **(E)** progressively increased during the 40h of continuous wakefulness and returned to baseline levels in the afternoon session following one night of recovery sleep. *P < 0.05, **P < 0.01, ***P < 0.001. Red and blue bars respectively indicate SD and recovery nights. *Figures and legends adapted from (Huber et al., 2013)*

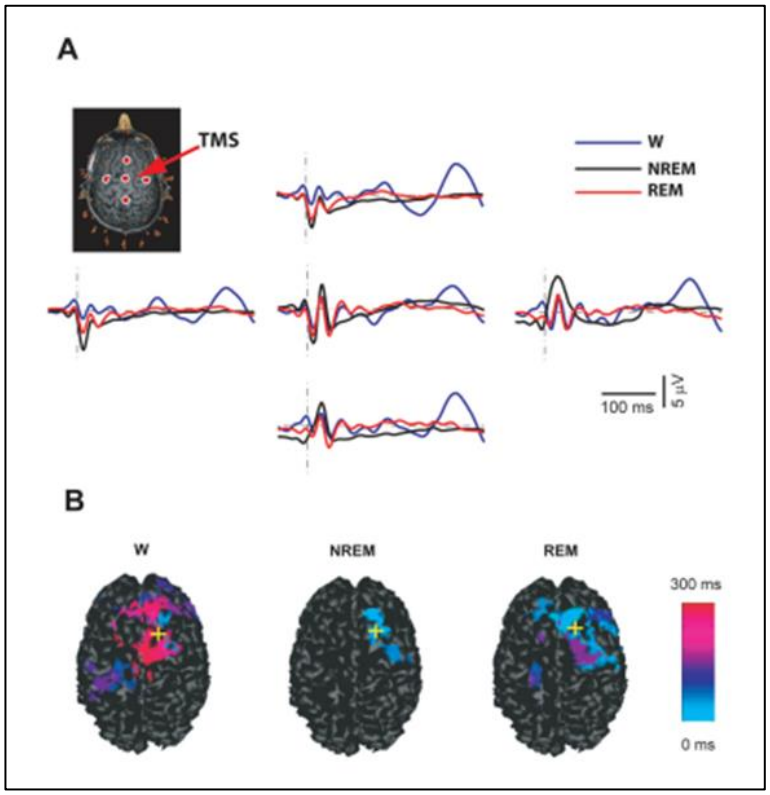


Fig. II.10 : Changes in TMS-evoked response during wakefulness, NREM sleep and REM sleep
(A) Average TMS-evoked EEG potentials recorded during wakefulness (blue trace), NREM sleep (black trace) and REM sleep (red trace) from one subject whom a long REM trace could be recorded. The TMS/EEG traces were recorded from the channels indicated by red dots in the upper left panel, where the site of stimulation on the subject's MRI is also indicated by a red arrow. See upper text for EEG traces descriptions and comparisons **(B)** Spatiotemporal cortical maps of TMS-evoked cortical activation during wakefulness, NREM and REM sleep. Maximum current sources are plotted and color-coded according to their latency of activation (light blue, 0 milliseconds; red, 300) for each significant sample. *Figure and legend from (Massimini et al., 2010)*

4.3.2 TMS/EEG changes induced pharmacological manipulations

Alcohol is known to have potential mood and behavioral altering effects. A TMS/EEG study showed that alcohol intake reduced human cortical excitability especially over the frontal and prefrontal cortex, as indexed by a decrease of the area under the

global mean field amplitude of TMS/EEG early (30-270 msec) response over this region (Kähkönen et al., 2002).

Midazolam is a drug which targets GABA-A receptors increasing inhibitory post-synaptic currents and possibly causing its anesthetic effect (Tanelian et al., 1993). Midazolam was shown to induce similar TMS/EEG changes as those observed during NREM sleep (more ample immediate response, rapidly extinguishing and which remains localized to the stimulation site (Ferrarelli et al., 2010))**[Figure II.11 B]**.

4.3.3 TMS/EEG changes in pathological neurological conditions

Disorders of consciousness (DOC)

Two studies assessed single pulse TMS/EEG responses of a total of 30 patients (Ragazzoni et al., 2013; Rosanova et al., 2012b). Fifteen, thirteen and two patients were respectively unambiguously diagnosed in unresponsive wakefulness state (UWS)[9], in minimally conscious state (MCS)[10], and in locked-in-syndrome (LIS)[11]. In UWS patients, 9 out of 15 showed a simple, local and slow response to TMS which was similar to ones observed during NREM sleep (Massimini et al., 2005, 2010) and general anesthesia (Ferrarelli et al., 2010) on healthy subjects **[Figure II.11C]**. The 6 remaining UWS patients did not show any TMS response. In MCS group, 12 out of the 13 showed a complex response with pattern of cortical activation changes over time **[Figure II.11C]**. Their response appeared similar to the two conscious LIS patients (Ragazzoni et al., 2013; Rosanova et al., 2012b). Facing only qualitative description TMS/EEG responses, Casali and colleagues elaborated several indices to

[9] Unresponsive wakefulness state (UWS) : state in which patients after falling into a coma recover arousal but not consciousness (anteriorly referred as "vegetative" state) (Laureys et al., 2010)
[10] Minimally conscious state (MCS): state in which patients show reproducible but fluctuating signs of consciousness (e.g. responses to command, visual pursuit) (Giacino et al., 2002)
[11] Locked-In-Syndrome (LIS): state in which patients are conscious but unable to produce any volitional motor output, except for eye movements. (Pellas et al., 2005)

quantitatively characterize the different aspects of the electrical brain response to TMS (i.e cortical excitability, connectivity (Casali et al., 2010) or response complexity (Casali et al., 2013)). The most recent is the perturbational complexity index (PCI) which aims at measuring the complexity of the spatiotemporal pattern of TMS-induced cortical activation. PCI is presented by its authors as a promising diagnosis tool because it reliably discriminates the level of consciousness in single individuals during wakefulness, sleep, and anesthesia, as well as in patients who had emerged from coma and recovered a minimal level of consciousness (Casali et al., 2013).

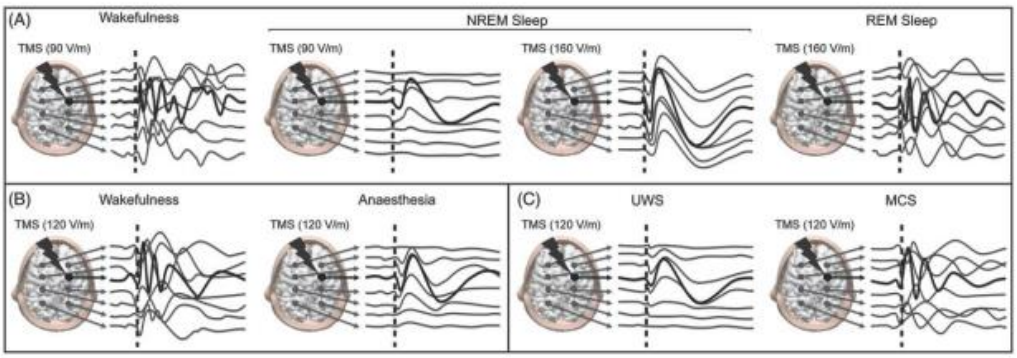


Fig. II.11 : Typical response to TMS in different physiological, pharmacological and pathological conditions. For each condition, cortical responses under the coil (bold black trace) and in seven other brain areas (lighter black traces) following right premotor stimulations (black lightning) are shown. See upper text for TMS EEG responses descriptions and comparisons. *Figure from (Napolitani et al., 2014)*

Alzheimer Disease

By means of TMS/EEG, patients suffering from Alzheimer Disease (AD) were shown to have reduced levels of cortical excitability as compared to healthy aged subjects. Their local EEG response to TMS delivered over the frontal cortex was indeed of lower amplitude (Casarotto et al., 2011 **[Figure II.12]**; Julkunen et al., 2008). This observation is consistent with the previously reported loss of EEG synchronization

putatively associated with cognitive impairment in those patients (Koenig et al., 2005). However, it contrasts with the lower motor threshold reported in those patients, as shown in multiple studies using TMS combined to EMG recording (Ferreri et al., 2003; Di Lazzaro et al., 2003; Pennisi et al., 2011). The reasons of opposing effects associated with AD on EMG and EEG responses triggered by TMS remain unclear. Nonetheless, TMS/EEG may represent a promising tool for better understanding neurodegenerative processes and a potential mean to early discriminate normal from pathological aging (Casarotto et al., 2011; Julkunen et al., 2008).

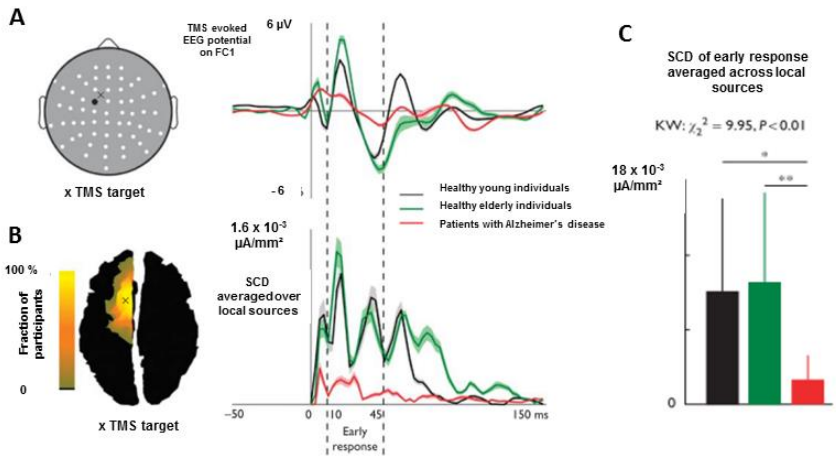


Fig. II.12 : Changes in TMS evoked EEG responses with normal and pathological (Alzheimer's Disease) aging.

(A) Grand average TMS-evoked potentials (TEPs) recorded from FC1 electrode in the three groups of participants. **(B)** Projection of the TMS target on the cortical surface (left); colored area contains the cortical sources located less than 3 cm (along the geodesic) from the individual TMS hotspot in any participant; the fraction of participants contributing to each source is color coded. Significant current density (SCD) is a synthetic index of cortical excitability obtained by summing all significant currents induced by TMS (Casali et al., 2010). The time course of the grand average SCD averaged over the cortical area under the stimulator is presented on the right. **(C)** Group comparison [Kruskal–Wallis analysis of variance (KW)] for the local mean SCD integrated over the temporal window of early (10 -45 msec) response to TMS [mean and standard deviation (SD)]. *Figure and legend adapted from (Casarotto et al., 2011)*

Depression

Electroconvulsive therapy (ECT) and total sleep deprivation (TDS) are used in Clinical Psychiatry for their short-term antidepressant effects (Bhattacharjee, 2007; UK ECT Review Group, 2003). However, how and why those treatments may improve depression remains unclear. Two TMS/EEG studies evaluated the impact of those interventions on cortical excitability over the prefrontal cortex in depressive patients. ECT (Casarotto et al., 2013) or TDS (combined to light therapy, Canali et al., 2014 **[Figure II.13]**)) were shown to increase cortical excitability as indexed by increases of the slope of the early TMS evoked potential and the local mean field power (LMFP, a measure of the local amount of electric activity induced by TMS). Furthermore, higher values of cortical excitability indices appeared to differentiate responders from non-responders in before, during and after TDS intervention. These observations go in line with the hypothesis that depression may underlie disruption in cortical excitability and especially in the synaptic homeostatic building. They also suggest that TMS/EEG may be used in the management of depressive patients to monitor the effects of therapies such as ECT, sleep deprivation or rTMS (Canali et al., 2014a; Casarotto et al., 2013).

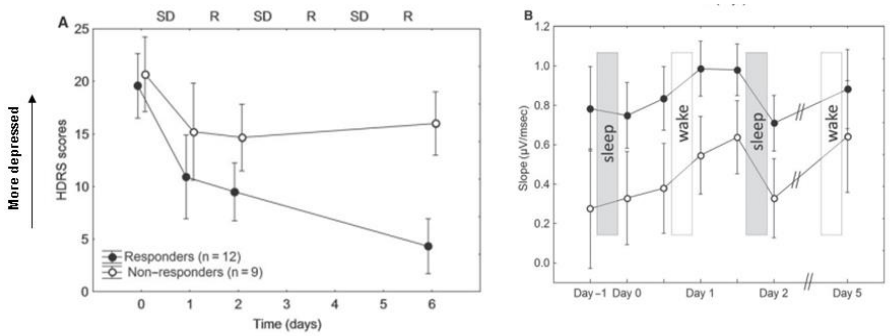


Fig. II.13 Changes of Hamilton Depression Rating Scale (HDRS) scores of depression severity (A) and of the slope of the first-evoked component (V/ms) (B) during treatment (sleep deprivation) in the sample divided according to final response to treatment (HDRS 50% reduction). *Figure and legend from (Canali et al., 2014a)*

Epilepsy

Epilepsy certainly stands out as the most obvious clinical example of pathological cortical hyperexcitability state. TMS/EEG may be used to identify distinct excitability changes associated with specific epileptic syndromes and reorient therapy-specific strategies. For instance, TMS can induce epileptiform discharges in patients suffering from genetic generalized epilepsy (Kimiskidis et al., 2015). The generation of these epileptiform discharges is inconstant and has been associated to interictal "high excitability" EEG states during the prestimulus period (Kimiskidis et al., 2015) . Unverricht-Lundborg disease (progressive myoclonus epilepsy, EPM1) is an inherited neurodegenerative disorder and the most common form of progressive myoclonus epilepsies, TMS/EEG responses of the left primary motor cortex in EPM1 (Julkunen et al., 2013) showed a significant increase of P30 and decrease N100/P180 [12] waveforms respectively suggesting an increase in cortico-cortical excitation (Rogasch and Fitzgerald, 2013) and a reduction in intracortical inhibition (Esser et al., 2006).

Stroke

To our knowledge, no TMS/EEG study has been yet performed in patients after stroke. This pandemic affection causes functional impairments which directly result from the ischemic loss of neurons combined with maladaptative brain reorganization (Taub et al., 1994). Conversely, proper reorganization and remodeling of networks connections contribute to stroke recovery (Grefkes and Fink, 2014). Concurrent TMS and EEG use for characterizing cortical activity in stroke may offer great opportunities (Sato et al., 2015). It could be used, for instance, to identify biomarker of stroke recovery and prospectively monitor changes in cortical excitability and

[12] P30, N100 and P180 respectively refer to the positive, negative and positive time-locked EEG waveforms after 30, 100 and 180 msec the TMS that are commonly distinguished from the TMS evoked EEG potentials of the motor cortex. P30 amplitude is calculated from the difference between P30 peak and baseline and N100/P180 from peak to peak.

connectivity in response to neurorehabilitation or pharmacologic therapy (Sato et al., 2015).

4.4 What are the advantages of EEG/TMS?

Like clinical and research tools, TMS/EEG has advantages and disadvantages. Those main are summarized in this table.

Advantages	Disadvantages
▪ **Good temporal resolution (msec)** ▪ **Bypass afferent sensory pathway** ▪ **Does not require functionning efferent pathways** ▪ **Does not require subject active participation** ▪ **Equipment can be made portable and is useable at patient's bedside** ▪ **Highly reproducible within subject[13]** ▪ **Sensitive to changes in stimulation parameters**	▪ **Limited whole brain spatial resolution (e.g. vs fMRI)** ▪ **Study limited to cortex/superficial brain areas** ▪ **Depend on scalp-to-cortex distance (e.g. reponse diminished with cortical atrophy)** ▪ **Response can be altered by drugs intake** ▪ **Requires stable state of wakefulness** ▪ **Subject's assessement limited by the presence of metallic implant** ▪ **Contraindicated in uncontrolled epilepsy** ▪ **Requires considerable logistic and subject preparation.**

Tab. II.1 : Advantages and disadvantages of TMS/EEG . *Modified from (Napolitani et al., 2014)*

[13] But TMS response may also be influenced by sleep-wake history (Huber et al., 2013) and the circadian phase (see chapter IV)

5 CONCLUSION

The simultaneous recording of EEG during TMS is technically demanding but has a potential great value to better understand human brain function. With its excellent time resolution and direct causal approach, TMS/EEG offers the opportunity to non-invasively probe cortical excitability and effective connectivity. Furthermore, it allows to index (rapid) changes in brain state in normal or challenged (e.g. sleep deprivation) physiological conditions, as well as in neurological disorders.

CHAPTER III

NEURONAL PLASTICITY: HOMEOSTATIC OR CIRCADIAN?

Sleep is a universal phenomenon that has been remarkably conserved through the entire evolution of the animal kingdom. And yet being plunged into such "off-line" state represents potential great danger since, for instance, a sleeping being becomes highly vulnerable to predators. So what makes sleep so necessary for brain activity? What is sleep function? There are number of evidences supporting that sleep and wake are accompanied with major changes in neuronal plasticity such that one sleep function would be to participate to learning and memory processes (Maquet, 2001; Rasch and Born, 2013; Tononi and Cirelli, 2014).

This third chapter addresses the putative role of sleep in neuronal plasticity. Its first part presents the synaptic homeostasis hypothesis which probably stands as the most influent and popular theory for the homeostatic role of sleep in regulation of brain function. In the second part of this chapter, we will assess evidences which suggest that other non-homeostatic processes, including the circadian timing system, may interplay in this fine-tuning.

1 THE SYNAPTIC HOMEOSTASIS HYPOTHESIS

1.1 Basic principles

The synaptic homeostasis hypothesis (SHY) claims that the fundamental function of sleep is the restoration of synaptic homeostasis (Tononi and Cirelli, 2003) **[Figure**

III.1]. SHY proposes that the increase in sleep pressure is brought by learning-related synaptic strengthening during wakefulness. Conversely, the same sleep pressure is dissipated during NREM sleep by synaptic downscaling through slow wave activity (SWA). The synaptic weakening gradually attenuates with time asleep until synaptic strength returns to suitable baseline level[14]. This process may present the main advantage to keep track of previous experience because synapses that were strengthened repeatedly during wake may be more "protected from depression" as compared to weaker ones. The system remains efficient, although at a recalibrated level of total synaptic strength, thus restoring selectivity of neuronal responses and preventing saturation of learning capacity during subsequent wake. In other words, SHY claims that wakefulness brings stronger synapses which have costs in terms of space, cellular and energy requirements. Sleep may be the price to pay for waking neuroplasticity (Tononi and Cirelli, 2014).

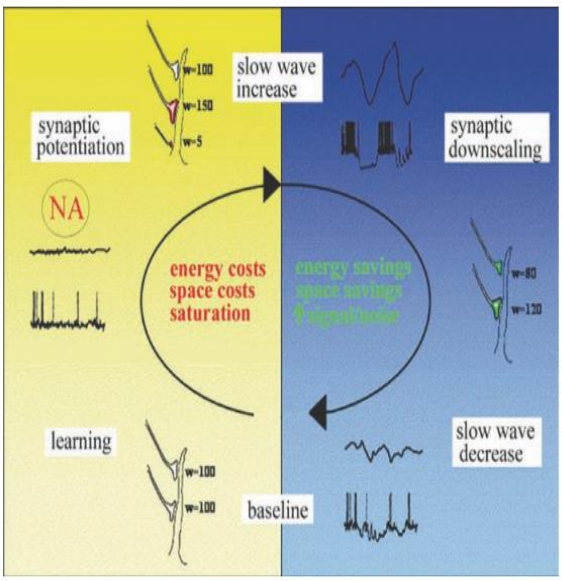


Fig. III.1 : The synaptic homeostasis hypothesis. Left yellow background: learning happens by synaptic potentiation during wake when the organism interacts with its environment. Right blue background: renormalizaton of synaptic strength happens during sleep when the brain is spontaneously "off-line". This renormalization operates through a competitive "down selection" whereby all synapses decrease in strength proportionally but those that end up below a minimal threshold become virtually ineffective. This downscaling is operated through slow waves sleep that attenuate with time asleep until the system returns to a global suitable baseline level. *Figure from (Tononi and Cirelli, 2006)*

[14] According to the SHY, the synaptic potentiation occuring during wakefulness is considered as ***Hebbi*an** because it involves changes in specific synapses mediated by coordinated activity in pre- and postsynaptic neurons. By contrast synaptic downscaling occurring during NREM sleep is considered as ***non-Hebbian*** referring as a type of plasticity that adjusts all synapses in a neuron (or network of neurons) upward or downward in response to global changes in activity (Burrone and Murthy, 2003; Turrigiano, 2011)

1.2 Evidence for SHY

SHY claims to efficiently integrate multiple experimental findings in the field, which are summarized in this subsection.

1.2.1 Molecular evidence

Insertion and removal of the GluR1 subunit of AMPA[15] receptors (GluR1 AMPARs) at the synaptic membrane are considered primary mechanisms for synaptic potentiation and depression respectively. Those receptors are permeable to calcium and powerfully affect synaptic strength (Kessels and Malinow, 2009). Expression of GluR1 AMPARs has been shown to be higher after wakefulness than after sleep in rats (Qin et al., 2005; Vyazovskiy et al., 2008). Furthermore, phosphorylation changes reported for these same receptors are consistent with net synaptic potentiation during wake and depression during sleep (Vyazovskiy et al., 2008). On a second hand, brain-derived neurotrophic factor (BDNF), another major marker of neuronal plasticity, sees its expression increase during wakefulness in rats. Furthermore, wake-related large increase in BDNF is associated with larger increase in SWA during subsequent sleep (Huber et al., 2007a). Finally, cortical unilateral microinjections of BDNF in awaken rats result in a reversible ipsilateral increase in SWA (Faraguna et al., 2008).

1.2.2 Structural evidence

In Drosophilae, the number or size of synapses in three different neuronal circuits increase after a few hours of wakefulness and declines if flies are allowed to sleep (Bushey et al., 2011). In young developing mice, few hours of sleep and wake can

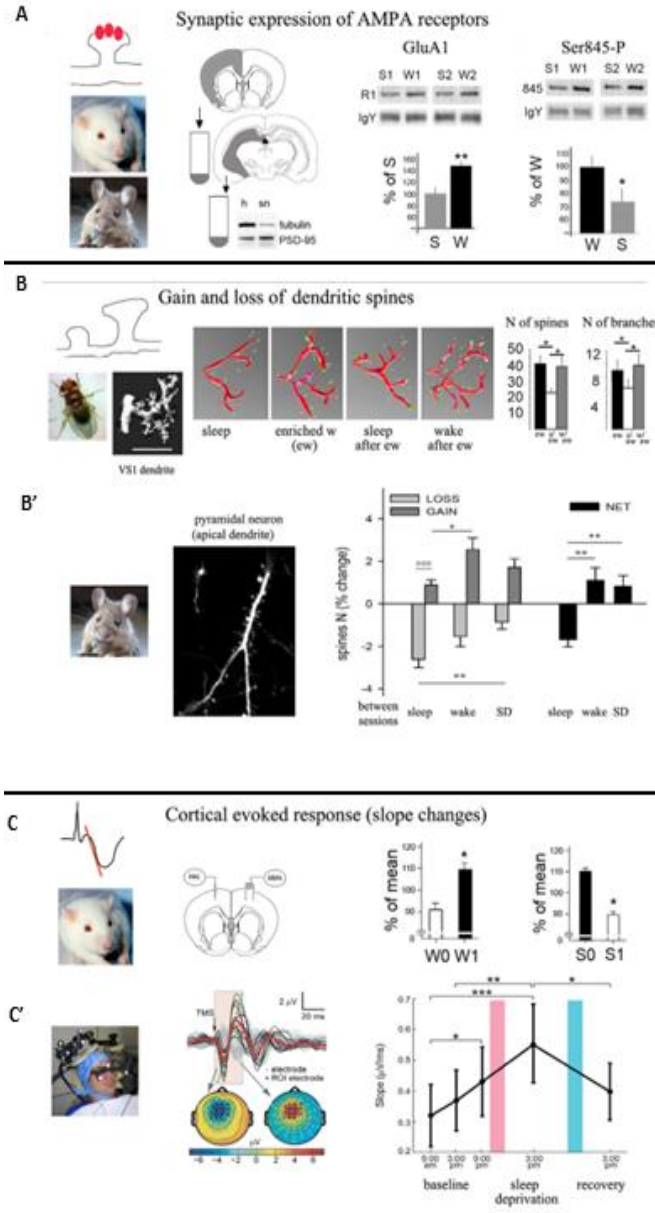
[15] AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid

affect density of cortical synapses. Formation of spines and filipodia within the somatosensory cortex was shown greater during the dark period, when mice are mostly awake. Conversely, more elimination was found during the light period, when mice are mostly asleep (Yang and Gan, 2012). These results were confirmed on adolescent mice but not on adult ones which showed limited spine turnover and no impact of sleep and wake. Authors argued that synaptic homeostasis after adolescence may be primarily mediated by changes in synaptic strength rather than number (Maret et al., 2011).

1.2.3 Electrophysiological evidence

The amplitude and particularly the slope of the early cortical response evoked by electrical stimulation are classical measures of synaptic strength and neuronal synchrony. In rats, the amplitude and slope of the local field potential evoked by electrical cortical stimulation (ECS) increase in the frontal cortex with time spent awake (Vyazovskiy et al., 2008). Conversely, still in rats, the slope of the response evoked by ECS in hippocampal CA3 region declines after sleep (Lubenov and Siapas, 2008). In humans, the amplitude and slope of the early EEG response evoked by transcranial magnetic stimulation was shown to increase progressively during 40h of sustained wakefulness to return to baseline level after one night of recovery sleep (Huber et al., 2013).

Fig. III.2 : Evidence Supporting SHY.
Figures and legends adapted from (Tononi and Cirelli, 2014)



Molecular evidence

In rats and mice, number and phosphorylation levels of GluA1-AMPARs increase after wake *(data from rats: Vyazovskiy et al., 2008)*

Structural evidences

(B) In flies, the number of spines and dendritic branches in the visual neuron VS1 increase after enriched wake (ew) and decrease only if flies are allowed to sleep (*from Bushey et al., 2011*). **(B')** In adolescent mice, a net increase and decrease in cortical spine density were respectively shown after wake and sleep deprivation (SD) and after sleep (*from Maret et al., 2011*).

Electrophysiological evidences

Cortical evoked responses using electrical stimulation (**C;** rats data *from (Vyazovskiy et al., 2008)*) and TMS (**C' ;** humans data *from (Huber et al., 2013)*) show increased slope after wake and decreased slope after sleep.

(C) W0 and W1 indicate onset and end of ~ 4 hr of wake; S0 and S1 indicate onset and end of ~ 4 hr of sleep, including at least 2 hr of NREM sleep *(Vyazovskiy et al., 2008)*.

(C') : see chapter 2 fig II.9 for legend details *(Huber et al., 2013)*

1.3 Synaptic homeostasis and slow wave activity

1.3.1 Regional regulation of synaptic homeostasis and SWA

According to the SHY, changes in synaptic strength induced during wakefulness result in a change in SWA during subsequent sleep. In other words, the amount of slow-wave activity during NREM sleep reflects the amount of sleep pressure accumulated during wakefulness. Although SWA enhancement occurs at a global level (Tobler, 2000,) there is great evidence that it is regionally affected by previous activity. For instance, when a subject's hand is exposed to a vibratory stimulus for several hours before going to bed, the balance of SWA is shifted to the hemisphere contralateral to the stimulated hand during subsequent sleep (Kattler et al., 1994). Furthermore, local manipulations of synaptic strength lead to specific local change in SWA in humans. Training to a visuomotor learning task (but not to a kinematically equivalent motor control task that does not require learning) increases sleep SWA in the right parietal lobe encompassing Brodmann areas 40 and 7 (Huber et al., 2004); regions which are known to receive converging visual and proprioceptive inputs and to be involved in spatial attention (Cohen and Andersen, 2002). Moreover, the amount of this local SWA increase correlated to visuomotor task improvement after sleep (Huber et al., 2004). By contrast, a short-term arm immobilization induces motor performance deterioration through synaptic depression, as indicated by decreased sensory and motor evoked responses over contralateral sensorimotor cortex. This is also associated with a local decrease in sleep SWA over the same area (Huber et al., 2006).

In those experimental paradigms, synaptic strength manipulation remained indirect and its local specificity was only relative since depending on a task. Thus, the same group used TMS for direct and more focused modulation of synaptic strength and SWA. In a first step, they reported that rapid 5 Hz rTMS applied over the premotor

cortex could induce local long term potentiation (LTP) in humans, as it increases amplitude of EEG response evoked by single pulse TMS over the same region (Esser et al., 2006). In a second step, they showed that SWA during subsequent NREM sleep is similarly affected over the previously stimulated/potentiated premotor cortex (Huber et al., 2007b). Finally, they enforced their finding in a paired associated stimulation (PAS)[16] protocol, in which median nerve stimuli were followed at different intervals (25 or 10 msec) by transcranial magnetic stimulation (TMS) pulses to the contralateral cortical hand area. They showed SWA local increase in subjects whose TMS-evoked cortical responses had increased after PAS (i.e. LTP), and decrease in subjects whose cortical responses had decreased (i.e. LTD) (Huber et al., 2008) .

1.3.2 SWA as a major actor of synaptic downscaling during sleep

SWA would not be a simple reflect of stronger synapses brought by wake-related experiences. It may also play active role in synaptic downscaling. Indeed, a patch-clamps recording of rats cortical neurons study showed that burst firings, which are common in slow wave sleep, favor long-lasting depression of excitatory postsynaptic potentials (Czarnecki et al., 2007). The same group reported later that those discharge patterns, which are characteristic of NREM sleep, may induce long-term depression (LTD) through removal of AMPARs at synapses of rats neocortical pyramidal cells (Lanté et al., 2011). Using a large-scale model of the corticothalamic network, a computational study demonstrated that stronger synapses increase SWA while SWA may contribute to the decrease of synaptic strength during sleep; realizing a control loop of synaptic strength regulation (Olcese et al., 2010).

[16] see chapter 2

The SHY is challenging in its implications. It may notably shed light on the enigmatic process that makes animals sleep longer (or more intensely) with function of prior time spent awake. In a TMS/EEG study, <1Hz TMS pulses were shown to be able to trigger slow waves during NREM sleep (Massimini et al., 2007) **[Figure III.3]**. Thus, according to SHY, it opens the doors to possible non pharmacological ways to improve brain synaptic restoration and maybe memory consolidation (Massimini et al., 2007).

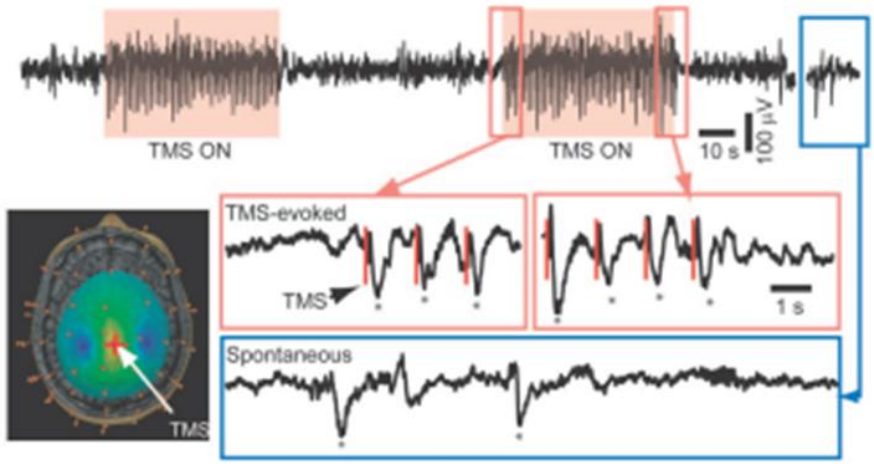


Fig III.3 - TMS during sleep triggers slow waves that resemble ones that spontaneously occur during NREM sleep. *Upper panel* - The signal recorded from a channel (Cz) located under the stimulator during two TMS-ON blocks over a background of spontaneous NREM sleep (single-subject data). Each TMS-ON block consisted of 40 stimuli at 0.8 Hz. *Left bottom panel* - The stimulation site (hotspot) is marked by a red cross on the cortical surface. *Right bottom panel*s - The red highlighted sections show the slow waves triggered at the beginning and at the end of one block. Spontaneously occurring slow waves recorded from the same subject a few minutes later are depicted in the blue highlighted section. *Figure and legend from (Massimini et al., 2007)*

2 SYNAPTIC PLASTICITY IS NOT SLEEP HOMEOSTASIS "ONE MAN SHOW"

As just presented, the synaptic homeostasis hypothesis is supported by an impressive number of experimental data obtained in insects, rodents and humans. They were provided by an equally impressive variety of elaborated research tools including molecular, cellular, electrophysiological and computational techniques. However, a growing number of experiments do not meet SHY predictions. This chapter section addresses an illustrative (but non exhaustive) list of findings that challenges SHY and leads to consider a circadian role in the regulation of synaptic plasticity (for review see : Frank, 2012; Frank and Cantera, 2014).

2.1 Depending on its type, learnings may mainly require LTD

Learning does not necessarily mainly imply LTP as claimed by the synaptic homeostasis hypothesis. The underlying synaptical/neuronal mechanisms indeed depend on the type of learning. For instance, extinction, which is a form of learning allowing to change prior learned behaviors after new information, is associated with LTD and AMPAR endocytosis (Dalton et al., 2008). Other kinds of learning such as behavioral flexibility and spatial memory also appear to require LTD involving NMDA LTD-dependent receptor (Ge et al., 2010; Nicholls et al., 2008).

2.2 Sleep alone has not a single effect on synaptic efficacy or morphology

As claimed by the SHY, sleep is not only devoted to synaptic downscaling. Sleep may have no effect on synaptic morphology and cytoarchitecture. In rats, a 2-4-weeks total or selective REM sleep deprivation is lethal. However, it does not lead to microscopic significant changes in axons, dendrites, synaptic density, or organelles (Rechtschaffen and Bergmann, 2002). Another study showed that whole brain levels of synaptic proteins (e.g. synapsin, synaptotagmin, synaptobrevin II) did not differ in

adult mice either sacrificed during the active or the sleeping phase (Yelamanchili et al., 2006).

Synaptic increase in size or potentiation may also occur during sleep. These possible effects of sleep on synapses were both demonstrated in insects and other animal species.

- For instance, *in Drosophila melanogaste*r, the number of motor neuron synaptic boutons reaches its maximum in the sleep phase (Mehnert et al., 2007).
- *In developing chicks*, visual imprinting, a form of memory trace, is associated with increases in post synaptic density and excitatory glutamate receptors in the intermediate medial of the hyperstriatum ventrale (Horn, 1998). This sort of developmental plasticity requires sleep within a restricted period after learning (Jackson et al., 2008).
- *In adult mice*, sleep promotes postsynaptic dendritic spines formation in the motor cortex after motor learning (Yang et al., 2014) and leads to cortical potentiation in primary visual cortex following a visual experience (Aton et al., 2009; Cooke and Bear, 2010).
- *In monocular deprived developing cats*, sleep consolidates ocular dominance plasticity by long term potentiation in visual cortex of the non-deprived eye (Aton et al., 2009). In adult cats, evoked electrophysiological potentials in somatosensory cortex increase after short episodes of NREM sleep (Chauvette et al., 2012).

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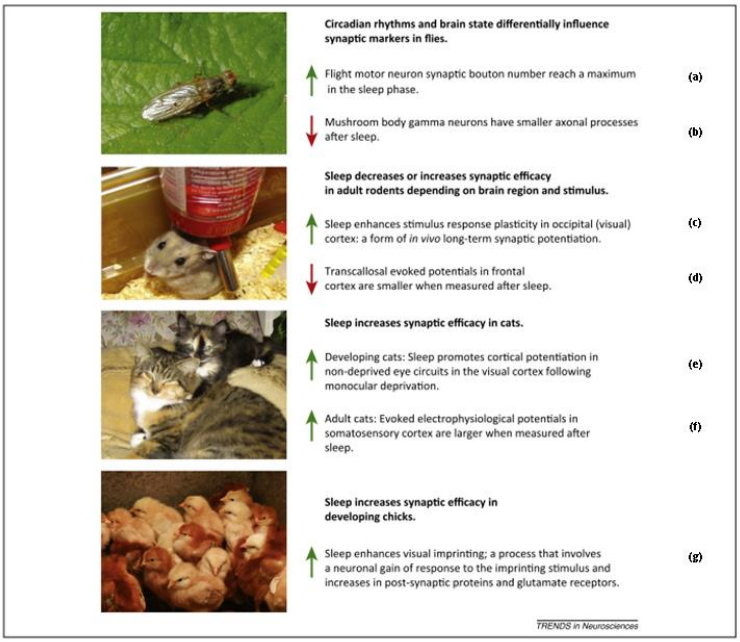

Fig III.4 : Sleep does not have a single effect on synaptic efficacy or morphology. Direct and indirect measures of synaptic plasticity after sleep show changes consistent with increases or decreases in synaptic strength depending on several factors. These include the animal species, the type of circuit under examination, the presence or absence of strong circadian rhythms, and the developmental age of the organism. References : (a) from (Mehnert et al., 2007); (b) from (Bushey et al., 2011); (c) from (Aton et al., 2009); (d) from (Vyazovskiy et al., 2008); (e) from (Aton et al., 2009); (f) from (Chauvette et al., 2012); (g) from (Horn, 1998). *Figure and legend from (Frank and Cantera, 2014)*

Thus, sleep may increase, decrease or have no effects on electrophysiological and morphological measures of synaptic strength. What determines sleep effects on a given synapse remains unclear. Nevertheless, the sleep homeostasis hypothesis appears to not fully explain the role of sleep in synaptic plasticity. Sleep itself is part of the circadian cycle and its function may be integrated in this circadian context.

2.3 The effect of SWA on synaptic strength is not so clear-cut

According to SHY, the activity of the sleeping brain, especially NREM SWA, mediates synaptic downscaling. However, no clear mechanism has been proposed to explain this effect and there is inconsistency in models that have simulated SWA exposition to neurons. 1 Hz stimulation that naturally approximates SWA, does not reliably lead to LTD in cortical neurons *in situ* (Perrett et al., 2001). Furthermore, the 1 Hz protocol which reliably induce LTD *in situ*, failed to reproduce this effect *in vivo* (Hager and Dringenberg, 2010). These conflicting results could mean that SWA has maybe more than one effect on synaptic strength (Frank, 2012).

2.4 There is a circadian rhythmicity in the control of synaptic efficacy and structural plasticity.

Increasing evidence supports a circadian intervention in the fine-tuned regulation of synaptic strength and morphology.

- *In Drosophila melanogaster*, olfactory responses demonstrate circadian rhythmicity with peak during light phase (active phase) and trough during dark phase (rest/sleep phase). This rhythmicity is abolished in clock-gene mutants and persists in situ (Mehnert et al., 2007; Tanoue et al., 2004).
- *In zebrafish* larvae, the number of synapses in hypocretin (HCRT) neurons axons displays a circadian rhythmicity. Mutation that leads to an arrhythmic overexpression of nptx2b, a protein implicated in AMPA receptor clustering, increases synapse number, abolishes HRCT axons rhythmicity and brings resistance to the melatonin sleep promoting effects (Appelbaum et al., 2010).
- *In mice,* LTP was shown to be induced more easily (or of greater magnitude) in hippocampal slices when the latter were obtained from rodents sacrificed in the dark (active) phase as compared to the light (inactive) phase.

Importantly, this time-of-day effect persists for several hours *ex vivo*, suggesting that the hippocampus is able to generate its own circadian rhythm of synaptic efficiency (Chaudhury et al., 2005). Still in mice, postsynaptic dendritic spine formation in the motor cortex that accompanies a motor skill learning is promoted by endogenous circadian peaks of glucocorticoids whereas troughs are required to stabilize newly formed spines that are important for long-term memory retention (Liston et al., 2013).

- In an early study (Barnes et al., 1977), *monkeys*, which are diurnal animals, showed a diurnal-nocturnal rhythm in hippocampal excitatory postsynaptic potentials (EPSPs) which was reversed as compared to rats, a nocturnal species.
- I*n human*, spinal and motor cortex excitability and the ability to generate torque during a maximum voluntary contraction vary with time-of-day and this variation is chronotype-dependent (Tamm et al., 2009).

2.5 The State-Clock model: a circadian alternative to SHY?

Evidence that sleep may have dual effects on synaptic strength which may be, at least partially, under circadian influence gave rise to the State-Clock model of synaptic plasticity **[Figure III.5]**. According to this model, biological clocks drive 24-h rhythms in synaptic plasticity. Experience-dependent changes are induced in wakefulness to be further consolidated during sleep. Nevertheless, the direction of the latter changes is not fixed and depends on the type of experience preceding sleep and the involved circuit. This regulation was proposed to be primary driven by the circadian oscillations in brain temperature and hypothalamic-pituitary axis (HPA) that accompany active (increased HPA and brain temperature) and inactive phases (decreased HPA and brain temperature). The State-Clock model stand as an alternative model to SHY which integrates a circadian synaptic dynamics and does

not require sleep to have only net or global downscaling effect on synapses (Frank and Cantera, 2014)

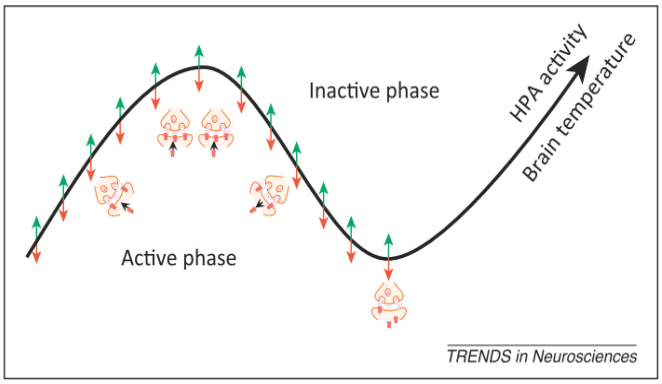

Fig III.5 – A State-Clock model of synaptic plasticity. See main text for explanations.
Figure from (Frank and Cantera, 2014)

3 CONCLUSION

The synaptic homeostasis hypothesis undoubtedly represents a valuable contribution to the study of sleep function and neuronal plasticity but certain aspects of its core theoretical concepts are puzzling. The idea that waking and sleep are respectively dominated by net increasing and weakening of synaptic strength may appear a little simplistic. It may require quite a narrow view of brain plasticity (and learning). As here argued, a circadian involvement in the regulation of cortical synaptic plasticity is more than very likely. However, its supporting evidences (or indices) are spread, fragmentary or even conflicting among the literature, thus explaining why no clear integrative model has yet emerged.

Sleep homeostasis and the circadian timing system interplay over time to set brain function. However, the neural electrophysiological correlates of this interaction remain elusive.

EXPERIMENTAL SECTION

CHAPTER IV

CIRCADIAN MODULATION OF HUMAN CORTICAL EXCITABILITY

1 SUMMARY

Prolonged wakefulness increases cortical excitability and is detrimental for cognitive performance. However, cognitive performance is modulated by a circadian signal such that cognition remains stable during the normal waking day before it deteriorates abruptly when wakefulness is extended into the biological night. Whether the circadian timing system achieves this fine-tuned regulation of cognition through an impact on cortical excitability is unknown. Here, we assessed cortical excitability using scalp EEG-responses to transcranial magnetic stimulation (TMS/EEG) in young individuals during 29h of continuous wakefulness. Data reveal a circadian modulation of cortical excitability over the 24h day-night cycle. This modulation appears strongest in those individuals with the strongest circadian drive and is associated with macroscopic EEG and behavioral changes. Our results suggest that cognition and system-level brain function are shaped by direct circadian modulation of cortical excitability and pave the way for future pharmacotherapy and chronotherapy development.

2 INTRODUCTION

Wakefulness is associated with molecular, cellular and systemic changes in human brain function, which are deemed to negatively impact on cognition (Schmidt et al.,

2007; Tononi and Cirelli, 2014). Nevertheless, the wake-related decrement in performance is not linear. During the first ~16 hours of a normal waking day, human cognitive performance remains remarkably stable, despite the concurrent buildup of sleep homeostatic pressure. However, if wakefulness is subsequently extended into the biological night, cognitive performance deteriorates abruptly (Cajochen et al., 1999a; Dijk et al., 1992). This non-linearity reflects the influence of the circadian timing system, which counters the detrimental effect of sustained wakefulness during the day, until the end of the so-called evening "wake-maintenance zone" (WMZ; Dijk and Czeisler, 1995; Strogatz et al., 1987). Conversely, at night, the circadian system switches to a sleep promoting signal, which favors sleep continuity, and opposes the progressive tendency to wake-up due to sleep pressure dissipation during sleep, up to the end of the early morning "sleep-promoting zone" (SPZ; Dijk and Czeisler, 1995). Behavioral, brain system and molecular correlates of the impact of the circadian timing system are being established (Curie et al., 2013; Gaggioni et al., 2014). However its neuronal bases remain elusive (Frank and Cantera, 2014).

Cortical excitability, as indexed by the strength of cortical neurons response to a stimuli, has been reported to increase with time awake in humans (Huber et al., 2013). This may underlie performance decrements and greater propensity for seizure (Gastaut and Tassinari, 1966) or hallucination (Babkoff et al., 1989) under sleep deprivation. Changes in human cortical excitability corroborate to rodent data showing linear increases with time awake in the firing rate and synchronization of cortical neurons (Vyazovskiy et al., 2009) and in the amplitude and slope of the local field potential evoked by electrical cortical stimulation (Vyazovskiy et al., 2008). Nonetheless, molecular markers of synaptic function and structure, as well as the level of synchrony among neuronal populations, show a marked circadian dependency (Cajochen et al., 2002; Frank and Cantera, 2014; Hung et al., 2013). Therefore, it can be hypothesized that cortical excitability, as an index of synaptic

strength and neuronal synchrony, reflects the influence of both elapsed time awake and circadian phase.

Here we investigated whether the circadian timing system impacts on human cortical excitability and explored whether this influence underpins the circadian fluctuations in cortical dynamics and behavior. We used transcranial magnetic stimulation coupled to high-density electroencephalography (TMS/EEG) (Ilmoniemi et al., 1997), as a non-invasive way to gauge, *in vivo,* the time course of human cortical excitability during 29h of continuous wakefulness. We hypothesized a circadian influence on cortical excitability to be most evident near the wake-maintenance (WMZ) and sleep-promoting zones (SPZ) and that individual variability in circadian signal strength, as derived from endocrine markers, to be related to dynamics of cortical excitability. We further postulated cortical excitability to be associated with spontaneous waking EEG measures and performance assessments.

3 RESULTS

Following an 8-hour nocturnal baseline sleep episode with polysomnography, 22 healthy young men (22 y.o. ± 2.61 ; **Table 1 for complete characteristics**), underwent 8 TMS/EEG recordings during ~29-hour of continuous wakefulness. This was conducted under strictly controlled behavioral and environmental conditions (constant routine protocol) to minimize external and internal factors that may mask circadian rhythmicity (Duffy and Dijk, 2002) **[Figure 1]**. The frontal cortex was chosen as stimulation target because of its high sensitivity to sleep deprivation (Cajochen et al., 1999b) and TMS sessions were scheduled with increased frequency around the putative WMZ and SPZ. During TMS/EEG recordings, participants performed a simple visual vigilance task to assess simultaneous performance as well as to remove from analyses TMS/EEG segments during vigilance lapses (Makeig et al., 2000). All data

were realigned according to circadian phase determined from individual melatonin profiles (Dijk et al., 2012).

N	22		
AGE	22.82	±	2.61
ETHNICITY	Caucasians		
BODY MASS INDEX	22.23	±	2.05
ANXIETY LEVEL (BDII)	1.23	±	1.93
MOOD (BECK)	1.68	±	2.12
DAYTIME PROPENSITY TO FALL ASLEEP (ESS)	3.73	±	2.73
CHRONOTYPE (HO)	52.41	±	5.03
RIGHT HANDED	17/22		
SLEEP QUALITY (PSQI)	4.09	±	0.15
SEASONALITY (SPAQ)	0.64	±	0.79
CAFFEINE (cup/day)	0.41	±	0.50
ALCOHOL (unit/week)	3.41	±	0.20
CHRONOTYPE (MCTQ)	4.76	±	0.16
SLEEP TIME (Sleep diary)	23:25	±	0:20
WAKE TIME (Sleep diary)	7:30	±	0:17
SLEEP DURATION (Sleep diary)	8:10	±	0:15
SLEEP TIME (Actigraphy)	23:30	±	0:15
WAKE TIME (Actigraphy)	7:30	±	0:20
SLEEP DURATION (Actigraphy)	8:00	±	0:20

Table 1 : Sample demographics (Mean ± Standard deviation), and sleep-wake timing during 7 days preceding the laboratory experiment based on sleep diary and actigraphy data (Median ± Standard deviation).

ANXIETY LEVEL was measured on the 21 item Beck Anxiety Inventory (BAI≤14) (Beck , 1988); CHRONOTYPE was assessed by the Horne-Ösberg Questionnaire (Horne and Ostberg, 1976); Daytime propensity to fall asleep in non-stimulating situations was assessed by the Epworth Sleepiness Scale (ESS≤11) (Johns, 1991); MOOD was assessed using the 21-item Beck Depression Inventory II (BDI-II≤14) (Steer et al., 1997); SLEEP QUALITY was determined by the Pittsburgh Sleep Quality Index Questionnaire (PSQI≤7) (Buysse et al., 1989). SEASONALITY is based on the Seasonal Pattern Assessment Questionnaire (SPAQ<11) (Rosenthal N, Bradt G, 1984). The Edinburgh Inventory (Oldfield, 1971) was administered to verify that the participants were right-handed. Sleep parameters are presented in hours.

Each TMS/EEG acquisition was preceded by a 2-minute eyes-open spontaneous waking EEG recording to extract theta frequency band power (4.5-8Hz), an established marker of alertness and sleep need (Dijk et al., 1987; Vyazovskiy and Tobler, 2005). In between TMS/EEG sessions, participants also completed an auditory psychomotor vigilance task (PVT) (Dinges and Powell, 1985) to probe sustained attention. Subjective sleepiness and affect were assessed hourly **[see appendix 2 for detailed experimental procedures]**. All these classical alertness-related measures exhibited typical and statistically significant variations during the protocol [p<.002]. Relatively stable values were observed during the normal waking day period followed by decrements during the biological night and subsequent partial recovery in the next day (Cajochen et al., 1999a) **[Figure 2]**.

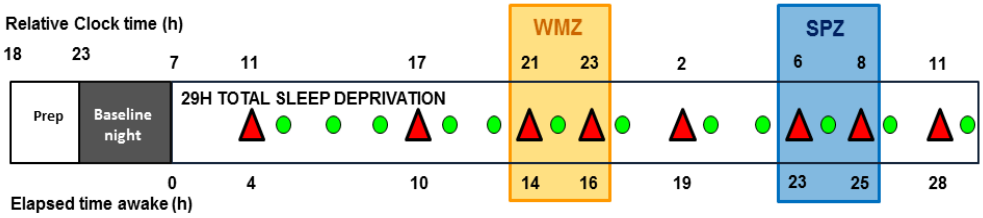

Figure 1: Experimental protocol.

Participants underwent a 29h sustained wakefulness protocol under stringent constant routine conditions [no time-of-day information, constant dim light (< 5 lux), external temperature and semi-recumbent posture, regular liquid isocaloric intake, sound proofed rooms]. TMS-evoked EEG potential (TEP) were recorded 8 times (ca. 250 trials per session; red triangles ▲) and test batteries including the psychomotor vigilance task (PVT; green circle ●) were completed 12 times. TMS/EEG sessions were scheduled throughout the 29-h period with higher frequency around the wake maintenance (WMZ) and sleep promoting zones (SPZ), the timing of which was predicted based on habitual sleep times (data realigned a posteriori). During TMS/EEG sessions, volunteers performed a visual vigilance task consisting in maintaining a constantly moving cursor in the center of a computer screen to assess simultaneous performance and exclude vigilance lapses. Saliva samples were collected hourly for melatonin and cortisol assays, together with subjective sleepiness as well as affects using visual analogic scales (VAS). Relative clock time displayed is for a participant with a 7 am – 11 pm wake-sleep schedule. Prep: 5 preparatory hours, including test battery task practice (< 5 lux). Baseline night: 8h night of sleep in darkness at habitual sleep times and under EEG recording.

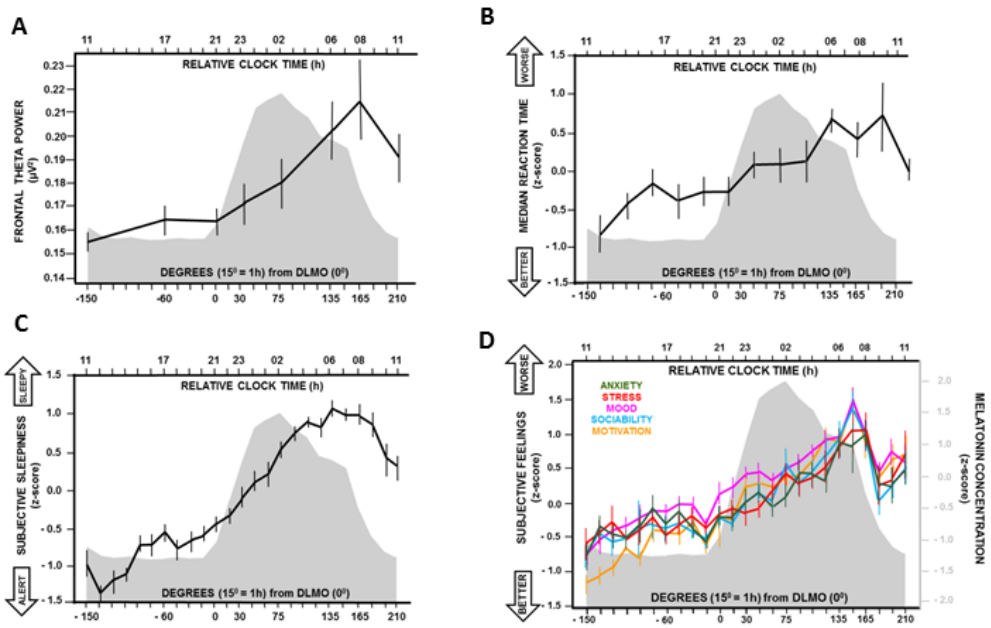

Figure 2 : Time course (mean ± SD) of waking EEG frontal theta activity (A), median reaction time to the psychomotor vigilance task (B), subjective sleepiness (C) and subjective affects (D) across the protocol**.** Mean z-scored melatonin profile is displayed in background light grey. The top x axis indicates the relative clock time for a participant with an 11 pm-7am sleep wake schedule. All variables underwent typical significant variations during the protocol [n= 22 ; Theta: $F_{(7,103)}$ =3.73, p= .0012; PVT: $F_{(11,209)}$ =3.78, p< .0001; sleepiness : $F_{(24,366)}$ =17.72, p< .0001; subjective affects: $F_{(24,368)}$>4, p< .0001]. Relatively stable values were observed during the normal waking day period followed by a decrement during the biological night and subsequent partial recuperation (multiple post-hoc analyses – not shown). All data are realigned to melatonin onset secretion. Due to slight delays in PVT sessions between participants, PVT was performed, as represented here, at 13 different circadian phases although there were only 12 PVT sessions per participant.

3.1 Non-linear changes in cortical excitability with extension of wakefulness

Cortical excitability was inferred from the amplitude and slope of the first component of the TMS-evoked EEG potential (TEP; 0-25msec post-TMS) (Huber et al., 2013), measured at the electrode closest to the maximally stimulated brain location (hotspot). Both TEP amplitude and slope significantly changed with time awake [$p<.0001$] **[Figure 3]**. Post-hoc analysis showed that cortical excitability increased globally from the first to the last session of the protocol [amplitude: $p_{corr}=.025$; slope: $p_{corr}=.064$]. However, the dynamics of this increase was not linear. A marked significant local decrease was observed from the afternoon session (S2) to the evening session (S3), close to the melatonin secretion onset, in the wake-maintenance zone [amplitude: $p_{corr}=.037$; slope: $p_{corr}=.058$]. Both amplitude and slope then significantly increased up to the seventh session (S7) around the onset of cortisol secretion **[Figure 5D]**, at the end of the putative early morning sleep-promoting zone [$p_{corr}<.0001$]. This sharp increase appeared to subsequently cease 3 hours later in the last session (S8) of the protocol, which was no longer significantly different from the previous one [$p_{corr}>.8$].

Importantly, changes in estimated cortical excitability followed a similar pattern when inferring amplitude and slope of the TEP first component from a dipole computed at the hotspot, following EEG source reconstruction, i.e. based on separate analyzes using signals from all available EEG electrodes **[Figure 4].**

These results confirm that human cortical excitability varies with wakefulness extension (Huber et al., 2013) but reveal local non-linear variations compatible with a strong influence of the circadian timing system, in addition to a linear trend likely related to sleep homeostasis.

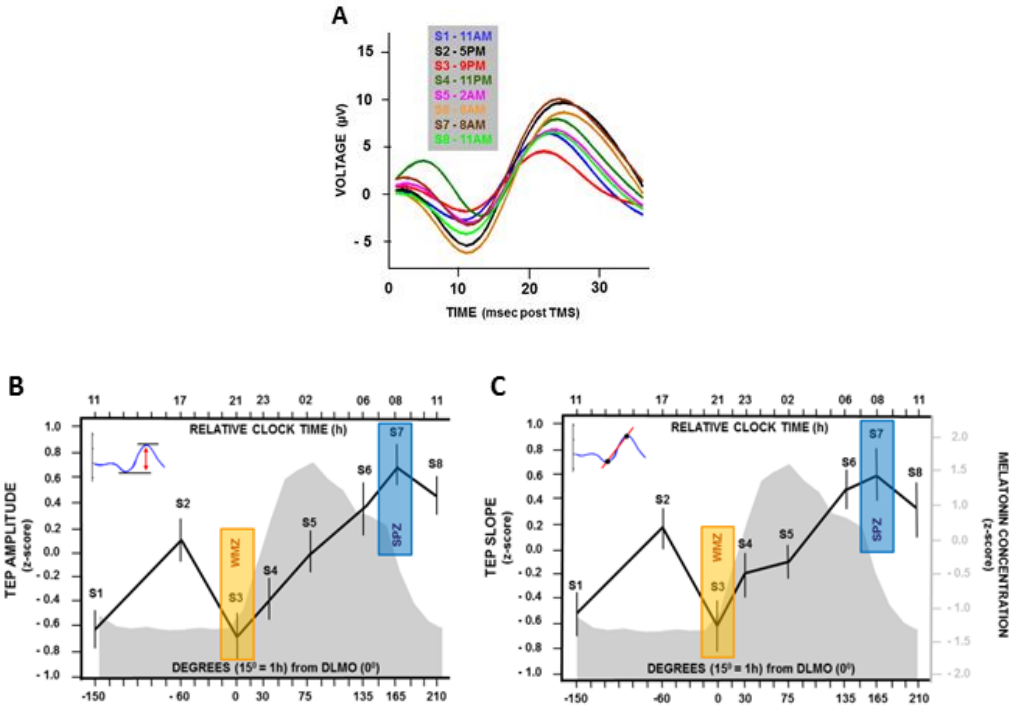

Figure 3 : Non linear changes in cortical excitability with wakefulness extension.

A. TMS-evoked potentials (TEP; 0-35 msec post TMS) measured at the electrode closest to the hotspot, averaged in each of the 8 TMS/EEG sessions, in a representative participant with habitual bed and wake-up times at 11 pm and 7 am, respectively. Hotspot location was provided by the neuronavigation system.

B-C. Time course of TEP amplitude and slope. Data were averaged after normalization (z-score) and realignment with individual circadian phase (n=22; melatonin secretion onset = 0°). Mean z-scored melatonin profile is displayed in grey. The top x axis indicates relative clock time for a participant with an 11 pm-7am sleep wake schedule. Both TEP amplitude and slope significantly changed across the 29h of sustained wakefulness [time effect: amplitude $F_{(7,128)}$ = 8.17 , p <.0001 ; slope : $F_{(7,/129)}$ = 5.91, p <.0001]. Post-hoc analysis revealed 1) a significant increase from the first to the last session [S1 vs. S8: amplitude: p_{corr}=.025; slope : p_{corr} =.0635], 2) a local decrease from the second afternoon session (S2) to the third evening session (S3) in the hypothetical WMZ [S2 vs. S3: amplitude: p_{corr}=.037; slope: p_{corr}=.058], 3) a sharp increase during the biological night [S3 vs. S7: amplitude and slope: p_{corr}<.0001], 4) ceasing after the seventh session, at the end of the theoretical SPZ [S7 vs. S8: amplitude and slope: p_{corr} >.8].

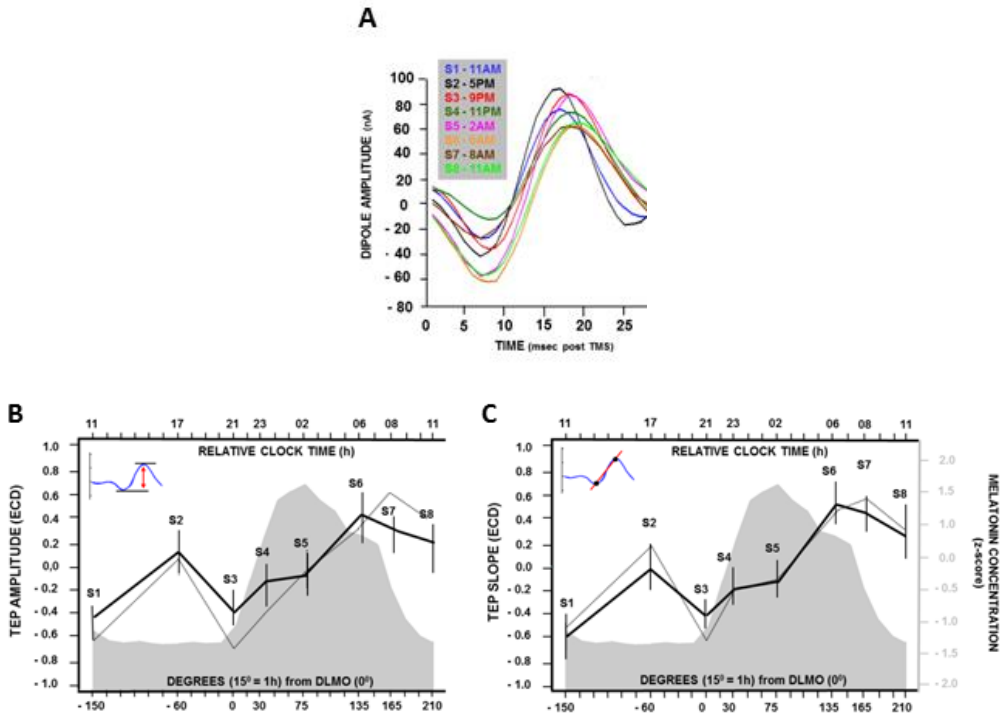

Figure 4: Cortical excitability dynamics inferred following EEG source reconstruction.

A. TMS-evoked potential (TEP) responses (0-25 msec post TMS) computed at the hotspot after equivalent current dipole (ECD) source reconstruction, i.e. based on information for all available EEG electrodes, in a representative participant with habitual bed and wake-up times at 11 pm and 7 am, respectively.

B-C. Time course of TMS evoked response amplitude (B) and slope (C) at the hotspot based on dipole amplitude and orientation. Mean z-scored melatonin profile is displayed background in light grey. The top x axis indicates the relative clock time for a participant with an 11 pm-7am sleep wake schedule. Both indices showed significant variation with time [n=23; time effect: amplitude $F_{(7,137)} = 2.96$, $p<.0001$; slope : $F_{(7,/137)} = 4.66$, $p <.0001$]. Dashed line: TEP z-scored amplitude (cf. Figure 2).

3.2 Cortical excitability dynamics is shaped by individual differences in sleep homeostasis and circadian parameters

To further investigate this dual influence, we compared the predictive value of 2 different models to explain the observed time-course of cortical excitability. The first fit consisted of a linear function representing the progressive build-up of sleep pressure (Dijk et al., 2012). The second fit comprised a 24h-period sine-wave function, centered on individual melatonin secretion onset, aimed at modelling the circadian signal (Viola et al., 2012b) **[Figure 5A]**. Both fits turned out to be good predictors of observed data, as indexed by low error sum of squares indices.

In a next step, we related cortical excitability to specific measures of sleep homeostasis and circadian system. We first associated cortical excitability to a well-established marker of sleep homeostatic pressure: sleep slow wave activity (SWA) (Tononi and Cirelli, 2003).

Individual dissipation rate of SWA reflects individual sleep homeostasis efficacy to eliminate sleep pressure (Tarokh et al., 2012). In our protocol, the first and the last TMS session were recorded 24h apart, at the same circadian phase but with large difference in sleep pressure. We hypothesized that change in cortical excitability between those two sessions would be related to the homeostatic build-up of sleep pressure. Regression analysis confirmed this hypothesis and showed that SWA dissipation rate during the baseline night prior to sleep deprivation **[Table 2 for baseline night characteristics]** was positively associated with the overall build-up in cortical excitability [$r^2 > .22$; $p < .037$] **[Figure 5B-C]**.

Cortisol profile is a reliable endocrine marker of circadian rhythmicity, typically associated to wakefulness, with peak at the habitual wake period and nadir in the evening WMZ (Morris et al., 2012b). A possible link between cortisol and cortical

excitability dynamics was therefore evaluated. We found that cortisol levels covaried positively with increased TEP amplitude and slope over the entire protocol [**Figure 5D**] [ANCOVA: $r^2>.24$, $p<.0001$]. We then hypothesized that cortisol secretion amplitude may reflect the strength of the circadian signal (Dijk et al., 2012) and that the latter may be related to the non-linear change in cortical excitability observed around the evening. Regression analysis revealed a significant positive association between individual estimates of cortisol amplitude during the protocol and the decrease in cortical excitability from the afternoon session to the evening WMZ session [$r^2>.21$; $p<.023$] **[Figure 5E]**. We found similar tendency when regressing cortisol amplitude with the overnight increase in cortical excitability (from S3 to S7), which however did not reach significance [$r^2>.18$; $p=.06$].

Baseline night Sleep Structure			
Total Sleep Time (h)	6.4	±	0.15
Sleep Efficiency (%)	79.5	±	1.9
Wake (min)	27	±	4.3
NREM stage 1 (min)	63	±	4.5
NREM stage 2 (min)	220	±	7.9
NREM stage 3 (min)	78	±	6.2
REM (min)	85	±	4.1

Table 2: Characteristics of the 8h baseline sleep immediately preceding the sleep deprivation protocol (n = 22 ; mean ± Standard Error of the Mean).

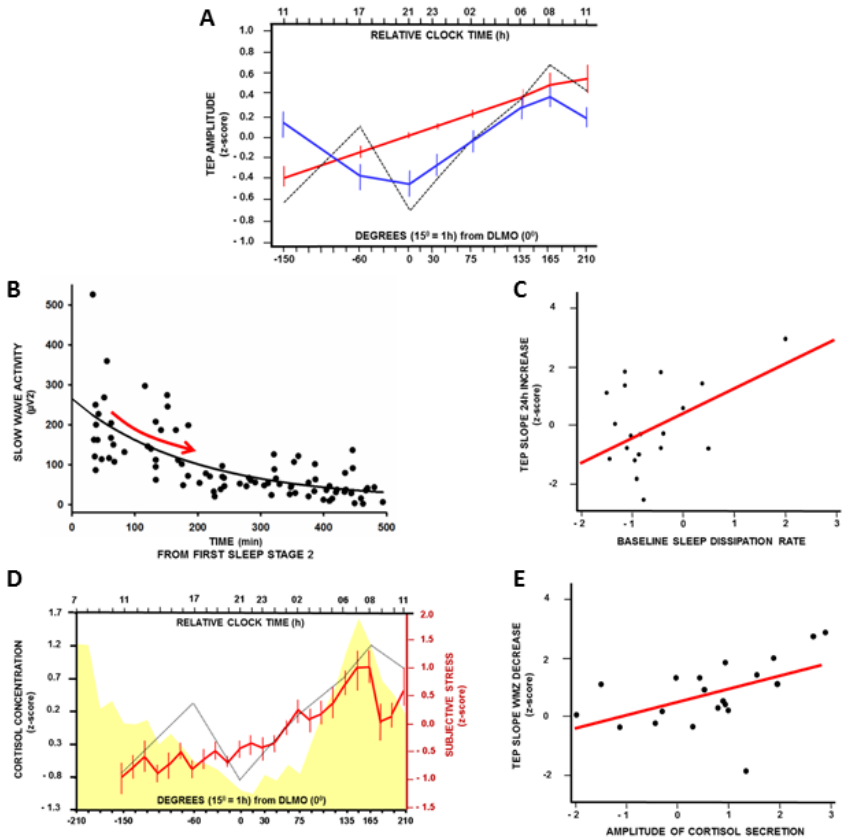

Figure 5 : The circadian system modulates cortical excitability.

A. Cortical excitability was fitted with linear (**red**) and 24h-period sine wave (**blue**) functions to mimic sleep pressure build-up and the circadian signal respectively. Error sum of squares (ESS) was <10 for both indices [amplitude: ESS linear fit= 4.9, p<.0001; ESS sine fit= 4.1, p<0.0001; slope: ESS linear fit= 5.19, p<0.0001; ESS sine fit= 4.24, p<.0001]. Dashed line: TEP z-scored amplitude (cf. Figure 2).

B. Slow wave activity across the first 4 cycles of sleep baseline night was fitted to compute individual dissipation rate (schematically shown by **red arrow**). Each dot represents SWA of an individual sleep cycle (4 per participant).

C. Regression analysis showed that individual dissipation rate was positively associated with the increase in cortical excitability from first to last session, recorded 24h apart, at the same circadian phase, following a normal night of sleep and after sleep deprivation [n=18; amplitude : p=.044; r^2=.23 ; slope : p=.036 , r^2=.25].

D. Cortisol (**yellow**) and subjective stress (**red**) levels. Salivary cortisol level was not significantly different between the first and the last protocol samples, collected 24h apart, but at the same circadian phase, following a normal night of sleep and after sleep deprivation [n=22 ; $F_{(28,482)}$=13.44 ; p<.0001]. Dashed line: TEP z-scored amplitude (cf. Figure 2).

E. Regression analysis revealed that individual fitted amplitude of cortisol secretion over time was positively associated with the decrease in cortical excitability measured around the wake-maintenance zone. [n=20; amplitude: p=.017; r^2=.24 ; slope : p=.023 , r^2=.21].

Collectively, our data speak to a critical role for sleep homeostasis on the dynamics of cortical excitability in conjunction to a classical "hand-of-clock" endocrine marker which putatively reflects individual circadian strength.

3.3 Cortical excitability dynamics sets changes in spontaneous waking EEG power and behavior

Lastly, we investigated whether the dynamics in cortical excitability, which arguably reflect a circadian influence, could constitute the neuronal bases for variations in individual brain system level and behavioral measures, for which a circadian influence is widely accepted (Cajochen et al., 1999b; Schmidt et al., 2007). We found that TEP amplitude and slope significantly covaried with theta power over the frontal region across the 29h of sustained wakefulness, with high cortical excitability associated with high theta power [ANCOVA; r^2=.19: p<.001] **[Figure 6A-C]**.

We then focused on the vigilance task which was performed simultaneously with the TMS/EEG recording. Task performance showed non-linear changes over time and was significantly linked to cortical excitability dynamics such that higher indices of cortical excitability associated with worst performance [ANCOVA; r^2=.23, p<.03] **[Figure 6D-E]**. Dynamics of cortical excitability also appeared to translate to the dynamics of subjective feelings. Increases in subjective sleepiness **[Figure 6A-B]** and negative affect (anxiety, stress, fatigue) and reductions in positive affect (mood, motivation, sociability) were related to increases in TEP amplitude and slope [ANCOVA; r^2>.4, p<.0001]. Altogether, these findings point towards a direct relationship between cortical excitability profiles and brain system-level or behavioral measures dynamics.

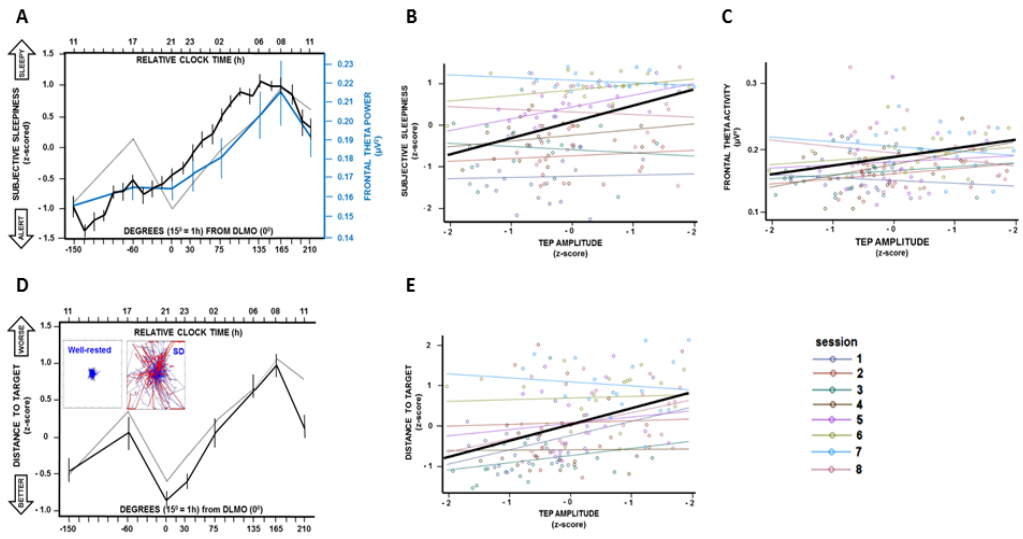

Figure 6: Cortical excitability dynamics is associated with changes in system level brain function measures and in behavior.

A. Time course of theta (4.5-8Hz) power in spontaneous waking EEG (**blue**) and subjective sleepiness (**black**). Both variables showed significant variation over the sleep deprivation protocol [n = 22; p<0.001; Figure S1 for details]. Dashed line: TEP z-scored amplitude (cf. Figure 2).

B-C. ANCOVAs showed that theta power (B) [n = 22; amplitude: r^2=.19, p=.0008; slope: r^2=.19.x, p=.0009] and subjective sleepiness (C) [n=22 ; amplitude: r^2=.69, p<.0001; slope: r^2=.69, p<.0001] were significantly and positively associated with both indices of cortical excitability. Amplitude/slope*circadian phase interactions were not significant (p>.28).

D. Time course of performance to the vigilance task performed simultaneously to TMS/EEG recordings. The task consisted of maintaining a constantly moving cursor in the center of a computer screen. Small inset depicts a representative well-rested and sleep-deprived (SD) session (lapses in red). Task performance (average distance kept from the target) significantly changed with time awake [n = 22; time effect: $F_{(7,122)}$=13.78; p<.0001]. Dashed line: TEP z-scored amplitude (cf. Figure 2).

Post-hoc analysis revealed 1) a local decrease from the afternoon session (S2) to the evening session (S3) in the WMZ [S2 vs S3: amplitude: p_{corr}=.0008], 2) a sharp increase during the biological night [S3 vs. S7: amplitude and slope: p_{corr}<.0001], and 3) a significant decrease from seventh session (end of SPZ) to last session [S7 vs. S8: p_{corr}=.0025].

E. An ANCOVA revealed that vigilance task performance impairment was associated to TEP amplitude/slope increase [n=22; amplitude: r^2=.44, p<.0001; slope: r^2=.43, p<.0001]**.** Amplitude/slope*circadian phase interaction was not significant (p>.69).

4 DISCUSSION

4.1 Non-linearity and timing of cortical excitability changes are compatible with a circadian modulation

To our knowledge, our study paradigm is the first to dissect out a putative modulatory role of the circadian timing system on human cortical excitability. The stringent constant routine conditions and melatonin measurements allowed for a precise assessment of individual circadian phase (Duffy and Dijk, 2002). Moreover, our relatively high sample of TMS/EEG acquisitions, particularly around the WMZ and the SPZ, unveiled marked non-linear changes in cortical excitability at these critical circadian time-windows.

The observed "afternoon" trough in cortical excitability **[Figure 3]** is not compatible with an exclusive sleep homeostasis dependency. Indeed, in the absence of any sleep episode, only a monotonic increase would be expected, which was not the case. A strong signal able to oppose the accumulation of sleep homeostatic pressure up to the evening WMZ may therefore co-exist. In the context of our protocol, this afternoon excitability reduction can only be arguably explained through an endogenous circadian influence independent of sleep, since participants did not nap, had no knowledge of clock time and all environmental and behavioral conditions were kept constant. Moreover, our data underlined a further sharp increase in cortical excitability starting around the WMZ and ending/stabilizing after the SPZ. It is remarkable that this time interval, under sleep deprivation (and entrained conditions), corresponds to a period when the circadian timing system and sleep homeostat promote sleep in synergy (Dijk and von Schantz, 2005).

4.2 The role of sleep on the regulation of cortical neuronal function recasted in a circadian context

We showed that cortical excitability reduction may occur in absence of any sleep episode. Thus, our findings support that sleep and sleep homeostasis are not the only processes that regulate and restores neuronal function, as previously pointed out (Frank and Cantera, 2014). It has been suggested that mammals with weak circadian rhythms (e.g. endotherm vs. ectotherm) do not show evident circadian variations in synaptic function over the sleep-wake cycle (Frank, 2012). This could explain in part why most previous studies have associated synaptic changes associated mostly with the sleep-wake rather than the circadian cycle (Frank, 2012). However, sleep itself is part of the circadian cycle and is accompanied with important behavioral changes influencing physiology. Here we argue that if the masking effect of such behavioral variations is attenuated, such as in a constant routine protocol, circadian variations in neuronal and synaptic function become evident also in humans.

Importantly, our results do not preclude a previously reported influence of sleep and sleep homeostasis on synaptic function (Huber, 2013). In our data, the overall build-up in cortical excitability, from the morning after a normal night of sleep to 24h later following continuous wakefulness, is related to the individual differences in the dissipation rate of SWA during sleep **[Figure 5 B-C]**. This dissipation is mainly related to sleep homeostasis, although for this variable, circadian influences are becoming evident (Dijk and Czeisler, 1995; Lazar, 2015). Our findings supports a link between cortical excitability build-up during wakefulness and sleep-induced excitability reduction, at least when considering time points ~24h apart during extended wakefulness, when the circadian influence is partly weighted-out.

4.3 Circadian cortisol secretion may trigger daily variations in cortical excitability

Our data show that non-linear variations in cortical excitability are most obvious in those individuals with highest amplitude of cortisol secretion, hypothesized to reflect the circadian signal strength (Dijk et al., 2012). We also pointed out a direct positive association between levels of cortical excitability and cortisol.

Cortisol secretion varies tonically with circadian rhythmicity but is also phasically triggered by stress exposure. Acute increases in plasma corticosteroids were reported to rapidly enhance synaptic function both in rodents (Liston et al., 2013; Yuen et al., 2009) and humans (Milani et al., 2010). Those changes were either circadian-driven (Liston et al., 2013), behaviorally stress-induced (Yuen et al., 2009) or mimicked by cortisol bolus injections (Milani et al., 2010). Importantly, corticosterone secretion was shown to increase in mice after 6 hours of total sleep deprivation and to contribute to the sleep-deprivation–induced changes in brain transcriptome (Mongrain et al., 2010). In humans, rise of evening concentrations of cortisol has been reported after 6 days of sleep restriction (Spiegel et al., 1999) and more recently during acute total sleep deprivation under constant routine conditions (Wright et al., 2015). However, we consider that stress and stress-induced cortisol secretion are unlikely to have significantly contributed to cortical excitability dynamics in our protocol. First, subjective stress variations were relatively limited in our sample, even though they did show previously reported significant circadian-related variations (Boivin, 1997) [$p<.0001$] **[Figure 5D]**. Second, the afternoon peak in cortical excitability did not correspond to punctual peak in stress at this phase. third, salivary cortisol levels of our participants did not exceed laboratory norms (Aardal and Holm, 1995). Finally, cortisol followed its typical circadian secretion profile (Morris, 2012) in our sample, as supported by the fact that cortisol levels were not significantly different at the beginning and end of the protocol, at same circadian phase but 24h apart [cf. **Figure 5D**; $p_{corr}=1$].

Collectively, our data suggest that cortisol secretion may constitute a circadian driven signal that triggers part of cortical excitability variations.

4.4 Circadian changes in cortical excitability subtends daily variations in performance and alertness level

Our results provide a strong link between cortical reactivity, system level measure of brain function (waking EEG theta power) and behavior (vigilance task performance, subjective feelings) **[Figure 6]**. Hence, the well-recognized non-linear variation in cognitive performance and subjective feeling during extended wakefulness (Cajochen, 1999a) appears to be grounded to basic output of neuronal function. For instance, cortical excitability decrease and return to baseline level observed in the afternoon may explain how cognitive performance (under entrained conditions) is maintained rather stable during the ~ 16 first hours of a normal waking day. Conversely, the marked increase in cortical excitability marked during night coincided with decrements in performance, subjective feelings and objective EEG measure of alertness **[Figure 6]**. Our data also suggest that the typical recovery observed in the morning of the second day of sustained wakefulness, as indexed by spontaneous waking EEG and behavioral measures, is mirrored by a decrease or at least a stabilization of cortical excitability.

The amplitude and particularly the slope of an EEG signal are considered to reflect neuronal synchrony and synaptic strength at the cortical level (Vyazovskiy et al, 2008). Variations in TMS-evoked EEG responses and their sharp overnight increase in TMS-evoked EEG responses could therefore reflect a loss of individual neuron discrimination/specificity and the impoverishment of firing repertoires of neuronal populations, which would jeopardize performance. This phenomenon would be particularly prominent in the night when both sleep homeostatic and circadian

processes are synergically promoting sleep (Dijk and von Schantz, 2005). On the other hand, reduction in cortical excitability may represent a previously unappreciated marker of the circadian mechanisms by which performance is maintained during the biological day despite growing homeostatic sleep pressure.

4.5 Circadian modulation of cortical activity may also be local

Global and local dynamics in neuronal synchrony have been demonstrated both during wakefulness and sleep (Cajochen et al., 2001; Huber et al., 2004; Hung et al., 2013; Sarasso et al., 2014). As we stimulated a single brain area, we can only speculate about this global/local aspect. We delivered TMS over the frontal cortex because this region shows the most pronounced impact of sleep-wake history based on lower EEG frequency power variations (Cajochen, 1999b; Lazar, 2015). The increase in these lower frequencies associated with wakefulness extension is global but also follows a fronto-occipital gradient (Cajochen, 1999b). This pleads for similar variations in cortical excitability over the entire brain that would be attenuated towards the occiput. Cortical excitability shows however region specific characteristics in the main frequency of a TMS-evoked EEG response in human (Rosanova, 2009). Both gradual and maybe quite focal brain variations in the dynamics of cortical excitability are therefore likely and their extent deserves further investigations.

We found a strong correspondence between cortical excitability and accuracy to the vigilance compensatory tracking task (CTT) whereas we did not find any significant association with performance to the auditory psychomotor vigilance task (PVT). Although they were compared at same circadian phases, TEP and PVT, in contrast to CTT, were not recorded simultaneously but rather separated by ~1 hour. Therefore, it is possible that measured cortical excitability levels changed in this time interval. Although cortical excitability may putatively set PVT performance, our PVT measures

were maybe not compared to the true corresponding instant values of cortical excitability. CTT and PVT may also imply different cognitive processes which may recruit different cortical networks. CTT consists of manually compensating the deviation of a constantly randomly moving cursor (Makeig et al., 2000) whereas, in PVT, participants are asked to press a button as soon as an auditory signal occurs (Dinges and Powell, 1985). Thus, as compared to CTT, PVT may arguably less involve motor and premotor cortical areas, including SMA which was our site of TMS stimulation and EEG response extraction.

The preferred timing to perform is clock-dependent but is also not homogeneous for all types of cognition (Schmidt et al., 2007). Whether different circadian regional changes in cortical excitability subtend different dynamics of cognitive performances sustained themselves by different cortical networks is unknown.

In behaviorally awaken rats, populations of neurons in different cortical areas have been reported to suddenly, transiently and asynchronously go 'OFF line' in a way that mimics the OFF periods of NREM sleep (i.e. recording of slow/theta wave on local field potentials in deeper layers of motor or parietal cortex) (Vyazovskiy et al., 2011). These phenomena, considered to reflect transient enhancement in neuronal synchrony, increase in occurrence with elapsed time awake and are associated with transient worsening of a task performance (Vyazovskiy et al., 2011).

In a similar vein, in humans, prolonged wakefulness increases specific regional waking theta power and amplitude of theta waves following practice to tasks targeting functionally and anatomically distinct cortical circuits (Hung et al., 2013). The authors argue that theta recruitment may be wake and use dependent and that it may reflect equivalents of "local sleeps" observed in awaken rats. Actually, when going back to their results **[Figure 7]**, theta power shows a similar profile amplitude and slope of our TMS evoked responses. Indeed, after an afternoon increase, it

returns to baseline levels in the evening before increasing overnight. According to our findings, a circadian modulation may have contributed to their results. Association between theta, slow oscillation evoked by TMS and local sleep during wake remains speculative. However, considering the hypothesis they would be reflecting each other, local sleeps may also happen in awaken humans and their occurrence may be influenced by the circadian signal.

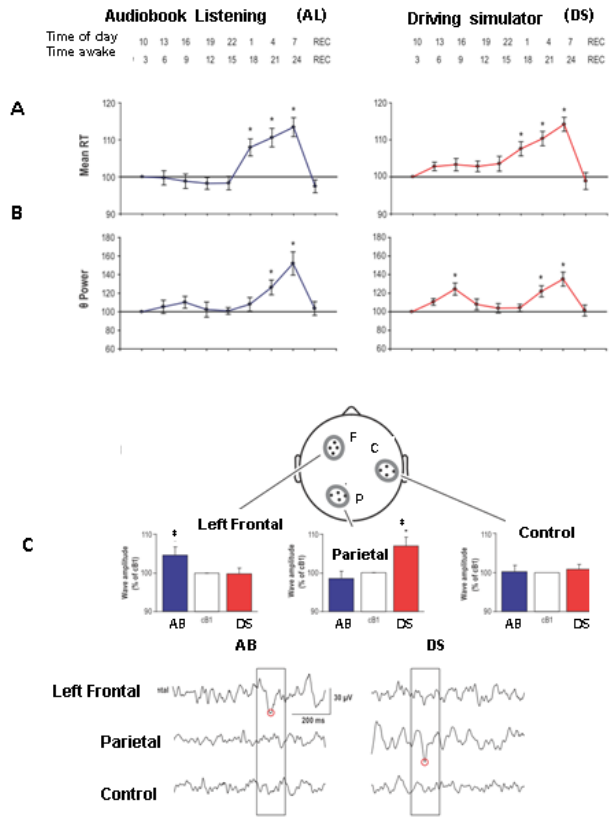

Figure 7 : Local Experience-Dependent Changes in the Wake EEG after Prolonged Wakefulness *from (Hung et al., 2013)* : In each experiment participants woke up at ~07:00 and underwent a baseline testing session (B1) at 10:00, followed by six 2-h tasks (Audiobook listening (AL) or Driving Simulator (DS)) interleaved by 1-h EEG recording sessions (T1–T7, total wake time: 24 h). A final testing session (R1) was scheduled 30 min after participants woke up from ~ 8 h of recovery sleep. In most experiments participants remained awake for 24 h and were allowed to sleep in the morning (starting at ~ 08:30). In 9 experiments, participants were awake for 36 h, and went to sleep in the evening. **(A)** Time course of task performance as indexed by mean reaction time (RT). Notice the circadian profile with relative stable levels during the day, sharp impairment during the night **(B)** Time course of theta power (average of 185 EEG derivations). Notice the typical that this profile has similar shape as our cortical excitability data (see figure 2) **(C)** comparison of theta waves amplitude across task specific brain regions (left frontal: AB ; parietal : DS; control). *Adapted from (Hung et al., 2013)*

This assumption is supported by recent findings of our group. Doctor Vincenzo Muto, in his very recent works (Muto et al. 2015, submitted), measured brain responses to a simple reaction time task (PVT) and a working memory task (3-back task) by functional magnetic resonance imaging (fMRI) in 33 young healthy volunteers 12 times during a 42h sleep deprivation protocol under constant routine conditions**.** He demonstrated that region-specific and task-dependent cortical responses are under the influence of local sleep need but also showed a circadian rhythmicity that may change from region to region **[Figure 8]**. In other words, their activity levels oscillate on a circadian manner but asynchronously.

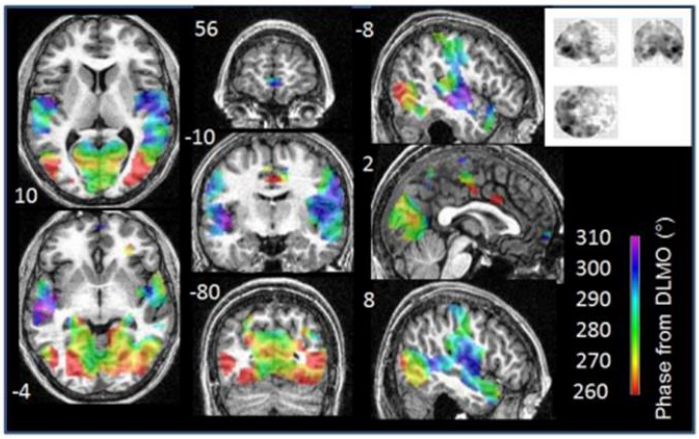

Figure 8 : Regional modulation of human brain responses by sleep need and circadian rhythmicity *from (Muto, 2015)* Analysis from 12 PVT fMRI sessions during a 42h sleep deprivation under constant routine (n=17) . Circadian phase map of brain responses to PVT are estimated with the Sandwich Estimator method (Guillaume et al., 2014) (p FDR < 0.05 over the whole brain; the False Discovery Rate is currently recommended with this method). The phase of the estimated maximum of brain responses to PVT, changing from region to region, is displayed according to the color scale (melatonin onset is = 0°) and overlaid over an individual normalized T1 MR scan. Coordinates in mm along x, y and z axes. Inset: Glass brain with areas showing significant 24-h periodicity in response.

4.6 Going non-invasively deeper in the comprehension of human cortical excitability temporal changes

Modifications in cortical excitability, which underlie behavioral fluctuations, imply changes in excitation/inhibition balance across subpopulations of neurons. This balance would therefore be under strong circadian influence, possibly through circadian changes in synaptic structure which is evident in many species other than humans (Bushey, 2011), through change in the extracellular milieu (Dash, 2009), via a glial contribution, or through changes in the influence of brainstem and mesencephalic structure of the ascending arousal system (Saper, 2010).

A key limitation in human neuroscience is the difficultly to isolate neuronal excitatory/inhibitory drive *in vivo*, due to the impossibility to knockout and/or invasively target specific neuromodulators involved in human brain function. Although it is quite superficial and focal, TMS cortical activation remains unspecific. Direct examination of TMS evoked scalp EEG potentials does not give access to the separate weights of excitatory (mostly glutamatergic) and inhibitory (mostly GABAergic) cortical neurons in generating the response. Computational models are therefore non-invasive approaches of choice to indirectly access hidden neuronal states.

Dynamic causal modelling (DCM) provides a well-validated physiological framework for inferences on neuronal architectures that generate hemodynamic or electromagnetic activity (Boly et al., 2011; Friston et al., 2003; Moran et al., 2014a) and ensures an efficient means to map from EEG evoked responses to causal neuronal states (Friston, 2009) in rats (Moran et al., 2014b) and humans (Boly et al., 2011; Moran et al., 2011).

In parallel analyzes, Doctor Sarah Chellappa used DCM to derive excitation/inhibition parameters across neuronal subpopulations from our set of TMS-evoked EEG responses. Accordingly, higher inhibitory cell-to-cell connectivity and glutamatergic receptor density levels were found to occur near the circadian evening WMZ. Conversely, higher excitatory cell-to-cell connectivity and GABAergic receptor density levels happened in the biological night, when wakefulness was extended beyond the normal waking day. Furthermore, the same sine-wave function we previously used for mimicking circadian variation also fitted very closely the temporal dynamics of excitation/inhibition indices relative to a linear function modelling sleep homeostasis **[Figure 9]**.

Together, these findings speak to a key role of the circadian timing system in the daily modulation of neuronal excitation/inhibition dynamics. Moreover, it posits cell-to-cell inhibition and GABAergic drive as putative circadian means to counteract wake-related increased cell-to cell inhibition and glutamatergic drive stabilize neuronal activity during the biological day. TMS/EEG combined with DCM analysis offer a unique window onto the multi-scale temporal organization of human brain function.

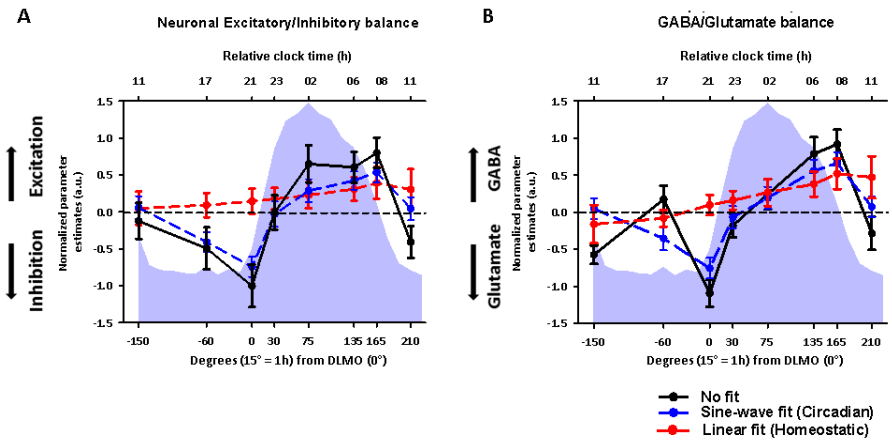

Figure 9: Dynamics of neuronal Excitation/Inhibition drive and GABA/Glutamate receptor density balances during normal waking and sleep deprivation. Figure and legend (see next page) from *(Chellappa, Gaggioni, Ly et al, 2015, submitted)*

A) Excitation/inhibition cell-to-cell connectivity parameter balance significantly varied across time ($F_{7,129}$=2.8, p=0.01), with relatively more inhibition around the circadian wake-maintenance zone and relatively more excitation during the biological night (multiple post-hoc corrections, Tukey-Kramer Test, p=0.02).

B) GABAa/Glutamate receptor density balance significantly varied with time ($F_{7,130}$=3.1, p=0.006), with relatively more glutamatergic drive around the circadian wake-maintenance zone and relatively more GABAergic drive during the biological night (multiple post-hoc corrections, Tukey-Kramer Test, p=0.002).

Both panels display a linear fit (dashed red) mimicking sleep homeostasis build up and a sine wave fit (dashed blue) reflecting the circadian timing system. Qualitatively, the sine wave fit appears to match better observed data. However, both models equally predicted the observed data, according statistical fit comparison using Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC (difference < 200).

4.7 Does photoperiod impact on daily dynamics of cortical excitability?

Our 22 participants performed our study from November 2012 to September 2013 at the Cyclotron Research Centre in Liège (Belgium). This period of acquisition thus encompassed different seasons associated with different amounts of daily natural light exposure. Christelle Meyer, from our group, stratified our population sample in two groups: those who started the experimental paradigm during short days (autumn-winter) and those who started during long days (spring-summer). Accordingly, eleven participants were classified in each group. Based on Chellappa's DCM analysis, Christelle Meyer found that the time course of inhibition/excitation balance, as indexed by GABA/Glutamate receptor availability, displayed a significantly earlier activity peak by nearly 3-hours during a short-day relative to a long-day **[Figure 10]**. In the main work presented in this thesis, we addressed the slow time-scale regulation of cortical excitability over a "normal" waking day and during sleep loss across 29 hours and showed that it was on sleep homeostasis and circadian dual influence. In addition, Christelle Meyer's results suggest that this dynamics in human cortical excitability is exquisitely plastic to critical changes over the slow passage of time across seasons.

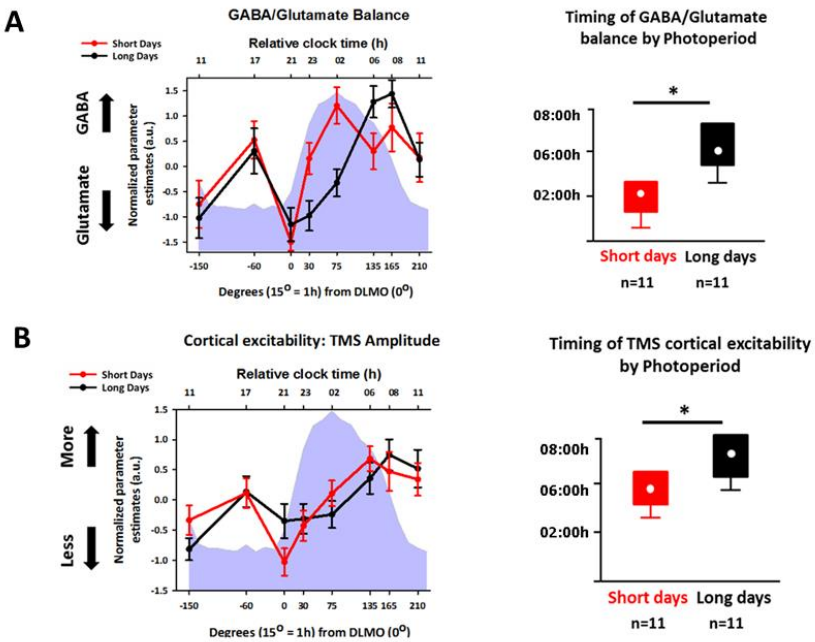

Figure 10 : Dynamics of neuronal GABA/Glutamate receptor availability balance and TMS cortical excitability during normal waking and sleep deprivation according to seasonal changes in photoperiod *from (Meyer, Gaggioni, Ly et al. 2015, on preparation)*

Data are presented as Mean ± Standard Error of Mean (SEM). Short days are represented in red (n=11) and long days in black lines (n=11).

A) $GABA_A$/Glutamate receptor availability balance (z-scored normalized amplitude) varied significantly with 'Time in hours' ($F_{7,123}$=7.9, p<0.001), with relatively more glutamatergic drive around the circadian wake-maintenance zone and relatively more GABAergic drive during the biological night (multiple post-hoc corrections, Tukey-Kramer Test, p=0.002). The two-way interaction of 'Time in hours' vs. 'Season' indicated a significant effect ($F_{7,123}$=2.1, p=0.04), with an earlier peak for GABAergic drive during the biological night for short days (surrounding melatonin midpoint) relative to long days (early morning circadian sleep promoting zone).

B) For TEP amplitude, main effect of 'Season' yielded a tendency for significance ($F_{7,120}$=2.1, p=0.08), and the two-way interaction of 'Time in hours' vs. 'Season' did not elicit significant differences.

4.8 Extending our comprehension of human cortical excitability dynamics to wider population

Our participants were all young (18-30 yo) healthy Caucasian men. Women were primarily excluded from our study because changes in ovarian hormones may influence cortical excitability in humans (Smith et al., 2002a). As our primary aim was to be the first to demonstrate a circadian modulation on cortical excitability, we chose to avoid this possible masking effect.

One of our secondary outpoints was to address the issue of interindividual variability in the impact of sleep need and circadian phase on cortical excitability dynamics. In our study, participants were selected based on their polymorphism in the clock gene *PERIOD3* (*PER3* variable number of tandem repeat, with 4 or 5 repeats) (Dijk and Archer, 2010, see chapter I sect 2.3). Only volunteers homozygous for $PER3^{4/4}$ or $PER3^{5/5}$ were recruited.

The initial genetic selection criterion was not considered in the analyses presented so far because we were only able to recruit 7 $PER3^{5/5}$ individuals (faced to 15 $PER3^{5/5}$). This limited statistical power to observe significant difference between genotypes. **[Figure 11]** shows cortical excitability profile over our 29h protocol in each genotype separately. No acceptable statistical threshold for significance was reached between the two groups. Despite this apparent similarity, PER3 genotype may be turn out to influence cortical excitability. In such that way, the results presented could be biased in a way or another. In addition, carriers of the form $PER3^{4/5}$ (~45 percents among general population (Lázár et al., 2012)) were excluded; which could introduce another bias in our results we cannot ignore. This is also applicable to our primary ethnic selection criteria.

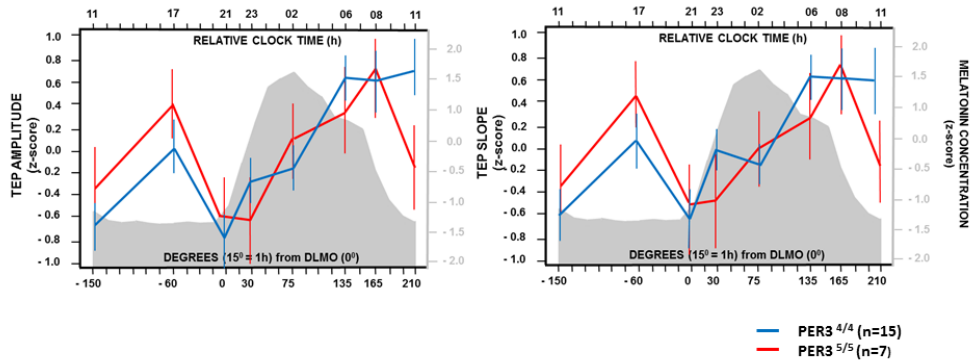

Figure 11 : Time course of TEP amplitude and slope in PER3 $^{4/4}$ (n=15; blue trace) **vs PER3 $^{5/5}$** (n=7; red trace) individuals. There is no statistically signicant difference between the two genotypes. Data were averaged after normalization (z-score) and realignment with individual circadian phase (n=22; melatonin secretion onset = 0°). Mean z-scored melatonin profile is displayed in grey. The top x axis indicates relative clock time for a participant with an 11 pm-7am sleep wake schedule.

4.9 Does aging impact on cortical excitability dynamics?

All our participants were young adults aged between 18 and 30 years (22 y.o. ± 2.61). Nonetheless, the weight of the interaction between sleep homeostasis and circadian processes is known to change with normal aging with typical slower increase in sleep need (Münch et al., 2004), weaker circadian signal strength (Münch et al., 2005) and circadian phase advancement (Duffy and Czeisler, 2002). This phenomenon is translated by shortening of sleep duration, earlier bed and wake times (Daneault et al., 2013) and diminished sensitivity to the negative impact of sleep deprivation on cognition (Duffy et al., 2009).

To determine the impact of normal aging on cortical excitability dynamics is the aim of a second study started in 2014 and which analyzes are still ongoing. 13 young (18-30 y.o.; 8 males) and 13 elderly healthy adults (55 -75 y.o.; 5 males) have completed the study. The protocol is quite similar to our first study presented in this work. It consists of a sleep deprivation protocol driven under constant routine conditions with TMS/EEG recordings scheduled with higher frequency around the WMZ and

SPZ. However we extended the duration of wakefulness from 29 to 35 hours adding a 9th TMS/EEG session in the afternoon (separated by 24h from the 2nd session) **[Figure 11A].**

Results presented here are preliminary and only include 22 participants (11 elderly) **[see appendix 3 for subjects characteristics]**. They must be taken very carefully since the analyzed participants sample is still incomplete and since the data have not been realigned to circadian phase because melatonin salivary measures are not yet available.

Preliminary results confirmed that cortical excitability temporal profile is non-linear **[Figure 11B-C]**. Indeed, TEP amplitude and slope tend to decrease in the first afternoon [$p_{corr\ S3\text{-}S2} < .1$]. They sharply increase in the biological night [$p_{corr\ S7\text{-}S3} < .037$] followed by partial recovering in the next morning [$p_{corr\ S7\text{-}S8} < .047$]. As expected, we observe a tendency of cortical excitability to further reincrease between the morning and the afternoon sessions both following one night of sleep deprivation [$p_{corr\ S8\text{-}S9}$ for amplitude =.052]. Those latter results were obtained by averaging TEP measures of all currently available participants without considering differences of age. They proved the robustness of our previous results by reproducing them on an independent population sample, which includes a larger scale of age, women and (at least in part) *PER3*$^{4/5}$ individuals since there were no genotype screening before recruitment.

Although differences between the two groups do not reach significance (yet), graphs tend to confirm our predictions. First, the temporal profile of cortical excitability appears flattened in the old group as compared to the young one. This is compatible with the well-reported weaker circadian signal strength and slower increase in sleep need observed with aging. Second, the night peak in cortical excitability seems to happen earlier in the older group. And third, cortical excitability levels tend to be

lower in older group at two moments: in the first morning (S1) and at the end of the putative SPZ (S7) [amplitude: p =.084; slope : p=.095] **[Figure 11D-E]**

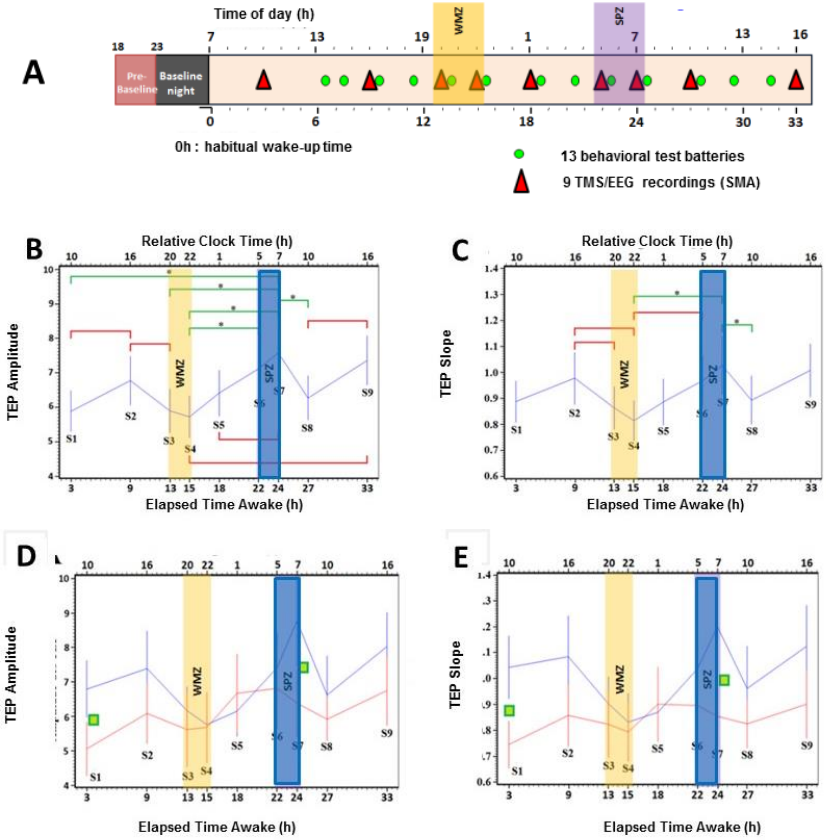

Figure 11: Impact of normal aging on dynamics of human cortical excitability

A) Study design (see main text)

B-C) Time course of TEP amplitude and slope averaged from all participants (n=22; 9 females) without considering differences of age. Green bars indicate significant post-hocs time intervals (p<.05). Red bars indicate post hocs that tend to significance (p<.1). See main text.

D-E) Time course of TEP amplitude and slope in young (blue trace, n=11; 3 females) **and elderly** (red trace, n=11; 6 females) groups. Green squares indicate sessions which tend to be significantly different (p<.1) between the two groups.

Data are presented here in absolute values. They have not yet been realigned to circadian phase (i.e. DLMO). Thus, the wake maintenance and sleep promoting zones (WMZ, orange rectangle; SPZ, blue rectangle) are only estimations based on usual sleep-wake schedule. *Figures and legend are adapted from the Master Thesis of (Delfosse, 2015) who acquired and analyzed the data*

5 PERSPECTIVES

Normal physiology does not equate to pathophysiology. However, it provides insights into some of the key mechanisms underpinning specific pathophysiological states.

In this thesis, we demonstrated that human cortical excitability varies during normal and under challenging physiological circumstances (i.e. sleep deprivation). We showed that these variations are intimately tied to the interplay between the hourglass sleep homeostasis process and the inner clock(s?) circadian timing system.

Appropriate excitability of cortical neurons is essential for proper brain function, including cognition. Not surprisingly, multiple neurological (and/or psychiatric) conditions show indeed important alterations in the fine-tuned regulation of cortical excitability.

Cortical excitability increases have been associated with chronic insomnia (Van Der Werf et al., 2010) and epilepsy (Kimiskidis et al., 2015). In contrast, decreases have been most obviously observed in stroke (Huynh et al., 2015) and disorders of consciousness (Rosanova et al., 2012b). Mixes of increases and decreases have been reported in neurodegeneration (Casarotto et al., 2011; Pennisi et al., 2011), depression (Bunse et al., 2014; Canali et al., 2014), possibly depending of the type and the stage of the disorder. Whether these abnormalities are sustained over the entire 24h sleep-wake cycle or are only transient is unclear. Likewise, whether the dynamics of cortical excitability over the circadian cycle is altered in those pathological conditions is also not known.

These uncertainties are problematic since circadian rhythmicity may play a role in clinical conditions. A circadian dysfunction is, for instance, very common in

Alzheimer disease and is deemed to contribute to cognitive impairment in those patients (Musiek et al., 2015). A time-of-day variation in occurrence of seizure is also well reported in certain forms of epilepsy (Mirzoev et al., 2012). More critically, depending on prior wake-history, there may be optimal times of day for neurorehabilitation approaches which attempt to restore normal cortical activity in neurological conditions either through cognitive intervention programs (Jean et al., 2010) or non-invasive neurostimulation (Di Pino et al., 2014; Wessel et al., 2015). A circadian influence on cortical excitability may in fact explain for instance why neurostimulation using TMS or transcranial electric stimulation (TES) fails to induce consistent improvement across clinical studies in Alzheimer's disease or stroke patients (Kadosh, 2013; Di Pino et al., 2014; Wessel et al., 2015). A full characterization of cortical excitability temporal profile in clinical populations is therefore warranted before TMS or TES neurorehabilitation can become an efficient therapeutic strategy.

As a whole, our study provides novel insights in the regulation of neuronal and synaptic function in healthy individuals and demonstrates that sleep-wake dependent cortical excitability dynamics is strongly influenced by the circadian machinery. This dynamics can arguably sustain the well-known changes in behavior associated with wakefulness extension and time-of-day. A full characterization of the temporal profile of cortical excitation holds promise for enhancement of human brain function in various healthy and clinical conditions (Kadosh, 2013; Luber and Lisanby, 2014).

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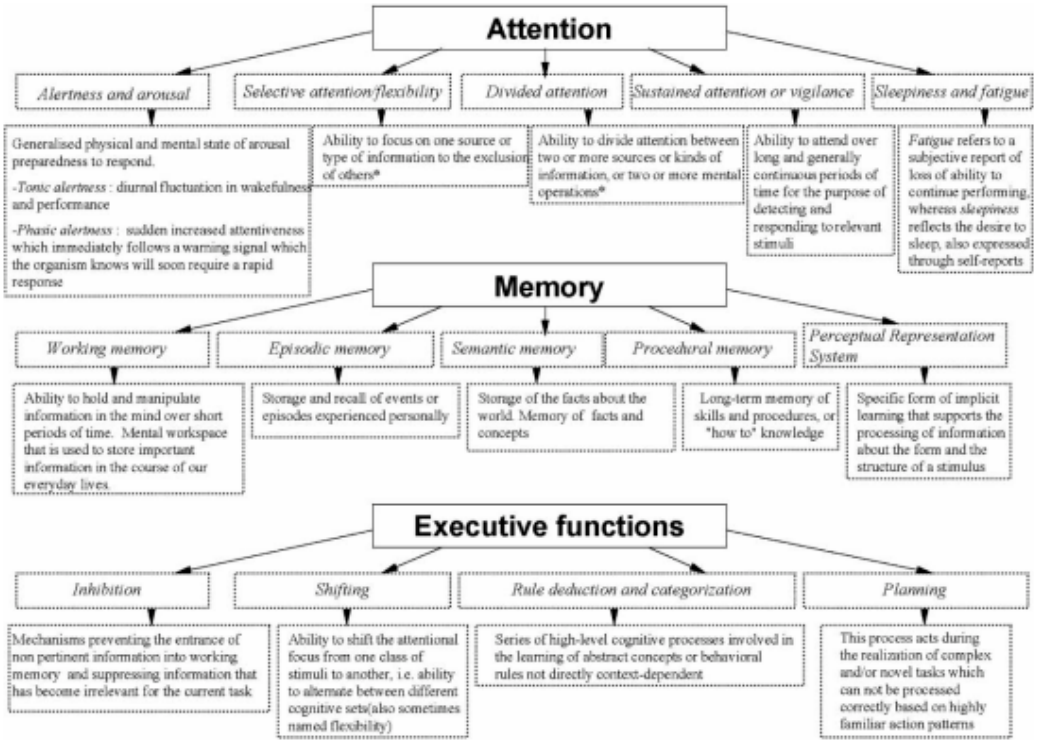
APPENDICES



Overview and global classification of main cognitive processes *from (Schmidt et al., 2007)*

EXPERIMENTAL PROCEDURES

1.1 Participants

The study was approved by the Ethics Committee of the Medicine Faculty of the University of Liège. Participants gave their written informed consent and received a financial compensation. Twenty-four healthy Caucasian men (18-30y), were enrolled. Women were excluded from the study as changes in ovarian hormones may influence cortical excitability in humans (Smith, 2002). Other exclusion criteria included: 1) BMI $\leq$ 18 and $\geq$ 25; 2) psychiatric history, severe trauma, sleep disorders; 3) addiction, chronic medication; 4) smokers, excessive alcohol (>14 doses/week) or caffeine (>3 cups/day) consumption; 5) night shift workers during the last year; 6) transmeridian travel during the last 2 months; 7) anxiety or depression; 8) poor sleep quality; 9) excessive self-reported daytime sleepiness. One participant was excluded due to a melatonin phase-delay >6h compared to the remainder of the sample, and one due to low EEG recording quality. Thus, data presented here include 22 participants. **Table 1** summarizes the demographic characteristics of the final study sample. Volunteers were recruited based on a polymorphism in *PERIOD3* (*PER3* variable number of tandem repeat, with 4 or 5 repeats) (Dijk and Archer, 2010), but genotype was ignored in the analysis given the limited sample size of $PER3^{5/5}$ genotype (7 $PER3^{5/5}$ for 15 $PER3^{4/4}$)

1.2 Experimental protocol

Participants first completed a "pretest" TMS/EEG session to determine the optimal TMS parameters providing artefact-free EEG recordings. The left or right supplementary motor area (SMA) was set as stimulation target for right or left-handed, respectively. This brain area was chosen for the following reasons: 1) similar to the entire frontal lobe, the SMA is exquisitely sensitive to sleep pressure, including

at the neuronal level, as indicated in a previous EEG-TMS experiment (Huber, 2013); 2) it plays a key role in cognitive performance, and is heavily connected to the prefrontal cortex (Bonini, 2014); 3) its stimulation does not trigger muscle activation, sources of EEG signal contamination.

The second step consisted of a laboratory polysomnography habituation night to exclude potential sleep disorders. During the 7 days preceding the study, volunteers kept a regular sleep-wake schedule of 8h sleep duration (+/- 30min). Compliance was verified using wrist actigraphy (Actiwatch, Cambridge Neuroscience, UK) and sleep diaries (Table 1). Participants were requested to abstain from all caffeine and alcohol-containing beverages and from intense physical activity for 3 days preceding the study.

For the experiment *per se*, participants arrived at the laboratory ~6h prior to their habitual sleep time. They were maintained in dim-light from there on (5<lux, except for sleep episode in complete darkness) and trained twice on the behavioral test battery. They then slept for an 8h sleep baseline episode starting at their habitual bedtimes (Table 2). The TMS-compatible electrode cap was placed upon awaking prior to the 29h of sustained wakefulness period (sleep deprivation) under constant routine conditions (i.e. light ca. 5 lux, temperature ca. 19°C, regular isocaloric liquid meals and water, semi-recumbent position and no time-of-day information, sound proofed rooms). These conditions aim to minimize external and internal factors masking circadian rhythmicity (Duffy and Dijk, 2002)**.**

Spontaneous quiet waking EEG and TMS-evoked EEG potentials (TEP) were recorded 8 times during sleep deprivation to cover the entire near-24h circadian cycle, with higher session frequency around the circadian wake maintenance and sleep promoting zones (11AM, 5PM, 9PM, 11PM, 2AM, 6AM, 8AM, 11AM, for a subject sleeping from 11PM to 7AM; **Figure 1**). Behavioral test batteries were carried out 12

times during the sleep deprivation period in between EEG sessions (12AM, 2PM, 4PM, 6PM, 8PM, 10PM, 12PM, 3AM, 5AM, 7AM, 9AM, 12AM). Subjective sleepiness and affect dimensions were assessed hourly by the Karolinska Sleepiness Scale (KSS; Akerstedt and Gillberg, 1990) and a visual analogical scale (VAS; Monk, 1989)respectively. Saliva samples for melatonin and cortisol assays were also collected hourly.

1.3 Data acquisition

1.3.1 TMS-evoked EEG responses

TMS delivery

TMS pulses were generated by a Focal Bipulse 8-Coil (Eximia; Nexstim, Helsinki, Finland; hotspot surface ~0.68 cm^2). Stimulation target (SMA) was located on individual structural MRI by means of a neuronavigation system (Navigated Brain Stimulation; Nexstim). This device allows for reproducible evoked EEG responses (Casarotto, 2010) and precise target location (FDA approval for presurgery). Distance to target threshold for TMS delivery was set at 2 mm. Each session included between 250 and 300 trials. Interstimulus interval was randomly jittered between 1900 and 2200 msec. Coil recharge was set at 900msec post-TMS. Total number of stimulations of the 8 EEG/TMS sessions was well below safety recommendations (Rossi, 2009).

EEG recording

TMS responses were recorded with a 60-channel TMS-compatible EEG amplifier (Eximia; Nexstim), equipped with a proprietary sample-and-hold circuit equipment guaranteeing TMS artifact free data 8 msec post TMS (Virtanen, 1999). Electrooculogram was recorded with 2 additional bipolar electrodes. Participants wore the EEG cap during the entire constant routine protocol and electrodes

impedance was kept below 5 kΩ. Signal was band-pass-filtered between 0.1 and 500 Hz and sampled at 1450 Hz. Each EEG/TMS session ended with a neuronavigated digitalization of the location of each electrode.

Masking noise

Auditory EEG potentials (AEP) evoked by the TMS and bone conductance were minimized by diffusing a continuous loud white masking noise through earplugs and applying a thin foam layer between the EEG cap and the TMS coil, respectively (Rosanova, 2012). Each session was followed by a "sham" session consisting in 30 to 40 TMS pulses delivered parallel to the scalp while white noise was diffused with the same level. Absence of AEP was checked online on Cz between 0-300 msec post TMS.

1.3.2 Waking EEG

Spontaneous quiet waking EEG was recorded prior to each TMS session using the same 60-channel TMS-compatible EEG (+ 2 EOG) amplifier (Eximia; Nexstim). Volunteers were instructed to fix a black dot during two minutes while relaxing and suppressing blinking.

1.3.3 Sleep EEG

Sleep EEG data were recorded using a V-Amp 16 amplifier (Brain Products GmbH, Gilching, Germany) according to 10/20 system). The habituation night montage consisted of a full PSG with 5 EEG channels (Fz, Cz, Pz, Oz, C3) referenced to left mastoid (A1), 2 bipolar electrooculogram (EOG), 2 bipolar electrocardiogram (ECG) channels, 2 bipolar electrodes placed on a leg to check for periodic movements and an oximeter for sleep related breathing disorder detection. Baseline night montage consisted of 11 EEG channels (F3, Fz, F4, C3, Cz, C4, P3, Pz, P4, O1, O2) referenced to

left and right mastoids (A1, A2), 2 bipolar EOG and 2 bipolar electromyogram (EMG) channels. EEG data were digitized at a sampling rate of 500 Hz.

1.3.4 Behavioral tests

TMS vigilance Task

While recording TMS-evoked EEG responses, volunteers performed a visual task (CTT) to monitor their vigilance level. The task consisted of keeping a constantly randomly moving cursor on a target located in the center of a computer screen, using a trackball device. Performance to the task was computed as the average distance, in pixels, between the cursor and the target during EEG/TMS recording (normalized according to the duration of the session). Transitory lapses of vigilance resulted in temporary increases of the target—cursor distance (Makeig, 2000) which could be automatically detected offline. A lapse was defined as a time when the cursor was located outside of a central 200 by 200 pixel box surrounding target following > 500msec from the last trackball movement. The lapse period included the period between the last trackball movement and the lapse detection. TMS evoked responses occurring during and < 1 sec from a lapse were discarded from the analyses.

Psychomotor Vigilance Task (PVT)

Volunteers were required to press a computer space bar as soon as an auditory signal, presented at a random interval of 3-7 seconds, occurred. The PVT lasted 5 minutes. Session performance was inferred from the median reaction time following lapses (> 500 msec), anticipation (< 100 msec) and error (>3000msec) removal (Dinges and Powell, 1985).

Karolinska Sleepiness Scale (KSS)

Participants were instructed to determine their subjective alertness level using a 9 ranks Likert scale: 1 and 2 correspond to “very awake”, 3 and 4 to “awake”, 5 to “neither awake nor tired” 6 and 7 to “tired”, 8 and 9 to “very tired” (Akerstedt and Gillberg, 1990).

Visual Analogic Scale (VAS)

Volunteers were asked to self-rate their affect using a visual analogic scale on different topics (mood, motivation, anxiety, stress, social contact) (Monk, 1989b).

1.3.5 Melatonin and Cortisol

Saliva samples were first placed at 4°C, prior centrifugation and congelation at -20°C within 12h. Salivary melatonin and cortisol were measured by radioimmunoassay (Stockgrand Ltd, Guildford, UK), as previously described (English, 1993). Of a total of 624 samples, 546 were analyzed in duplicate for melatonin concentration. The limit of detection of the assay for melatonin was 0.8 ± 0.2 pg/ml using 500 µL volumes. Of a total of 631 samples, 631 were analyzed in duplicate for cortisol concentration. The limit of detection of the assay for cortisol was 0.37 ± 0.05 nmol/L using 500 µL volumes (Read, 1977).

1.4 Data analysis

1.4.1 TMS/EEG data analysis

Data pre-processing

Data pre-processing was computed using Statistical Parametric Mapping 12 (SMP12, http://www.fil.ion.ucl.ac.uk/spm/) implemented in Matlab 2011a (The Mathworks

Inc, Natick, MA). Continuous EEG recordings were successively re-referenced to the average of all good channels, low-pass filtered at 80 Hz, downsampled from 1450 to 1000 Hz, and high-pass filtered at 1 Hz, split into epochs between –101 and 300 msec around TMS pulses, and baseline corrected -101 to -1.5 msec pre-TMS periods. Robust averaging was applied to compute the mean evoked response of each session (Leonowicz, 2005; Wager, 2005).

Cortical excitability

Cortical excitability was inferred from the amplitude and slope of the first EEG component (0-25 msec) of the TMS evoked potential (TEP) measured at the artifact free electrode closest from the hotspot (i.e. brain location with highest TMS-induced electrical field estimated by the neuronavigation system). The latter electrode was always located in the stimulated brain hemisphere. It could vary across subjects but remained constant at the individual level.

Source reconstruction

TEP amplitude and slope were also extracted from a reconstructed signal at the hotspot using localization of equivalent current dipole.

1.4.2 Waking EEG

Data were analyzed with MATLAB® (2011a, The Mathworks Inc, Natick, MA). Data preprocessing was performed using Statistical Parametric Mapping 12 (SPM12,http://www.fil.ion.ucl.ac.uk/spm/). Artefacted channels were rejected after visual inspection. Continuous EEG recordings were downsampled from 1450 to 50 Hz. Data were then manually and visually scored offline for artefacts (eye blinks, body movements, and slow eye movements). Power spectral densities were computed using a fast Fourier transform on artifact-free 4-s, overlapping by 2 second, using the Welch's method (pwelch function in MATLAB 7.5.0). EEG activity

was computed over frontal region (FP1, FPz, FP2, AF1, AFz, AF2, F7, F3, F1, Fz, F2, F4, F8) for delta (0.75-4Hz), theta (4.5-7Hz), alpha (8-12Hz), sigma (12-15Hz) and beta (16-25Hz) frequency bands over the entire 2-min recording.

1.4.3 Sleep EEG

Sleep EEG recordings were re-referenced to the average of both mastoids and bandpass filtered between 0.5 and 25 Hz. Data were visually inspected for artefact and manually scored for sleep stages on a 30-s epoch basis using FASST (an SPM compatible toolbox (Phillips, 2011)), according to AASM criteria (Iber, 2007). One baseline night was excluded from analyses because of poor quality of the recording (n=21). To avoid bias in sleep scoring, EEG data were scored by an automated sleep stage detection algorithm (Berthomier et al., 2007). NREM-REM sleep cycles were determined according to Feinberg and Floyd (Feinberg and Floyd, 1979). For one subject, an extremely long first NREM cycle was divided into two separate NREM cycles (Feinberg and Campbell, 2003) . Power spectra were computed using a fast Fourier transform on successive 4-s epochs, overlapping by 2 s and weighted by a Hanning window.

1.4.4 Statistics

All statistical analyses were performed with SAS version 9.3 (SAS Institute, Cary, NC, USA). For TMS evoked potential (TEP) amplitude and slope, cortisol and melatonin levels, KSS and PVT measures, normalization was provided by a z-score at individual level. TMS vigilance task was normalized by dividing performance to the duration of task and then z-scored. Frontal waking theta activity was normalized by dividing theta power by the sum of frequencies within 1 and 20 Hz over the same region. The time-course of cortical excitability (i.e. TEP amplitude and slope) was examined with mixed-model analyses of variance for repeated measures (PROC Mixed), with within-

subject factor "time". Contrasts were assessed with LSMEANS statement. All *p*-values were based on Kenward-Roger's corrected degrees of freedom. All data were realigned, at the individual level, to dim light melatonin onset (DLMO). Estimation of circadian phase (where 0° = individual DLMO) was determined using a nonlinear function: Var= Mesor + Amplitude * sin ((sample*ti-time)/24.2), where mesor, amplitude, and phase are free parameters, ti represents clock time at which a sample is collected (Dijk, 2012). Afterwards, phase of melatonin rhythm was based on the timing of the 20% of the fitted melatonin maxima (DLMO). Analysis of covariance (ANCOVA) were performed to estimate how TEP amplitude and slope were associated to theta EEG activity, subjective sleepiness and effects, cortisol level, and TMS vigilance task behavioral responses. To investigate the influence of sleep homeostasis and circadian rhythmicity on cortical excitability, TEP amplitude and slope were fitted with, respectively, linear and sine-wave functions:

Linear function: Var= (C+L*time), where C corresponds to initial constant and L is the linear increment across time. (Dijk, 2012)

Sine-wave function: Var= Mesor + Amplitude * sin ((sample*ti-time)/24.2), where mesor, amplitude, and time are free parameters, ti represents clock time i at which a sample is collected (Viola, 2012b).

Estimated fitted cortisol secretion profile was obtained using this same sine-wave function. The amplitude of cortisol estimated secretion, as a proxy of the circadian signal strength, was derived from the difference between the maximal and minimal cortisol predicted values.

An exponential decay function (PROC NLIN, SAS 9.3) was fitted to sleep delta data power (0.75– 4 Hz) of the first four sleep cycles (Mongrain, 2006) and derived from the frontal derivations, known to be more sensitive to sleep deprivation: SWA(t) =SWA0*exp(-r*epi) (Cajochen, 1999b; Mongrain, 2006). The amount of initial slow wave activity (SWA0) and its dissipation rate (r) were derived.

Regression (PROC REG) were also performed between individual estimated cortisol amplitude and the TEP amplitude and slope decrease from session 2 to session 3 [two participants were excluded from this latter analysis because one showed a cortisol amplitude more than 4 standard deviations inferior from sample mean and another because the TMS responses of session 2 were of poor quality]; 2) between individual estimated slow wave activity dissipation rate (r) and the TEP amplitude and slope increase from the first to the last session [four participants were excluded from this latter analysis because 2 showed dissipation more than 3 standard deviations superior to the sample mean and 2 have a TMS response during first or last session of poor quality].

	YOUNG			ELDERLY		
	Mean		SD	Mean		SD
Body Mass Index (BMI)	22,52	±	2,99	24,27	±	1,75
Anxiety (BDI – II)	3,45	±	3,17	4,36	±	5,05
Mood (BECK)	3,27	±	4,55	2,82	±	2,52
Daytime propensity to fall asleep (Epworth)	4,09	±	3,42	6,18	±	4,30
Chronotype (HO)	53,91	±	8,12	57,89	±	23,34
Sleep quality (PSQI)	2,64	±	1,55	4,27	±	2,45
Seasonality (SPAQ)	0,73	±	0,62	0,78	±	0,88
Cafeine (cup/day)	2,45	±	2,23	2,18	±	1,47
Alcohol consumption (unit/week)	3,18	±	3,86	5,73	±	5,41
Female	3/11			6/11		
Right handed	9/11			11/11		

Table 1 : Sample demographics (Mean ± Standard deviation), and sleep-wake timing during 7 days preceding the laboratory experiment based on sleep diary and actigraphy data (Median ± Standard deviation).

ANXIETY LEVEL was measured on the 21 item Beck Anxiety Inventory (BAI≤14) (Beck , 1988); CHRONOTYPE was assessed by the Horne-Ösberg Questionnaire (Horne and Ostberg, 1976); Daytime propensity to fall asleep in non-stimulating situations was assessed by the Epworth Sleepiness Scale (ESS≤11) (Johns, 1991); MOOD was assessed using the 21-item Beck Depression Inventory II (BDI-II≤14) (Steer et al., 1997); SLEEP QUALITY was determined by the Pittsburgh Sleep Quality Index Questionnaire (PSQI≤7) (Buysse et al., 1989). SEASONALITY is based on the Seasonal Pattern Assessment Questionnaire (SPAQ<11) (Rosenthal N, Bradt G, 1984). The Edinburgh Inventory (Oldfield, 1971) was administered to verify that the participants were right-handed. Sleep parameters are presented in hours.