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CICLO XX

High density EEG studies of local sleep changes following synaptic plasticity in humans

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List of Abbreviations

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid

ANOVA: analysis of variance

APB: abductor pollicis brevis

BDNF: brain-derived neurotrophic factor

CamKIV: calcium/calmodulin-dependent protein kinase type IV

CDR: current density reconstruction

CPP: [(6)-3-(2-carboxypiperazin-4-yl)-propyl-Lphosphonic acid]

EEG: electroencephalogram

EPSP: excitatory post synaptic potential

ERP: event related potential

FFT: fast Fourier transform

fMRI: functional magnetic resonance imaging

GABA: gamma-aminobutyric acid

GMFP: global mean field power

ISI: inter stimulus interval

LTD: long term depression

LTP: long term potentiation

MEP: motor evoked potential

MNI: Montreal neurological institute

MR: magnetic resonance

MRI: magnetic resonance imaging

NA: noradrenaline

NBS: navigated brain stimulation

NMDA: N-methyl-D-aspartate

PAS: paired associative stimulation

PET: positron emission tomography

REM: rapid eye movements

RMT: resting motor threshold

mRNA: messenger ribonucleic acid

rTMS: repetitive transcranial magnetic stimulation

s.e.m.: standard error mean

SEP: somatosensory evoked potential

sLORETA: standardized low resolution brain electromagnetic tomography

SnPM: statistical non parametric mapping

SNR: signal to noise ratio

SWA: slow wave activity

TEP: transcranial magnetic stimulation evoked potential

TMS: transcranial magnetic stimulation

TrkB: neurotrophic tyrosine kinase receptor type 2

Abstract

A general definition of sleep can be stated as a reversible behavioral state of perceptual disengagement from the environment that has been observed across all animal species studied so far. The behavior of sleep as well as the need for sleep are relatively constant throughout all human lifespan, and approximately one third of our life is spent in this behavioral state.

A glance at PubMed search engine for "sleep" reveals over 85000 published papers, giving a feeling of the growing body of literature for sleep research over the last fifty years. Many of those papers focused on the characterization of its behavioral, physiological and pathological aspects and its relationship with the waking behavior.

Despite the large body of research devoted to it, a clear definition of its role is still lacking. Many theories regarding its function have been proposed ranging from the physiological to the psychological (Horne, 1985; Rechtschaffen, 1998).

One idea in particular that has generated great interest and controversy is the theory that sleep facilitates learning and, by extension, synaptic plasticity (for a review see Frank, 2006).

Different approaches to the study of learning and its neural substrates led up to the development of different, though not mutually exclusive, hypotheses regarding the role that sleep plays in the learning process.

Early theories proposed a facilitating role of sleep behavior for consolidation of memories via the simple prevention of possible interference occurring after learning of new information (Jenkins and Dallenbach, 1924). Other approaches developed more recently assigned a more active role to sleep in the learning process.

Among the latter, the Synaptic Homeostasis Hypothesis proposed by Tononi and Cirelli (Tononi and Cirelli, 2003) accounts for daytime learning as a plastic process following long term potentiation (LTP) rules, thus involving neuronal changes at synapses, and sleep as a regulatory process for these neuronal changes. During the day the synapses in most cortical circuits are strengthened, resulting in a net increase in synaptic weight. Sleep is the process needed to downscale the total synaptic weight to a baseline every night. By doing this sleep brings about synaptic homeostasis preserving relative differences in synaptic strength between those synapses that have been potentiated and those that have not.

Methodologically, the development of new non-invasive research tools such as transcranial magnetic stimulation (TMS) and high density EEG (hd-EEG), allowed for a new wave of investigation into the neurophysiological mechanisms underpinning sleep and brain plasticity, and together with functional imaging they represent state of the art techniques for human neuroscience.

The present work will focus on the Synaptic Homeostasis Hypothesis, presenting it as a good candidate for the characterization of sleep function.

By means of quantitative analysis of EEG data we tested several specific predictions made by the hypothesis in order to provide support for the idea that local plastic changes in the brain lead to local changes in subsequent sleep.

A brief introduction on the basic mechanisms of synaptic plasticity followed by an exposition of the Synaptic Homeostasis Hypothesis will help to better understand its key features. A description of two experiments carried out during the period of my PhD program spent at UW-Madison together with a description of the methods adopted will provide support to this hypothesis.

Finally, a general discussion on the presented data will show the strengths as well as the limitations of this perspective and will suggest directions for future research on this topic.

SECTION 1:

INTRODUCTION

Synaptic Plasticity: a key feature of the Central Nervous System

Throughout the entire life spectrum, brain shows a striking ability to react in a proactive way to new environmental requests.

The adaptive process of learning that everybody experience everyday, is indeed based on modifications of the neural structure in order to create and store new memories.

These modifications concern the way neurons are interconnected through chemical synaptic transmission, a process known as synaptic plasticity.

A well characterized form of synaptic plasticity that fulfills many of the criteria for a neural correlate of memory and learning is the so called long-term potentiation (LTP) (Cooke and Bliss, 2006).

LTP results from coincident activity of pre- and post-synaptic elements, leading to a facilitation of chemical transmission lasting for hours or more (Bliss and Gardner-Medwin, 1973; Abraham et al., 2002).

Originally discovered by Bliss and Lomo (Bliss and Lomo, 1973) in the hippocampus of the anaesthetized rabbit, it has been studied in a variety of species and at a number of different synapses throughout the central nervous system, from the cerebral neocortex (Fox, 2002) to the spinal chord (Ji et al., 2003).

LTP is called associative, or 'Hebbian', if it occurs at an input to a postsynaptic cell conditional on (i) concomitant and synchronous activation of another input to the same cell, or (ii) concomitant and synchronous postsynaptic depolarization (Buonomano and Merzenich, 1998). This form of LTP has attracted attention because it provides a model of

how converging inputs from various sources (intracortical fibers, corticocortical and thalamocortical afferents), could interact to reshape local cortical patterns.

From the beginning, high-frequency trains of stimuli (tetani) delivered to Schaffer collateral/commissural fibers projecting from CA3 to CA1 pyramidal neurons have been used, ensuring sufficient synaptic input to induce action potentials post-synaptically.

Later experiments used intracellular techniques in order to depolarize post-synaptic cell paired to a simultaneous afferent stimulation, providing evidence that coincidence between pre- and post-synaptic activity is essential for LTP induction (Gustafsson et al., 1987; Markram et al., 1997; Bi and Poo, 1998).

Molecular Mechanism

Associative LTP is based on a property relying upon a mechanism detecting coincident pre- and post-synaptic activity.

N-methyl-D-aspartate (NMDA) sub class of glutamate receptor performs this function at most glutamatergic synapses of the central nervous system.

The mechanism works as follows:

Under condition of rest or low levels of input activity, the channel of the NMDA receptor, positioned on the glutamatergic postsynaptic membrane, is blocked by positive charged magnesium ions (Mg^{2+}) (Fig.1 A). Glutamate molecules released from the pre-synaptic terminal diffuse across the synaptic cleft and bind to both NMDA and α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptors, opening the latter type. The resulting inward current flow carried by Na^{+} ions depolarizes the post-synaptic membrane to produce an excitatory post-synaptic potential (EPSP). The resulting membrane depolarization is still not enough to relieve the Mg^{2+} -block of the NMDA receptor channel

(Fig.1 B). Only high concentrations of released glutamate at a strong active synapse produce strong depolarization of the post-synaptic membrane so to relieve the Mg^{2+} -block form the NMDA receptor channels and allowing influx of Na^{+} and Ca^{2+} ions (Fig.1 C). Calcium intake is thought to initiate the LTP induction (Lynch et al., 1983; Malenka et al., 1988).

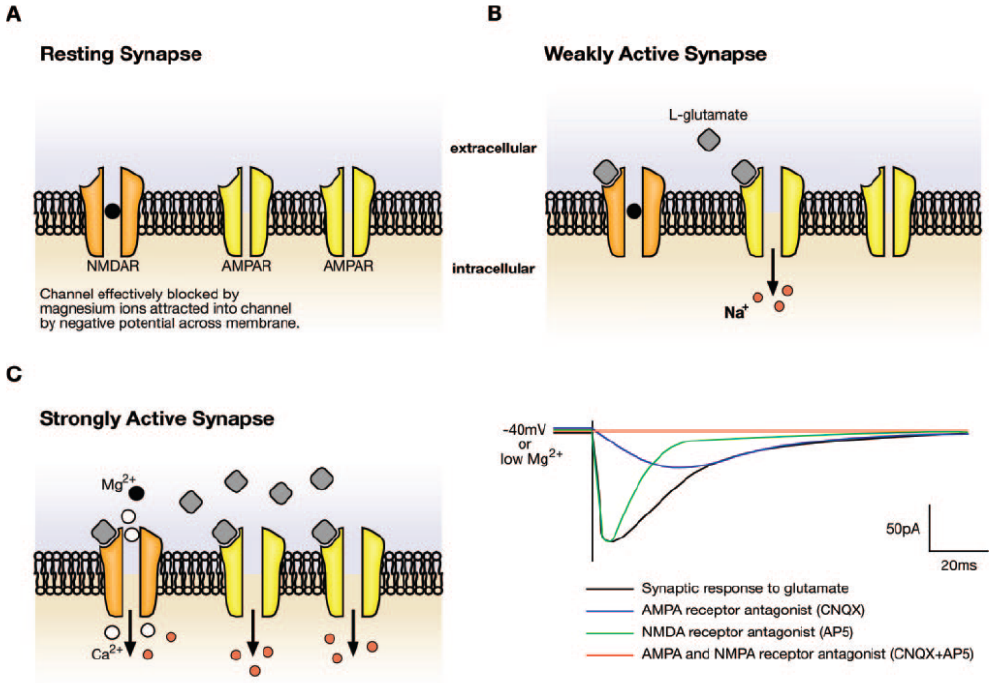

Fig. 1 NMDA receptor as a coincidence detector (from Cooke and Bliss, 2006).

Despite the above described detailed chain of events well known to occur at most glutamatergic synapses, it seems that the important properties of LTP, longevity, input specificity and associativity, can be implemented by a variety of receptors and signaling systems (Cooke and Bliss, 2006).

Low concentrations of post-synaptic calcium, conversely, seem to be responsible for the opposite process to LTP, long term depression (LTD).

This process results in a long-lasting decrease in synaptic efficacy. The standard protocol for inducing LTD uses long trains of low-frequency (1 Hz) stimulation (Dudek and Bear, 1992), or mismatching of pre- and post-synaptic action potentials (Markram et al., 1997). LTD may serve as a homeostatic mechanism to ensure that central nervous system synapses are not saturated by learning (enhancing signal-to-noise ratio, renormalizing synaptic weights after LTP has occurred) (Massey and Bashir, 2007). Alternatively, LTD may mediate learning in itself, forgetting or behavioral extinction.

LTP in humans

Although most of the research on LTP/LTD as been carried out based on animal models, recently, technical advances have presented the possibility of investigating these processes delivering tetanic stimulation to awake human subjects in different regions of cortex.

Auditory and photic stimulaton:

Recently, new methods have been used to induce long-lasting changes in neuronal excitability in human subjects, both in the auditory (Clapp et al., 2005a) and visual cortices (Clapp et al., 2005b; Teyler et al., 2005). By means of scalp electrodes they used ERPs recorded in either area to monitor responses to auditory or visual stimuli respectively. In these experiments, long-lasting enhancement of the amplitude of a component of either auditory-evoked or visual-evoked responses is achieved using a 13 Hz auditory tetanus, consisting in a sequence of tone pips, or a photic tetanus generated on a computer screen, which consists in a series of chequerboard stimuli delivered at a frequency of 9 Hz. Either of these tetani is sufficient to increase the amplitude of a component of ERPs in the respective area of cortex for at least 50 min afterwards.

Moreover, in the latter case, delivery of lower-frequency visual stimuli (1 Hz) reduces the amplitude back to baseline levels, suggesting a depotentiation-like process. The authors argue that the selective alteration of a single component of the ERP, which consists of electrical fields generated by a large number of neurons, constitutes a form of synaptic plasticity. The finding that a sensory tetanus alone can be used to induce long-lasting effects on neuronal responses in cerebral cortex, although fascinating, cannot be validated without more refined analysis, which, with the limits of current technology, is not yet possible.

Nevertheless, those findings complement animal studies in which LTP is induced at synapses made by fibres from projection neurons in the lateral geniculate nucleus on layer IV cells in the visual cortex pathway of rats (Heynen and Bear, 2001). Here LTP is induced by tetanic electrical stimulation, but subsequent to the tetanus, responses in primary visual cortex evoked by visual stimuli, such as light flashes and patterned gratings, are enhanced. Heynen and Bear demonstrate that the potentiation is NMDA-receptor-dependent, using the NMDA receptor antagonist CPP [(6)-3-(2-carboxypiperazin-4-yl)-propyl-Lphosphonic acid] and indicate that the site of plasticity is synaptic, as revealed using current source density analysis.

Transcranial Magnetic Stimulation (TMS) and repetitive TMS (rTMS):

TMS stimulation of motor cortex in humans using frequencies of 1–20 Hz produces effects on motor-evoked potentials variable from individual to individual. Generally, 1 Hz stimulation reduces neural activity and anything over 5 Hz increases activity and motor output. In both cases the effects of such stimulation appear to be transient, lasting around half an hour at most (Hallett, 2000). Interestingly, application of this low-frequency TMS to area M1 in the motor cortex can be used to block consolidation of motor skill acquisition

in normal human subjects without interfering with motor performance itself (Muellbacher et al., 2002). Although changes in evoked potentials persist after the higher frequency (5 Hz) trains of stimuli, the effect is not consistent and never lasts long enough to be comparable with LTP (Maeda et al., 2000). LTP is often induced in animals using repeated trains of high-frequency stimulation spaced at a frequency that mimics a spontaneous 5-7 Hz neural rhythm, the theta wave. Tetani of this sort via TMS can induce long-lasting changes in motor cortical output (Huang et al., 2005).

Frequency of stimulation never exceeds 50 Hz in this sort of experiment, and the stimulation intensity must be set well below motor threshold during the tetanus. Nevertheless, the amplitude of motor evoked potentials in the hand as a result of super-threshold baseline stimulation can increase by ~50% for at least 20 min after application of several theta burst-like tetani spaced 10 s apart. This finding strongly suggests that remote stimulation can be used to induce a long-lasting change in motor cortical output. It has yet to be demonstrated, however, that the site of such change is the synapse.

Recently, two experiment provided a direct demonstration for a potentiation effect of rTMS stimulation at 5 Hz on cortical responsiveness (Esser et al., 2006; Huber et al., 2007), a result that encourages using this technique in order to assess synaptic plasticity in humans.

Paired associative stimulation (PAS):

An alternative way of inducing LTP that does not require the application of high-frequency tetani, is to pair pre- and post-synaptic action potentials (Wigstrom et al., 1986; Markram et al., 1997; Bi and Poo, 1998). Pairing of this sort can potentially be modeled in humans by combining low-frequency TMS to the cortex whilst simultaneously stimulating a peripheral nerve, an approach known as paired associative stimulation (PAS). For example,

peripheral stimulation of the right median nerve can be followed by TMS directed at the hand representation area in contralateral primary motor cortex (M1), at a latency determined by the time-lag in evoking an M1 cortical potential via activation of somatosensory cortex (Stefan et al., 2000). Motor evoked potentials (MEPs) can again be used as an index of the resultant increase in motor cortical output, here in the abductor pollicis brevis muscle in the thumb.

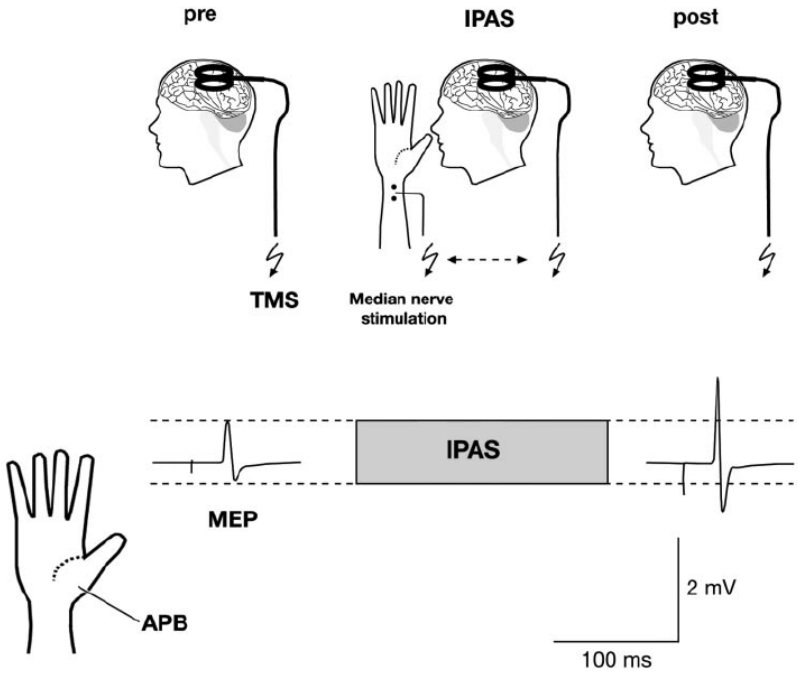

Fig. 2 Paired associative stimulation (from Cooke and Bliss, 2006).

Strength of this method is that it is more physiologically realistic and enables the testing of one of the key requirements for LTP: coincident pre- and post-synaptic activity.

While coincident pre- and post-synaptic stimulation in the cortex, using peripheral stimulation preceding TMS stimulation, results in an increase in cortical excitability lasting for at least an hour (Stefan et al., 2000), mis-timing of peripheral and TMS stimulation, by shortening the interval between the two, results in a depression of cortical excitability lasting for an hour and a half (Wolters et al., 2003). Both of these effects can be blocked by

NMDA receptor antagonists, a finding consistent with the involvement of LTP/LTD-like processes. Moreover, the plasticity is specific for those cortex patches receiving stimulation due to both peripheral stimulation and direct TMS, as demonstrated by the fact that there is no potentiation of motor-evoked responses in muscles controlled by neighboring regions of motor cortex, such as the biceps brachii, which receive TMS stimulation but not peripherally induced stimulation. Only those cells receiving paired input show the potentiation effect.

Another issue addressed by means of this technique is homeostatic regulation of synaptic plasticity.

Recent experiments reveal that motor learning prior to PAS stimulation can prevent induction of the LTP-like plasticity in motor cortex for a period of 6 h (Stefan et al., 2006). Again, this finding suggests that the early motor learning may have saturated plasticity, thereby occluding further potentiation. At the same time, the induction of LTD-like plasticity during this same period is facilitated (Ziemann et al., 2004), consistent with the idea that motor learning increases output from M1 through an LTP-like process, thereby allowing a greater extent for a reduction in the motor output, through depotentiation.

Furthermore, Muller and colleagues (Muller et al., 2007) used two consecutive sessions of PAS in order to clarify the issue about homeostatic regulation of synaptic plasticity in human motor cortex.

The first of those session could vary between a LTP inducing condition (interval between peripheral stimulation and TMS pulse: individual N20 somatosensory evoked potential + 2ms), a LTD inducing condition (interval N20 - 5ms), or a control condition (random alternation of the two intervals). The second session, 30 minutes later, was always LTP inducing.

The MEP of the abductor pollicis brevis following the second PAS session showed a LTP effect when conditioned by the control condition which increased if conditioned by the LTD inducing condition, but decreased if conditioned by the LTP inducing condition.

Based on the aforementioned studies, but avoiding possible confounding factors due to the interaction between two different experimental manipulations, they could provide evidence to the fact that a homosynaptic mechanism underpins homeostatic regulation of synaptic plasticity.

Summary

As it has been shown, different means of stimulation are capable to induce a long-lasting increase in responsiveness over several cortical areas in humans. As yet, however, investigators have not been able to establish the exact nature of the underlying neural plasticity. Among others, changes in synaptic efficacy or in the threshold for action potential generation in the excitatory output cortical cells are included.

It is important to stress that the only direct evidence for synaptic plasticity in human cortex comes from experiments on excised tissue like inferior and middle temporal lobe (Chen et al., 1996) and dentate gyrus (Beck et al., 2000).

In order to disentangle the nature of those changes it will be necessary to conduct experiments able to clarify the synaptic nature of the cortical response modulation, however all the studies described above using remote means to induce changes in the neuronal excitability and functional output, showed results consistent with the induction of LTP and LTD at synapses.

The aforementioned procedures, therefore are to be seen as good candidates for further non-invasive investigation of LTP/LTD in humans.

The Synaptic Homeostasis Hypothesis

Aim of this section will be to expose the general concepts of the Synaptic Homeostasis Hypothesis, together with some evidence from literature. First, a brief introduction will present the theoretical framework which it arises from.

Sleep Function: reviewing different theories

The debate on the role of sleep is one of the most peculiar situations in the sciences nowadays. Sleep scientists, for all their advances in different directions, remain in the awkward position of not knowing why we sleep (Frank, 2006).

The large body of theories - and evidences in behalf of one or another - developed in the last fifty years can be summarized as follows:

- Somatic theories of sleep: sleep facilitates anabolic processes or restores some bodily function worn down by wakefulness.
- Neural theories of sleep: sleep is primarily "for the brain". Among these we can include:
    - Neuro-metabolic theories: sleep detoxifies substances that accumulate during wakefulness, or repairs neural substrates degraded by wakefulness.
    - Neuro-cognitive theories: sleep serves higher-order functions such as neural development or memory, presumably by promoting synaptic plasticity.

Somatic theories of sleep function:

As reviewed by Akerstedt and Nilsson (Akerstedt and Nilsson, 2003), mortality rates in short and long sleepers indicate a link between sleep and physical well being.

People who sleep much more or much less than the average show higher mortality rates and greater incidences of myocardial infarction and type 2 diabetes mellitus (Akerstedt and Nilsson, 2003). Furthermore, animal research found that sleep loss could be fatal. Some of them showed how after 4 weeks or longer period of total or REM selective sleep deprivation rats became debilitated and eventually died. The cause of death is poorly understood but it might be caused by a combined collapse in many bodily systems (i.e. endocrine, immune) (Cirelli et al., 1999; Rechtschaffen and Bergmann, 2002; Rechtschaffen et al., 2002).

Even if one might suspect that this peculiar physical syndrome could be due to a general systemic failure induced by the stress of sleep deprivation procedure, the specific changes in autonomic output, skin and fur changes and energy metabolism seem inconsistent with the effects induced by chronic stress (Rechtschaffen and Bergmann, 2002; Rechtschaffen et al., 2002).

One possible explanation could be an interaction between sleep and endocrine system. Many events happening during sleep seem to modulate endocrine system and vice versa, notwithstanding these effects seem to be indirect and primarily correlative in nature (Brandenberger and Weibel, 2004).

There might also be interesting connections between the immune system and sleep (Bryant et al., 2004). Especially for endogenous released cytokine, however, the relationship between their levels and sleep is not so clear. Furthermore, the effects of sleep deprivation on immune function are not entirely consistent (Everson and Toth, 2000; Bryant et al., 2004).

All together, these results show how interestingly sleep is linked to endocrine and immune systems. Nonetheless, these relationships suggest reciprocal modulation, rather than direct functional connections.

Moreover, it is not clear why facilitation of anabolic processes or modulation of endocrine and immune functions would require the loss of consciousness and all the peculiar features of non-REM and REM sleep. For all this reasons, it is unlikely that sleep primary function concerns the body.

Neuro-Metabolic theories of sleep function:

In 1909, Ishimori proposed the "Hypnotoxin theory" claiming that waking brain metabolism is associated with an increase in sleep-inducing toxins (Ishimori, 1909). Modern version of the theory replaced the original one, proposing that sleep preserves or protects against neuronal damage caused by prolonged glutamate release or oxidative processes that occur during wakefulness (Inoue et al., 1995; Schulze, 2004). Two molecules seem to be linked with sleep and a reduction in synaptic activity. Namely, uridine and glutathione reduce synaptic activity by enhancing GABAergic and reducing glutamatergic synaptic transmission respectively. Also, glutathione has strong antioxidant properties and is hypothesized to protect cells form oxidative damage (Inoue et al., 1995). However, the extent to which waking or sleep influences oxidative processes in the brain is controversial, as confirmed by the absence of evidence coming from sleep deprivation studies (Cirelli et al., 1999).

Sleep has been also linked with neuronal restoration. Specifically, effects of normal sleep, wakefulness and sleep deprivation have been linked to both synthesis and degradation of a variety of macromolecules (nucleotides, proteins and mRNAs), as well as restoration of some important molecules for cerebral energy supply. Non-REM sleep amounts, indeed, showed positive correlation with cerebral protein synthesis in adult rats, monkeys and the ovine fetus (Ramm and Smith, 1990; Nakanishi et al., 1997; Czikk et al., 2003).

On the other hand, the link between sleep and energy supply has been proposed by Benington and Heller, who hypothesized that the function of sleep is to restore cerebral glycogen levels (Benington and Heller, 1995). Glial glycogen acts as a reserve glucose store for neurons and is depleted during wakefulness and restored during Non-REM sleep. Excitatory neurotransmitters release during wakefulness brings about the conversion of glycogen into glucose. States with lowered excitation such as Non-REM sleep, on the contrary, favor the restoration of glycogen. In this view, as glycogen is mobilized adenosine is released so triggering and maintaining Non-REM sleep.

Even if this hypothesis is able to provide an elegant connection between waking cerebral metabolism and sleep homeostasis, some evidence coming from total sleep deprivation studies in many animal species are contradictory (Franken et al., 2003).

In general, neuro-metabolic theories have weak experimental support. Removal of some toxic byproduct of wakefulness as well as brain damage following sleep loss does not seem to show any convincing evidence. Moreover the functional consequences of the sleep-related changes in macromolecules are still unknown.

Cognitive theories of sleep function:

The idea that sleep is related to learning and memory has been widespread among scientists as well as the general public. Thus this issue has been investigated by decades and, especially in the last ten years, led to many proofs of significant association between the two domains (Smith, 1995; Stickgold et al., 2001; Rauchs et al., 2005).

Recently, new approaches combining learning paradigms with measurement of brain activity and metabolism allowed the investigation of some outcome related to plastic events taking place in the brain. For example, Huber *et al.* have shown a learning-

dependent enhancement of EEG slow wave activity (1-4 Hz) during Non-REM sleep which correlated with improved learning (Huber et al., 2004).

Form the other hand, since LTP is thought to be the cellular correlate of memory, a number of investigations have been carried out in order to fulfill the need for a convincing demonstration that sleep directly influences synaptic plasticity. Specifically, some investigated the role of sleep states in influencing tetani-induced LTP, finding that stimulation during Non-REM sleep was not effective or produced high variable results also including LTD (Leonard et al., 1987; Bramham and Srebro, 1989), while stimulation during REM sleep induced an LTP-like effect (Bramham and Srebro, 1989).

Other investigations showed that sleep loss can affect LTP-like induction and maintenance. Especially, Campbell showed how LTP can be reduced *in vitro* after 12 hour of sleep deprivation in hippocampal CA1 by stimulation of Shaffer collaterals (Campbell et al., 2002).

Despite these findings should be interpreted with some caution, many neurophysiological and molecular evidences suggest that sleep actually has a role in regulating synaptic plasticity.

From a neurophysiological standpoint, several works have reported changes in single neurons or circuits during sleep showing reactivation or "reverberation" of wake-active circuits in rodents, primates and humans (Pavlides and Winson, 1989; Laureys et al., 2001; Hoffman and McNaughton, 2002; Lee and Wilson, 2002). These findings are consistent with a secondary reprocessing of information in sleep in order to consolidate the changes in neuronal circuitry triggered by waking experience.

A fascinating connection between "replay" in sleep and memory has been proposed by Buzsaki. Underpinning on temporal relationship between cortical and subcortical activity, he states that, during sleep, it is possible that information flows out from the hippocampus

to the cortex. Moreover, it is likely that such a flow occurs during Non-REM sleep when the hippocampal activity is consistent with outflow (Buzsaki, 1996).

More recently, Ji and Wilson showed a more detailed scenario of the complex relationship between hippocampus and visual cortex during Non-REM sleep in rats (Ji and Wilson, 2007).

Both neocortex and hippocampus showed organized periods of increased neuronal population activity defined as "frames" by the authors. After recording multicell firing sequences evoked by awake experience, they found a replay of these sequences during sleep frames in both cortex and hippocampus. Deepening the investigation on the temporal sequence of activation, they also found a sequence similar to the one seen during waking behavior in both cortex and hippocampus. This simultaneous reactivation of coherent memory traces in both these structures during sleep may be the reflection of the memory consolidation process. However, it is still unclear whether the correlation between hippocampal and cortical activity during sleep reflects an actual transmission of information between these structures (Pelletier et al., 2004).

Another idea, with strong experimental support is that synaptic plasticity may be promoted during slow wave sleep by rhythmic spike burst and spike trains fired by thalamic and neocortical neurons (Timofeev et al., 2002). For example, experimentally induced thalamic volleys and anesthesia-induced spindles indeed are able to augment cortical responses persisting for several minutes in cats *in vivo*.

More recently, the same group showed local cortical synaptic plasticity modulation -i.e. induction of potentiation or depression- in cats during simulated sleep rhythms depending on the level of background neuronal activity (Crochet et al., 2006).

Specifically, high levels of background neuronal activity achieved using intact cortex under ketamine-xylazine anesthesia preparation reduced the induction of plasticity (a

ceiling–like effect[1]) but favored potentiation, whereas under the condition of reduced spontaneous activity achieved using intact cortex under barbiturate anesthesia preparation, the number of neurons showing plastic changes was significantly higher and depression was induced in the vast majority of the cases. This effect was even greater when tested using an isolated cortical slab.

Investigating the specific neurophysiological mechanisms associated with different vigilance states, a recent work showed that pairing the neocortical rhythmic burst action potentials -occurring predominantly during slow wave sleep- with excitatory postsynaptic potentials (EPSPs) leads to long-term depression in rat neocortical pyramidal cells *in vitro*. Conversely, pairing individual action potentials -prevalent during wakefulness- with EPSPs leads to a long-term potentiation (Czarnecki et al., 2007).

Thus, sleep -and especially NREM sleep- seems to be associated with specific plastic changes in the neocortex, reflecting the specific neuronal activity that characterizes this vigilance state.

From a molecular standpoint, many evidences suggest that sleep holds a clear role in regulating synaptic plasticity.

There is a compelling pool of evidences showing a marked difference between wakefulness and sleep in the expression of genes related to synaptic plastic processes.

For example, synthesis of some macromolecules such as cortical mRNA transcripts for two genes implicated in LTD processes (calcineurin and camKIV), is specifically up-regulated during sleep (Cirelli et al., 2004; Cirelli, 2005). Moreover, during sleep, the expression of LTP-related molecules known to be up-regulated during wakefulness and sleep deprivation

[1] These findings are in general agreement with the concept of metaplasticity, which imply that the activity at a synapse modifies the thresholds for induction of plasticity.

reaches a low level. Specifically, genes for subunits of the AMPA receptors, calmodulin, BDNF, and TrkB receptor are down-regulated during sleep (Cirelli and Tononi, 1998).

This difference is in line with the fact that, from an evolutionary perspective, potentiation of cortical circuits should take place during wakefulness when the animal is exposed to the external environment and behaves in a active way. Supporting this view, both the quantity and the quality of wakefulness also showed to play an important role over the expression of LTP-related genes. Recently, indeed it has been shown that animals whose wakefulness was prolonged by gentle handling, or engaged in an extensive exploration of their environment, as well as animals exposed to enriched environments show an increased expression of molecular markers of LTP compared to those left to a "spontaneous" wakefulness (Cirelli and Tononi, 2000b; Pinaud et al., 2002; Vazdarjanova et al., 2002; Cancedda et al., 2004; Huber et al., 2007).

A possible explanation for this selective induction of LTP-related genes during wakefulness is that the firing of noradrenergic system is high during wakefulness and low or absent during sleep (Aston-Jones and Bloom, 1981). If the noradrenergic innervation to the cortex is destroyed, the expression of a number of macromolecules related to LTP decreases towards the levels seen in sleep even if the animal is awake and its EEG is unchanged (Cirelli and Tononi, 2000a).

In general, all these molecular results suggest that sleep plays a role in the regulation of synaptic plasticity compatible with a depotentiation/depression process. Furthermore, aforementioned neurophysiological evidences suggest that such a role is likely to take place during NREM sleep.

The Synaptic Homeostasis Hypothesis

In order to give an integrated view of the features characterizing the link between sleep and plasticity, Tononi and Cirelli (Tononi and Cirelli, 2003) proposed a novel hypothesis claiming that sleep plays a role in the regulation of synaptic weight in the brain.

Based on the two-process model of sleep regulation (Borbely and Achermann, 1999), the hypothesis focuses on the process S -the homeostatic component- which accumulates exponentially during wakefulness and is discharged during sleep. Since the time course of Process S was derived from a physiological variable, EEG slow-wave activity (SWA) in the EEG of NREM sleep, a restorative aspect of sleep is suggested by the homeostatic regulation of SWA but its characterization remained elusive. The present hypothesis links the Process S with the process of synaptic homeostasis. The curve representing Process S indeed can be interpreted as reflecting the total amount of synaptic strength, thus increasing during wakefulness and reaching the peak just before going to sleep (Fig. 3). As soon as sleep begins, total synaptic strength starts to decrease reaching a baseline level by the time sleep ends.

More than a simple link between Process S and homeostatic regulation of synaptic strength, the hypothesis can account for several aspects of sleep and its regulation, and makes several specific predictions. Specifically (Tononi and Cirelli, 2003):

- Wakefulness is associated with synaptic potentiation in several cortical circuits.
- Synaptic potentiation is tied to the homeostatic regulation of SWA.
- SWA is associated with synaptic downscaling.
- Synaptic downscaling is tied to the beneficial effects of sleep on neural function.

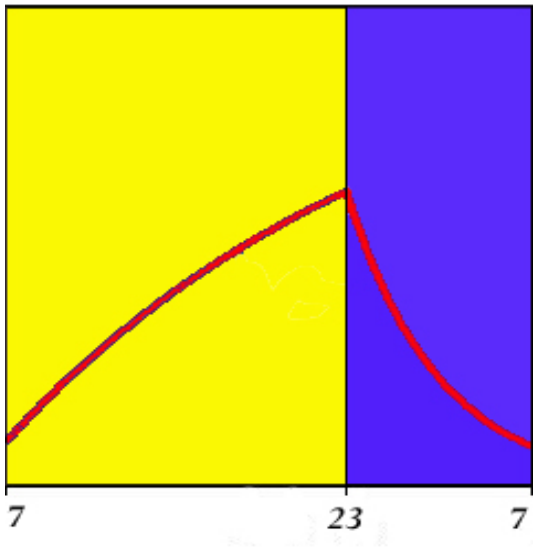

Fig. 3 Graphic representation of Process S, the homeostatic component of the two-process model of sleep regulation (Borbely and Achermann, 1999). The yellow background represents waking , while the blue is sleep. (Modified from the Internet).

Before discussing in detail the above predictions, an overview illustration of their relationship and temporal dynamics will help in understanding the key features of the hypothesis (Tononi and Cirelli, 2006).

Starting from the bottom of Fig. 4, during wakefulness (yellow background) we interact with the environment and acquire new information. The EEG is activated, and the neuromodulatory milieu (for example, high levels of noradrenaline, NA) favors the storage of information, which occurs largely through long-term potentiation of synaptic strength. This potentiation occurs when the firing of a presynaptic neuron is followed by the depolarization or firing of a postsynaptic neuron, and the neuromodulatory milieu signals the occurrence of salient events. Strengthened synapses are indicated in red, with their strength given by a number. Note that one synapse grows to a strength of 150, while another synapse does not change and stays at 100. Note also the appearance of a new synapse with a strength of 5.

Due to the net increase in synaptic strength, waking plasticity has a cost in terms of energy requirements, space requirements, and progressively saturates our capacity to learn. When we go to sleep (blue background), we become virtually disconnected from the environment.

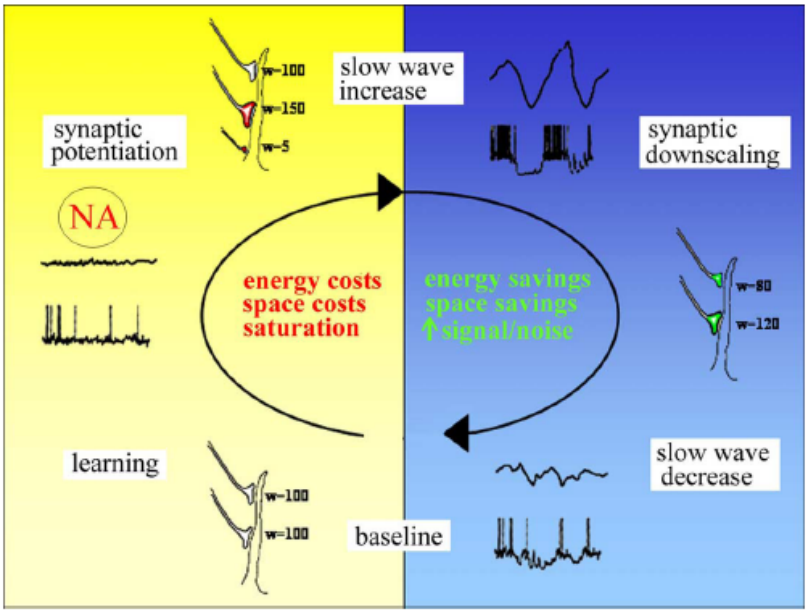

Fig. 4 Schematic illustration of the synaptic homeostasis hypothesis. (From (Tononi and Cirelli, 2006))

Changes in the neuromodulatory milieu trigger the occurrence of slow oscillations in membrane potential, comprising depolarized and hyperpolarized phases, which affect every neuron in the cortex, and which are reflected in the EEG as SWA. The changed neuromodulatory milieu (for example, low levels of noradrenaline) ensures that synaptic activity is not followed by synaptic potentiation, which makes adaptive sense given that synaptic activity during sleep is not driven by interactions with the environment. Because average synaptic strength at the end of the waking period is high, neurons undergoing sleep slow oscillations are highly synchronized. As a result, the EEG of early sleep shows slow waves of high amplitude. The slow waves, however, are not just an epiphenomenon of the increased synaptic strength, but have a role to play. The repeated sequences of

depolarization-hyperpolarization cause the downscaling of the synapses impinging on each neuron, which means that they all decrease in strength proportionally, here by 20%. Thus, a synapse that after wakefulness had strength of 100 is downscaled to 80, another synapse, which had been potentiated to 150, is downscaled to 120 (green color). The synapse with a strength of 5, having being downscaled below a minimum strength, has been 'downselected' or removed altogether. The reduced synaptic strength reduces the amplitude and synchronization of the slow oscillations in membrane potential, which is reflected in a reduced SWA in the sleep EEG. Because of the dampening of the slow waves, downscaling is progressively reduced, making the process self-limiting when synaptic strength reaches an appropriate baseline level. Indeed, total synaptic strength, which had increased from 200 (100+100) at the beginning of wakefulness to 255 (100+150+5) at the end of wakefulness, is downscaled back to 200 (120+80) by the end of sleep. By returning total synaptic weight to an appropriate baseline level, sleep enforces synaptic homeostasis. This has benefits in terms of energy requirements, space requirements, and learning and memory. Thus, when we wake up, neural circuits do preserve a trace of the previous experiences, but are kept efficient at a recalibrated level of synaptic strength, and the cycle can begin again.

Wakefulness and synaptic potentitation:

As we already mentioned in the former paragraph, plastic changes would occur through much of our waking life, whether or not we are specifically engaged in a learning task. After all, synapses and neurons do not know whether they are engaged in a learning task, but only whether strong presynaptic discharge is followed by a postsynaptic depolarization or firing in the presence of an appropriate neuromodulatory milieu. Such a

situation is very likely to occur during wakefulness, when we are alert and actively interacting with the external environment.

Due to the fact that the molecular changes associated with LTP regularly occur during wakefulness (Cirelli and Tononi, 2000a; Ying et al., 2002; Cirelli et al., 2004) when the state-dependent firing of noradrenergic system (Aston-Jones and Bloom, 1981) is compatible with the storage of information via LTP-like processes, these plastic changes are likely to result in LTP more than LTD, thus producing a net increase of synaptic strength.

Synaptic potentiation and slow wave homeostasis:

According to the synaptic homeostasis hypothesis, the homeostatic regulation of slow wave activity (SWA) during sleep is tied to the amount of synaptic potentiation brought about by wakefulness. The two measures are linked by a directly proportional relation so that the higher the amount of synaptic potentiation in cortical circuits, the higher the increase in SWA.

As discussed in the former paragraph, both the duration and the quality of wakefulness should impact the regulation of markers of synaptic potentiation, and a recent work (Huber et al., 2007) showed how an up-regulation of LTP markers due to exploration of the environment led to an increase in SWA in rats. Moreover, as predicted by the hypothesis, a reduced expression of LTP related macromolecules in animals with lesioned noradrenergic system results in a dampening of the SWA peak normally seen in the morning hours after the nocturnal activity phase as much as the SWA rebound following sleep deprivation (Cirelli et al., 2005).

It is worth noting how the amount and timing of sleep were unchanged thus suggesting that is not wakefulness *per se*, but the induction of LTP-related molecules associated with it that is responsible for the homeostatic increase in SWA.

Another prediction of the hypothesis is that, to the extent that synaptic potentiation is particularly strong in a specific brain area, the resulting increase in SWA in subsequent sleep should be disproportionate in that area. Searching for signs of local SWA homeostasis, Huber (Huber et al., 2004) investigated sleep after a visuomotor learning task by means of high density EEG.

In order to reach visual targets on a screen using a handheld cursor, subjects had to unconsciously adjust their perceived trajectories adapting them to systematic rotation experimentally imposed. A previous PET study (Ghilardi et al., 2000) showed that rotation adaptation learning involves a circumscribed region of the right parietal cortex. One week earlier or later, subjects performed a control motor task with no experimentally imposed rotation. So, the only difference between the two tasks was the implicit learning in the rotation adaptation task, presumably involving a synaptic potentiation in the right parietal area.

Comparing the two conditions brought to light a local increase of SWA over a small cluster of electrodes localized over the right parietal cortex, confirming that the presumed induction of plastic changes associated with practicing a visuomotor task is associated with a local induction of SWA in subsequent sleep.

These results are in line with previous literature on local SWA homeostasis in humans (Kattler et al., 1994) and rats (Vyazovskiy et al., 2000) and with other suggestion for a local regulation of sleep (Krueger and Obal, 1993; Krueger et al., 1995).

The mechanism linking synaptic potentiation during wakefulness with increased SWA during subsequent sleep lies in the neurophysiological nature of slow waves recorded in the sleep EEG.

SWA is the reflection of oscillations in neuronal membrane potentials, the most important of which is a slow oscillation generated by cortical neurons and synchronized by cortico-cortical connections (Steriade, 2003).

At a single cell level, the slow oscillation comprises a depolarized up-phase, during which neurons fire at relative high rates and a hyperpolarized down-phase during which neurons are silent. A sodium-dependent potassium current activated as a function of neuronal firing is probably underpinning the induction of the down-phase, so that stronger cortico-cortical connections cause a stronger activation of the sodium-dependent potassium current leading in turn to a longer and more hyperpolarized down-phase, resulting in a slow oscillation of increased amplitude. Moreover, stronger cortico-cortical connections following synaptic potentiation are responsible for an increase of the degree of synchronization of slow oscillations (Hill and Tononi, 2005). Both mechanisms results in a larger amplitude of slow waves at EEG level.

Slow wave homeostasis and synaptic downscaling:

According to the hypothesis, the increased amplitude of slow waves during sleep following a net potentiation of synaptic strength occurring during wakefulness has a role to play and is not to be seen as a mere epiphenomenon. This role is to promote a generalized downscaling of synaptic strength. Downscaling refers to a proportional reduction of the strength of all synapses converging onto the same neuron (Tononi and Cirelli, 2003). Thus, downscaling is to some extent conceptually different from long-term depression which affects only select groups of synapses, or depotentiation which affects only recently

potentiated ones. Nevertheless, like depotentiation or LTD, downscaling depends on molecular mechanisms linked to the dephosphorylation and subsequent internalization of AMPA receptors, leading to a reduction in synaptic efficacy.

Proportionally reducing the strength of all synapses impinging on the same neuron, downscaling lowers total synaptic weight while preserving relative differences in synaptic strength and therefore memory traces.

The proposed sleep-dependent homeostatic regulation of the total synaptic strength is in line with the activity-dependent mechanism observed both *in vivo* and *in vitro* in cortical and hippocampal neurons (Turrigiano, 1999; Desai et al., 2002). Such activity-dependent mechanism of synaptic scaling ensure that neurons maintain a regulated firing level facing possible uncontrollable changes in their inputs.

The hypothesized downscaling function of SWA is also in line with the well-established exponential decrease of SWA itself during sleep (Borbely and Achermann, 1999). If the amplitude and synchronization of slow oscillations reflects the strength of cortico-cortical connections, the reduction of SWA power across the night is likely to be linked to its dampening.

Also, as showed by Dijk and co-workers (Dijk et al., 1987), if SWA is disrupted by auditory stimulation during the first three hours of sleep, the resulting EEG power in the second part of the night shows a remarkable amount of SWA compared to a baseline night, thus showing that slow waves are needed for sleep SWA to decline and thereby they are not a mere epiphenomenon of sleep.

A role for sleep in downscaling is also in line with the aforementioned molecular evidences regarding the low LTP-related molecules expression together with the upregulation of molecules implicated in depotentiation/depression (Cirelli and Tononi, 2000a; Cirelli et al., 2004).

Looking for a suitable mechanism linking SWA and synaptic downscaling, great attention should be paid to the temporal features of the cellular phenomenon underpinning SWA, the slow oscillation. Indeed it occurs at a frequency -less than 1 Hz- that is likely to produce depression in stimulation paradigms, probably through changes in calcium dynamics, a crucial mechanism for depression (Kemp and Bashir, 2001).

Another possible mechanism is suggested by the unique neuromodulatory milieu present during NREM sleep -characterized by low levels of acetylcholine, noradrenaline, seroronin and histamine- as well as low levels of BDNF, a neurotrophic factor preventing depression (Sheng and Hyoung Lee, 2003).

However, the sequence of up and down-phases of the slow oscillation is likely the most significant factor promoting downscaling. The close temporal pairing between generalized spiking at the end of the up-phase and following hyperpolarization at the beginning of the down-phase could represent for synapses a signaling mechanism for the failure of the presynaptic input in driving the postsynaptic activity. Another possibility could be that depression may be triggered by temporal pairing between generalized hyperpolarization at the end of the down-phase and the generalized spiking at the beginning of the up-phase. Alternatively, depolarization-hyperpolarization sequences could also bring about downscaling themselves.

The functional significance of synaptic downscaling during sleep:

Synaptic downscaling is first important in keeping very basic brain resources such as energy and space at an efficient level.

By scaling synaptic strength down to a baseline level it interrupts synaptic overload due to an increased metabolism as well as synaptic growth due to morphological changes. All the features of the increased synaptic weight brought about by wakefulness -increased average

firing rate, increased size of terminal boutons and spines as well as number of synapses- are indeed leading to a condition progressively saturating the system capability of both energy and space.

In addition to this basic function and mainly relying on it, downscaling is also important for high cognitive functions such as learning and memory. The ability of the brain to acquire new information is indeed linked to the availability of energy and space resources of the system. A saturated condition is not consistent with this ability, and a mechanism taking care of the recalibration of synaptic weight is therefore needed.

By downscaling synaptic strength, sleep is thought to operate also a mechanism of downselection of synapses that fail to reach a certain threshold. This mechanism is beneficial in terms of promoting synaptic competition during development -a time of exuberant synaptic growth- and of increasing the signal to noise ratio (SNR) in the relevant brain circuits in the adult -a fine tuning process very important in learning and highly correlated with behavioral performance (Huber et al., 2004)-.

An opened question: is sleep necessary for synaptic homeostasis?

After illustrating the main features of the synaptic homeostasis hypothesis, this remarkable question is still not completely answered. All the molecular and cellular phenomena differentiating sleep from waking cannot completely rule out the issue regarding the possibility of downscaling during wakefulness.

Since sleep is a potentially dangerous behavior defined by disengagement from the environment, whatever the function it could fulfill, it should be extremely specific to this behavioral state in order to explain its universality. Going by the very mechanisms

characterizing the synaptic homeostasis hypothesis, the authors provided evidence supporting the specificity of sleep state for downscaling (Tononi and Cirelli, 2006).

Indeed, in order to determine the degree of downscaling needed, a neuron should be able to assess its total synaptic input in an unbiased manner, potentially offered by sleep only. Being a off-line state, independent of behavioral requirements, sleep allows a correct estimate of synaptic strength, and therefore of the appropriate level of downscaling needed. Such a process is difficult to accurately take place during wakefulness. The high synaptic input brought about by the interaction with the outside world could indeed overestimate the dose of downscaling needed.

Another reason why synaptic homeostasis might be confined to sleep is that, at the molecular level, the depolarization-hyperpolarization sequences leading to downscaling are perfectly compatible with sleep but could seriously interfere with behavior, if occurring during wakefulness.

SECTION 2:

STUDIES

Spike timing dependent plasticity predicts changes in EEG slow-wave activity during subsequent sleep

Introduction

Increasing evidence indicates that sleep and neural plasticity are linked. Sleep after learning promotes memory consolidation for declarative and non-declarative tasks (Karni et al., 1994; Gais et al., 2000; Maquet, 2001; Gais and Born, 2004; Walker and Stickgold, 2004; Stickgold, 2005). Sleep deprivation impairs new learning (Yoo et al., 2007) and compromises long-term potentiation (LTP) (Davis et al., 2003; McDermott et al., 2003).

On the other hand, learning can lead to changes in sleep duration and intensity (Meier-Koll et al., 1999; Gais et al., 2002; Schmidt et al., 2006).

The best characterized marker of sleep intensity is the amount of slow wave activity (SWA, EEG power density 0.5-4.5 Hz) during non rapid eye movement (NREM) sleep (Borbély and Achermann, 2000), which increases as a function of prior wakefulness and declines during sleep. However, the mechanisms underlying the increase in SWA with sleep pressure remain unknown. An important clue has come from work showing that sleep SWA can be regulated locally in the cerebral cortex, pointing to a link between SWA regulation and synaptic plasticity. In a high density (hd)-EEG study, sleep SWA was locally increased after a visuomotor learning task involving right parietal cortex (Huber et al., 2004). In another study, subjects received 5 Hz transcranial magnetic stimulation (TMS) over left premotor cortex to potentiate cortical circuits, and during subsequent sleep they showed a local increase in SWA (Huber et al., 2007b). In a third study, subjects underwent arm immobilization during the day, leading to a decrease in motor performance

and in somatosensory and motor evoked potentials. This was followed by a local decrease of sleep SWA over sensorimotor cortex (Huber et al., 2006).

Altogether, these experiments suggest that sleep SWA is affected by plastic changes in local cortical circuits and, more specifically, that SWA may increase after manipulations that favor synaptic potentiation and decrease after those that promote synaptic depression (Tononi and Cirelli, 2003, 2006). Computer simulations indicate that stronger synapses lead to increased SWA by enhancing neuronal synchronization, whereas weaker synapses have the opposite effect (Hill and Tononi, 2006; Esser et al., in press). To test this hypothesis directly, it is important to investigate whether established paradigms for inducing LTP and LTD yield the predicted changes in sleep SWA. To reduce variability, it is essential to use very similar paradigms for LTP and LTD and compare the effects within subjects. We employed a paired associative stimulation (PAS) protocol (Classen et al., 2004) based on spike timing dependent plasticity (Abbott and Nelson, 2000): presynaptic activity that precedes postsynaptic firing induces LTP, whereas reversing the order causes LTD. In the PAS protocol, plastic changes are induced by combining peripheral somatosensory stimuli and TMS pulses applied to contralateral sensorimotor cortex and manipulating the latency between the two stimuli, such that either pre- or post-synaptic neurons are stimulated first. To directly evaluate changes in cortical excitability, we recorded, in addition to motor evoked responses (MEP), EEG responses evoked by test TMS pulses using a TMS-compatible EEG system (Massimini et al., 2005) equipped with optical tracking to ensure reproducibility and coregistration with magnetic resonance images.

Methods

Design:

Nineteen healthy right-handed male subjects (mean age 25.2±1.0 years) gave informed consent to participate in the study, which was approved by the local ethics committee. We performed a neurological screening to exclude subjects with conditions that could predispose them to potential adverse effects of TMS. The TMS sessions took place in the evening (between 10 and 11 pm). A 60-channel EEG cap was prepared on each subject with scalp impedances less than 5 KOhm. The design of the experiment is depicted in Figure 1. Briefly, when the subjects were ready, we collected motor evoked potentials followed by TMS evoked potentials in the pre PAS period. The 30 min paired PAS was then followed by the collection of motor evoked potentials and TMS evoked potentials in the post PAS period. After a 5 to 10 min break, the room was darkened and subjects were allowed to sleep in a bed while their EEG was recorded. Our EEG electrode system permitted only the acquisition of a first sleep cycle (1 to 2 hours). The sleep recording was therefore terminated at the first occurrence of REM sleep or when the subject woke up. All reported satisfactory, restful sleep. Subjects underwent 3 experimental sessions, PAS with an interstimulus interval (ISI) of 10 ms, PAS with an ISI of 25 ms and sham. Sessions were separated by one week and their order was randomized.

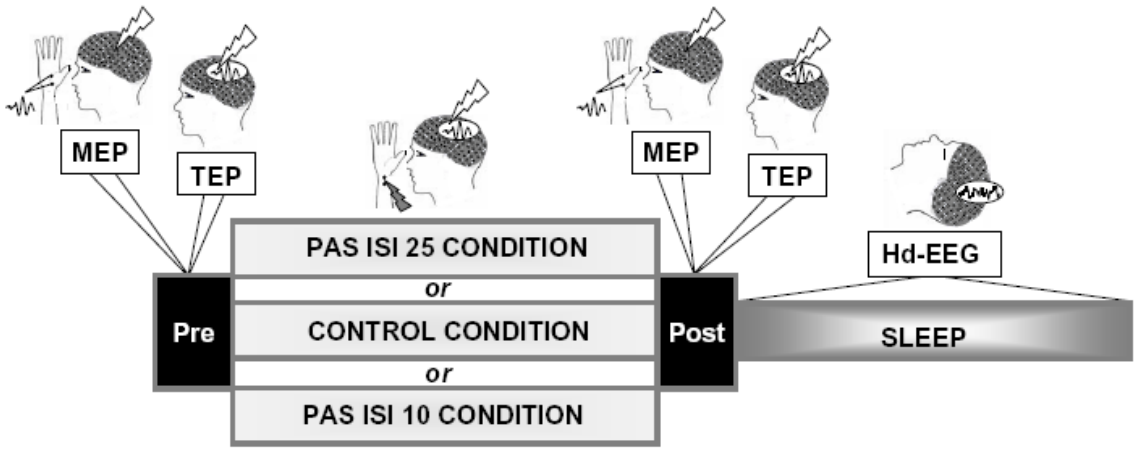

Fig. 1 (previous page). Study design. During the pre and the post test sessions we collected motor evoked potentials (MEP) followed by TMS evoked EEG potentials (TEP). The three conditions, 1) paired associate stimulation (PAS) with an inter stimulus interval (ISI) of 25 ms, 2) a PAS with ISI of 10 ms and 3) a sham control, were separated by a week with randomized order. Subjects were blind to the condition. In the darkened room, the first sleep cycle was recorded using high-density EEG(hd-EEG).

Transcranial magnetic stimulation:

The stimulation setup consisted of a Magstim Rapid magnetic stimulator (Magstim Ltd, Whitland, UK) and a figure-of-eight TMS coil (Magstim Double 70mm). Precision and reproducibility of the stimulation were achieved by means of a Navigated Brain Stimulation (NBS) system (Nexstim Ltd, Helsinki, Finland). The NBS device uses an optical tracking system to locate the TMS coil relative to the subject's co-registered MR image and allows a digitization of the location of the EEG electrodes. The NBS system delivered trigger pulses that synchronized the TMS, EMG and EEG systems. To locate the optimal site of TMS, subjects' primary motor cortex was extensively mapped around the anatomically defined "motor knob" to find the area evoking the largest response in right abductor pollicis brevis (APB muscle), which was chosen as the hot spot. The coil was placed tangentially to the scalp with the handle pointing backwards and 45 degrees away from the midline. The scalp hot spot varied across subjects, encompassing an area of left central electrodes (number 27, 28, 37, 38). Stimulus intensity was set relative to resting motor threshold (RMT), which was determined using a maximum likelihood threshold hunting procedure and as the TMS intensity producing at least five motor evoked potentials (MEPs) N50 OV (peak-to-peak) out of 10 consecutive trials (Rossini et al., 1994; Awiszus, 2003). The RMT was 64.2 ± 2% of the maximal stimulator output. All stimuli were delivered to the same cortical target during the experiment. The electric field induced on the cortex at RMT was estimated at 102.5±7.7 V/m. The pre and post PAS test phases consisted of 200 TMS pulses delivered every 0.5-0.7 sec at 90% RMT.

Somatosensory evoked potentials (SEP):

SEPs were evoked by electric stimulation of the median nerve at the right wrist using constant current square-wave pulses (0.5 ms) with intensity just above the thenar motor threshold.

Motor evoked potentials (MEP):

Continuous on-line EMG and MEPs were measured from an electrode pair attached to the skin overlying the APB muscle and the first metacarpophalangeal joint by means of the Nexstim amplifier (see below). We assessed changes in MEP amplitude in a MEP test phase before and after the paired associate stimulation. MEPs were generated by delivering 20 TMS pulses, one every 10 sec at 130% RMT.

TMS evoked potentials:

EEG responses to TMS were recorded by means of a cap with 60 carbon electrodes and a specifically designed TMS-compatible amplifier (Nexstim Ltd). TMS was targeted to the hot spot of the APB muscle of the left hemisphere at 90% RMT. The EEG signals were filtered (0.1-500 Hz) and sampled at 1450 Hz (for details see (Massimini et al., 2005). Confounding factors such as auditory evoked responses and attentional effects on evoked responses were reduced by noise masking and by engaging the subject in a simple oddball task. In this task, interspersed within the noise masking, tones were played at irregular intervals (10-60 sec) and the subject had to respond as fast as possible with a mouse button click with the left hand. Differences were assessed by paired t-tests. Offline, for the analysis of evoked responses, the data were average referenced, baseline corrected (100 ms prestimulus), band pass filtered (5-100 Hz) and averaged for each subject. Total EEG activity was assessed using the global mean field power (GMFP) (Lehmann and Skrandies,

1980). TMS evoked potentials were assessed by delivering 200 TMS pulses delivered every 0.5-0.7 sec at 90% RMT.

Paired associate stimulation (PAS):

PAS consisted of 90 electrical stimuli of the right median nerve at the wrist (see SEP) paired with a single TMS pulse over the motor representation of the right APB muscle of the left hemisphere (hot spot) at 130% RMT (see MEP) every 15 sec. The interstimulus interval (ISI) between peripheral and TMS stimulus was either 10 ms (ISI 10) or 25 ms (ISI 25), which was shown previously to induce long-lasting decreases (LTD-like) or increases (LTP-like) in MEP amplitude, respectively (Stefan et al., 2000; Stefan et al., 2002). For safety reasons, the subjects'EEG was carefully monitored online during the PAS sessions. We found no epileptiform EEG abnormalities. Furthermore, subjects were interviewed immediately following and one week after the experiment and reported no adverse effects. For sham PAS, the coil was rotated 90° around the axis of the handle and separated from the head using a 2 cm plastic spacer cube to ensure an indirect contact between the coil and the head. The right median nerve stimulation was applied as in the experimental conditions. At debriefing at the end of the experiment subjects did not report any difference between the three conditions.

Sleep recording:

Sleep EEG recordings for the first sleep episode were band-pass filtered between 0.1 and 40 Hz, downsampled to 128 Hz, and average-referenced. Sleep stages were visually scored for 20-sec epochs according to standard criteria (Rechtschaffen and Kales, 1968). For a quantitative analysis of the sleep EEG, spectral analysis of consecutive 20-sec epochs was performed for all 60 channels (FFT routine, Hanning window, averages of five 4-s epochs).

Visual and semi-automatic artifact removal were performed (Huber et al., 2000). Significant topographical differences in hd-EEG power during the first 20 min SWS sleep were assessed by statistical nonparametric mapping (SnPM) using the single threshold test (Nichols and Holmes, 2001; Huber et al., 2004; Huber et al., 2006). This method takes advantage of the actual data distribution and accounts for multiple comparisons testing in hd-EEG recordings. Briefly, EEG readings at each electrode for an experimental condition and the control condition were shuffled according to all possible permutations for all subjects. Based on the statistics obtained from the permutation data, we calculated a t-value for each electrode, and found the maximal t-value over all electrodes for each permutation. The t-value threshold was taken as the 95th percentile of the permutation-derived t-values, and electrodes exceeding that threshold were taken as showing a significant difference between the two conditions. T-values presented in the figures are based on paired t-tests. For the topographical display of EEG activity we used the topoplot function of the EEGLab Matlab toolbox (Delorme and Makeig, 2004).

Source localization:

Source localization was performed on the average pre-conditioning TMS-evoked EEG response using the Curry software package (Curry 5.0, Neuroscan). Electrode positions were digitized and co-registered to each subject's MRI by means of an infrared positioning system (Nexstim). We then estimated the current density on the cortical surface by using the sLORETA algorithm (Pascual-Marqui, 2002). The current density of the average evoked response was then projected onto the Montréal Neurological Institute (MNI) standard brain.

Results

The design of the experiment is depicted in Figure 1 (see Methods for details). After PAS, changes in cortical excitability were first evaluated using MEPs to TMS stimuli. To directly evaluate changes in response strength depending on stimulation parameters, we also recorded the EEG responses evoked by test TMS pulses using a dedicated, TMS compatible EEG system (Massimini et al., 2005). To ensure the precise localization and reproducibility of stimulation sites and the coregistration with EEG responses, we also employed an optical tracking system based on each subject's magnetic resonance images. In this way, we could: i) employ the same paradigm to induce either LTP- or LTD-like changes by merely varying the PAS interval from 10 to 25 ms; ii) compare the effects of LTP-PAS and LTD-PAS and of sham TMS in the same subject, assuring precise reproducibility of the site of stimulation; iii) obtain a topographic map of changes in cortical evoked responses in addition to traditional MEPs; iv) correlate the changes in TMS-evoked EEG responses with local changes in sleep SWA.

TMS response before and after PAS:

We first examined each subject's peripheral and cortical responses to TMS before and after PAS. As expected, we found that motor responses to TMS (measured as the amplitude of MEPs) were significantly increased or decreased following PAS ISI 25 or 10, respectively (Fig. 2). However, it is noteworthy that responses varied considerably among individuals, such that some subjects even showed reversed responses, i.e. increased MEP amplitude after ISI 10 and decreased amplitude after ISI 25. In a subgroup of subjects we also recorded SEPs, which showed the classical cortical components with the first negative deflection around 20 ms (N20) and the largest amplitude between 35 and 45 ms (n=10).

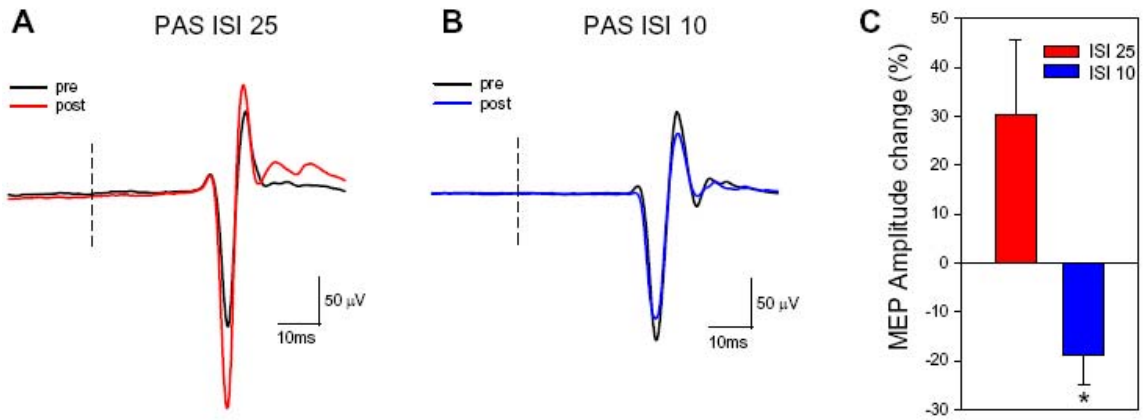

Fig. 2. Motor evoked potentials (MEP). Responses to TMS before and after paired associate stimulation with an inter stimulus interval of 25 ms (ISI 25) and 10 ms (ISI 10). A-B. Average of 20 MEPs produced by stimulation at 130% RMT before (in black) and after PAS (in color). C. MEPs were significantly increased in amplitude following PAS ISI 25 and decreased following PAS ISI 10 ($p<0.05$, paired t-test). The asterisk indicates a significant difference between the two PAS conditions ($p<0.01$, paired t-test).

Next, we analyzed the cortical responses to TMS targeted to the hot spot of the APB muscle of the left hemisphere, directly probing the excitability of the underlying cortex. We first performed source localization of the activity occurring during each peak in the GMFP to visualize which cortical areas were activated by TMS. Figure 3 (next page) shows the TMS induced activity in a single subject before the sham condition (the first two deflections were omitted due to TMS artifacts). TMS produced large deflections in scalp voltage primarily near the site of stimulation but also on the contralateral side. This activity lasted for about 150 ms. Very similar spatial activation patterns were obtained after the sham stimulation as well as after the two active TMS conditions.

We then determined the time course of the total EEG response to TMS by calculating the GMFP for the two experimental conditions across all subjects. Under both conditions, the GMFP contained distinct peaks, which had similar latencies when compared between the pre and post TMS test phases.

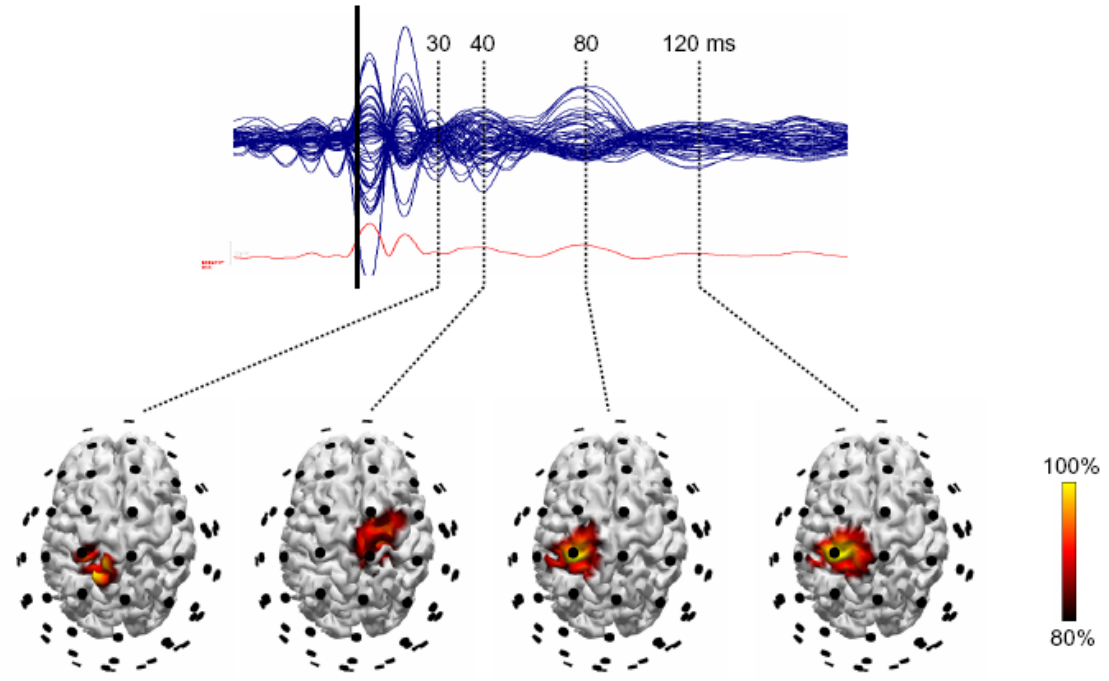

Fig. 3. Source localization of TMS evoked EEG potentials. Top. Averaged TMS evoked potentials recorded at all electrodes, superimposed in a butterfly diagram (black vertical bar represents the time point of TMS application). The red curve below represents the corresponding GMFP. Bottom. Source localization of the activity occurring during each peak in the GMFP (due to TMS artifact contamination the first two peaks were omitted). The top 20% of current produced is shown.

Figure 4 shows the average GMFP difference between the pre and post phase: marked differences can be observed between the two conditions, i.e. responses of higher amplitude after PAS ISI 25 and of lower amplitude after ISI 10.

For a topographical analysis of the TMS induced responses we integrated activity in the time range 50–100 ms for each subject. For all conditions, we observed the strongest evoked activity in electrodes just anterior and posterior to the site of stimulation (Fig. 5). When contrasting the pre and post map of the integrated activity we found reduced activity anterior to the stimulation site after the PAS ISI 10 (n.s.) and increased activity spanning the stimulation site after the PAS ISI 25 ($p<0.05$ for electrodes 17, 28).

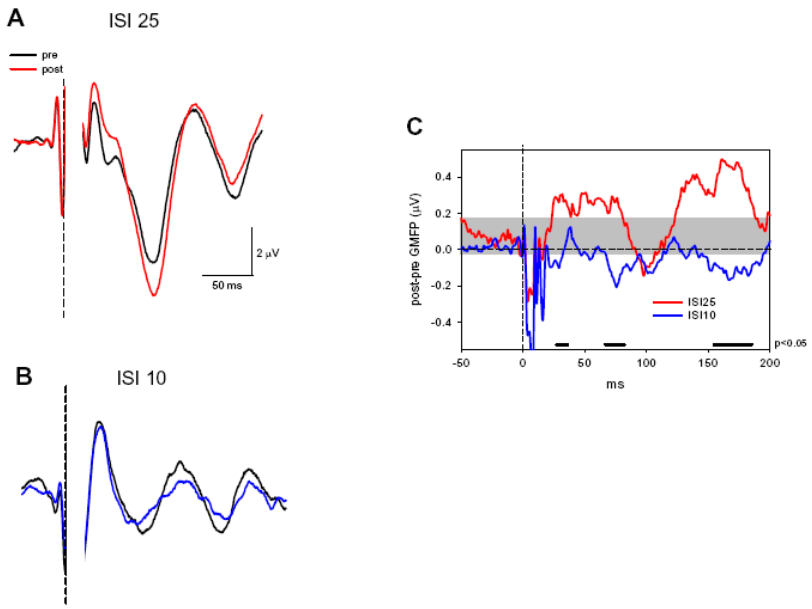

Fig. 4. Change in TMS evoked potentials. A-B. Average TMS evoked potential at electrode 28 for one individual, before (in black) and after (in color) paired associate stimulation with an inter stimulus interval of 25 ms (A, ISI 25) and 10 ms (B, ISI 10). C. Average GMFP difference between the post and pre test for PAS ISI 25 (red) and ISI 10 (blue). Bottom horizontal black lines indicate significant differences between ISI 25 and ISI 10 ($p<0.05$, paired t-test).

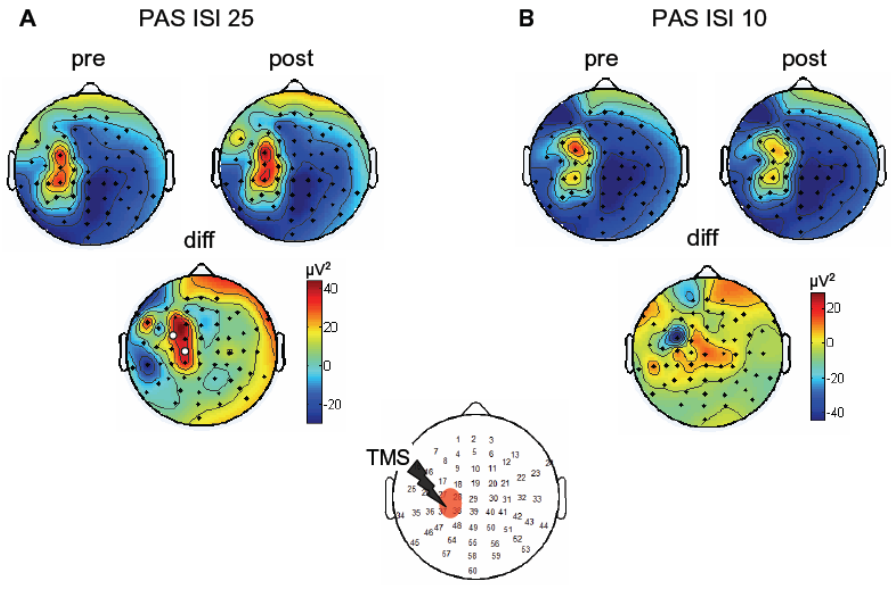

Fig. 5. Topographic distribution of the TMS evoked activity. Average integrated evoked response between 50 and 100 ms before and after paired associate stimulation with an inter stimulus interval of 25 ms (A, ISI 25) and 10 ms (B, ISI 10). Contrasting the pre and post test topography revealed increased activity underlying the stimulation site after PAS ISI 25 and decreased activity after PAS ISI 10. The time range of 50 to 100 ms was chosen because it was not affected by stimulation artifacts but still considered an early component.

Similar to the observed interindividual differences in changes of the MEP amplitude, the induced change in total EEG activity after PAS was highly variable across subjects. This is illustrated in Figure 6A, where the change in the integrated GMFP between 50 and 100 ms after PAS is depicted for each individual. Activity after PAS shows a trend for an increase after ISI 25 and a decrease after ISI 10, however with a large overlap. Therefore, we grouped the subjects for further analysis according to whether they increased or decreased their EEG response, as measured by a change in GMFP, irrespective of the protocol applied.

Sleep SWA after PAS:

After the control and the PAS sessions the subjects were allowed to sleep and we recorded their sleep EEG. Subjects showed the usual progression of sleep stages in all three sessions, with sleep onset occurring about 20 to 30 min after the end of the sessions (sleep latency: control 8.9±1.3, ISI 25 6.8±1.2, ISI 10 10.2±1.8 min; total sleep time: control 86.9±5.1, ISI 25 81.4±4.2, ISI 10 76.8±5.5 min; percentage wakefulness: control 15.5±3.2, ISI 25 14.2±3.5, ISI 10 13.7±2.9 %; percentage NREM sleep: control 73.7±4.2, ISI 25 75.4±4.4, ISI 10 73.4±4.5 %).

For a visualization of the initial topographic distribution of SWA, for each electrode we calculated the average power in the SWA frequency range during the first 20 min of slow-wave sleep (SWS, NREM stages 3 and 4). In accordance with previous studies (Werth et al., 1997; Finelli et al., 2001) we found that SWA was prevalent in anterior regions and highly reproducible across nights (Fig. 6B). Next, we contrasted the conditions to highlight local differences between the PAS and the control session. During sleep after PAS, all subjects (either ISI 25 or ISI 10) showing increased GMFP activity compared to the control condition showed a significant increase of SWA at a cluster of four left central electrodes

(Fig. 6C top; electrodes 27, 28, 37, 38; SnPM, $p<0.05$). The same comparison revealed an additional significant decrease of SWA at a cluster of right central electrodes (Fig. 6C top; electrodes 31, 32, 41, 42; SnPM, $p<0.05$).

For an anatomical localization of these local changes, all electrodes were digitized and co-registered with the subject's magnetic resonance images. The electrodes showing a significant increase or decrease of SWA were then projected onto the brain. The cluster of electrodes showing increased SWA after PAS was localized to the left sensorimotor cortex (Brodmann areas 4 and 3, 1, 2). Similarly, the cluster of electrodes with decreased SWA was localized to right sensorimotor cortex (Brodmann areas 4 and 3, 1, 2).

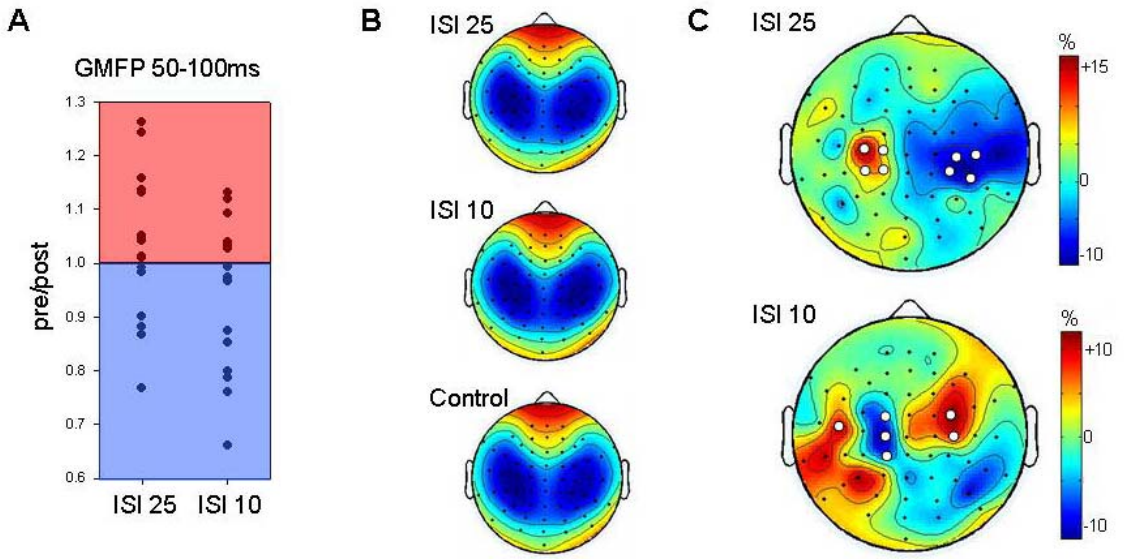

Fig. 6. Local SWA changes after PAS. A. Difference of the integrated GMFP between 50 and 100 ms before and after PAS. All subjects showing increased or decreased total EEG activity, irrespective of the condition, were pooled for further analysis. B. Topographic distribution of SWA after paired associate stimulation with an inter stimulus interval of 25 ms (ISI 25) and 10 (ISI 10) and the sham control condition. Average EEG power density at 1-4.5 Hz (n=19 subjects) for the first 20 minutes of SWS. Values were normalized by total power for the recording, color coded, plotted at the corresponding position on the planar projection of the scalp surface, and interpolated (biharmonic spline) between electrodes (dots). C. Topographic distribution of the ratio of SWA between the ISI 25 (top) or ISI 10 (bottom) condition and control condition. White dots indicate significant differences.

We also compared the distribution of SWA during sleep in all subjects (either ISI 25 or ISI 10) showing decreased GMFP activity after PAS, and found decreased SWA in a cluster of left central electrodes (Fig. 6C bottom; electrodes 18, 28, 38; SnPM, $p<0.05$), which was associated with increased SWA in contralateral electrodes (Fig. 6C bottom; electrodes 21, 31; SnPM, $p<0.05$) and ipsilateral electrodes (electrodes 26, 47; SnPM, $p<0.05$). Again the local changes were localized to left and right sensorimotor cortex (Brodmann areas 6, 4 and 3, 1, 2 for the left decrease; 6, 4 for the right and 4 and 43 for the left increase). We also computed SWA topography changes for subgroups of subjects with the same directional change in GMFP activity within each PAS condition. We found similar local changes for subjects showing increased GMFP after PAS with ISI 25 and ISI 10 condition. Similarly, subjects showing decreased GMFP after PAS showed comparable SWA topographies, irrespective of whether they were subjected to PAS with ISI 25 or ISI 10 (data not shown).

Next, we asked whether the local changes of SWA were predicted by the changed activity of the TMS-evoked responses induced by PAS. Figure 7 illustrates, topographically across all electrodes, the correlation values between the change in GMFP of the evoked response between 50 and 100 ms and the change in SWA during subsequent sleep. We found positive correlations for three electrodes (27, 28 and 38) overlying the site of stimulation. Negative correlations were found for electrodes further left (26 and 36), as well as for contralateral electrodes (12, 20 and 21).

Homeostatic changes in sleep pressure as a function of prior wakefulness are reflected in the global sleep EEG power spectrum mainly as changes in SWA, (Borbély and Achermann, 2000).

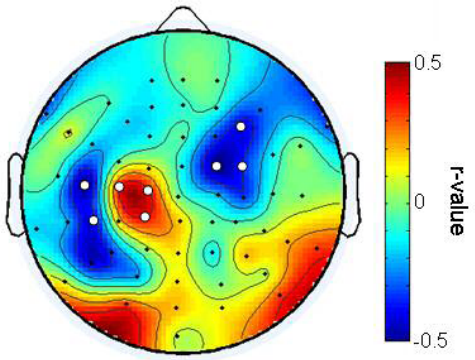

Fig. 7. The change in TMS-evoked responses predicts the local change of SWA. Topographic distribution of the correlations between the SWA change and the change in the TMS-evoked response after PAS. For each subject the change in the integrated global mean field power between 50 and 100 ms before and after PAS was calculated and correlated with the change in SWA at each electrode. White dots indicate electrodes showing a significant correlation ($p<0.05$).

To examine whether the local EEG changes after PAS shared key features with the global homeostatic response observed in the sleep EEG we examined, for each frequency bin, whether there was a correlation between the PAS induced change in EEG power and the PAS related change in GMFP. Consistent with a homeostatic response, we found that significant correlations occurred exclusively in the low SWA frequency range (Figure 8).

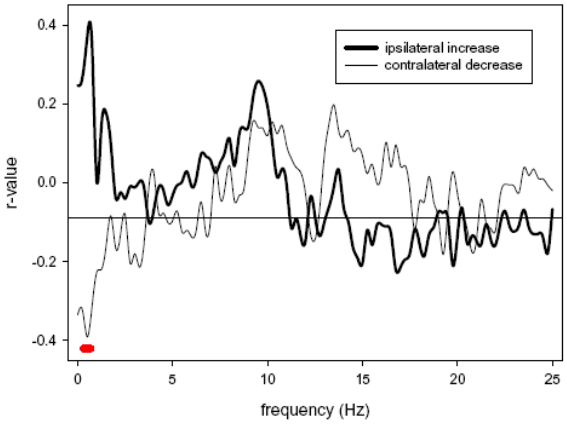

Fig. 8. Best correlation between TMS-evoked response and sleep EEG power in the slow-wave frequency range. The integrated global mean field power between 50 and 100 ms was correlated with the average EEG power within the clusters of electrodes showing a significant change in SWA topography (as in Fig. 6) during the first 20 min of slow-wave sleep for each 0.25 Hz frequency bin. Red line indicates a significant correlation coefficient.

In summary, PAS protocols with different ISI caused local changes in the amplitude of EEG responses to TMS pulses, indicative of potentiation or depression of sensorimotor circuits. These changes were followed, during subsequent sleep, by corresponding local changes in SWA: increased SWA was found in subjects whose cortical responsiveness had

increased, and decreased SWA in subjects whose cortical responsiveness had decreased after PAS. Moreover, the change of the EEG response to TMS pulses and the change in sleep SWA were localized to a similar cortical region and were positively correlated. Together, these results indicate that changes in synaptic efficacy lead to changes in local sleep regulation, as reflected by SWA, and thus provide evidence for a tight relationship between synaptic plasticity and sleep intensity.

Discussion

Effects of PAS on cortical excitability during wakefulness:

In vitro studies have shown that presynaptic activity that precedes postsynaptic firing or depolarization by a few tens of ms can induce LTP, whereas reversing this temporal order causes LTD (Levy and Steward, 1983; Gustafsson et al., 1987; Debanne et al., 1994).

Spike timing dependent plasticity appears to be a key mechanism governing moment-to moment changes in synaptic efficacy in cortical circuits (Magee and Johnston, 1997; Markram et al., 1997; Karmarkar et al., 2002; Watanabe et al., 2002). Although the mechanisms that make synaptic plasticity sensitive to spike timing are not fully understood, they appear to depend on NMDA receptor activation (Linden, 1999; Karmarkar et al., 2002; Watanabe et al., 2002).

The PAS protocol was devised with the explicit purpose of exploiting the mechanisms of spike timing dependent plasticity in order to induce lasting changes in cortical excitability in humans (Classen et al., 2004). In practice, PAS employs median nerve stimulation in conjunction with TMS of contralateral sensorimotor cortex at different ISI to induce lasting changes in cortical excitability (up to one hour), which are demonstrated by comparing TMS-induced MEPs before and after PAS (Stefan et al., 2000). Most likely, PAS protocols

in humans have complicated effects both in sensorimotor cortex and in connected areas. Nevertheless, the effects of PAS on MEPs reverse sign just as predicted by spike timing dependent plasticity. Moreover, antagonizing NMDA receptors by dextromethorphan blocks the increase in MEP amplitude triggered by PAS, whereas intracortical inhibition is unaffected (Stefan et al., 2002). These findings suggest that the main effects of PAS on cortical excitability are likely due to LTP-like (or LTD-like) mechanisms and not to changes in the balance between inhibition and excitation (Stefan et al., 2002).

In the present study, we confirmed that at longer ISI (25 ms) PAS produces an average increase in MEPs, whereas at shorter ISI (10 ms) average MEPs are reduced. As noted by others (Fratello et al., 2006), however, the effects of PAS on MEP show great interindividual variability. Indeed, our subject-by-subject analysis found that in some subjects a 25 ms delay could potentiate MEP responses and a 10 ms delay could depress them. Unlike previous PAS studies, we also recorded TMS-evoked cortical responses by combining TMS with simultaneous hd-EEG. Our results thus provide the first direct evidence that PAS protocols cause lasting changes in TMS-evoked cortical EEG responses, presumably due to underlying changes in the excitability of cortical circuits.

Reports of increased EEG responses to peripheral stimulation after PAS protocols (Tsuji and Rothwell, 2002; Wolters et al., 2005) are consistent with our findings. By analyzing hd-EEG maps of TMS-evoked responses, we also found that the most significant changes in cortical excitability after PAS occur near the site of the TMS application over sensorimotor cortex. This site of maximal plasticity overlaid precisely the cortical region where somatosensory evoked potentials induced by median nerve stimulation overlapped with TMS-evoked EEG responses induced by TMS pulses to the motor hot spot (Fig. 9), thereby supporting the rationale behind the PAS paradigm.

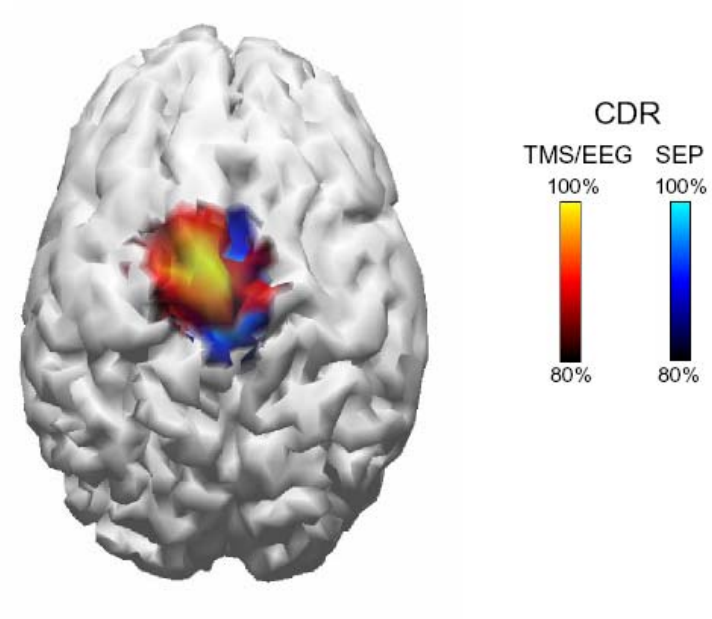

Fig. 9. Overlap between somatosensory evoked potentials (SEP) and TMS evoked potentials. Current density reconstruction (CDR) shows the top 20% of activity.

In line with our findings, a recent PAS study targeted to the somatosensory cortex localized changes in somatosensory evoked potentials to the area underlying the coil, and showed that these changes were correlated with changes in tactile discrimination (Litvak et al., 2007). A recent study using 5 Hz repetitive TMS conditioning over motor cortex also found maximal changes in TMS-evoked EEG responses near the site of stimulation (Esser et al., 2006).

Hd-EEG recordings also showed that the effects of PAS were not confined exclusively to the sensorimotor regions underlying the TMS coil. Specifically, changes in excitability extended to the contralateral sensorimotor cortex, though with the opposite sign. A possible explanation for this finding is that the arrival of somatosensory volleys triggered by median nerve stimulation and that of volleys triggered, directly or indirectly, by TMS, reach different brain regions at different time intervals, so that in some areas the predominant effect may be potentiation, and in other areas it may be depression.

Consistent with this interpretation, source localization showed that single pulse TMS targeted to the motor hot spot leads to an initial activation of the ipsilateral primary motor

cortex, next the activation spreads to the contralateral motor cortex and eventually returns to the ipsilateral motor cortex (Fig. 3). Secondary activation of connected cortical areas have also been demonstrated by combined TMS-PET and TMS-fMRI studies (Fox et al., 1997; Bestmann et al., 2003; Ferrarelli et al., 2004). Transcallosal inhibitory connections may also play a role (Ferbert et al., 1992), as suggested by fMRI studies in which unilateral hand movements were associated with contralateral activation and ipsilateral deactivation (Allison et al., 2000). It should be mentioned that PAS-induced potentiation or depression of TMS-evoked EEG responses also showed considerable interindividual variability with respect to ISI, presumably as a function of the specific set of fibers that were stimulated and their conduction delays. This variability in the site of potentiation and depression may also underlie the variability in MEP effects.

Effects of PAS on sleep SWA:

The main purpose of this study was to examine whether plastic changes induced through a PAS protocol would be reflected in local changes in sleep SWA, the best characterized marker of sleep homeostasis. In previous work, it was shown that learning a visuomotor task involving right parietal cortex led to a local increase in SWA during the first sleep cycle (Huber et al., 2004). Importantly, this increase was correlated with improvement in performance after sleep. In another study, a difficult declarative learning task led to increased sleep SWA and spindle activity at left frontal locations during post-training sleep. Again, there was a positive correlation between sleep EEG changes and changes in memory performance (Schmidt et al., 2006). Furthermore, a recent study showed that boosting slow oscillations by transcranial application of oscillating potentials has beneficial effects on the retention of hippocampus-dependent declarative memories (Marshall et al., 2006). By contrast, arm immobilization, a procedure that leads to a

deterioration in motor performance and to a decrease in somatosensory responses and MEPs, was followed by a local decrease in sleep SWA over right sensorimotor cortex (Huber et al., 2006). Finally, high-frequency TMS (5 Hz) over right premotor cortex was followed by a local increase in sleep SWA (Huber et al., 2007b). These experiments suggest that sleep SWA is affected by plastic changes in local cortical circuits and, more specifically, that SWA may increase after manipulations that favor synaptic potentiation and decrease after those that promote synaptic depression (Tononi and Cirelli, 2003, 2006). However, none of these paradigms could test directly, in the same subjects, whether opposite manipulations of cortical plasticity during wakefulness would lead to corresponding changes in SWA during sleep.

In this study, regional changes in cortical excitability triggered by PAS at different ISIs were compared to subsequent changes in sleep SWA within the same subject. Due to the interindividual variability of the effects of different PAS ISIs, subjects were grouped on the basis of the actual changes observed in TMS-evoked EEG responses (potentiation or depression) rather than on the basis of ISIs (25 or 10 ms). The results indicate that when PAS produced an increase in cortical excitability during wakefulness, as indicated by an increased amplitude of TMS-evoked EEG responses, it was followed by a local increase in sleep SWA. By contrast, when PAS produced a decrease in cortical excitability during wakefulness, as indicated by a decreased amplitude of TMS-evoked EEG responses, it was followed by a local decrease in sleep SWA. Thus, there is a direct relationship between the sign of plastic changes induced by PAS protocols and that of SWA changes during subsequent sleep. Notably, the only difference between the two PAS protocols is a 15 ms shift in the pairing between somatosensory and TMS stimuli (for a total of just 90 pairings). Thus, it is difficult to see how the observed differential modulation of sleep SWA could result from aspecific effects of TMS or somatosensory stimulation. In

particular, it is unlikely that the effects on sleep SWA can be accounted for by difference in the extent of neuronal activation ("use"), the depletion of energy resources, or harmful consequences on the stimulated tissue, since the number of TMS pulses and somatosensory stimuli received by each subject was the same in both sessions.

Instead, it is likely that the differential effects are due to differential plastic changes induced in local cortical circuits, as predicted by spike timing dependent plasticity. The present results also show that changes in the amplitude of TMS-evoked EEG responses during wakefulness were positively correlated with changes in local SWA during subsequent sleep. This positive correlation was strongest in the frequency range of the cortical slow oscillations (< 1 Hz, Fig. 8), which are most prominent during slow wave sleep (Steriade et al., 1993). Furthermore, the spatial location of changes in cortical excitability corresponded with that of changes in sleep SWA. Depending on the PAS interval, the amplitude of TMS evoked cortical responses increased or decreased over the stimulated sensorimotor cortex compared to the sham condition, and behaved oppositely on the unstimulated sensorimotor cortex. Sleep SWA followed the same pattern, showing a positive correlation with TMS evoked cortical responses over the stimulated cortex and a negative correlation over the unstimulated cortex (Fig. 7).

Possible mechanisms underlying the link between neuronal plasticity and sleep SWA:

These findings provide important new evidence for a tight link between plastic processes and sleep, confirming and expanding previous studies in humans (Huber et al., 2004; Huber et al., 2006; Schmidt et al., 2006; Huber et al., 2007b). In particular, they indicate that local changes in sleep SWA can reflect both increases and decreases in cortical excitability, and suggest that sleep SWA is sensitive to neural plasticity above and beyond

possible changes in activity (the amount of induced activity being presumably similar for both PAS protocols).

The link between waking plasticity and sleep SWA is also supported by studies in animals. For example, in mice dark rearing results in a reversible decrease of SWA over visual cortex (Miyamoto et al., 2003). Moreover, adult rats that showed more exploratory activity (for the same duration of wakefulness) had a larger increase in SWA during subsequent sleep (Huber et al., 2007a). Importantly, the amount of exploration and the increase in SWA were positively correlated with the induction of BDNF in the cerebral cortex. Thus, under physiological conditions BDNF may be involved in mediating cortical plastic changes (in this case synaptic potentiation) that subsequently lead to changes in sleep SWA. Indeed, local infusions of BDNF in the cerebral cortex of rats during wakefulness also cause a local increase in SWA during subsequent sleep, whereas BDNF antagonists or anti-BDNF antibodies block SWA increases (Faraguna et al., submitted). Conversely, chronic lesions of the noradrenergic system, which reduce the expression of BDNF and other molecular markers of synaptic potentiation during wakefulness, blunt the SWA response during sleep (Cirelli et al., 2005).

The actual mechanisms by which changes in synaptic efficacy can produce changes in sleep SWA have yet to be explored experimentally. However, large scale computer simulations of slow wave sleep in thalamocortical circuits demonstrate that an increase in the strength of excitatory corticocortical connections is sufficient to produce a marked increase in sleep SWA, and vice versa (Esser et al., in press). The simulations also show that the effects of synaptic strength on SWA and other slow wave parameters are mediated by changes in the amplitude of single-cell oscillations due to increased excitatory postsynaptic potential size, in the dynamics of network synchronization due to increased neuronal coupling, and in the rate of neuronal recruitment and decruitment. Consistent with

this interpretation, the coherence of slow oscillations also increases after learning tasks in humans (Molle et al., 2004). Nevertheless, it should be emphasized that the local regulation of sleep SWA is compatible with other mechanisms and alternative accounts are possible. For example, use-dependent changes in the efficacy of inhibitory circuits, accumulation or depletion of substances altering neuronal excitability, or alterations of intrinsic excitability may have similar consequences for the generation and synchronization of sleep slow waves or may at least contribute to the observed effects.

Altogether, these studies are consistent with the notion that SWA, and presumably sleep need, are increased by events leading to synaptic potentiation and decreased by events leading to synaptic depression, and that their regulation can occur locally in cortical circuits (Tononi and Cirelli, 2003, 2006).

Visuomotor learning and local slow wave activity changes during sleep after 12 hours

Introduction

Recent research concerning sleep and its function has brought about several findings supporting a close link between sleep and learning. Sleep following learning has shown to promote consolidation of both declarative and non declarative memories (Karni et al., 1994; Gais et al., 2000; Maquet, 2001; Gais and Born, 2004; Walker and Stickgold, 2004; Stickgold, 2005) as well as learning has shown to produce changes in sleep duration and intensity (Meier-Koll et al., 1999; Gais et al., 2002; Schmidt et al., 2006).

SWA (EEG power density between 0.5 and 4.5 Hz) during non-REM sleep seems to be the most sensitive marker of sleep intensity (Borbély and Achermann, 2000), increasing depending on previous wakefulness and gradually dissipating during sleep. Several studies illustrated a close relationship between sleep intensity and plastic changes in local cortical circuits, showing that SWA can be regulated following cortical plasticity. In particular, a study showed how learning induced by performing a visuomotor task right before sleep time brings about local SWA increase (Huber et al., 2004). The physiological mechanisms underpinning this kind of learning is likely to be explained by plastic changes increasing synaptic efficacy in local cortical circuits. The increase in synaptic efficacy, in turn, is the neurophysiological mechanism thought to be responsible for SWA regulation. By exploiting a task whose plastic effects have shown the involvement of a circumscribed area over the right parietal cortex (Brodmann areas 7 and 40) (Ghilardi et al., 2000), it was possible to compare the topographic representation of plastic changes induced by the task and of the subsequent SWA modification in sleep, showing a large topographical overlap.

Furthermore, the positive correlation between local increase in SWA and performance improvement at the task after sleep is consistent with the idea of a beneficial effect of sleep over learning (Tononi and Cirelli, 2003). These data nicely illustrate a close link between learning process and sleep regulation and are referred to the first sleep cycle only, where sleep intensity has been proved to be at its peak (Borbély and Achermann, 2000).

With the present whole night hd-EEG study we wanted to investigate SWA regulation dynamics across sleep cycles exploiting the same visuomotor learning task. Spontaneous waking EEG recordings have been also performed in order to keep track of the potential learning trace produced by the execution of the task during the 12 hours elapsing between task execution and sleep recording.

Finally, a correlation between the SWA change and the modification in task performance after sleep will validate the close relationship between sleep behavior and learning brought about by previous literature.

Methods

Design:

Sixteen healthy right-handed subjects (aged 20-38 years, mean age 28.4 years, 9 men) gave informed consent to participate in the study, which was approved by the local ethics committee. They presented no history of psychiatric or neurological disease or sleep disorders. Two of them were excluded from sleep EEG analysis due to technical reasons. The sleep data presented here are thus from 14 subjects (aged 20-38 years, mean 28.4 years, 8 men). A 256-channel EEG system (Electrical Geodesic, Inc.) was used for all EEG recordings. The design of the experiment, consisting of two conditions separated by at least one week, is depicted in Figure 1.

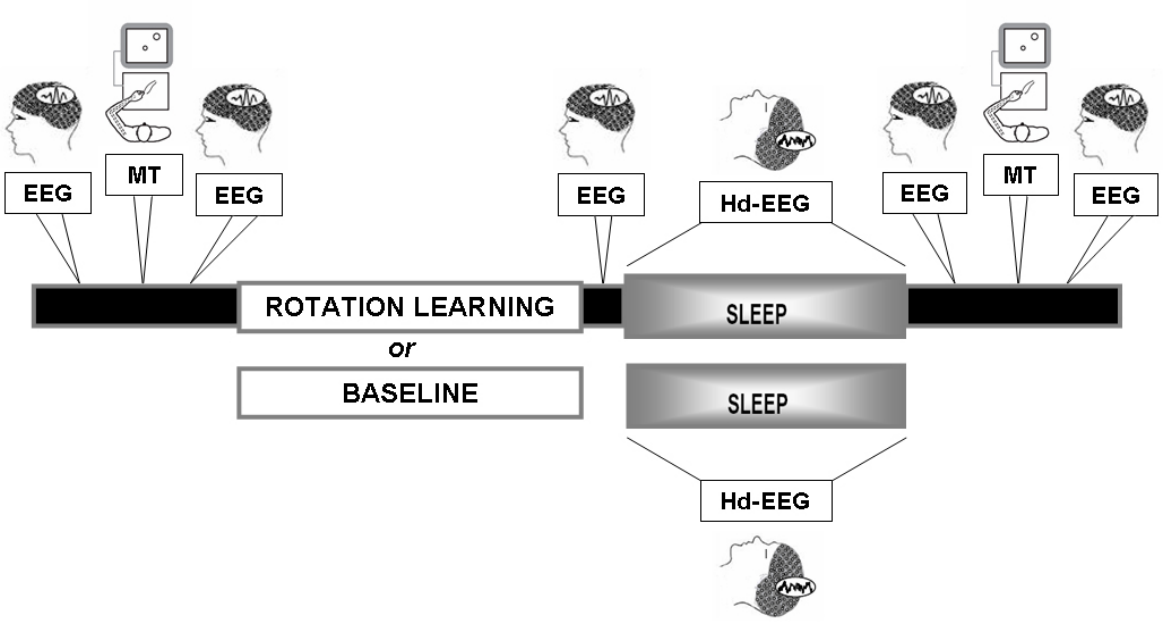

Fig. 1. Study design. During the rotation learning session we collected spontaneous waking EEG before and after the motor learning task the first morning, at night before subjects went to bed and before and after the re-test the day after in the morning. The two conditions, rotation learning and baseline, were separated by at least a week and were counterbalanced in order. The sleep recording took place in a isolated sound-proof darkened room, and high-density EEG (hd-EEG) was used in both conditions.

1. In the rotation learning condition, the subjects completed a one-hour motor learning task in the morning. Thereafter, they were allowed to leave the laboratory and perform daily activities. In the night, they returned to the laboratory, where they slept undisturbed while EEG was recorded. The following morning, half an hour after waking up, subjects completed a short re-test of the motor task. In the learning condition, we also recorded spontaneous waking EEG activity a) before and after the learning task in the morning, b) before the subjects went to bed in the evening, and c) before and after the re-testing the following morning.
2. In the control condition, we recorded sleep EEG in the laboratory after a day of normal activities. Subjects arrived in the laboratory about one hour before their usual bedtime. Upon awakening, after electrode removal, they were free to leave the laboratory.

Starting one week before the experiment, we instructed subjects to maintain their usual sleep-wake schedule. During the experiments, they were then were allowed to go to bed and wake up at their habitual times. In both conditions, the subjects were required to avoid daytime napping, and their compliance was confirmed by wrist actigraphic recordings (Actiwatch 64, MiniMitter). In the rotation learning condition, the timing of the motor task in the morning was adjusted so that each subject had approximately 12 hours of waking between completing the motor task and going to sleep. The order of the two conditions was counterbalanced across subjects.

Rotation adaptation learning task:

During rotation adaptation learning task subjects moved a handheld cursor on a digitizing tablet, executing out-and-back target reaching movements from a central starting point to one of eight targets (distance of 4.2 cm) displayed on a computer screen together with the cursor position. An opaque shield prevented subjects from seeing their arm and hand at all times.

Targets were randomly highlighted at regular 1-s intervals and, unbeknown to the subjects, the cursor position was rotated clockwise or anticlockwise (counterbalanced) relative to the hand position by a fixed angle (Fig. 2). Subjects performed the task at different rotation angle steps (15° to 60°) progressively and unconsciously adapting their movements to the systematic imposed rotations on the perceived cursor trajectory.

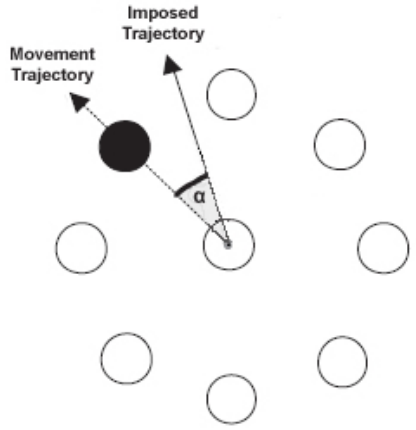

Fig. 2. Rotation learning task. The imposed trajectory is resulting from the rotation angle (α) systematic control. Subjects need to adjust their movement trajectory to counterbalance the effect of the imposed rotation. The figure depicts a clockwise imposed rotation.

Sleep recordings:

Sleep EEG recordings for the whole night were acquired at 500 Hz with scalp impedances kept below 50 KOhm and referenced to the vertex. Signals were off-line band-pass filtered between 0.5 and 50 Hz, downsampled to 128 Hz, artifact rejected and average-referenced. Artifact removal was performed by means of visual and semi-automatic detection based on the power in 0.75-4.5 Hz and 20-40 Hz bands (Huber et al., 2000). Sleep stages were visually scored for 20-sec epochs according to standard criteria (Rechtschaffen and Kales, 1968). For a quantitative analysis of the sleep EEG, 71 electrodes (face and neck electrodes) out of the original 256 of the net were excluded and spectral analysis of consecutive 20-sec epochs was performed (FFT routine, Hanning window, averages of five 4-s epochs) for all included channels after subsequent bad channels rejection. Significant topographical differences in SWA (0.75-4.5 Hz) EEG power during the first 30 min SWS sleep of each of the first three cycles (all subjects presented three complete sleep cycles) between the two conditions were assessed by statistical nonparametric mapping (SnPM) using the suprathreshold cluster test (Nichols and Holmes, 2001; Huber et al., 2004). This method takes advantage of the actual data distribution and accounts for multiple comparisons testing in hd-EEG recordings. For the topographical display of EEG activity we used the topoplot function of the Matlab (The MathWorks, Natick, MA) EEGLab

toolbox (Delorme and Makeig, 2004). In all other comparison, we used appropriate designs of the analysis of variance (ANOVA), and post hoc tests (t-tests, Tukey's HSD) were applied to determine the sources of the significant effects. The level of significance was set at p=0.05. Statistical analyses were computed both with Matlab and STATISTICA (Statsoft) statistical software.

Waking EEG recordings:

Waking EEG recordings were acquired during rotation learning session only. Recordings were performed during an auditory oddball task execution, so to control for vigilance levels. Subjects had to count for odd tones and to report their number after each of four sessions lasting three minutes. Signals were sampled at 1000 Hz with scalp impedances kept below 50 KOhm and referenced to the vertex. Signals were off-line band-pass filtered between 0.5 and 50 Hz, artifact rejected and average-referenced. Quantitative analysis procedures were the same as in sleep recordings (see above). Power spectra for each electrode was here then decomposed into standard EEG frequency bands (delta (0.75-4.5 Hz), theta (4.5-8 Hz), alpha (8-12 Hz), sigma (12-15 Hz), beta (15-25 Hz), gamma (25- 40 Hz)) and topographical differences between recordings were assessed for each frequency band separately by statistical nonparametric mapping (SnPM) using the suprathreshold cluster test (Nichols and Holmes, 2001; Huber et al., 2004). As for the sleep recordings, topographical display of EEG activity was performed using the topoplot function of the Matlab (The MathWorks, Natick, MA) EEGLab toolbox (Delorme and Makeig, 2004).

Preliminary Results

SWA local changes:

We recorded high-density EEG during sleep 12 hours after learning task in the rotation learning condition and at usual bedtime in the baseline condition. All subjects had at least three complete sleep cycles in both conditions. The two conditions did not differ with respect to sleep latency, total sleep time, or the amount of NREM or REM sleep, thus assuring that the two conditions were fully comparable (Table 1).

	Rotation Learning	Baseline
Total Sleep Time (min)	440,5 ±14	418,7 ±18,8
Sleep Latency (min)	16 ±2,4	20,5 ±5,1
% n-REM 1st Cycle	78,9 ±3,4	77,6 ±1,9
% REM 1st Cycle	18,5 ±2,1	20,6 ±1,6
% n-REM 2nd Cycle	71,5 ±3,9	72 ±4,6
% REM 2nd Cycle	28,4 ±3,9	25,1 ±3,4
% n-REM 3rd Cycle	72,1 ±2,6	78,5 ±3,8
% REM 3rd Cycle	27,8 ±2,6	21,3 ±3,8

Table 1. Sleep features in the two conditions for each of the three cycles. Mean ± s.e.m. are reported. T-tests have been carried out to test for significant differences but always failed to reach significance.

Furthermore, the temporal evolution of SWA during NREM sleep, computed across the three cycles, was similar between the 2 nights and showed a significant decline in the amount of SWA from the first to the second and third sleep cycle (Fig. 3), thus following the homeostatic component behavior in the two-process model of sleep regulation (Borbély

and Achermann, 1999) as proposed by the hypothesis (Tononi and Cirelli, 2003). Average power spectra of consecutive 20-s epochs during NREM sleep (stages 3 and 4) in the 0.75-4.5 Hz frequency range calculated for each electrode showed that that SWA was prevalent in anterior regions (see Fig. 5A, C, E) in both conditions. Also, average power spectra in the 12-15 Hz frequency range showed that sleep spindle activity was prevalent over the central regions (data not shown). These topographic patterns were reproducible across cycles, nights and subjects in accordance with previous studies (Werth et al., 1997; Finelli et al., 2001).

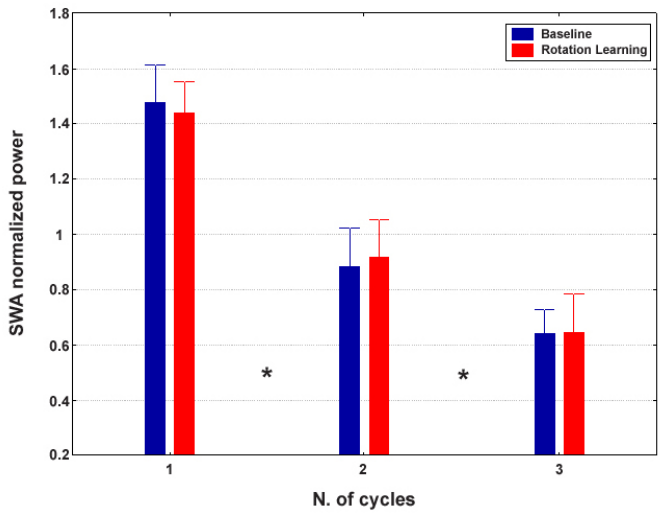

Fig. 3. SWA evolution across cycles. SWA amount for each cycle is normalized for the mean of the three cycles in the corresponding condition. The two conditions show the same amount of SWA for each cycle. Asterisks indicate statistical significant differences across cycles (p<0.005; HSD post-hoc test) showing a significant decrease in SWA amount in both conditions.

In order to highlight local differences, we contrasted the two condition for the first 30 minutes of non-REM sleep of each cycle separately (Fig. 4). These comparison showed a significant local increase of SWA only after rotation learning in the first 30 minutes of non-REM sleep in the second cycle over a cluster of five right central electrodes (Fig. 4D, electrodes 143, 154, 155, 163, 184, SnPM, p<0.05).

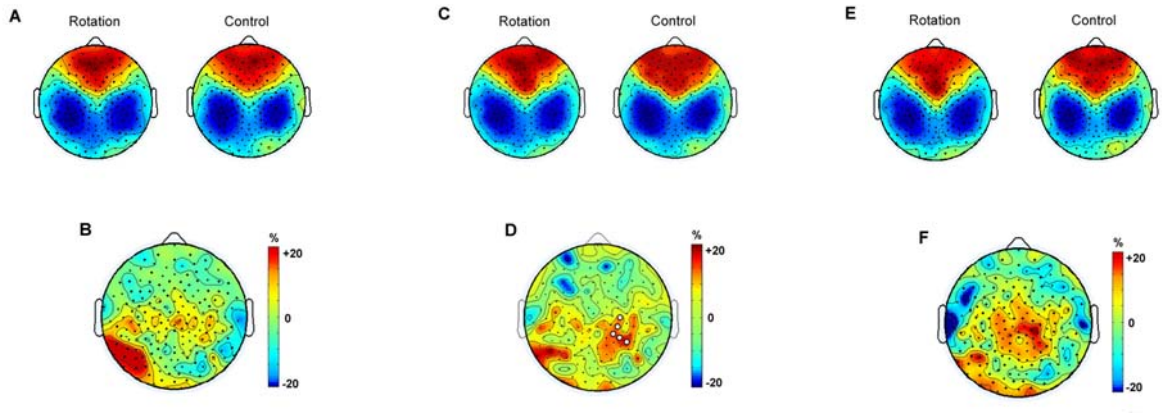

Fig. 4. Local SWA changes after rotation learning. **A, C and E:** Topographic distribution of SWA in the two condition for each of the three cycles. Average EEG power density at 0.75-4.5 Hz (n=14 subjects) for the first 30 minutes of non-REM sleep for each cycle. Values were normalized by total power for the recording, color coded, plotted at the corresponding position on the planar projection of the scalp surface, and interpolated (biharmonic spline) between electrodes (dots). **B, D and F:** Topographic distribution of the ratio of SWA between the rotation learning condition and baseline condition for the three cycles. White dots indicate significant differences (SnPM, $p<0.05$).

For an anatomical localization of these local changes, all electrodes were digitized and co-registered with the subjects' magnetic resonance images by means of an optical tracking system (Nexstim Ltd, Helsinki, Finland). The electrodes showing a significant increase of SWA were then projected onto the brain. The cluster of five electrodes showing increased SWA for the first 30 minutes of the second cycle after rotation learning was localized over the right parietal lobe encompassing Brodmann areas 40 and 7.

For reference purpose, electrodes 143, 155 and 184 project onto Brodmann area 7 (Talairach coordinates: x= 29, y= -63, z= 55; x= 31, y= -71, z= 47 and x= 33, y= -75, z= 47, respectively), and electrodes 154 and 163 project onto Brodmann area 40 (Talairach coordinates: x= 43, y= -57, z= 51 and x= 51, y= -37, z= 56, respectively).

In order to assess frequency specificity for these local changes we computed a power spectral density analysis showing a significant increase for low frequencies in the first 20 minutes of non-REM sleep in the second cycle of the rotation learning condition. The increase was especially evident within the low delta band (< 2 Hz) and at frequencies

corresponding to the slow oscillation (< 1 Hz). Also the fastest frequencies of the sigma range (13.25-15.5 Hz) with a peak at 14 Hz showed a significant increase (Fig. 5).

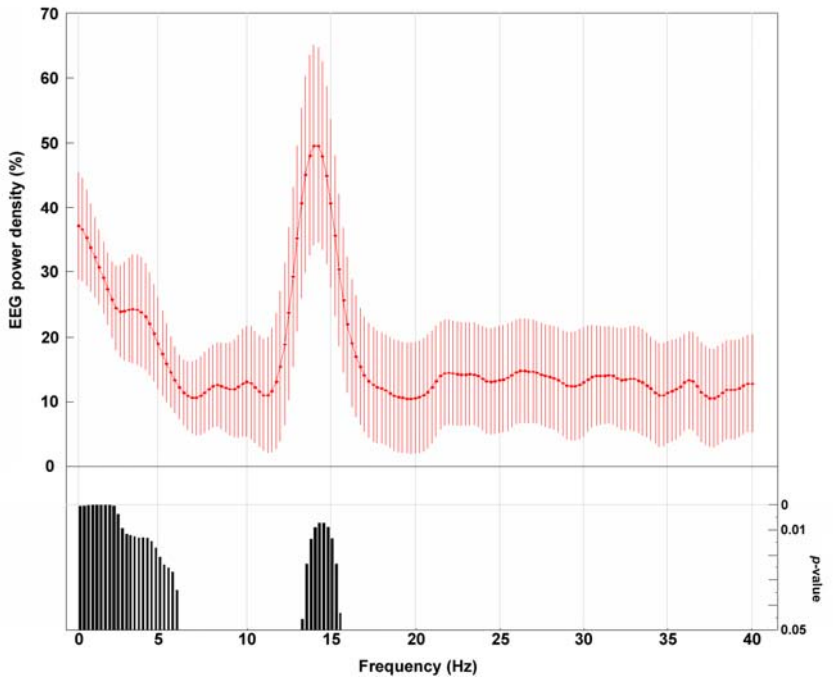

Fig. 5. Frequency specificity of power changes. EEG power spectral density for the first 20 min of non-REM sleep during the second cycle. Values represent the percentage change of the rotation learning condition with respect to the baseline one (mean ± s.e.m. for 0.25-Hz bins, n=14 subjects). Red curve represents the average power change across subjects for the electrode yielding the peak SWA increase for each subject in the significant cluster. Bottom bars indicate frequency bins for which power in the rotation learning condition differed significantly from the baseline condition (paired t -test).

Discussion

Main goal of the present work was to assess local SWA changes due to a visuomotor learning task performed 12 hours before sleep time. A previous PET experiment exploited the same task showing an activation of Brodmann areas 7 and 40 in a rotation condition compared with a no-rotation one (Ghilardi et al., 2000). Moreover, a recent experiment investigated the changes in SWA -a well established synaptic plasticity marker- after the

rotation adaptation task, showing a local increase in SWA in the same two areas (Huber et al., 2004). Thus this SWA regulation during sleep seems to be related to neural processes specific to rotation adaptation involving right parietal areas. Both areas 40 and 7 indeed receive converging visual and proprioceptive inputs, and are involved in processing sensory information relevant for spatial attention (Cohen and Andersen, 2002). Such a task is therefore suitable for inducing local changes in synaptic efficacy in order to test homeostatic response in SWA. The direction of the above illustrated changes (increase in SWA) is then compatible with a LTP-like phenomenon occurring locally and involving the cortical circuits proved to be linked with the execution of the rotation adaptation learning task. In the present work our subjects performed the task 12 ours prior to sleep and a preliminary analysis on sleep data showed an increase in SWA during the first 30 minutes of the second cycle, thus confirming previous findings about local changes in synaptic efficacy and SWA regulation during sleep.

Considering this study as the first attempt to investigate systematically the whole night dynamics of local sleep changes following a learning task execution, we can reasonably presume that the long time interval elapsed in between task execution of and the sleep recording is likely to lie beneath the absence of local changes in SWA during the first sleep cycle, when sleep intensity has been proved to be at its peak (see Fig. 3). Synaptic downscaling during the first cycle, by acting globally on all the plastic changes that have taken place during wakefulness, allows the considerable potentiation of cortical circuits involved in task execution to be visible and significant during the second cycle.

Although lacking of the necessary pool of evidence, and therefore speculative at best, this interpretation is in line with the Synaptic Homeostasis Hypothesis predictions about signal to noise ratio increase following downscaling process.

Concerning the power spectral density analysis, they showed a significant specificity for low frequencies in the delta range, especially for frequencies within the slow oscillation range which have been demonstrated to be most prominent during slow wave sleep (Steriade et al., 1993). This finding supports the idea proposed by large scale computer simulations of slow wave sleep in thalamocortical circuits (Esser et al., in press). As already mentioned, these simulations showed that the effects of synaptic strength on SWA and other slow wave parameters are mediated by changes in the amplitude of single-cell oscillations due to increased excitatory postsynaptic potential size, in the dynamics of network synchronization due to increased neuronal coupling, and in the rate of neuronal recruitment and decruitment. Importantly, consistent with this interpretation, the coherence of slow oscillations has also been proved to increase after learning tasks in humans (Molle et al., 2004). Nevertheless, as already mentioned in the previous study (see Discussion section), it should be emphasized that the local regulation of sleep SWA is compatible with other mechanisms and alternative accounts are possible.

Moreover, the ongoing analyses concerning behavioral task performance, and in particular the comparison between the two morning session (learning and re-test) together with the correlation between the illustrated increase in SWA and the potential increase in performance after sleep, will be needed to confirm the specific predictions brought about by the Synaptic Homeostasis Hypothesis concerning the beneficial effects of downscaling process over learning. If confirmed, these data would be in line with previous findings exploiting the same learning task (Huber et al., 2004).

Finally, the ongoing analyses on spontaneous waking EEG activity will allow the investigation of possible changes in EEG spectral content topographically consistent with SWA changes in sleep, so to keep track of the local changes in synaptic efficacy throughout the day.

SECTION 3:

CONCLUSION AND FUTURE DIRECTIONS

Conclusion

Aim of this work was to investigate the relationship between cortical plastic changes at synapses and sleep regulation by means of high density EEG (hd-EEG) technique.

Sleep EEG slow wave activity (SWA; 1-4.5 Hz) has been taken into account as a suitable marker of sleep intensity thought to be strictly related to plastic processes in cortex.

In the first study, a paired associative stimulation (PAS) TMS protocol, allowed a highly standardized manipulation of synaptic plasticity, capable of inducing both long term potentiation (LTP) and long term depression (LTD) underpinning spike timing dependent plasticity mechanisms. Quantitative analysis of sleep EEG showed local changes in SWA topographically consistent with TMS induced changes in synaptic efficacy.

In the second study, sleep SWA changes have been investigated exploiting a visuomotor learning task increasing synaptic efficacy in a precise local cortical circuit. Quantitative analysis of sleep EEG, again, showed a local increase in SWA circumscribed to the same cortical area where plastic changes occurred.

By referring to the theoretical framework offered by the Synaptic Homeostasis Hypothesis (Tononi and Cirelli, 2003), both studies thus showed a tight link between induced changes in synaptic efficacy and subsequent sleep regulation resulting in slow wave activity (SWA) power changes at the EEG level.

As predicted by the hypothesis, local regulation of sleep intensity suggests a role for sleep at the cellular level, and local SWA induction triggered by a learning task (as reported in the second study) supports previous findings suggesting that local plastic changes associated with learning may be involved, directly or indirectly in the sleep regulation process.

The actual mechanisms by which changes in synaptic efficacy can produce changes in sleep SWA have yet to be explored experimentally.

Despite large scale computer simulations of slow wave sleep in thalamocortical circuits demonstrating that changes in the strength of excitatory corticocortical connections produce a marked increase in sleep SWA, local regulation of sleep SWA is compatible with other mechanisms and alternative accounts are possible. For example, use-dependent changes in the efficacy of inhibitory circuits, accumulation or depletion of substances altering neuronal excitability, or alterations of intrinsic excitability may have similar consequences for the generation and synchronization of sleep slow waves or may at least contribute to the observed effects.

Therefore, further research is needed in order to enlighten the specific mechanisms involved in such a process, particularly at the molecular level.

Future directions

My research on this topic will continue investigating changes in sleep SWA following plastic changes induced by rehabilitation of cognitive functions in stroke patients. Functional recovery after stroke is indeed strongly dependent on the adaptive plasticity of the cerebral cortex.

Thus, referring to synaptic homeostasis hypothesis predictions, it should be possible to investigate plastic changes underlying functional recovery by means of sleep hd-EEG, so to collect further evidence supporting the idea that sleep is the mechanism favoring synaptic homeostasis in a condition where cortical plasticity is undoubtedly involved.

Preliminary results from the first patient recorded after one day of intensive speech therapy are the only one available so far, but are reported here (Fig.1 and 2) because they offer the chance to look at the potential of this investigation.

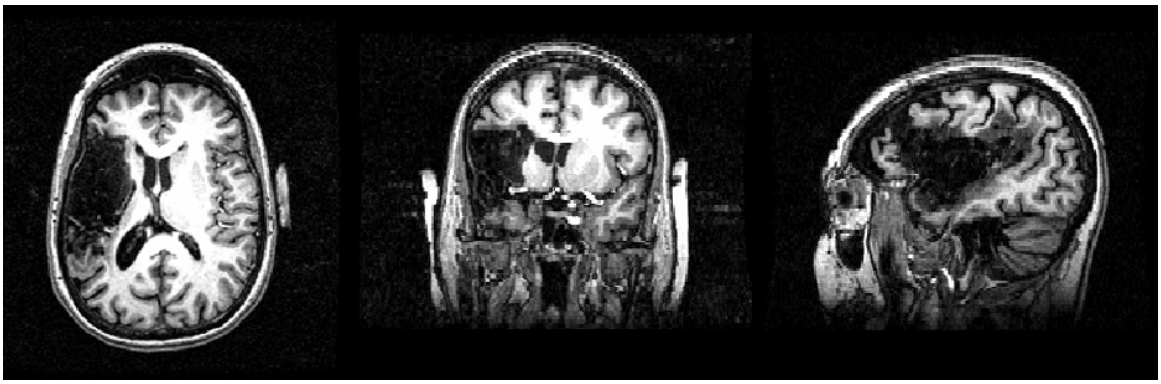

Fig. 1. Axial, coronal and sagittal magnetic resonance images of the patient's brain. Left hemispheric 9 month old ischemic vascular lesion. Functional deficit circumscribed to non-fluent aphasia.

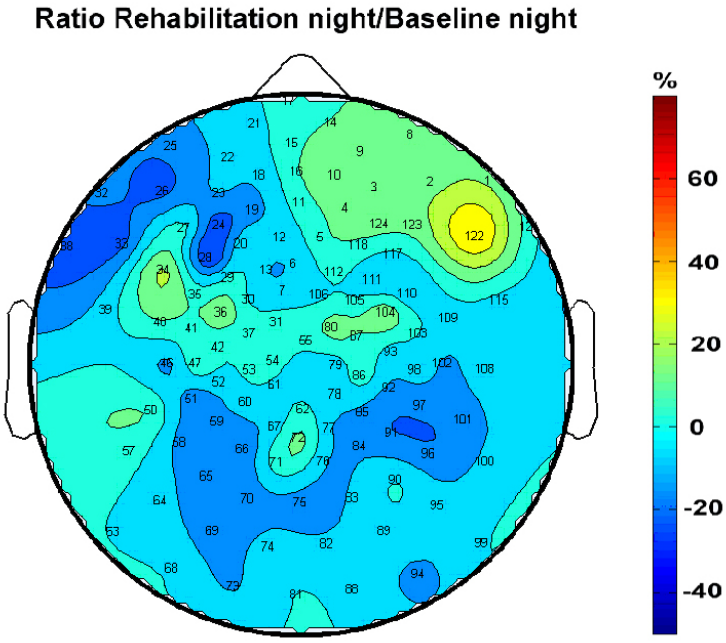

Fig. 2. Topographic distribution of the ratio of SWA between the night following rehabilitation (oral motor observation-execution matching and imitation) and a previously recorded baseline night. Electrode number 122 showed the most prominent increase in SWA (~30%). Digitization and co-registration of electrode with magnetic resonance image indicates that electrode 122 projects over the right inferior frontal gyrus (Brodmann's area 44, 45, 47) corresponding to the contralateral homologue of Broca's area.

SECTION 4:

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