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The effects of γ-hydroxybutyrate, baclofen and $GABA_B$ receptors on brain activity and sleep in mice and humans

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par

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The effets de y-hydroxybutyrate, baclofen and $GABA_B$ receptors on brain activity and sleep in mice and humans

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"If sleep does not serve an absolutely vital function, then it is the biggest mistake the evolutionary process has ever made."

Allan Rechtschaffen

1. Acknowledgments

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2. Abstract

Currently, there is an increased interest in γ-hydroxybutyric acid (GHB) and its effects on sleep. This compound, sometimes referred to as 'rape drug', was recently approved as a treatment for the sleep disorder narcolepsy. Although several studies suggest that GHB induces slow-wave sleep duration and improves sleep quality by increasing EEG slow-wave activity, others question its ability to induce physiological sleep. GHB's mechanism of action is still unclear, although *in vivo* and *in vitro* it seems to act at high doses as a low-affinity agonist of $GABA_B$ receptors. Furthermore, the role $GABA_B$ receptors play in sleep and the electroencephalogram (EEG) is largely unknown.

The aim of this project was therefore to investigate the effects of GHB on sleep and EEG, the involvement of $GABA_B$ receptors in mediating these effects, as well as the intrinsic role of each $GABA_B$ receptor subunit in the regulation of sleep. Thus, we administered GHB and baclofen (BAC, a high-affinity agonist at $GABA_B$ receptor) to mice lacking the different $GABA_B$ receptor subunits and to healthy human volunteers.

Our results, both in mice and humans, showed that GHB produced slow waves exclusively through the stimulation of $GABA_B$ receptors, but did not induce physiological sleep necessary to reduce sleep need and to increase cognitive performance. Unlike GHB, BAC affected the homeostatic regulation of sleep (sleep need) and induced a delayed hypersomnia. Finally, $GABA_B$ receptor and its subunits seem to play an important role in sleep and in particular its circadian distribution.

3. Résumé

L'acide γ-hydroxybutyrique (GHB) suscite actuellement un grand intérêt quant à son effet sur le sommeil. Cette substance, également appelée 'la drogue du violeur', a été récemment acceptée en tant que traitement de la narcolepsie, une maladie du sommeil. Bien que plusieurs études suggèrent que le GHB augmenterait la quantité et la qualité du sommeil lent (caractérisé par une grande densité d'ondes lentes sur l'électroencéphalogramme (EEG)), d'autres mettent en doute sa capacité à induire du sommeil physiologique. Son mécanisme d'action est encore indéterminé bien que, *in vivo* et *in vitro,* il semblerait agir à haute dose comme agoniste à faible affinité des récepteurs $GABA_B$. De plus, le rôle joué par les récepteurs $GABA_B$ sur le sommeil et l'EEG est très peu connu.

Le but de ce travail a été d'étudier les effets du GHB sur le sommeil et l'EEG, l'implication des récepteurs $GABA_B$ dans ces effets, ainsi que le rôle intrinsèque de chaque sous-unité des récepteurs $GABA_B$ sur le sommeil. Nous avons administré du GHB, du baclofen (BAC ; un agoniste à haute affinité des récepteurs $GABA_B$), et un placebo à des souris n'exprimant pas l'une ou l'autre des sous-unités des récepteur $GABA_B$ ainsi qu'à des volontaires sains.

Nos résultats chez l'homme et chez la souris ont montré que GHB produisait des ondes lentes exclusivement par l'intermédiaire des récepteurs $GABA_B$, mais n'induisait pas de sommeil physiologique nécessaire à la réduction du besoin de sommeil et à l'augmentation de la performance cognitive. Au contraire de GHB, BAC a affecté la régulation homéostatique du sommeil (besoin de sommeil) et induit une hypersomnie retardée. Finalement, les récepteurs $GABA_B$ et ses sous-unités semblent jouer un rôle très important dans le sommeil et en particulier sur sa distribution circadienne.

4. Résumé pour un large public

L'acide γ-hydroxybutyrique (GHB) semble jouer un rôle très important dans le sommeil. Cette substance, également appelée 'drogue du violeur', a été récemment acceptée en tant que traitement de la narcolepsie, une maladie du sommeil. Bien que le mécanisme d'action de GHB ne soit pas encore élucidé, il semble activer les récepteurs $GABA_B$ qui exercent une action inhibitrice dans le cerveau des mammifères. De plus, l'implication des récepteurs $GABA_B$ dans le sommeil est encore méconnue. C'est pourquoi nous avons étudié les effets sur le sommeil du GHB, des récepteurs $GABA_B$ et leur interaction chez la souris et chez l'homme.

Les récepteurs $GABA_B$ jouent un rôle important dans le sommeil et notamment sur sa composante circadienne. En effet, la perte de ces récepteurs chez la souris conduit à une distribution très différente du sommeil et de l'éveil sur 24h. Nous avons également montré que le GHB agissait uniquement à travers les récepteurs $GABA_B$ pour modifier l'activité électrique générée par le cerveau et le comportement de la souris. Finalement, bien que le GHB augmente les ondes lentes du cerveau (caractéristique du sommeil profond), il ne semble pas induire du sommeil physiologique, comme suggéré par d'autres études scientifiques, ni chez la souris, ni chez l'homme.

5. Introduction

Why do we sleep? This seemingly simple question turns out to be much more complex in reality. Sleep has fascinated poets, writers as well as scientists since the dawn of time. However, still today, sleep remains an intriguing mystery. In his book "Why do we sleep?" James Horne claimed: "despite 50 years of research, many think that the only thing we can say about sleep is that it prevails over drowsiness"[1].

Sleep is a natural, periodically recurring state of rest, during which there is a decrease of responsiveness to external stimuli and a minimal processing of sensory information. All mammals and birds as well as certain invertebrates spent a substantial portion of their life in this behavioral state. Given the conservation of this behavior throughout evolution and the fact that lack of sleep or disturbed sleep lead to strong negative consequences on health and performance, and even death[2,3], sleep appears to fulfill a vital function comparable to feeding and reproduction. Surprisingly, the neurobiology and the molecular basis of sleep have attracted little attention until recently. Modern sleep research is usually dated back to the early 1950s when rapid-eye movements (REMs) and muscle atonia during sleep have been discovered leading to the characterization of a new vigilance state; i.e. REM sleep [4]. Since then, substantial progress has been achieved in our understanding of the neurobiology of expression and regulation of sleep. Lesion and pharmacological studies show that sleep and wakefulness are controlled by multiple neuronal systems using different chemical neurotransmitters such as glutamate, acetylcholine, noradrenaline, dopamine, serotonin, histamine, adenosine, orexin, and γ-aminobutyric acid (GABA)[5]. However, the precise mechanisms by which each neuronal system regulates sleep and wakefulness remain to be discovered[6]. The investigation of these mechanisms is crucial not only to better understand the function of sleep but also to improve the treatment of sleep disorders.

Indeed, pathophysiology of insomnia, narcolepsy, idiopathic hypersomnia and many other sleep disorders are still unclear and their treatments far for optimum.

A first aim of the present work is to improve our understanding of the mechanism by which the GABAergic system regulates sleep. This system is the predominant inhibitory system in mammalian brain and is known to play a key role in sleep. Two distinct types of GABAergic receptors, $GABA_A$ and $GABA_B$ receptors, modulate respectively the fast and slow inhibition of the neuronal GABAergic target systems. The implication of $GABA_A$ receptors and their ligands in sleep regulation are well studied and the majority of sleep-promoting drugs are $GABA_A$ receptors modulators. However, very little is known about the effects of $GABA_B$ receptors and their ligands on sleep and electoencephalographic (EEG) activity. Thus, the first aim of my PhD project was to investigate the effects of $GABA_B$ receptor subunits and their role in sleep regulation and EEG activity. Furthermore, as a pharmacist, I have a major interest in pharmacology and I was particularly drawn to understand sleep and EEG effects as well as the mechanism of action of a recent drug used to treat narcolepsy; i.e. γ-hydroxybutrate (GHB). Among the potential mechanisms of action of GHB, there is its ability to stimulate $GABA_B$ receptors. Therefore, I also investigated the role of $GABA_B$ receptors in the response to GHB by using animal and human models, and by comparing GHB with a high-affinity agonist of $GABA_B$ receptors, baclofen (BAC). This work may contribute to the improvement of narcolepsy treatment, to a better understanding of the pathophysiology of narcolepsy, and bring some new insights into the mechanisms by which the GABAergic system regulates sleep.

Before the experimental work is introduced, the neurobiology and regulation of vigilance states, sleep pharmacology with special focus on GHB, and scientific knowledge about $GABA_B$ receptors will be summarized.

5.1. Vigilance states:

5.1.1. Organization

Three major vigilance states are easily distinguishable in all normothermic mammals and birds: Wakefulness, Non-Rapid Eye Movement Sleep (NREMS), Rapid-Eye Movement Sleep (REMS). According to a simple behavioral definition, sleep is a reversible behavioral state of perceptual disengagement from and unresponsiveness to the environment[7]. More precisely, sleep has to fulfill the following criteria: (1) specific sleeping site, (2) typical body posture, (3) physical quiescence, (4) elevated arousal threshold, (5) rapid state reversibility, and (6) regulatory capacity, i.e., compensation after loss[8]. Within sleep two separate states are distinguished on the basis of different physiological parameters: NREMS and REMS, which are also called slow-wave sleep (SWS) and paradoxical sleep, respectively (more commonly used in animal research). These two states exist in virtually all mammals and birds and are distinct one from another as each is from wakefulness[9]. Note that SWS can also refer to deep sleep stages in humans (see Figure 5.1) and it is under this definition that it will be used below.

In any typical sleep episode the two sleep states, NREMS and REMS, alternate. This NREMS-REMS cycle has a period of 12 minutes in rats[10], and 90-100 minutes in humans[4]. Because this period is shorter than 24 hours and occurs 4-5 times a night in humans, it is also referred to as an ultradian sleep-dependent rhythm. The two substrates are distinguished by different patterns in the EEG, electromyogram (EMG) and electrooculogram (EOG). Other physiological parameters change during sleep, such as arterial blood pressure, body temperature, cardiac and respiratory rhythms, and the production of different hormones (melatonin, growth hormone, etc.). However, the more complete description is derived from the EEG and the EMG.

EEG is a complex signal resulting from postsynaptic potentials of cortical pyramidal cells which can be recorded by electrodes placed on the scalp[11]. This signal summarizing cortical activity in the brain is visualized in terms of line tracings called brain waves. These brainwave tracings vary in amplitude (height) and frequency (cycles per second, Hz). Frequently, EEG is divided into several frequency bands, named after letters in the Greek alphabet: delta (1-4 Hz), theta (5-7 Hz), alpha (8-11 Hz), sigma (12-15 Hz), and beta (16-30 Hz)[12]. As the EEG, the EMG and EOG are also recorded by electrodes, but they summarize the electrical activity of muscles (muscle tone) and eye movements, respectively.

NREMS in humans is traditionally subdivided into stages I to IV[13], corresponding roughly to increasing depth of sleep. NREMS occupies about 70 – 80% of sleep of human adults[14], whereas REMS represents the remaining 20 – 30% (Figure 5.1).

The conventional method of sleep scoring, which is used to differentiate sleep stages in humans and in animals is inadequate for quantitative EEG analysis because the definition of sleep stages is based on rather general and arbitrary criteria. Thus, the quantitative analysis of the EEG is usually performed by computer-aided methods of signal analysis, such as spectral analysis. Spectral analysis is a mathematical approach allowing the decomposition of the EEG signals into its frequency components. The fast Fourier transformation (FFT) is a widely applied method for obtaining the EEG power spectrum, which displays the distribution of power over the frequency components of the signal[11].

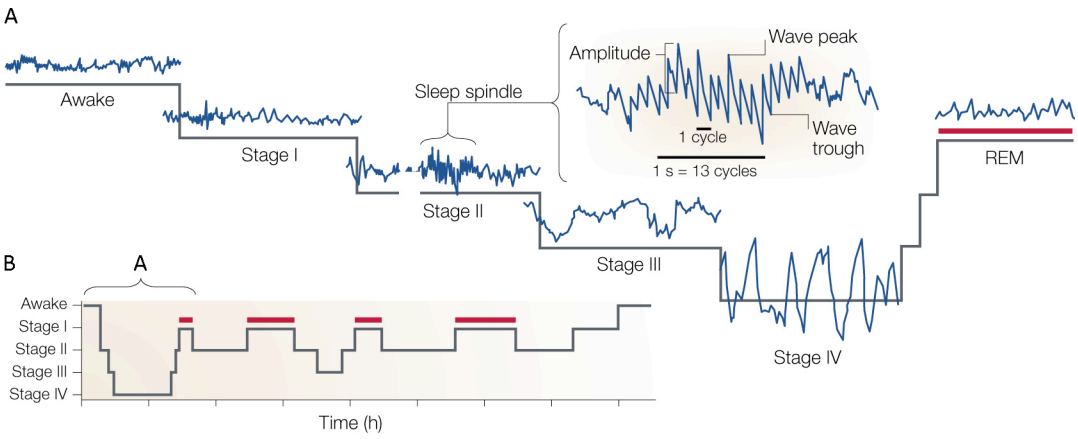


Figure 5.1: Sleep parameters and measures.

Panel (A) shows characteristic brainwave tracings (*blue*) of wakefulness, NREMS and REMS. In humans, NREMS is divided into four stages, corresponding to increasing depth of sleep as indicated by progressive dominance of the slow-wave activity (delta activity). NREMS and REMS alternate in each of the four or five cycles that occur in a typical adult human sleep. Early in the night, NREMS is deeper and occupies a disproportionately large amount of time, while the REMS episode is short or aborted. Later in the night, NREMS is shallow, and more of each cycle is devoted to REMS (red bars). Panel (B) illustrates these changes over the course of a night's sleep and panel (A) depicts, in detail, features of an early-night sleep cycle. Total sleep time, number of awakenings, and the time to reach a stage are some of the variables that can be measured after EEG/EMG/EOG analysis (polysomnography).

REMS is characterized by the followings: high-frequency, low-amplitude (wake-like or 'desynchronized') activity in the EEG; singlets and clusters of REMs in the EOG; and very low muscle tone (atonia) in the EMG[4]. NREMS Stage I is a light and drowsy sleep, dominated by low-amplitude and theta frequency EEG activity (4-8 Hz) and displaying slow eye oscillations. People drift into unconsciousness from NREMS Stage II, which shows EEG characteristics such as sleep spindle and K-complex waveforms, as well as slow oscillations. Stages III and IV, also termed SWS, represent the deepest stages of NREMS, with high-amplitude and low-frequency ('synchronized' or delta (0.5-4.5 Hz)) EEG activity. This EEG delta activity occupies less than 50% of the time in stage III and more than 50% in stage IV. In rodents, sleep stages are usually divided in REMS and NREMS. Sometimes, NREMS is divided into SWS-1 and SWS-2, mimicking stage I-II and III-IV, respectively. *(Figure modified from ref. [15])*

5.1.2. Sleep/wake neuronal pathways and neurochemistry

This part is dedicated to the description of the brain circuitry that regulates sleep and produces wakefulness, including cell groups in the brainstem, hypothalamus and basal forebrain (BF) that are crucial for arousing the cerebral cortex and thalamus. These neurons are inhibited during sleep by a system of GABA-containing neurons, amongst which the ventrolateral preoptic nucleus (VLPO) seems to have a key role. Mutual inhibition between

the arousal- and sleep-producing circuitry results in switching properties that define discrete wake and sleep states, with sharp transitions between them. This switch is supposed to be stabilized by hypocretin (orexin, Orx) neurons in the lateral hypothalamus[5] (Figure 5.2).

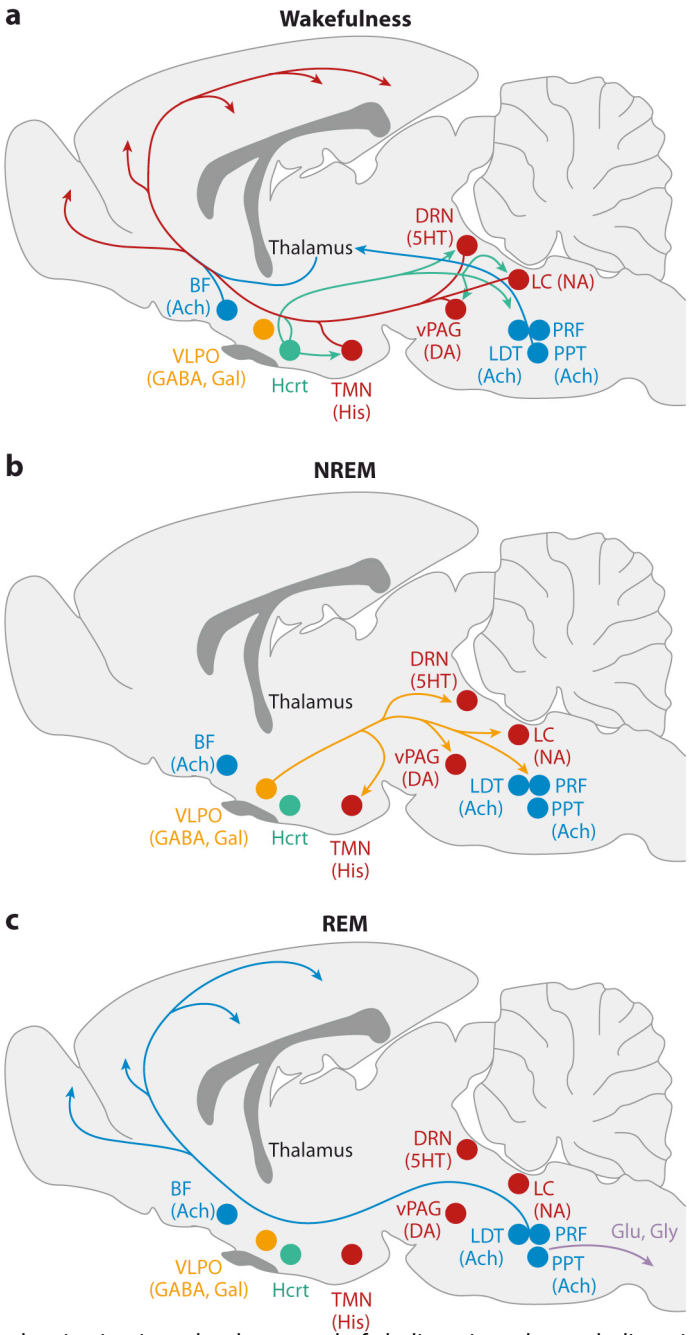


Figure 5.2 : Schematic sagittal view of the rodent brain showing the major structures and neurotransmitters involved in the regulation of the vigilance states.

(a) Wakefulness is maintained by cholinergic (Ach: acetylcholine; in *blue*) ascending inputs from the brainstem (PFP: pontine reticular formation, LDT: laterodorsal tegmentum, and PPT: pendonculopontine tegmentum) to the thalamus, which in turn activates the cortex, and from the basal forebrain (BF). Additionally, monoaminergic [in *red*; i.e., serotonergic (5-HT) DRN: dorsal raphe nucleus), noradrenergic (NA) (LC: locus coeruleus), dopaminergic (DA) (vPAG: ventral periaqueductal gray matter), histaminergic (His) (TMN: tuberomammillary nucleus)], and orexinergic/hypocretinergic (Hcrt; in *green*) inputs, contribute to the waking cortical activation. (b) NREM sleep may be initiated and maintained by inhibitory inputs (GABA and Galanin (Gal); in *orange*) from the ventrolateral preoptic nucleus (VLPO) to all wakefulness-promoting brain sites. (c) REMS cortical activation is under the control of cholinergic and non-cholinergic structures arising from the brainstem, while REMS atonia is under the control of the glutamatergic (Glu) and glycinergic (Gly) projections to the spinal cord (in *purple*). *(Figure from ref. [6]).*

5.1.2.1. Ascending reticular activating system

In the 1910s, Baron Constantin von Economo, a Viennese neurologist, began to see patients with a new type of encephalitis that specifically attacked regions of the brain that regulate sleep and wakefulness (unknown at that time). Based on his work, he proposed that there was an ascending arousal system originating in the brainstem that kept the forebrain awake[5]. Since then, scientists have investigated further this hypothesis and currently the concept of the 'ascending reticular activating system', which begins in the rostral pons and runs through the midbrain reticular formation, is well accepted[16,17,18]. The ascending arousal system largely originates from a series of neuronal populations using different chemical neurotransmitters such as acetylcholine (Ach), noradrenaline (NA), dopamine (DA), serotonin (5-HT) and histamine (His). All of these neurons have widespread or diffuse projections to the cortex, thalamus and brainstem or spinal cord[6]. This network of fibers ensures cortical alertness and the ability to modulate reactions to surrounding stimuli. The input to the cerebral cortex is augmented by neurons in the lateral hypothalamus containing melanin-concentrating hormone (MCH) or Orx, and by BF neurons containing Ach or GABA. Lesions along this pathway produce the most profound and long-lasting forms of sleepiness or even coma[19].

Ach-containing neurons of the brainstem: These neurons localized in pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT), as well as in the pontine reticular formation (PFP) project to the thalamus which activates the thalamic relay neurons crucial for transmission of information to the cerebral cortex[20,21]. They discharge rapidly during stages accompanied by cortical activation: wakefulness and REMS[21,22]. These cells are much less active during NREMS, when cortical activity is low. Injection of carbachol, an Ach agonist into the

pontomesencephalic tegmentum causes cortical activation with muscle atonia, a state similar to REMS[23].

NA-containing neurons of the locus coeruleus (LC): These neurons project through the forebrain, brainstem and spinal cord and stimulate cortical activation. They are essentially active during wakefulness, slow down during NREMS and are totally silent during REMS. Two types of adrenoreceptors are involved: the α_1 are associated to depolarization and α_2 to hyperpolarization. Thus, NA can either stimulate other cell groups involved in wakefulness or inhibit sleep-promoting systems. Drugs acting through the stimulation of the release or the blocking of the reuptake of NA are used to treat sleepiness (amphetamine, modafinil)[24,25,26].

There is a balance between Ach-mediated and NA-mediated neurotransmission. The activation of both maintains a waking state accompanied by muscle tone and cortical activation. REMS can occur when Ach-containing neurons stay active and NA-containing neurons become inactive. For instance in the 1970s, it was shown that administration of acetylcholinesterase inhibitors induced wakefulness by increasing Ach activity. However, if these drugs are administrated following catecholamine (NA and DA) depletion the effect is totally different: REMS is stimulated[23]. Moreover, when administrated during wakefulness, the acetylcholinesterase inhibitors stimulate cortical activation, while their administration during sleep, when the arousal system such as NA system are inactive, provokes REMS[27].

DA-containing neurons in ventral periaqueductal grey matter (vPAG): These neurons play a major role in arousal, projecting to the striatum, BF and cortex. They seem to discharge in bursts of spikes in association with aroused and often positive rewarding states[28,29]. However, recently, controversial opinions about the function of DA on sleep–wake regulation

emerged. The debate resides on the demonstration that DA is a substance dramatically related to sleep processes, and not associated exclusively with wakefulness. It appears that REMS neural pathways are triggered when D_2 dopaminergic receptors are activated[30] and selective lesion of the substantia nigra pars compacta (SNpc) neurons elicits a remarkable disruption of REMS[31]. Additionally, the overall mean firing rate of the ventral tegmental area (VTA) neurons (which are largely dopaminergic), presents a large increase in the burst firing during REMS episodes. Such evidence prompts to speculate that DA neurons could be considered essential for sleep regulation, in particular for triggering and maintenance of REMS[32].

5-HT-containing of the dorsal raphé nucleus (DRN): These neurons discharge, like the NA-containing neurons, maximally during wakefulness, decrease discharge during NREMS and cease firing during REMS[33]. However, unlike Ach-containing neurons, 5-HT-containing neurons appear to be active in association with less-aroused wakefulness states, such as grooming and rhythmic movement in animals. They also attenuate cortical activation through inhibitory influences on other neurons of the activating systems and particularly on Ach-containing neurons[34].

His-containing neurons of the tuberomammillary nuclei (TMN): These neurons which are located in the posterior hypothalamus stimulate cortical activation through diffuse projections[35]. They seem to discharge maximally during wakefulness, diminish during NREMS and cease firing during REMS. Through H_1 (His type 1) and H_2 receptors, His excites multiple neurons of the arousal systems as well as cortical neurons. Interestingly, it does not inhibit putative sleep-promoting neurons of the preoptic region[36]. Antihistamine drugs are widely

prescribed to alleviate allergies. However, these H_1-antihistamines, particularly from the first generation, are also used as hypnotic agents due to their side-effects of somnolence[37].

Orx-containing hypothalamic neurons in the hypothalamus: Orx, also called hypocretin (Hrct), is a recently discovered peptide in the brain. Like its receptor, Orx is necessary for the maintenance of wakefulness as shown with transgenic mouse models lacking either Orx or Orx receptors[38,39,40]. These mice showed clear symptoms of narcolepsy. Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness and attacks of cataplexy (sudden loss of muscle tone triggers by strong emotion)[41]. Moreover, these patients also show unnatural short REMS latency and high number of arousals during their sleep leading to a poor sleep quality[42]. The biological basis of this disease is Orx deficiency. The posterior and lateral hypothalamus, which has long been known to be important in maintaining wakefulness, is the location of the Orx-containing neurons. Orx stimulates cortical activation, behavioral arousal and autonomic changes by diffuse projections and excitatory influences on the cortex, BF Ach-containing neurons, His-containing TMN neurons, NA-containing LC neurons, and spinal cord motor and sympathetic neurons[43]. Orx-containing neurons have been found to tonically discharge during wakefulness, decrease firing during NREMS and cease firing during REMS[44,45]. Thus, they are assumed to stimulate arousal, and antagonize cortical deactivation and loss of muscle tonus, which occurs in their absence in cases of narcolepsy. By diminishing the discharge frequency of REM-on cholinergic neurons located in the PPT/LDT, they block the occurrence of REMS episodes during wakefulness[46]. They have a central role in stimulating and maintaining wakefulness, given their innervation and excitatory role on all the other arousal systems. However, Orx does not inhibit sleep-promoting neurons in the preoptic region[5].

Ach-containing neurons of the BF: These neurons have an important role in cortical activation[47]. Local delivery of agonists of the arousal systems, such as NA, evokes fast cortical activity, especially high-frequency EEG called gamma activity. Conversely, local administration of blockers, such as lidocaine produces a loss of fast cortical activity and predominance of low-frequency EEG called delta activity. These Ach-containing neurons are found to be active during continuous wakefulness enforced by sleep deprivation[48] and also during REMS. Their discharge rate correlates positively with the EEG power of fast gamma (30-60 Hz) and theta (4–8 Hz) across sleep–wake states, and correlates negatively with delta activity (1–4 Hz). Thus, they stimulate high-frequency gamma and theta activity during both wakefulness and REMS, through an activation of cortical neurons mediated by nicotinic and/or muscarinic Ach receptors[20].

For instance, Nicotine, one of the most common stimulants, provokes cortical activation and blocking nicotinic Ach receptors diminishes this cortical activation. Muscarinic Ach receptor antagonists such as scopolamine or atropine block fast cortical activity and induce a predominance of EEG delta activity even while animals appear to be awake[49]. Thus, BF Ach-containing neurons have a crucial role in stimulating and maintaining cortical activation during both wakefulness and REMS.

5.1.2.2. Sleep-generating system

The action of the ascending arousal system is counter-balanced by several other neuronal populations which have been identified as being sleep-active and are involved in promoting and maintaining natural sleep. These neurons mainly releasing GABA, provide inhibitory control of many arousal nuclei[50].

GABA-containing neurons in the BF and preoptic area: Since the 20th century, it has been known that neurons in the BF and preoptic area have an important role in promoting sleep, because lesions in these areas result in insomnia. Moreover, it was shown that neurons located in these regions discharge at higher rates during sleep than during wakefulness[51,52]. The first neuronal population to be identified was neurons in the VLPO[53], which provides inhibitory control of many of the arousal nuclei. These cells mainly release GABA, but some also release the small inhibitory peptide galanin. VLPO neurons increase their firing just before the onset of EEG synchronization and progressively increase their activity with sleep depth, a pattern that is consistent with them being involved in both causing and stabilizing natural sleep[52]. A second population of GABA-releasing neurons in the median preoptic nucleus (MnPO) also displays enhanced firing during both REMS and NREMS[54], with firing increasing in anticipation of sleep but then slowly declining during prolonged periods of NREM sleep, implying a role for these neurons in sleep initiation[55]. A last group of sleep-active GABAergic neurons is interspersed among cholinergic cells in the magnocellular regions of the BF[56]. The firing of these neurons, like VLPO neurons, is associated with sleep depth, but in this case the firing rates are higher during NREM sleep than during REM sleep[52]. Thus, this suggests that sleep initiation and maintenance is an active process that exerts inhibitory control over the ascending arousal nuclei, predominantly through GABAergic inhibition from the hypothalamus and the BF. Importantly, arousal nuclei can also send reciprocal inhibitory feedback to the sleep–promoting nuclei[57,58,59]. Thus, when the arousal nuclei are inhibited, this positive feedback disinhibits the sleep-promoting centers, further enhancing their firing. This results in a bi-stable system that can only flip-flop between sleeping and waking and cannot normally rest in some intermediate state[59].

GABA-containing neurons in brainstem and thalamus: GABA-containing neurons in other areas also appear to be active selectively during sleep to inhibit wake-active neurons in many areas, including the brainstem reticular formation and LC[60,61]. GABA-containing neurons in the caudal medullary reticular formation, together with glycine (Gly)-containing neurons[62] are active during REMS. Their projections to the spinal cord might inhibit spinal motor neurons directly during REMS producing atonia.

In the thalamus, GABA-containing neurons inhibit thalamocortical relay neurons to dampen cortical activation by diminishing the sensorial influx going through the cortex[63]. Their burst pattern of discharge during NREMS triggers spindle activity (12–14 Hz). With increasing level of hyperpolarization of the thalamocortical relay neurons, spindles are progressively replaced by delta oscillations (1-4 Hz). This delta rhythm of the entire cerebral cortex is not only imposed by the thalamus but also intrinsically by pyramidal cells from the V cortical layer[64,65].

In summary, GABAergic transmission is therefore involved in the induction and maintenance of NREMS by inhibiting the ascending arousal nuclei and by sustaining the thalamocortical burst creating spindles and delta activity.

Adenosine (AD): AD has long been thought to play a role in sleep since caffeine, the major stimulant used around the world, was found to act as an antagonist at AD receptors. AD antagonists increase wakefulness and decrease sleep along with EEG delta activity in humans and rodents[66,67]. Conversely, AD and its analogs increase sleep and enhance delta activity in a way suggesting that AD could serve as an endogenous sleep promoting substance. Moreover, mice with a loss of function of A_{2a} receptors have reduced sleep and reduced response to sleep deprivation and caffeine[68,69] and human polymorphism of the

catabolic enzyme, AD desaminase, directly affected homeostatic regulation of sleep[70]. This leads to the conclusion that adenosinergic modulation may play an important role in sleep.

5.1.2.3. REM-on and -off neurons

During the past decade, evidence from both rat and cat studies has suggested that each of the events of REMS is executed by distinct cell groups located in the brainstem[71]. These cell groups are discrete components of a widely distributed network, rather than a single REMS "center". One of the most important experiment in the field was the discovery that many neurons of the perilocus coeruleus α (peri-LCα) show a tonic firing selective to REMS; i.e. REM-on neurons[72,73]. Two types of REM-on neurons were recognized: (1) the cholinergic ones projecting to rostral brain areas potentially involved in cortical activation during REMS, and (2) the non-cholinergic ones proposed to generate muscle atonia during REMS through descending excitatory projections to Gly pre-motoneurons. These non-cholinergic neurons were thought to be glutamatergic (Glu) [74]. Another achievement in research for REMS regulatory mechanisms was the finding that 5-HT-containing DRN neurons, NA-containing LC neurons, His-containing TM neurons and Orx neurons from hypothalamus cease firing specifically during REMS, i.e, show a REM-off firing activity, reciprocal to that of REM-on neurons. Nowadays, the hypothesis suggesting that REMS onset is gated by reciprocal inhibitory interactions between REM-on and REM-off neurons is well accepted. Recent findings have led to update models of the mechanisms controlling REMS onset and maintenance involving various neurotransmitters such as Glu, GABA, MCH and Gly [75].

5.2. Regulation of sleep

The discovery of specific pathways involved in governing sleep has improved our understanding of the differences in brain activity while we are asleep and awake. As seen previously, the mutual "inhibitory-excitatory loop" between the arousal system (active during wakefulness) and the sleep-generating system (active during sleep) is predisposed to a "flip-flop" switch mechanism, being in either one state or the other, with a very rapid transition[5]. This rapid transition state is highly conserved across species and is an important survival mechanism.

Factors that influence this circuit have been the topic of considerable research, providing new insights and opportunity for treating sleep-related disorders. Two different factors have a major impact on sleep/wake distribution. First, homeostatic influences, such as the accumulation of sleep debt following prolonged period of wakefulness, and second, the endogenous circadian rhythm, which produces the 24h cycle and governs many biological processes.

5.2.1. Circadian control of sleep regulation

The regulation of sleep is strongly influenced by circadian rhythms. Indeed, we are all conscious of bedtime and wake time, especially when the quality of our sleep is disrupted. This occurs, for example, in response to shift work and jet lag and leads to decrements in quality of life, performance, and health[76].

In mammals, a master circadian pacemaker critical for circadian organization of sleep/wake states has been localized to the suprachiasmatic nucleus (SCN), a cluster of neurons bilaterally distributed in the anterior hypothalamus[77]. This pacemaker is interconnected with the cell groups specialized for the induction and maintenance of wakefulness, REMS and NREMS previously described in "sleep/wake neuronal pathways and neurochemistry"[5,78].

Thus, SCN may control this system by actively promote arousal during the active phase and sleep during the rest phase[79].

Several studies in animals supported the hypothesis that SCN has intrinsic oscillatory properties and can affect sleep/wake organization. For instance, SCN transplantation of arrhythmic hamsters, whose own nucleus had been ablated, restored circadian rhythms, confirming the endogenous properties of the SCN[80]. Lesions of the SCN in rodents leads to total arrhythmicity in the sleep/wake distribution[81,82]. In addition to having a control over the sleep/wake distribution, SCN produces a rhythmic output that influences, activity, temperature, drug metabolism, heart rate, regulation of stress and hormones (melatonin, cortisol, growth hormones), immunity and even the timing of the cell-division cycle[83].

Most mammals have a circadian period very close to the 24h day and highly stable; longer than 24 h in human (24.18 h[84]) and shorter than 24 h in mice (22.88 h and 23.61 h in C57BL/6J and BALB/cJ inbred mouse strains respectively[85]. Kleitman performed the first experiment demonstrating that the endogenous circadian rhythm of body temperature continues to exhibit a near-24-hour circadian rhythm in a human subject living on a non-24h routine, deep within a cave shielded from all known periodic stimuli from the external environment[86]. Moreover, studies of naps, short and ultrashort sleep/wake schedules, short-term constant routines, long-term temporal isolation, and forced desynchrony have produced a coherent body of evidence consistent with the view that the circadian clock in humans plays a bidirectional role in sleep/wake regulation, alerting at some phases, and promoting sleep at other phases[87,88]. These studies also clearly demonstrate that the onset and duration of the main daily sleep episode and of REMS are strongly controlled by circadian phase.

Importantly, environmental cues called Zeitgebers (German, literally "Time Givers") are necessary to reset the endogenous clock each day in order to keep a perfect 24h rhythm. One of the most important zeitgebers is light. The SCN receives information about

illumination through the eyes; the photopigment melanopsin sensitive to light (particularly blue light) and expressed in specific photosensitive ganglion cells of the retina detects and transmits light information through the retinohypothalamic tract to the SCN[89].

Overall, this supports the view that the distribution of sleep over the 24h day is strongly determined by the circadian system.

5.2.2. Homeostatic aspects of sleep regulation

The homeostatic process tracks sleep need. Sleep need and the propensity to initiate sleep increase during wakefulness and decrease during sleep. This homeostatic process of sleep also appears as a 24h oscillation with the important distinction that this oscillation is imposed or driven by the sleep/wake distribution whereas the circadian rhythm is self-sustained. To emphasize this distinction, the oscillation generated by the homeostatic process is sometimes referred to as a "hour-glass" and the oscillation generated by the circadian process as a self-sustained oscillation. The homeostatic process is seen as a process which strives to maintain a preferred or required level of sleep. Thus, the quantification of sleep homeostasis might be assessed by the average level to which a specific sleep variable returns after a perturbation, such as sleep deprivation. Dependent variables may include total sleep time, REMS, NREMS and specific EEG variables such as EEG delta (1-4 Hz) power during NREMS or EEG theta (4-8 Hz) power during wakefulness.

In this work, sleep homeostasis is indexed by delta power in NREMS. Delta power is the quantification of EEG delta activity through EEG spectral analysis. It is negatively correlated with the response to arousing stimuli[90] and NREMS fragmentation[91]. Therefore, delta power seems to be the best electrophysiological indicator of sleep depth or sleep intensity in humans and in animals. Sleep loss evokes a proportional increase in delta power, while a

sleep excess induces a decrease[92,93]. Delta power during a night sleep decreases with the number of NREMS episodes.

5.2.3. The two-process model of sleep regulation

A dominant and well-validated model for sleep regulation today is the two-process model[94,95]. These two processes – the homeostatic process and the circadian process – sometimes work together and sometimes against each other, and the situation and wishes of the person can often override the process and keep us awake when we should be sleeping.

Conceptually, the model considers the alternation of wakefulness and sleep to result from the interaction of two processes, *S* and *C* (Figure 5.3). The saturated exponential *process S* represents sleep need; the homeostatic process of sleep as seen previously. It increases during waking and decreases during sleep. Functionally, this implies that sleep would serve a recovery function. *Process S* is entirely determined by the temporal sequence of behavioral states. The sinusoidal *process C* (circadian process), in contrast, is totally controlled by the circadian pacemaker (SCN), irrespective of behavioral state, and is proposed to set limits to process *S*. Those limits vary with time of day. As soon as *S* reaches the lower limit during sleep, subjects will wake up. If S reaches the upper limit during waking, sleep will be initiated. Delta power behaves as predicted for a measure representing the decline of *S* during sleep[96,97] and its increase during waking[92]. This relationship alone turned out to be sufficient to quantify the dynamic properties of both processes (Figure 5.3)[94].

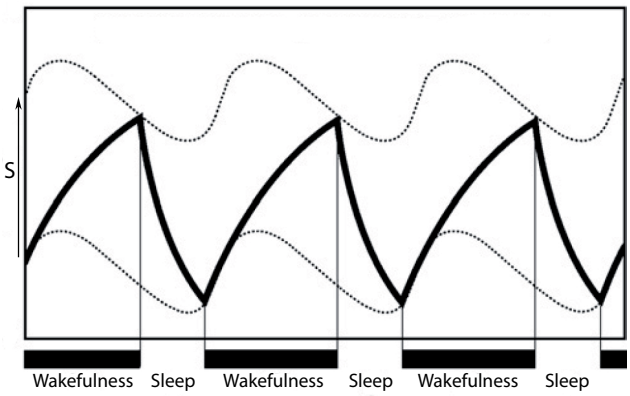


Figure 5.3: The two-process model of sleep regulation.

Process S represents sleep need. It increases during wakefulness and decreases during sleep. Its variation is restricted to a range of values determined by the circadian *process C* (dotted lines), which is not constant over time but varies with the time of day. In addition to the 24h variation of process C, there is an influence of conscious decisions, which can temporarily modify the positions of the process C thresholds. *(Figure from ref. [98])*

The 2-process model reliably predicts increased delta power and sleep time in a sleep episode following a prolonged period of wakefulness, as seen after sleep deprivation (Figure 5.4) [99,100].

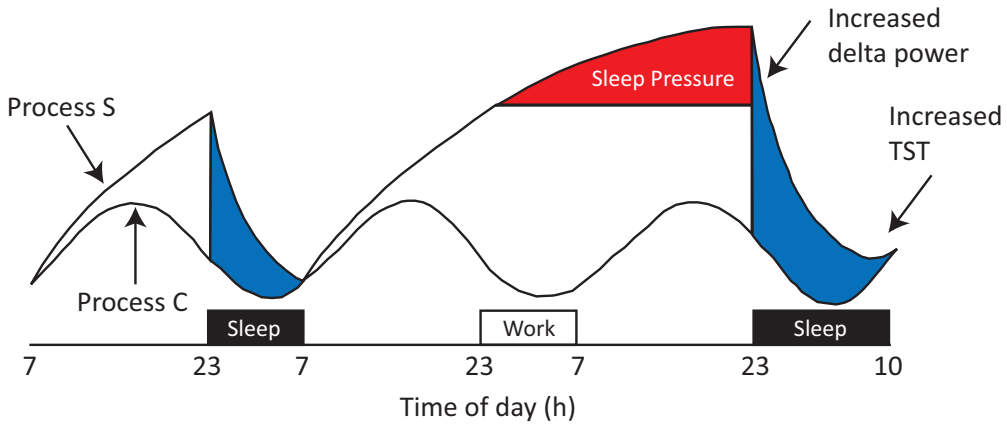


Figure 5.4: Effect of sleep deprivation simulated by the two-process model.

When subjects are sleep deprived (2nd 24h period), additional sleep pressure builds, leading to greater delta power and total sleep (TST) time on the subsequent night of recovery sleep. *(Figure modified from ref. [101])*

Evidence showed that the homeostatic and circadian processes are controlled by distinct mechanisms. In arrhythmic SCN-lesioned rodents, increases in delta power and NREMS amount after sleep deprivation are not obliterated[102], and animals exposed to different photoperiods show dramatic changes in the distribution of sleep and wakefulness, whereas

the homeostatic component of sleep (delta power) is unaffected[103]. In human, alertness and cognitive performance kept a clear circadian rhythm even during 88h sleep deprivation[104].

To summarize, the two processes are generated independently but their interaction determines the timing, duration and quality of both sleep and wakefulness[105].

5.3. Sleep-promoting drugs

In the adult population, about one third is suffering from occasional sleep disturbances. About one in ten is suffering from a chronic sleep disorder, which is also affecting the person's mood and daytime performance. This is a major social, medical and economic problem in modern society. This part reviews the pharmacology of sleep with special emphasis on sleep-promoting drugs.

5.3.1. History

While the effects of opium were known for thousands of years, it became a patent medicine product in the 1800s when it was combined with alcohol and sold as laudanum (tincture of opium). Although effective, this treatment had serious safety problems, including dependency and respiratory depression. Chloral hydrate, another abusable sedating medicine employed to induce sleep, became widely used in the mid-19th century. Barbiturates were developed in the early 20th century and remained the primary prescribed hypnotic medications until the 1960s. Other past hypnotics included glutethimide, ethchlorvynol, paraldehyde and bromide preparations. For all of these, there have been serious safety concerns[106].

In recent decades, pharmaceutical approaches to the treatment of sleep disturbance led primarily to major improvements in safety, however, none of the molecules currently used is

capable of producing “natural physiological” sleep.

5.3.2. Benzodiazepines (BZD)

BZD medications became available in the 1960s and were promoted first for the treatment of insomnia in the early 1970s with the introduction of flurazepam. Importantly, BZDs lacked the toxicity of barbiturates, as well as the risk of overdoses due to their pharmacokinetics[107]. They improve insomnia symptoms by binding to the $GABA_A$ receptors on postsynaptic neurons in the central nervous system, thus inhibiting neuronal excitation through increased neuronal chloride permeability. Indeed, $GABA_A$ receptors are pentameric chloride channels (the five subunits form a rosette around a transmembrane ion channel) widely expressed in the central nervous system including in the thalamus and cortex, two key areas for sleep physiology[108]. The complex $GABA_A$ receptors heterogeneity, derived from the large variety of its subunits, influences the cellular and subcellular localization of these receptors as well as electrophysiological properties[109,110,111]. In addition to their sedative effect, BZDs produce anxiolytic, muscle relaxant, anticonvulsant effects and impair cognition[112]. The results of a meta-analysis published in 2000 revealed that BZDs increased total sleep time but did not significantly affect sleep latency[113]. However, BZDs reduce REMS and prolong REMS latency[114]. Daytime sleepiness or “hangover” effect, dizziness, and impaired memory are often reported as adverse effects. Anterograde amnesia has also been associated with BZDs, particularly BZDs with short half-life like midazolam (Dormicum®) and tiazolam (Halcion®) while agents with longer half-lifes like flurazepam (Dalmadorm®) and flunitrazepam (Rohypnol®), increase the risk of “hangover” effect, confusion, dizziness and falls[115]. Moreover, their long-term use could also lead to tolerance and dependence, as well as rebound symptoms at withdrawal[116]. These side-effects have stimulated research on novel, more selective compounds.

5.3.3. Z-drugs

A new generation of sleep-promoting compounds, following the BZDs was launched in the 1980s. This new class of drugs, called Z-drugs (Zolpidem (Stilnox®), (S)-Zopiclone (lunesta®), and Zaleplon (Sonata®)), is structurally different from BZDs although they act on the same $GABA_A$-receptor binding site. Z-drugs, as well as BZDs, are positive modulators, i.e. they enhance the GABA-induced chloride current. They have little intrinsic activity in absence of GABA[117]. Overall, Z-drugs have similar effects on sleep as BZDs, including the shortening of sleep latency and reduction of REMS[118]. They are reported to cause less dependence and "hangover" effects compared with BZDs[115], despite a lack of convincing evidence showing benefit of newer Z-drugs compared to BZDs[119]. Importantly, the prescription of both Z-drugs and BZDs should be restricted for short-term use to avoid side-effects such as amnesia, tolerance and dependence which showed increasing risk with long-term treatments[120].

Currently, two modes of $GABA_A$-mediated transmission are distinguished: "phasic" and "tonic" transmission. Traditionally, $GABA_A$-mediated transmission refers to phasic inhibitory postsynaptic currents following activation of synaptic receptors by high concentration of GABA released from presynaptic nerve terminals[121]. The persistent tonic inhibition, recently discovered, is thought to be mediated by extrasynaptic $GABA_A$ receptors, which are continuously activated by low concentration of GABA. Thus, phasic conductance rapidly and transiently inhibits neuronal excitability, while tonic conductance slowly reduces the capability of neurons to be excited. This property to establish a baseline excitability level of neuronal network raises the possibility that such a mechanism may be involved in sleep[117]. Molecules mediating this tonic inhibition, such as gaboxadol (THIP) and tiagabine, were investigated for the treatment of insomnia. It is important to mention that, conversely, BZDs and Z-drugs target $GABA_A$ receptors mediating phasic inhibition.

5.3.4. Drugs modulating tonic inhibition

THIP is a selective $GABA_A$ agonist and its effects on sleep differ substantially from those induced by allosteric modulators such as BZDs and Z-drugs. THIP increases NREMS EEG slow oscillations (<10 Hz), decreases of NREMS EEG spindle frequency range, and does not affect REMS amount or cognition, diametrically opposing the effects of BZDs and Z-drugs[117]. However, THIP was discontinued in March 2007 while in a Phase III clinical trial, because of an overall unfavorable therapeutic profile, including lack of efficacy in a three-month study and a higher incidence of psychiatric side effects[122].

Tiagabine is a GABA uptake inhibitor (inhibition of GABA transporter GAT-1) launched initially as an anticonvulsant in the treatment of epilepsy. Recently, it has been investigated for its potential as a hypnotic[123,124]. Its effects on sleep was similar than those found for THIP.

Currently, new compounds targeted specific subunits of $GABA_A$ receptors either located in sleep neuronal networks or involved in tonic conductance are under investigation as potential hypnotic agents[125,126].

5.3.5. Anti-histamines and antidepressants

First-generation antihistamines, such as diphenylhydramine and doxylamine, are frequently used as nonprescription sleeping aids, although not much of the existing data support their use. They can give temporary relief for sleep problems, but long-term treatment are not recommended due to tolerance development[127].

The prescription of antidepressants as sleep-promoting drug is not uncommon, but typically more beneficial in patients with comorbid depression. Sedative effects of trazadone, amitriptyline and mirtazapine for example are well known[128].

5.3.6. Serotonin antagonists

The 5-HT_2 receptors have been recently among the most promising targets in the search of effective and well-tolerated novel medications for the treatment of sleep disorders[129]. Ritanserin has been found to increase the amount of delta activity and deep NREMS (SWS) in healthy volunteers and young poor sleepers[130,131], and in rats[132]. Ketanserin another 5-HT_{2A}/5-HT_{2C} receptor antagonist, has also been demonstrated to promote SWS, although to a lesser extent than ritanserin[133]. Epilvanserin, considered as a selective 5-HT_{2A} receptor antagonist[134], is in late Phase III clinical development for chronic insomnia characterized by difficulties with sleep maintenance. This compound appears to increase EEG delta and theta power in NREMS but does not produce physiological EEG sleep[135]. Antagonists and inverse agonists of 5-HT_2 receptors are promising sleep-promoting drugs, which in addition, appear to be well tolerated with a lack of abuse potential.

5.3.7. Melatonin agonists

The hormone melatonin has a strong circadian rhythm[136] and serves as a sleep-anticipating cue in humans. The ability of melatonin to shift circadian rhythms is well known. As a result, melatonin has been used in the treatment of various circadian rhythm sleep disorders, such as advanced and delayed sleep phase disorders, jet lag and shiftwork disorder. However, the current evidence for melatonin being effective in the treatment of primary insomnia is less compelling[137]. Melatonin appears to have a clear beneficial effect on sleep in individuals with a low endogenous level of melatonin such as elderly or those suffering from circadian-rhythm disorders. However, melatonin receptors agonists, which have a longer half-life than melatonin, represent a novel approach in the therapeutic management of insomnia. Ramelteon, the first melatonin-receptor 1 and 2 agonist on the market, appears to be well tolerated and efficient especially to induce sleep[138]. Evidence supports that ramelteon can

also increase total sleep time (TST)[139], however subjective measures of sleep quality are not consistent[140,141]. Further comparative studies with others hypnotic agents are needed to assess the real impact of this treatment in insomnia.

5.3.8. Orx antagonists

Expectedly, in light of the wake-promoting effects of Orx, antagonists of Orx receptors are under investigation as a potential new class of sleep-promoting drugs. Almorexant (ACT-078573), a selective, dual Orx_1 and Orx_2 receptor antagonist, showed to increase NREMS, delta power in NREMS and REMS without producing symptoms of narcolepsy such as cataplexy[142,143], although several important points to further investigate has been raised[144].

5.3.9. GHB

Several studies in humans and in animals suggest that GHB may promote "physiological" NREMS and particularly SWS[145,146,147] which makes GHB highly interesting, because the other sleep-promoting drugs induced a "pharmacological" or "non-physiological" sleep (except perhaps Orx and melatonin receptor antagonists) and appear to have much more severe side-effects than GHB. As already mentioned, one of the aim of this thesis was to investigate whether GHB is able to induce physiological sleep and better understand its mechanisms. The following part introduces GHB and its potential mechanisms of action.

5.4. GHB

GHB is a short-chain fatty acid derivative of GABA, the major inhibitory neurotransmitter in the brain[148](Figure 5.5). Early studies on GABA function were hampered by its inability to cross the blood-brain barrier. In an attempt to produce a GABA analogue that could more

easily enter the brain, H. Laborit introduced GHB in 1960[149], which showed sedative properties. Since then, GHB has been used as an anaesthetic for minor surgical procedures in the laboratory[150,151] and in the clinic[152], and is marketed in Italy for treating alcoholism (Alcover®)[153,154] and in Europe and USA for treating narcolepsy with cataplexy (Xyrem®)[155,156]. GHB is also currently under investigation for potential treatment of different disorders such as fibromyalgia[157,158], depression and anxiety[159,160].

However, GHB is notoriously known as a recreational drug of abuse. Indeed, GHB and its precursors (γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD); Figure 5.5) are currently abused for their recreational and pleasurable properties (heightened sexual pleasure, stress reduction, sedative, anti-anxiety, and antidepressant effects) by club attendees (rave parties); anabolic effects by body builders; and disinhibitory and sedative effects by sexual predators. Of interest, GHB's physiochemical properties (colorless, odorless, and slightly salty taste) have been exploited as an "ideal" date rape drug[160].

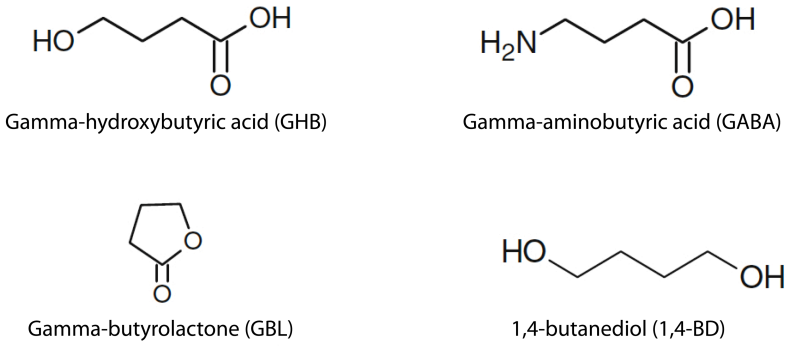


Figure 5.5: Structure of GABA, GHB and the two prodrugs of GHB: GBL and 1,4-BD.

The chemical structure of these four molecules are very similar. The prodrugs of GHB, GBL and 1,4-BD, are rapidly converted into GHB in humans and rodents. GHB is a metabolite of GABA. Both are found naturally in the mammalian brain. *(Figure modified from ref.[161]).*

5.4.1. GBL, a prodrug of GHB

Since the GHB banning and series of warnings from different health agencies, the interest of GHB users for its recreational properties has shifted to GHB prodrugs, in particular GBL, due to its readily availability as a common solvent in numerous industrial processes[162]. The *in vivo* pharmacological properties of GBL are thought to be secondary to its final conversion into GHB[163]. Evidence showed that GBL is biologically inactive[164] and all its biologic and behavioral effects are due to its rapid conversion to GHB by peripheral lactonases or by nonenzymatic hydrolysis[165,166]. The half-life of conversion of GBL to GHB has been estimated to be less than 1 minute[164]. Moreover, GBL has a greater lipid solubility than GHB, allowing uniform and rapid absorption. This greater liposolubility might also explain why several studies show that GBL has a better bioavailability, and is slightly more potent than GHB itself[167,168,169,170].

5.4.2. GHB: Neurotransmitter and metabolism

GHB is unequivocally a naturally substance occurring in the mammalian brain[171], which is present in micromolar concentration (1-4 μM)[172]. Regional distribution studies revealed that the substantia nigra, thalamus, and hypothalamus contain the greatest amount of GHB, whereas the cerebellum and certain areas of cerebral cortex have the lowest concentrations[173,172]. The distribution of GHB is not limited to the nervous system. Indeed, it is normally present in other extraneural tissues like kidney, heart and skeletal muscle, containing markedly greater concentrations than the brain[174].

GHB is derived from the conversion of its parent neurotransmitter, GABA, to succinic semialdehyde through mitochondrial GABA transaminase. Succinic semialdehyde is then reduced to GHB by cytosolic succinic semialdehyde reductase[175,176]. GHB may be metabolized through the action of GHB dehydrogenase to succinic semialdehyde, which may be further

metabolized either to GABA, by GABA transaminase or to succinate through the action of mitochondrial succinic semialdehyde deshydrogenase to finally enter into the Krebs cycle (Figure 5.6).

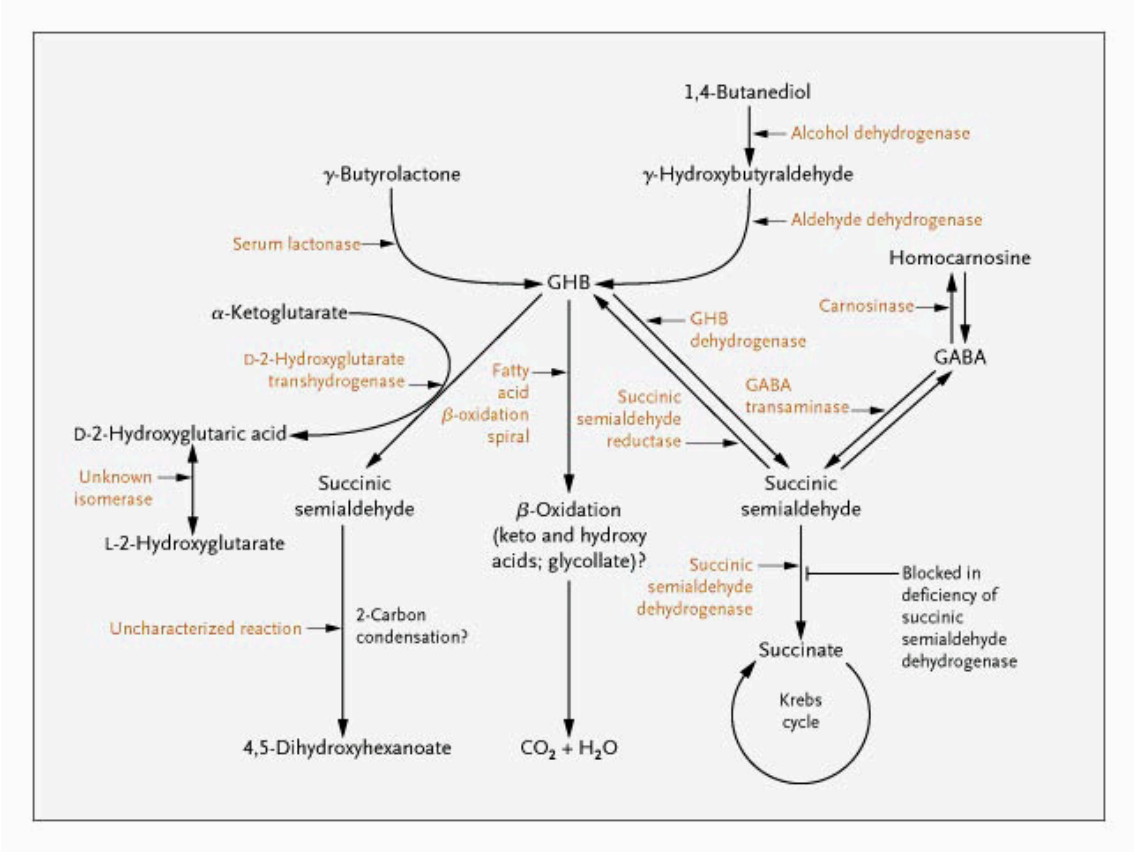


Figure 5.6: Metabolism of GHB and interrelationship with GBL and 1,4-BD.

The most important synthetic pathway for GHB entails conversion of GABA to succinic semialdehyde by mitochondrial GABA transaminase, followed by reduction of succinic semialdehyde to GHB by cytosolic succinic semialdehyde reductase. Mitochondrial succinic semialdehyde dehydrogenase, converting succinic semialdehyde to succinate, couples neurotransmitter metabolism to mitochondrial energy production. This is the enzyme missing in clinical and experimental deficiency of succinic semialdehyde dehydrogenase. A minor pathway for GHB production involves partial oxidation of 1,4-butanediol. Systemically administered GBL is converted by a circulating lactonase to GHB. This lactonase is not present in the brain tissue. The most significant catabolic pathway for GHB degradation is the oxidation of GHB to succinic semialdehyde by $NADP^+$-linked succinic semialdehyde reductase. The resultant succinic semialdehyde undergoes further metabolism to either GABA or succinate. A mitochondrial $NADP^+$-independent transhydrogenase is capable of metabolizing GHB to succinic semialdehyde with the production of D-2-hydroxyglutaric acid from L-2-hydroxyglutarate and an end-product of 4,5-dihydroxyhexanoate. There is disagreement as to whether there is significant metabolism of GHB through a β-oxidation scheme. *(Figure from ref.[177])*

GHB is thought to be a neuromodulator or neurotransmitter because it shows many of the requisite properties, including a discrete, subcellular anatomical distribution in neuronal presynaptic terminals, along with its synthesizing enzyme[178]. Furthermore, a release of GHB after calcium-dependant neuronal depolarization, a sodium-dependent GHB-uptake system and an active vesicular uptake system have all been reported[178,179,180].

5.5. GHB receptors

The existence of specific GHB receptor is suggested by specific, high-affinity GHB-binding sites that are observed in the brains of rats and humans[181,182,183,184]. The kinetics of GHB receptors are related to the 1-to-4 μM concentration of GHB that is typically found in mammalian brain tissue[172,185]. Maitre's lab even cloned a GHB receptor in the rat and two other GHB receptors in humans[186,187]. Although there are contradictory data[188] (the reported receptor may just represent cloning artifacts), some evidence suggests that the GHB receptor is presynaptic and G-protein-coupled[189] and that it may inhibit the release of GABA[190].

5.6. $GABA_B$ receptors

Despite data showing that GHB may be biologically active in its own right, compelling evidence suggests that most of the physiological and pharmacological effects of systemically administrated GHB are mediated by the $GABA_B$ receptors in humans and in animals. Data showed that the systemic administration of GHB induced different molecular changes including an increase of serotonin turnover[191], acetylcholine level (as other anaesthetics)[192] and dopamine level[193], but also a decrease of glucose use in the brain[194] and binding to NMDA receptors[195]. Moreover, it was shown that GHB altered presynaptic release of GABA and glutamate[190,196]. At the physiological level, after ingestion of GHB, the different effects

appear as hypothermia[197], hypertension, tachycardia, increase of the renal sympathetic nerves activity[198], decrease of minute ventilation and intestinal motility, secretion of growth hormone[145], impairment of spatial learning[195], increase of protection against neurotoxicity[199] and changes in EEG and behaviour[145,200]. All of these GHB effects seem to be due to the direct activation of $GABA_B$ receptors or their indirect activation after GHB conversion into GABA.

Thirty years ago, metabotropic $GABA_B$ receptors were first identified based on the receptor's distinct pharmacological profile compared to ionotropic $GABA_{A/C}$ receptors[201,202]. A few years later, it was shown that $GABA_B$ receptors are G-protein coupled receptors (GPCR) that usually inhibit adenylyl cyclase (AC) activity via the $G_{\alpha i/o}$ subunits of the activated G-protein[203,204]. The physiological consequences of inhibiting AC activity via $GABA_B$ receptors are not well understood but include effects on transcription factors (CREB2 (cAMP responsive element binding protein-2) and kinases (extracellular signal-regulated kinase 1 and 2 (ERK1/2))[205,206,207]. Electrophysiological experiments on $GABA_B$ receptors revealed that these receptors modulate potassium and calcium channels activity, thereby controlling presynaptic transmitter release and postsynaptic silencing of excitatory neurotransmission[208]. Presynaptic $GABA_B$ receptors are present on inhibitory and excitatory terminals where they function as auto- and heteroreceptors, respectively. Stimulation of presynaptic $GABA_B$ receptors suppresses neurotransmitter release by inhibition of voltage-sensitive Ca^{2+}-channels, but a direct modulation of vesicle priming was also proposed[209]. Postsynaptic $GABA_B$ receptors induce a slow inhibitory postsynaptic current by gating Kir3-type K^+-channels, which hyperpolarizes the membrane and shunts excitatory currents[210].
$GABA_B$ receptors are expressed throughout the mammalian tissues and appear to be quite widely distributed in brain with however some regional variations[211,212].

Although biochemical and pharmacological studies have long suggested the presence of diverse $GABA_B$ receptor subtypes[213], molecular cloning has only identified two genes encoding receptor subunits: $GABA_{B1}$ and $GABA_{B2}$[208]. It is now well accepted that most functional $GABA_B$ receptors in the brain are formed as $GABA_{B1}$ and $GABA_{B2}$ heterodimers[214,208]. In the heteromeric receptor, $GABA_{B1}$ is responsible for binding of all known $GABA_B$ ligands, whereas $GABA_{B2}$ is necessary for surface trafficking and G-protein coupling[215,216].

The only firmly establish molecular diversity in $GABA_B$ system thus far arises from the two isoforms of the $GABA_{B1}$ subunit: $GABA_{B1a}$ and $GABA_{B1b}$[217]. The transcripts of these two subunits are generated from a single $GABA_B$ gene by differential promoter usage [218,219] Structurally, the isoforms differ in their N-terminal ectodomain by pair of sushi domains (SDs) that are present in $GABA_{B1a}$ but not in $GABA_{B1b}$. SDs, also known as complement control protein modules, or short consensus repeats, are found in other GPCR as well and mediate protein-protein interactions in a wide variety of adhesion proteins[209]. The SDs in $GABA_{B1a}$ bind to auxiliary proteins that can modify receptor subcellular location[220,221].

To improve understanding about the localization and the functions of each $GABA_B$ receptor subunit *in vivo* different knock-out and knock-in mice were generated: $GABA_{B1}^{-/-}$, $GABA_{B2}$, $GABA_{B1a}^{-/-}$ and $GABA_{B1b}^{-/-}$ mice. $GABA_{B1}^{-/-}$ mice do not exhibit detectable electrophysiological, biochemical or behavioral responses to $GABA_B$ agonists[222], suggesting that $GABA_{B1}$ subunit is absolutely necessary for the $GABA_B$ receptor operation. These mice and $GABA_{B2}^{-/-}$ mice suffer from spontaneous seizures, hyperlocomotor activity, severe memory impairment, hyperalgesia, altered anxiety and depression-related behavior[222,223]. This clearly demonstrates that the lack of heteromeric $GABA_{B1,2}$ receptors underlies these phenotypes. This finding also renders the existence of additional obligatory receptors subunits unlikely. However, $GABA_{B1}$ exhibits a broader cellular expression pattern than $GABA_{B2}$. Indeed, $GABA_{B2}$ mRNA was found only expressed in neurons, while $GABA_{B1}$ mRNA is expressed both in

neurons and glia. This suggests that $GABA_{B1}$ could be functional in the absence of $GABA_{B2}$[224]. Atypical electrophysiological $GABA_B$ responses and $GABA_{B1}$ protein relocation from distal neuronal sites to the soma and proximal dendrites $GABA_{B2}^{-/-}$ mice support this view. These data on genetically modified mice suggest that association of $GABA_{B2}$ with $GABA_{B1}$ is essential for receptor localization in distal processes but is not absolutely necessary for signaling. It is therefore possible that functional $GABA_B$ receptors exist in neurons that naturally lack $GABA_{B2}$ subunits. Apart from this atypical electrophysiological $GABA_B$ response and the subcellular relocalization of $GABA_{B1}$ protein in $GABA_{B2}^{-/-}$ mice, the two knock-out mice are relatively similar; they have similar phenotypes and pharmacological responses, and show a down-regulation of the $GABA_B$ subunit, which they are still able to produce. The requirement of one subunit for the stability of the expression of the other subunit supports again the view that in WT mice, virtually all $GABA_{B2}$ protein is associated with $GABA_{B1}$[222,225,226]. In conclusion, it remains unclear whether these atypical $GABA_{B1}$ responses are of physiological relevance or represent an artifact of the knock-out condition which would be in accordance with numerous *in vitro* studies showing that $GABA_{B2}$ subunit is necessary for activating the G-protein and for receptor trafficking to the cell surface.

$GABA_{B1a}^{-/-}$ and $GABA_{B2}^{-/-}$ mice were generated a few years after $GABA_{B1}^{-/-}$ and $GABA_{B2}^{-/-}$ mice to determine whether the two isoforms of $GABA_{B1}$ contribute to distinct native $GABA_B$ functions[220]. These mice allowed to show that $GABA_{B1a}$ and $GABA_{B1b}$ have distinct physiological properties and function due to their differential subcellular localizations but not due to their binding pharmacology, which is similar. $GABA_{B1b}$ was mostly localized to dentritic spines opposite to glutamatergic terminals and mediates the postsynaptic inhibition, whereas $GABA_{B1a}$ was largely found in distal axons and formed presynaptic heteroreceptors inhibiting glutamate release[220,227,228]. In agreement with the differential localization of the two isoforms of $GABA_{B1}$, $GABA_{B1a}^{-/-}$ and $GABA_{B1b}^{-/-}$ mice display dissimilar

phenotypes. $GABA_{B1a}^{-/-}$ mice have a deficit in long-term potentiation (LTP), which is correlated with an impairment of nonspatial hippocampal memory formation (object recognition task)[220]. This lack of LTP caused by the absence of presynaptic $GABA_{B1a,2}$ receptor inhibition in amygdala give rise to a generalization of conditioned fear to nonconditioned stimuli[229]. In contrast to $GABA_{B1a}^{-/-}$ mice, $GABA_{B1b}^{-/-}$ mice display no LTP and object recognition impairment, but fear conditioning was totally impaired in these mice. Other reports assessing memory and anxiety also showed differences between $GABA_{B1a}^{-/-}$ mice and $GABA_{B1b}^{-/-}$ mice[230,231,232]. Furthermore, it was recently shown that baclofen (BAC), a high-affinity agonist of $GABA_B$ receptors, and GHB decreased temperature and locomotion similarly in both genotypes, but to a lesser extent than WT mice. However, in baseline conditions, $GABA_{B1b}^{-/-}$ mice exhibit higher locomotor activity in a novel environment compared to $GABA_{B1a}^{-/-}$ and WT mice[233].

Thus, the differential distribution of $GABA_{B1}$ isoforms may underlie some of the differences in $GABA_B$ physiological function and agonists' potencies, but not all. Very interestingly, two recent studies open new exciting perspectives for the understanding of native $GABA_B$ response diversity. First, it was shown that regulators of G-protein signaling (RGS), and particularly RGS2 protein, are able to decrease the activation of $GABA_B$ agonists. This complex mechanism can modulate agonist activation differently according to the type of neuronal populations[234]. Second, a proteomic study showed that C-terminal domain of the $GABA_{B2}$ subunit can bind tetrameric proteins, which present distinct but overlapping distribution pattern in the brain. These associated tetramers seem to determine both pharmacological properties and the kinetics of the receptor response. Specifically, they alter agonist potency, onset and desensitization of the $GABA_B$ response. Since most if not all $GABA_B$ receptors in the brain are associated with these tetramers, they qualify as auxiliary subunits of $GABA_B$ receptors[235].

5.7. GHB and sleep

A number of reports suggest that GHB may promote NREMS, particularly SWS, and decrease sleep latency both in patients with a history of impaired sleep[236] and in healthy subjects[145,237]. Studies in animals also suggest that GHB may promote SWS and decrease wakefulness (e.g., in rats[147,238] and cats[146,239]).

On the other hand, several reports indicate that GHB has behavioral and EEG side effects that complicate the interpretation of these findings. GHB not only promotes delta oscillations during sleep, but can induce EEG hypersynchrony during wakefulness as well, both in humans[145,236] and animals[200,238,239,240]. Thus, in studies that only rely on EEG recordings, it may be difficult to distinguish between overall EEG synchronization (including during wakefulness) and an increase in SWS.

In addition, it appeared that the effects of GHB were $GABA_B$ receptor dependent. Indeed, for example $GABA_{B1}^{-/-}$ mice, which did not express any functional $GABA_B$ receptors but kept normal binding to GHB receptors, showed neither the hypolocomotion, hypothermia, increase in striatal dopamine synthesis nor electroencephalogram delta-wave induction seen in wild-type mice[241].

Taken together, this suggests that GHB may have potential sleep-promoting effects by acting through $GABA_B$ receptors, but some of these effects may be difficult to distinguish and separate from its side effects (e.g. EEG synchronization).

The currently most prescribed sleep-promoting drugs act through $GABA_A$ or H_1 receptors and induce the so-called "non-physiological" sleep with less deep sleep and well-known side effects (e.g. dependence, tolerance). In this context, it is particularly interesting to investigate new substances such as GHB, which may induce "physiological sleep" and thus improve the treatment of different sleep disorders.

6. Research outline

6.1. Animal study

The aim of the present mouse study is to investigate the GHB effects and the role of each known subunits of $GABA_B$ receptors in vigilance states and EEG spectra by using different $GABA_B$ subunit knock-out mice: $GABA_{B1}^{-/-}$, $GABA_{B2}^{-/-}$, $GABA_{B1a}^{-/-}$ and $GABA_{B1b}^{-/-}$ mice. This will determine whether GHB (known to act as a low-affinity agonist of $GABA_B$ receptors) can induce "physiological" sleep and thus influences homeostatic regulation of sleep (and delta power) or not. Furthermore, GHB effects were compared with those of BAC, a high-affinity agonist of $GABA_B$ receptors.

EEG and EMG of the different genotypes were recorded, three different experiments were performed. First, vigilance states and EEG spectra were analyzed during 24h baseline conditions. Second, 6h sleep deprivation followed by 18h recovery were performed. Finally, four and three different doses of GBL (precursor of GHB) and BAC respectively were administrated in the middle of the 12h light period when physiological delta power is low in order to see in what extent GHB and BAC can induce delta power by themselves and how that will influence physiological sleep. This work has been published in 2010 in Journal of Neuroscience.

6.2. Human study

The human study is a continuation of the mouse study. Its aim is to investigate whether GHB can induce physiological sleep and influence homeostatic process of sleep in humans. Furthermore, because two potential adverse effects of GHB are memory impairment and sedation and because sleep is known to consolidate memory, GHB effects on vigilance and memory are assessed by two vigilance tasks and three different memory tasks, respectively.

In order to determine the role of $GABA_B$ receptors in GHB response, BAC is administrated as well and its effects are compared to those of GHB.

This study is a monocentric, placebo-controlled, double-blind and crossover study performed in young health volunteers. This human project has not yet been published. Please not that this part is not the final form and data analysis is still on going.

7. Animal study:

Differential effects of $GABA_B$ receptor subtypes, GHB, and baclofen on EEG activity and sleep regulation

7.1. Abstract

The role of $GABA_B$ receptors in sleep is still poorly understood. GHB (γ-hydroxybutyric acid) targets these receptors and is the only drug approved to treat the sleep disorder narcolepsy. $GABA_B$ receptors are obligate dimers comprised of the $GABA_{B2}$ subunit and either one of the two $GABA_{B1}$ subunit isoforms $GABA_{B1a}$ and $GABA_{B1b}$. To better understand the role of $GABA_B$ receptors in sleep regulation, we performed EEG recordings in mice devoid of functional $GABA_B$ receptors ($1^{-/-}$ and $2^{-/-}$) or lacking one of the subunit 1 isoforms ($1a^{-/-}$ and $1b^{-/-}$). The distribution of sleep over the day was profoundly altered in $1^{-/-}$ and $2^{-/-}$ mice suggesting a role for $GABA_B$ receptors in the circadian organization of sleep. Several other sleep and EEG phenotypes pointed to a more prominent role for $GABA_{B1a}$ as compared to the $GABA_{B1b}$ isoform. Moreover, we found that $GABA_{B1a}$ protects against the spontaneous seizure activity observed in $1^{-/-}$ and $2^{-/-}$ mice. We also evaluated the effects of the GHB-prodrug GBL (γ-butyrolactone) and baclofen (BAC), a high-affinity $GABA_B$ receptor agonist. Both drugs induced a state distinct from physiological sleep that was not observed in $1^{-/-}$ and $2^{-/-}$ mice. Subsequent sleep was not affected by GBL while BAC was followed by a delayed hypersomnia even in $1^{-/-}$ and $2^{-/-}$ mice. The differential effects of GBL and BAC might be attributed to differences in $GABA_B$-receptor affinity. These results also indicate that all GBL effects are mediated through $GABA_B$ receptors while these receptors seem not to be involved in mediating the BAC-induced hypersomnia.

7.2. Introduction

$GABA_B$ receptors are involved in epilepsy[222,242], anxiety and depression[230,243], nociception[244], memory[232,245], addiction[246,247,248], and potentially sleep[249,250]. While a prominent role of $GABA_A$ receptors in sleep is firmly established and is central in the pharmacological management of disturbed sleep[117], little is known about the importance of $GABA_B$ receptors in regulating sleep and the electroencephalogram (EEG). Although the effects of specific $GABA_B$ agonists, like BAC, on rapid eye movement sleep (REMS) remain unclear[249,251], available data indicate that BAC increases non-REMS (NREMS) and promotes EEG slow (delta) waves (0.75-4.5 Hz) during NREMS[251,252,253].

GHB is a GABA metabolite found in low concentrations throughout the mammalian brain[171,176,254]. Since its synthesis in the 1960s[149], GHB has been used as an anesthetic, sedative, and hypnotic agent[150,151]. Because of its abuse potential GHB is banned in many countries. GHB is approved as a treatment for narcolepsy with cataplexy[155,255]. Although the mechanism of action is still unclear, GHB decreases excessive daytime sleepiness and attacks of cataplexy in narcolepsy patients[256,257]. Despite conflicting results suggesting that GHB acts through specific GHB receptors[172,258], compelling evidence suggests that most of the physiological and pharmacological effects of exogenous GHB are mediated through $GABA_B$ receptors[197,241,259,260,261].

Both in patients and in healthy subjects, GHB decreases sleep latency and promotes deep NREMS evidenced by the marked increase in the prevalence and amplitude of EEG delta waves[145,236,237]. Animal studies also suggest that GHB promotes NREMS[147,238,239]. However, it was also reported that GHB and its GBL can induce paradoxical EEG slow/delta waves in awake humans[145,236] and animals[200,238]. This finding challenges the claimed physiological sleep-promoting effects of GHB. A first aim of the present study was to investigate the role of each of the known $GABA_B$ receptor subunits in sleep-wake regulation and in mediating the

effects of GHB. A second aim was to perform a detailed sleep and EEG analysis to investigate whether the delta waves induced by GHB contribute to normal physiological sleep.

7.3. Materials and Methods

Animals and housing conditions

All experiments were performed in accordance with the protocols approved by the Ethical Committee of the State of Vaud Veterinary Office, Switzerland.

$GABA_{B1}^{-/-}$ (*1*$^{-/-}$), $GABA_{B2}^{-/-}$ (*2*$^{-/-}$), $GABA_{B1a}^{-/-}$ (*1a*$^{-/-}$), $GABA_{B1b}^{-/-}$ (*1b*$^{-/-}$) mice were generated on a BALB/c background as described previously[220,222,225]. Adult male mice of the four genotypes along with their wild-type controls (WT) were used in baseline conditions, 6h sleep deprivation, and in experiments with GBL and saline injections (*n*=8-9/genotype, age: 10-15 weeks, weight: 24-31g). For the BAC experiments, BALB/cJ (WT) mice were purchased from Jackson Laboratory (Maine, USA). All mice were kept individually in polycarbonate cages (31 x 18 x 18 cm) under a 12h light/dark cycle (lights-on at 9:00 a.m.) at an ambient temperature of 24.5–25.5 °C. Food and water were available *ad libitum*.

Surgery and sleep recordings

EEG and electromyogram (EMG) electrodes were implanted under deep anesthesia as previously described[262]. Four to six days of recovery from surgery were allowed before connecting animals to the recording leads. A minimum of 6 adaptation days (or 10 including recovery from surgery) were scheduled before data collection. The analog signals were digitized at 2 kHz and subsequently stored at 200 Hz on hard disc. The EEG was subjected to a discrete–Fourier transformation yielding power spectra (range: 0.75–90 Hz, frequency resolution: 0.25 Hz, time resolution: consecutive 4s epochs, window function: hamming). Hardware (EMBLA®) and software (Somnologica–3®) were purchased from Medcare/Flaga

(Iceland). Activity in the 50 Hz band was discarded from further analysis because of power line artifacts in the EEG of some of the animals.

Based on the EEG and EMG signals, the animal's behavior was classified as REMS, NREMS, or wakefulness [263]. In addition to these three behavioral states, seizures, and drug (i.e., GBL or BAC)-induced states were also assessed (for description see below "GBL and BAC administration"). All states were scored by visual inspection of the EEG and EMG signals displayed on a PC monitor. 4s epochs containing EEG artifacts were marked, and excluded from EEG spectral analyses.

Five to twelve animals were recorded together in one experimental session (*1b*$^{-/-}$ mice: *n*=9; *1a*$^{-/-}$ mice: *n*=8; WT mice: *n*=8; *1*$^{-/-}$ mice: *n*=8; *2*$^{-/-}$ mice: *n*=8). At least two genotypes were included per session in an attempt to equally distribute the environmental variation over genotypes. Overall, eight sessions were necessary to complete the study.

Baseline and sleep-deprivation experiments

EEG and EMG signals were recorded continuously for at least 48 hours with the first 24 hours serving as baseline followed by 6h sleep deprivation (SD) starting at light onset and 18 hours of recovery sleep. SD was achieved by 'gentle-handling' consisting of introducing novel objects into the cage, approaching a pipette next to the mouse, or gentle cage-tapping, as soon as a sleeping behavior was observed. Note that due to health deterioration, particularly when disturbed, *1*$^{-/-}$ and *2*$^{-/-}$ mice were not included in the sleep deprivation protocol. One subset of mice was used for the baseline conditions protocol (*n*=8/genotype), and one other subset of mice was included in the drug protocol (see below).

Mean EEG spectra were calculated over 4s epochs scored as artifact-free NREMS, REM, or wakefulness to construct behavioral state-specific spectral EEG profiles for baseline. EEG delta power (a measure of homeostatic sleep need) was calculated by averaging EEG power density in the 1-4 Hz range for 4s epochs scored as NREMS. Time-course analysis of EEG delta

power during baseline and after SD was described in detail elsewhere[264]. In short, the recording was divided into sections to which an equal number of 4s epochs scored as NREMS contributed (i.e., percentiles). The first 6 hours of the baseline light period was divided into 6 such sections; the second 6 hours into 4. The second 6 hours of the recovery light period was divided into 6 sections, the dark periods of both the baseline and recovery dark periods into 8 sections. The choice of the number of sections per recording period depended on NREMS prevalence. Delta power values were normalized by expressing them as a percentage of the individual mean value reached over the last 4 hours of the main rest period when delta power is minimal during baseline.

The main rest period was calculated as described previously[265] with modifications. Mean sleep duration was calculated over a 2h moving average at 15min increments within individual mice. Fifteen-minute intervals in which mice slept more than their individual 24h baseline mean were termed as 'rest'. Fourteen or more 15min 'rest' intervals interrupted by < 6 non-'rest' intervals constituted a rest period. Applying this algorithm to mice, generally one main rest period is obtained associated with the light period.

Sleep quality was assessed by analyzing its consolidation by counting the number of brief awakenings and the number of short and long NREMS episodes as previously described[265].

GBL and BAC administration

Five days after the sleep deprivation experiment, EEG and EMG signals were recorded continuously for 6 consecutive 24h periods, starting at lights-on. 24h baseline was followed by a "saline" day and 4 days with administration of four different doses of GBL (50, 100, 150, and 300 mg/kg) or 3 days with administration of three different doses of BAC (5, 7.5, and 10 mg/kg). WT mice taking part in the BAC experiment and $1^{-/-}$ and $2^{-/-}$ mice were not previously used in the sleep deprivation experiment. Out of concern of health condition in $1^{-/-}$ and $2^{-/-}$ mice the drug protocol was slightly simplified, i.e., only saline and the highest drug

dose were tested (*n*=3/genotype/drug). To exclude any carry-over or tachyphylaxis due to our increasing dosing protocol, eighteen wild-type BALB/cJ mice (*n*=9/drug) were studied in a randomized cross-over experiment with GBL and BAC at the lowest and highest doses and saline. Mice were included in one of 3 conditions: (A) administration of the highest dose followed 24 hours later by the lowest dose and 24 hours later by saline, (B) administration of the highest dose followed by 48h washout, then the lowest dose and 24 hours later saline and, (C) administration of saline followed 24 hours later by the lowest and 24 hours later by the highest dose. The results indicated that the order of dose or duration of washout did not significantly affect the results for three main and tested sleep phenotypes: amount of drug-induced state, time course of delta power in NREMS following the drug-induced state, and amount of NREMS after drug administration (rANOVA factor 'condition'; data not shown).

Drug doses were chosen according to the literature to cover a large range of sedative/hypnotic effects that could be compared between drugs[200,222,266,267]. Saline, GBL, and BAC were i.p. administrated 6 hours after lights onset at Zeitgeber Time (ZT) 6 (light onset being ZT0). At least 18 hours were recorded after the last injection. BAC- and GBL-induced states were characterized by an increase of hypersynchronous slow waves and/or spiky EEG pattern following drug injection. The drug-induced state can be readily distinguished from the three classical behavioral states, and were therefore analyzed separately. The drug-induced state was determined as follows: at the onset of the drugs effects animals were awake while large amplitude short lasting (2-4s) burst of hypersynchronous slow waves appeared and progressively dominated the EEG until 'normal' EEG activity could no longer be discerned. The first waking 4s epoch in which abnormal EEG activity was observed was taken as drug-induced state onset. Towards the end of the drug-induced state this alternation between 'normal' waking and drug-induced state reappeared. The last 4s epoch

with abnormal EEG was taken as the end of the drug-induced state. Please note that the amount of drug-induced state was the sum of 4s epochs scored as drug-induced state.

Four-second epochs of NREMS, BAC-, and GBL-induced states were subjected to spectral analysis to calculate the EEG power density in the delta frequency range (1-4 Hz). Time-course analysis of the delta power on the saline day and the three or four days with injections of BAC or GBL, respectively, were performed similarly to the baseline condition. Delta power during the BAC- and GBL-induced state is presented as a single time-point as its duration was too short to reliably estimate a time course.

Spectral content of the EEG during NREMS, BAC-, and GBL-induced state was quantified as described above. EEG spectra were normalized to be directly comparable as follows: EEG power in each frequency bin for each mean NREMS or drug-induced state spectrum was expressed as a percentage of the mean NREMS EEG power determined over all artifact-free 4s epochs during 4 hours of the rest period in baseline within individual mice.

Analysis tools

TMT Pascal Multi-Target®5 (Software, TMT Development Corp, Brighton (MA), U.S.A) was used to manage the data, SigmaPlot® 10.0 (Systat Software Inc., London, UK) for graphics, and SAS Institute (Cary, NC) software, Ver. 9.1 for statistical analyses.

Drugs

Placebo was a saline solution (NaCl 0.9%, B. Braun Medical AG, Emmenbrücke, Switzerland). GBL and racemic BAC (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were freshly diluted in saline solution to obtain different solutions of GBL (50, 100, 150, and 300 mg/kg) and BAC (5, 7.5, and 10 mg/kg) with an injection volume of 5ml/kg of body weight.

GBL has a greater lipid solubility than GHB, allowing uniform and rapid absorption[268]. The *in vivo* pharmacological properties of GBL are secondary to its final conversion into GHB[163]. GBL

is biologically inactive[164] and all its physiologic and behavioral effects are due to its rapid conversion (< 1 min) to GHB by peripheral lactonases or by non-enzymatic hydrolysis [165,166].

7.4. Results

Spontaneous epileptiform activity in $1^{-/-}$, $2^{-/-}$, and $1a^{-/-}$ mice

Both *1*$^{-/-}$ and *2*$^{-/-}$ mice lack functional $GABA_B$ receptors while *1a*$^{-/-}$ mice still have functional $GABA_{B1b,2}$ receptors and *1b*$^{-/-}$ mice $GABA_{B1a,2}$ receptors. As previously observed [222,225], all *1*$^{-/-}$ and *2*$^{-/-}$ mice displayed spontaneous seizures. Over the 24h baseline recording period, 5 out of 8 mice of both *1*$^{-/-}$ and *2*$^{-/-}$ genotypes showed at least one seizure. Health status of *1*$^{-/-}$ and *2*$^{-/-}$ mice gradually deteriorated manifested as weight loss, ruffled fur, and hunched posture accompanying the increasing number of seizures (up to 20 seizures per day). Only data from healthy animals were included in the analyses.

Interestingly, 4 out of 8 *1a*$^{-/-}$ mice also exhibited similar spontaneous epileptiform activity (Figure 7.1A), a phenotype never described before for this genotype. This epileptiform trait was, however, less severe with the number of seizure never exceeding 4 per day and without affecting their overt health status. Almost all seizures observed in the three genotypes were of the clonic type lasting between 12 seconds and 1.5 minutes. On occasion, tonic-clonic seizures were observed after audiogenic stimuli, handling, or cage change. In addition, epileptic mice displayed high-voltage EEG spikes generally in the hours prior and/or following a seizure (Figure 7.1B,C). Seizures could occur in all behavioral states and during both light and dark periods. No epileptiform activity was observed in any of the *1b*$^{-/-}$ or WT mice.

Loss of $GABA_B$ receptors delays the rest period, reduces delta and theta activity in the NREMS EEG, and increases theta activity in the waking EEG.

Although genotype did not affect behavioral state duration under baseline conditions (Table 7.1) a lack of functional $GABA_B$ receptors greatly altered the sleep-wake distribution (Figure 7.1D). While the main rest period in *1a*$^{-/-}$, *1b*$^{-/-}$, and WT mice was initiated several hours before light onset (-4.6 ± 0.7, -6.9 ± 0.4, and -6.2 ± 0.8 h, respectively; mean ± SEM throughout the text) which is typical for male BALB/c mice [85,265], in *1*$^{-/-}$ and *2*$^{-/-}$ mice the onset of the rest period was delayed by 6 h compared to WT and became closely associated with light-onset. The end of the rest period was similarly delayed in *1*$^{-/-}$ and *2*$^{-/-}$ mice compared to *1a*$^{-/-}$, WT, and *1b*$^{-/-}$ mice (11.7 ± 0.2, 11.8 ± 0.1, 10.0 ± 0.3, 9.0 ± 0.4, and 8.9 ± 0.3 h after light onset, respectively; one-way ANOVA for rest-onset and rest-end: $p<0.0001$, Paired t-test: $p<0.05$). *1a*$^{-/-}$ mice displayed an onset and end of their rest period intermediate between *1*$^{-/-}$ and *2*$^{-/-}$ mice on the one hand, and WT and *1b*$^{-/-}$ mice on the other. The delay of the end of the rest period was also reflected in the increased time-spent-asleep in the second half of the light period with highest values reached in *1*$^{-/-}$ and *2*$^{-/-}$ mice, intermediate in *1a*$^{-/-}$ mice, and lowest in WT and *1b*$^{-/-}$ mice (Figure 7.1D, Figure 7.2A,C).

Genotype difference in the duration of the rest period, calculated as the time-span between the onset and end of the rest period, also distinguished *1*$^{-/-}$ and *2*$^{-/-}$ mice, from *1a*$^{-/-}$, WT, and *1b*$^{-/-}$ mice, the former two genotypes showing a significantly shorter rest period (one-way ANOVA for total rest duration: $p<0.0001$, *1*$^{-/-}$ = *2*$^{-/-}$ < *1a*$^{-/-}$ = WT = *1b*$^{-/-}$; Tukey's test: $p<0.05$; Figure 7.1D). Also for this phenotype, *1a*$^{-/-}$ mice appeared intermediate between mice completely lacking $GABA_B$ receptors and WT and *1b*$^{-/-}$ mice. While all other genotypes displayed only one rest period per 24 h, *1b*$^{-/-}$ mice showed a consistent 3.0 ± 0.2 h 'gap' interrupting the rest period (Figure 7.1D). Apart from this marked re-distribution of sleep and waking over the day, we also noticed genotype differences in sleep architecture at the level

of individual sleep episodes. Judged by the increased number of brief awakenings (< 16 s), sleep in *1a*$^{-/-}$ mice was more fragmented compared to all other genotypes (Figure 7.1E) consistent with the fragmented sleep recently reported in mice lacking functional $GABA_B$ receptors in orexin neurons specifically[269]. In contrast, we found that an overall lack of functional $GABA_B$ receptors (i.e., in *1*$^{-/-}$ and *2*$^{-/-}$ mice) lead to a greater number of longer periods of sleep (> 1 min) compared to the other genotypes (Figure 7.1E). Thus, brain site-specific effects of $GABA_B$ receptors and subcellular localization of $GABA_B$ receptors subunits[220,270] can have a profound impact on the consolidation of sleep.

$GABA_B$ receptor genotype also affected EEG activity and the main spectral changes were found in frequencies below 20 Hz. During NREMS, *1b*$^{-/-}$ mice exhibited a reduced EEG activity in theta frequency range compared to WT (3.75-7.5 Hz, Figure 7.3). This decrease became more pronounced both in terms of amplitude and frequency range in *1*$^{-/-}$ and *2*$^{-/-}$mice, which showed a strong decrease over a broad frequency range (1.75-10 Hz), including both delta and theta frequencies, compared to WT mice. This decrease is reminiscent of the reduction in EEG synchronization observed after the thalamic administration of a $GABA_B$ receptor antagonist[250] underscoring the crucial role of $GABA_B$ receptors in thalamocortical oscillations characteristic of NREMS[271,272].

The waking EEG spectra of the latter two genotypes also markedly differed from *1a*$^{-/-}$, *1b*$^{-/-}$, and WT mice in that theta activity, especially at around 7 Hz, was more pronounced (Figure 7.3; statistics not shown for *1a*$^{-/-}$ and *1b*$^{-/-}$ mice). The increase in theta power during wakefulness might suggest an increase in active and exploratory behavior which is associated with hippocampal theta oscillations[273]. Alternatively, $GABA_B$ receptors seem to be directly involved in theta rhythm generation[274] although the REMS spectral signature, with its characteristic theta peak around 7 Hz, remained unaffected by genotype.

The homeostatic regulation of sleep is not affected in mice lacking $GABA_B$ receptor subunits

A 6h sleep deprivation (SD) was performed to assess whether $GABA_B$ receptor subunits contribute to sleep homeostasis. Due to the health deterioration of $1^{-/-}$ and $2^{-/-}$ mice during SD these two genotypes were excluded from this experiment. Recovery of sleep loss in the three remaining genotypes was evident by increases in both NREMS duration and in EEG delta power in the first 6 hours after SD (i.e., recovery light period). This response did not differ among $1a^{-/-}$, $1b^{-/-}$, and WT mice (Figure 7.2). EEG delta power steeply declined over the course of recovery and fell below baseline in the subsequent recovery dark period. During this period levels of delta power in $1a^{-/-}$ mice were higher than those observed in $1b^{-/-}$ and WT mice. This genotype difference was observed also in the dark period of baseline after a spontaneous period of wakefulness (Figure 7.2B). Although the effect of SD could not be evaluated in $1^{-/-}$ and $2^{-/-}$ mice (see Materials and Methods section), EEG delta power during baseline also decreased during the rest period and increased over the course of the active period. Like for $1a^{-/-}$ mice, delta power levels reached in the baseline dark period in $1^{-/-}$ and $2^{-/-}$ mice seemed higher than WT and $1b^{-/-}$ mice (Figure 7.2B). These results together with the effects observed after SD in the other three genotypes suggest that $GABA_B$ receptors do not play a major role in sleep homeostasis as indexed by EEG delta power.

GBL, through $GABA_B$ receptors, induces an anesthetic-like state distinct from physiological sleep

We tested the effects of GBL and BAC on sleep and the EEG at various doses. Order of dose did not affect the main drug effects presented here (see Materials and Methods). Administration of GBL or BAC did not affect behavior or the EEG in $1^{-/-}$ and $2^{-/-}$ mice. In contrast, GBL and BAC dose-dependently affected the EEG and behavior in WT, $1a^{-/-}$, and $1b^{-/-}$ mice without noticeable behavioral or EEG differences among these three genotypes. At

low doses of GBL (50 and 100 mg/kg), EEG slow waves appeared (Figure 7.4A) and locomotor activity decreased, while animals remained behaviorally awake with eyes open and responded normally when stimulated. At higher GBL doses (150 and 300 mg/kg), mice became immobile with an unnatural flat body posture with hind limbs stretched sideways while eyes remained open. Their EEG displayed hypersynchronous slow waves and a spike-like pattern, which was more abundant after the highest dose (Figure 7.4B). Importantly, at the highest GBL dose, animals became completely unresponsive to stimulation resembling a state of deep anesthesia. BAC, administered in WT mice, also induced hypersynchronous slow waves and decreased locomotor activity. However, even at the highest dose (10 mg/kg), BAC did not induce the spike-like EEG pattern observed after 300 mg/kg of GBL (Figure 7.4C,D). Moreover, although at 10 mg/kg animals were also immobile with abnormal flat posture and open eyes, they still responded to tactile stimuli. The EEG patterns combined with behavioral observations indicated that the state induced by the drugs could not be interpreted as either normal sleep or wakefulness. We therefore scored periods with abnormal EEG following drug administration as "drug-induced state" (see Materials and Methods). While the GBL-induced state appeared 4 to 9 min after injection in *1a*$^{-/-}$, *1b*$^{-/-}$, and WT, the BAC-induced state appeared significantly later (13 to 17 min, one-way ANOVA: $p<0.001$) in WT mice. The amount of both drug-induced states varied according to dose and both *1a*$^{-/-}$ and *1b*$^{-/-}$ mice displayed an overall shorter GBL-induced state amount than WT mice (Figure 7.5E,F).

Because of the induction of slow waves, especially at lower drug doses, reminiscent of those present during NREMS, we contrasted delta power during drug-induced state to the levels usually obtained during NREMS. Delta power during GBL-induced state increased from 50 to 100 mg/kg but did not further increase at higher doses (Figure 7.6A). In *1a*$^{-/-}$ and *1b*$^{-/-}$ mice delta power reached at the highest three doses was significantly higher compared to after 50

mg/kg of GBL (Figure 7.6A). Furthermore, in *1a*$^{-/-}$ mice, levels reached at the three highest doses were around 2-fold higher compared to *1b*$^{-/-}$ and WT mice and 3-fold higher than the baseline reference reached in NREMS. During the BAC-induced state, delta power levels remained within the baseline range determined for NREMS and did not differ among doses (Figure 7.6B).

Similar to the analysis of delta power, we contrasted the full EEG spectra during the drug-induced state to the EEG spectra obtained during NREMS over the last four hours of the baseline rest period. In addition, because like GBL and BAC also SD increased delta power (Figure 7.2B), we compared the drug-induced state EEG spectra to the EEG spectra obtained during NREMS after 6h SD. Results for the highest GBL and BAC doses are illustrated in Figure 7.6A,B (for all doses see Suppl 7.1). Spectral analyses revealed that the abnormal EEG activity following the injection of the highest GBL dose (300 mg/kg; Figure 7.4B) was due to a large increase of EEG activity in the low delta frequencies (0.75-1.5 Hz) reaching 3 to 4-fold higher levels than those reached after BAC and saline injections and ~1.5-fold higher compared to the effects of SD (Figure 7.6A). An equally large suppression of EEG activity was observed at frequencies over 3 Hz with the largest reduction reached at around 13 Hz (Figure 7.6A). The GBL effects on the EEG spectra were dose-dependent (two-way ANOVA in WT mice: factor 'dose': $p<0.0001$, factor 'bin' $p<0.0001$, interaction $p<0.0001$) with a progressive increase with dose in the low delta frequencies (0.75-1.75 Hz) and a decrease with dose for frequencies above 3 Hz (Suppl 7.1).

EEG spectra during the BAC-induced state revealed that only fast delta activity (4-5.25 Hz) contributed to the slow waves induced by this drug (Figure 7.6A). Although the increase in this frequency range was similar to the increase observed in the NREMS EEG after SD, the effect of SD also included slower delta oscillations (Figure 7.6A). EEG activity in higher frequencies (10.75-37.5 Hz) encompassing the sigma and beta ranges, was clearly reduced

by BAC compared to the NREMS spectrum after saline injection (Figure 7.6A, statistics not shown). Dose-dependent spectral differences for BAC were restricted to 3.25-4.5 Hz range, where 5 mg/kg has a significantly smaller effect than 10 mg/kg (Suppl 7.1D). Decreases in power density at higher EEG frequencies in the NREMS EEG were not observed after SD. Together these results suggest that neither the GBL- nor the BAC-induced state reflect physiological sleep found after 6h SD or saline injection.

Genotype affected the drug-induced changes in EEG spectra. The most salient of these genotype differences are illustrated for the highest dose of GBL (300 mg/kg) in Figure 7.6B. In *1a*$^{-/-}$ mice, the increase in low frequencies during the GBL-induced state was more pronounced compared to WT and *1b*$^{-/-}$ mice and significant increases in EEG power density extended to 2.75 Hz (high doses vs. the lowest dose; Suppl 7.1A,B,C). Moreover, the decrease in EEG power density for frequencies >3 Hz, equally observed in *1b*$^{-/-}$ and WT mice, was less pronounced in *1a*$^{-/-}$ mice. In general, *1b*$^{-/-}$ mice displayed a GBL EEG signature very similar to that observed in WT mice (Figure 7.6B and Suppl 7.1A,B). The same held true for the EEG spectra during subsequent NREMS (Figure 7.6D and Suppl 7.2A,B).

BAC induces hypersomnia similar to that observed after sleep deprivation

After the acute effects of the drugs on behavior and the EEG waned, normal behavioral states could again be assigned. We quantified the longer-term effects of both drugs on sleep and the EEG in WT as well as in *1*$^{-/-}$ and *2*$^{-/-}$ mice. Compared to individually-matched recording periods after saline injections, both BAC and GBL initially suppressed REMS. This loss in REMS time was fully compensated over the course of the final 12 h of the recording period (Figure 7.7A and Suppl 7.3). The effect on NREMS amount importantly differed between the two drugs, illustrated for the highest doses of BAC and GBL in Figure 7.7B (For other doses see Suppl 7.3). Over the entire recovery period after BAC injection mice spent 40 min more in NREMS than calculated over the same period after saline injection (Figure 7.7B).

Especially during the dark period, extra NREMS was accumulated. In stark contrast, GBL was followed by an immediate decrease of NREMS in the recovery light period (Figure 7.7B and Ref[200]). During the subsequent dark period no differences in NREMS time were observed. As a result of these opposing drug effects, at the end of the recording period, WT mice treated with BAC gained 1.0 h of NREMS compared to WT mice treated with GBL, indicating that BAC induced a long-term hypersomnia (Figure 7.7A,B; ANOVA: p=0.0005). Interestingly, hypersomnia was also observed after BAC administration in $1^{-/-}$ and $2^{-/-}$ mice now concerning both NREMS and REMS (Figure 7.7C,D). This indicates that, in contrast to the acute effects of BAC, BAC-induced hypersomnia might not be mediated through $GABA_B$ receptors.

GBL affected REMS in $1a^{-/-}$ mice in a similar fashion as observed for WT mice with an initial decrease that was compensated during the recovery dark-period (Suppl 7.3). In $1b^{-/-}$ mice the extra REMS occurring during the dark period was somewhat more pronounced resulting in an overcompensation of REMS at the end of the recording period for the lowest two GBL doses (Suppl 7.3). In contrast to WT and $1a^{-/-}$ mice, $1b^{-/-}$ mice spent significantly more time in NREMS during the dark period relative to the saline condition.

Besides sleep amounts we also quantified the distribution and consolidation of sleep but did not observe significant changes in sleep fragmentation after any dose of GBL or BAC compared to saline conditions (Suppl 7.4). However, similar to baseline conditions, sleep in $1a^{-/-}$ mice was more fragmented compared to $1b^{-/-}$ and WT mice. Moreover, as pointed out above for baseline (Figure 7.2C), $1a^{-/-}$ mice spent more time asleep during the last 6h of the light period than $1b^{-/-}$ and WT mice throughout the 5-day drug experiment (one-way ANOVA for each day: p<0.05, Tukey's test: $1a^{-/-}$>$1b^{-/-}$=WT, data not shown).

Recovery from drug effects was also assessed at the level of delta power in NREMS. Despite the pronounced increase in EEG delta power during the GBL-induced state (Figure 7.5A,

Figure 7.6A), the time course of delta activity during subsequent recovery sleep remained unaffected in the genotypes tested (i.e., *1a*$^{-/-}$, *1b*$^{-/-}$, and WT; Figure 7.5A). As expected, GBL also failed to alter the time course of EEG delta power during NREMS in *1*$^{-/-}$ and *2*$^{-/-}$ mice (Suppl 7.5). In stark contrast to the lack of an effect of GBL, the BAC-induced state was followed by an immediate increase in NREMS delta power, independent of dose (Figure 7.5B). Delta power quickly decreased in the presence of NREMS and, in the dark period, values below those obtained during the same period after saline injection were reached. This decrease became more pronounced with increasing dose (one-way ANOVA factor 'dose' $p<0.0001$, Tukey's test: saline > 5 = 7.5 > 7.5 = 10 mg/kg) consistent with the dose-dependent BAC-induced increase in NREMS time during the dark period (Suppl 7.3). In contrast to the BAC-induced hypersomnia (see above), the BAC-induced increase in delta power during NREMS was not observed in *1*$^{-/-}$ and *2*$^{-/-}$ mice (Suppl 7.5), suggesting that only the latter effect involves the $GABA_B$ receptor. The effects of BAC in WT mice on NREMS time and especially on the dynamics of delta power are very similar to the effects of SD (Figure 7.2).

We analyzed these drug effects on the NREMS EEG in further detail by comparing NREMS spectra obtained immediately after the end of the drug-induced states with the NREMS spectra obtained immediately after a 6h SD and after a saline injection in WT mice. EEG spectra were calculated over the first 20 minutes of NREMS following each of these conditions and expressed as a percentage of the individual mean NREMS spectra over the 4 last hours of the rest period (same reference was used for evaluating the EEG during the drug-induced state; see Figure 7.6). The similarity between the EEG effects of BAC and SD were not restricted to the delta frequencies. EEG spectra calculated over the first 20 minutes of NREMS following the BAC-induced state and SD were similar over a broad frequency range and differed only in the low delta frequencies (1-2 Hz; Figure 7.6C) independently of

dose (Suppl 7.2D). EEG power density during NREMS after GBL was significantly lower in the delta and low theta frequency range (1-5.75 Hz) and higher in gamma and higher frequency ranges (30-90 Hz) compared to the NREMS spectra obtained after both BAC and SD (Figure 7.6C). Compared to the saline conditions GBL increased EEG activity in the theta (7.25-9.75 Hz) and in the higher beta, gamma, and higher frequency ranges (18.5-90 Hz; Figure 7.6, statistics not shown). Note that in WT mice, relative NREMS spectra after any dose of GBL and BAC were significantly different from those after saline (Suppl 7.2A,D). In WT and *1b*$^{-/-}$ mice relative NREMS spectra after GBL exhibited a significant dose-dependent increase in theta, beta and gamma activity (7-10 Hz and 18-90 Hz; Suppl 7.2A,B). In *1a*$^{-/-}$ mice, although a similar tendency was observed no significant difference among doses were obtained (Suppl 7.2C). This decreased EEG response in *1a*$^{-/-}$ mice was illustrated for the highest GBL dose (300 mg/kg); the relative increase in NREMS EEG activity in the 8-10 Hz range was lower in *1a*$^{-/-}$ mice compared to that observed for the two other genotypes (Figure 7.6D).

7.5. Discussion

We studied the role of $GABA_B$ receptors in sleep in mice lacking functional $GABA_B$ receptors or one of the two $GABA_{B1}$ receptor subtypes. We identified a number of sleep and EEG phenotypes under baseline conditions and after the administration of $GABA_B$-receptor agonists that not only separated *1*$^{-/-}$ and *2*$^{-/-}$ mice from *1a*$^{-/-}$, *1b*$^{-/-}$, and WT mice but also *1a*$^{-/-}$ from *1b*$^{-/-}$ and WT mice. Among the most salient phenotypes we observed in *1*$^{-/-}$ and *2*$^{-/-}$ mice are the presence of clonic seizures, the marked delay in the distribution of sleep over the 24h day, the altered spectral composition of the NREMS and waking EEG, and the complete lack of the acute response to GBL and BAC. *1a*$^{-/-}$ mice differed from *1b*$^{-/-}$ and WT mice in that they showed seizures, their sleep was more fragmented and more prevalent in the second half of the light period, and after GBL administration responded with a larger

increase in EEG delta power. For several sleep and EEG phenotypes *1a*$^{-/-}$ thus seemed intermediate between *1b*$^{-/-}$ and WT mice, on one hand, and *1*$^{-/-}$ and *2*$^{-/-}$ mice on the other, suggesting functional differences between the two $GABA_{B1}$ receptor isoforms. These differences are likely to be due to differential subcellular localizations of the two isoforms because binding pharmacology showed similar properties[220,221].

The $GABA_{B1a}$ receptor subunit protect against seizures

Spontaneous epileptiform activity has been reported in mice lacking functional $GABA_B$ receptors[222,225]. We discovered that mice lacking subunit $GABA_{B1a}$ also display spontaneous seizures indicating a specific role for $GABA_{B1a}$ subunit in preventing seizures. $GABA_{B1a}$ and $GABA_{B1b}$ subunits localize to distinct synaptic sites thereby conveying separate functions. Of relevance for the epileptiform trait is the fact that at hippocampal synapses, $GABA_{B1a,2}$ receptors inhibit glutamate release, while $GABA_{B1b,2}$ receptors predominantly mediate postsynaptic inhibition[220]. The lack of presynaptic inhibition of glutaminergic neurons in *1a*$^{-/-}$ mice might have contributed to the presence of seizures. Functional differences between these two subunits might also have contributed to the sleep and EEG genotype differences we report here.

$GABA_B$ receptors determine the diurnal organization of sleep

The distribution of sleep and wakefulness over the 24h day markedly differed among genotypes. BALB/c and BALB/cByJ mice initiated their main rest period in the middle of the dark period (Figure 7.1; Ref [85,265]) while the rest period in *1*$^{-/-}$ and *2*$^{-/-}$ mice coincided largely with the light period, which is common for most other inbred strains. We and others attributed the earlier rest onset and resulting compression of the active period to the shorter endogenous circadian period length observed in BALB/c mice[85,265,275]. Several studies implicate $GABA_B$ receptors in circadian timing. Activation of $GABA_B$ receptors in the

suprachiasmatic nucleus (SCN), the master circadian clock, phase-shifts circadian rhythms both *in vitro* and *in vivo*[276,277], and the effects of light on circadian phase are blocked by BAC[278,279]. It remains to be established whether the large delay in the timing of the rest period we report here is due to a role of $GABA_B$ receptors at the level of the (light) input to the SCN or at the level of rhythm generation itself.

$GABA_B$ receptor agonists do not promote physiological sleep

The lack of any behavioral and EEG effects of GBL in $1^{-/-}$ and $2^{-/-}$ mice, clearly indicates that exogenous GHB acts through $GABA_B$ receptors only. A similar lack of effect in $1^{-/-}$ mice has been reported for other variables such as the GHB-induced decrease in locomotor activity and hypothermia[197,241]. Our behavioral and EEG observations show that GBL does not induce physiological sleep, but a sub-anesthetic state with EEG hypersynchrony consistent with reports by others[200,238]. Also BAC did not initially induce physiological sleep and its acute effects in WT mice had some similarities with the acute effects of GBL. However, BAC even at the highest dose failed to induce the spiky EEG pattern characteristic of the GBL-induced state while the amount of the drug-induced state was comparable between the two drugs. First evidence of spiky EEG patterns appeared at an extreme high BAC dose (50 mg/kg) but at this dose the drug-induced state lasted around 5 h (data not shown) demonstrating that the drug dynamics for EEG and behavioral aspects greatly differ.

Delta power during NREMS is in a quantitative and predictive relationship with prior wakefulness and is therefore thought to reflect a need or pressure for NREMS and its underlying homeostatically regulated recovery process[264]. Delta power during NREMS is also considered a measure of the efficiency with which sleep need decreases during NREMS[97,280]. The profound increase in EEG delta activity during the GBL-induced state did not affect the dynamics of delta power in subsequent NREMS indicating that functionally, GBL-induced delta oscillations differ from those expressed during physiological NREMS.

The changes evoked by BAC on subsequent NREMS were even more remarkable than the lack of response observed after GBL; delta power importantly increased and the subsequent recovery dynamics were highly similar to those observed after SD. This similarity was true for the entire NREMS EEG spectrum supporting the puzzling conclusion that the BAC-induced state is functionally similar to intense wakefulness. Nevertheless, we cannot rule out that the increase in delta power is a residual direct effect of BAC on EEG synchronization rather than reflecting increased homeostatic drive. Also the pattern of NREMS recovery with its largest increase in the dark period is reminiscent of the effect of SD[265]. This delayed hypersomnia was observed also in $1^{-/-}$ and $2^{-/-}$ mice suggesting that this aspect of the sleep response is most probably not mediated through $GABA_B$ receptors. Studies in human subjects reported a BAC-induced increase in NREMS[251,281] and somnolence as a side effect[252,282]. In contrast and similar to our findings in mice, GHB given at night did not increase total sleep time in healthy men and narcolepsy patients[145,283] and did not induce daytime somnolence and, importantly, reduces excessive daytime sleepiness in narcolepsy patients[257].

Although $GABA_B$ receptors mediate the acute effects of both GBL/GHB and BAC and the two drugs have several effects in common (e.g. hypothermia, catalepsy, sedation[284]), the underlying mechanisms may not be identical[267,285] For instance, in mice, NMDA receptor antagonists enhanced the cataleptic effects of GHB but not those of BAC[286] suggesting a differential role of glutamate in $GABA_B$ receptor-mediated effects of GHB and BAC. Moreover, BAC inhibited both dopaminergic and GABAergic neurons in the ventral tegmental area, while GHB inhibited only GABAergic[246]. This discrepancy may be explained by the fact that GHB is a full, low-affinity agonist and BAC a full, high affinity agonist of $GABA_B$ receptors[287]. Thus, low-affinity compounds can have very different or even opposite effects compared to high-affinity agonists. These differences in drug kinetics could be further modulated by potassium channel tetramerization domain-containing proteins that function as auxiliary

subunits of $GABA_B$ receptors[235].

Conclusions

It is believed that GHB, by consolidating sleep and promoting EEG delta oscillations, reduces excessive daytime sleepiness and cataplexy associated with narcolepsy. Although it has been reported that GHB consolidates sleep in narcolepsy patients[288] and that BAC promotes sleep efficiency in healthy subjects[251], we found no evidence for increased sleep consolidation after GBL or BAC in mice. Given the contradictory effects of both drugs on EEG and sleep among the various studies, species differences and potentially the dose used might play a role. Our in depth quantitative EEG analyses show that, at least in the mouse, GBL and BAC do not promote physiological sleep at the doses used and that delta oscillations during the drug-induced state functionally differ from those during NREMS. We further identified several functional differences between the two $GABA_{B1}$ isoforms, the most salient of which concerns the role of the $GABA_{B1a}$ subunit in epileptogenesis and sleep consolidation. Finally, BAC, but not GHB, seems to mobilize a sleep homeostatic mechanism comprised of hypersomnia and increased EEG delta power. Identifying the cellular mechanism contributing to this differential response might gain insight into the elusive sleep homeostatic process.

7.6. Tables and Figures

Table 7.1: Behavioral states in baseline; 12 and 24h values.

	Waking (min)	NREMS (min)	REMS (min)	TS (min)
24h period				
WT	753.9 ± 5.6	608.6 ± 8.9	77.5 ± 5.2	686.1 ± 8.9
$1a^{-/-}$	752.1 ± 18.4	607.9 ± 19.3	79.9 ± 4.6	687.8 ± 19.3
$1b^{-/-}$	796.7 ± 14.5	561.6 ± 16.7	81.7 ± 3.5	643.3 ± 16.7
$1^{-/-}$	781.5 ± 29.6	570.6 ± 32.5	81.3 ± 6.0	651.8 ± 32.6
$2^{-/-}$	812.4 ± 27.1	543.0 ± 30.1	82.7 ± 4.7	625.7 ± 29.6
p	0.28	0.12	0.95	0.25
12h light period				
WT	328.5 ± 13.3 bc	352.0 ± 15.9 ab	39.5 ± 3.7 a	391.5 ± 15.9 ab
$1a^{-/-}$	283.3 ± 7.8 ab	386.8 ± 8.6 bc	49.7 ± 4.5 ab	436.5 ± 8.6 bc
$1b^{-/-}$	350.9 ± 10.6 c	325.1 ± 12.1 a	44.0 ± 2.7 a	369.1 ± 12.1 a
$1^{-/-}$	244.5 ± 18.9 a	413.4 ± 20.5 c	58.8 ± 4.3 b	472.2 ± 19.9 c
$2^{-/-}$	244.7 ± 16.6 a	429.2 ± 16.4 c	64.4 ± 2.9 b	493.6 ± 16.5 c
p	<0.0001	<0.0001	0.0001	<0.0001
12h dark period				
WT	425.4 ± 14.4 a	256.5 ± 17.5 b	38.0 ± 4.0 b	294.6 ±17.5 b
$1a^{-/-}$	468.8 ± 16.9 ab	221.0 ±19.4 bc	30.2 ± 2.7 ab	251.2 ± 19.4 bc
$1b^{-/-}$	445.8 ± 11.5 ab	236.5 ± 14.9 b	37.7 ± 4.1 b	274.2 ± 14.9 b
$1^{-/-}$	537.0 ± 24.7 bc	157.2 ± 29.7 ac	22.4 ± 5.1 ab	179.7 ± 29.5 ac
$2^{-/-}$	587.7 ± 28.3 c	113.8 ± 18.2 a	18.2 ± 4.7 a	132.1 ± 33.0 a
p	<0.0001	<0.0001	0.0048	<0.0001

Mean (±SEM; *n*=8-9) artifact free recording time in 24h baseline, 12h light, and 12h dark period for the time spent in waking, NREMS, REMS, and total sleep time (TS; NREMS+REMS). Behavioral states varied among genotypes (*p* values of one-way ANOVA indicated). *a–c*, Tukey's test, $p<0.05$; genotypes for which mean values significantly differed do not share the same character.

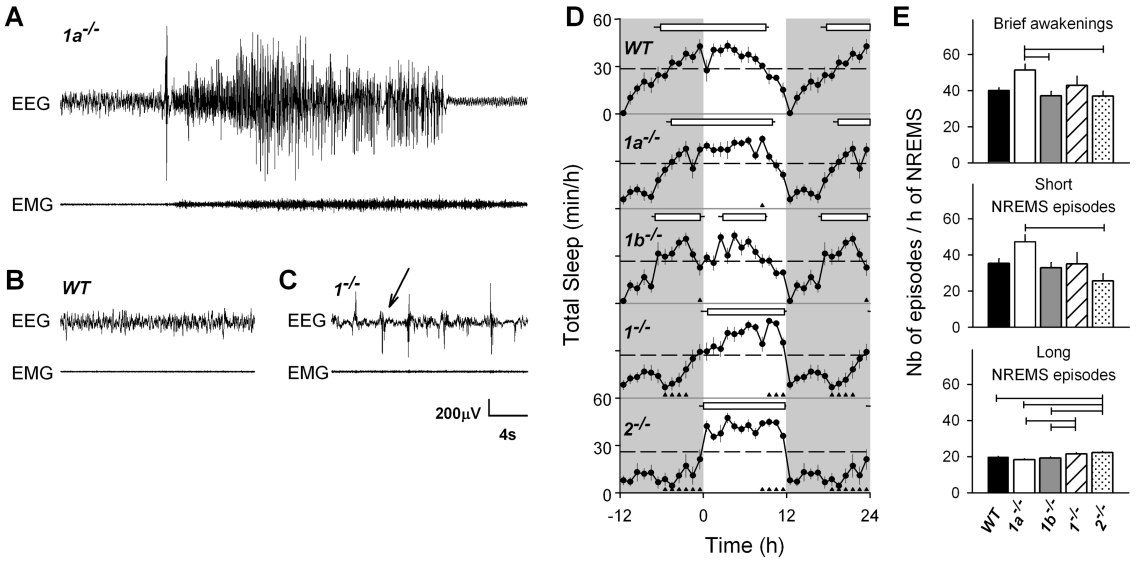


Figure 7.1: Sleep and EEG phenotypes for *1a*$^{-/-}$, *1b*$^{-/-}$, *1*$^{-/-}$, *2*$^{-/-}$, and WT mice.

A, EEG and EMG signals illustrating a spontaneous clonic seizure in *1a*$^{-/-}$ mouse during undisturbed baseline conditions. This seizure occurred during NREMS (2 s before seizure onset). Both EEG amplitude and frequency were increased as well as muscle tone (EMG). Animal showed rearing and bilateral clonus of the forelimbs during the seizure. ***B***, Twenty seconds of typical NREMS in a WT mouse characterized by high amplitude of low-frequency EEG oscillations (delta waves) and reduced muscle tone. ***C***, Example of abnormal EEG during well identified NREMS in a *1*$^{-/-}$ mouse. Arrow points to an abrupt EEG sharp wave. This epileptiform activity during NREMS was seen only in *1*$^{-/-}$ and *2*$^{-/-}$ mice, and was present during >20% of their NREMS. Waking and REMS were also affected to a lesser extent. These abnormal EEG events were excluded from the spectral analysis depicted in Fig.8. ***D***, Time course of hourly mean values of total sleep amount (NREMS+REMS; ±SEM, n=8-9) during baseline. Values of the dark period (gray areas) were depicted twice to illustrate the changes at the dark-to-light transition. Horizontal dashed lines mark the mean baseline (0-24h) value for total sleep. Genotype did not affect sleep amount but its distribution changed (two-way ANOVA for factors 'genotype' p=0.11, 'hour' p<0.0001 and their interaction p<0.0001). Triangles below each curve indicate hourly intervals for which values differed from WT mice (Dunnett's two-tailed t test, p<0.05). For each genotype, the main rest period is indicated by a horizontal bar connecting rest onset and end (mean±SEM, n=8-9). Rest periods were determined individually by selecting intervals in which NREMS and REMS were above the individual baseline mean (see Materials and Methods). In all *1b*$^{-/-}$ mice, the main rest period was interrupted by a 3h gap. ***E***, Sleep fragmentation was quantified by counting the number of brief awakenings (<16 s; 1, 2, 3, or 4s epochs of waking; top) interrupting sleep and the number of short (<1min; <15 consecutive 4s epoch of NREMS; center) and long (>1min; bottom) NREMS episodes according to previously published criteria [289]. Variables were expressed per hour of NREMS to correct for differences in total NREMS amount. Calculated over the 24h of baseline, *1a*$^{-/-}$ mice had more short NREMS episodes than *2*$^{-/-}$ mice and more brief awakenings compared with *1b*$^{-/-}$ and *2*$^{-/-}$ mice (one-way ANOVA for factor 'genotype' p=0.017 and p=0.0089, respectively). The number of long NREMS episodes was generally higher in *1*$^{-/-}$ and *2*$^{-/-}$ mice compared with *1a*$^{-/-}$, *1b*$^{-/-}$, and WT mice (one-way ANOVA for factor 'genotype' p<0.0001). Horizontal lines connect genotypes for which significant differences were observed (Tukey's test, p<0.05).

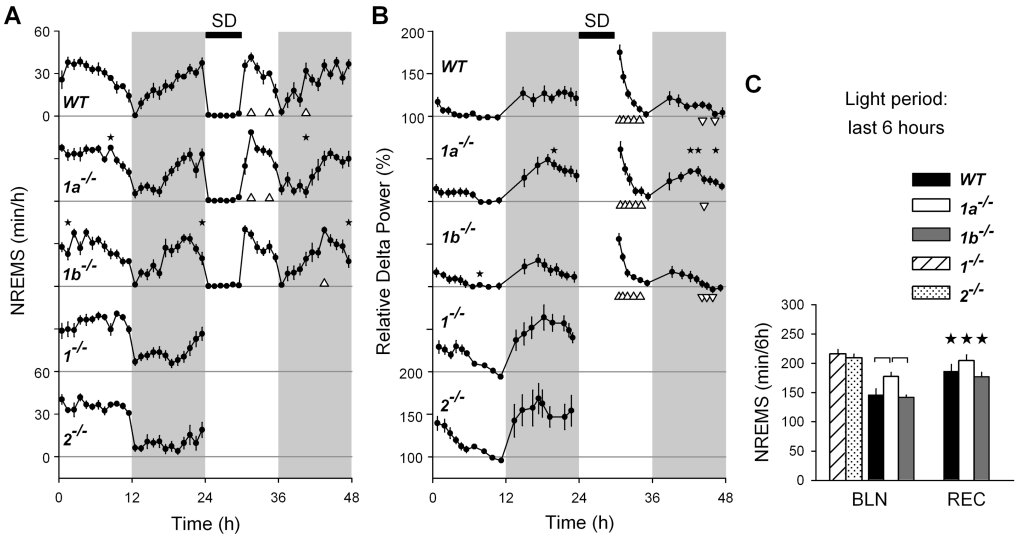


Figure 7.2: Time course of the amount of NREMS and EEG delta power (1– 4 Hz) in NREMS.

A, ***B***, Mean 1h values for NREMS (***A***) and mean values for delta power (±SEM; ***B***) during 24h baseline (BLN; 0-24h), 6h sleep deprivation (SD; 24 –30h) and 18h recovery (REC; 30–48h) in WT, *1a*$^{-/-}$, and *1b*$^{-/-}$ mice (*n*=8, *n*=8, and *n*=9, respectively). For comparison, baseline results were also shown for *1*$^{-/-}$ and *2*$^{-/-}$ mice (*n*=8 per genotype; these mice were not sleep deprived; see Materials and Methods). Delta power was expressed as a percentage of individual mean NREMS delta power over the last 4 h of the rest period. Gray areas mark the dark periods, white areas the light periods, and the black bar on the top indicates the 6h SD. Stars above the curves of *1a*$^{-/-}$ and *1b*$^{-/-}$ mice indicate significant differences from WT (one-way ANOVA; Dunnett's two-tailed *t* test, $p<0.05$). In recovery, triangles below the curves indicate hours at which values differed from baseline (*t* tests, $p<0.05$, up-pointing triangles>baseline, down-pointing triangles<baseline). ***C***, Mean values for NREMS amount during the last 6 h of the light periods of baseline and recovery in *1b*$^{-/-}$ (*n*=9), *1a*$^{-/-}$ (*n*=8), and WT (*n*=8) mice. These two values were compared among the three genotypes. Black stars indicate significant recovery-baseline differences (one-way ANOVA; Tukey's test, $p<0.05$). Horizontal connecting lines indicate significant differences among genotypes (one-way ANOVA; Tukey's test, $p<0.05$). Amount of NREMS during the last 6 h of the baseline light period was shown also for *1*$^{-/-}$ and *2*$^{-/-}$ mice (*n*=8 per genotype), but values were not included in the statistics.

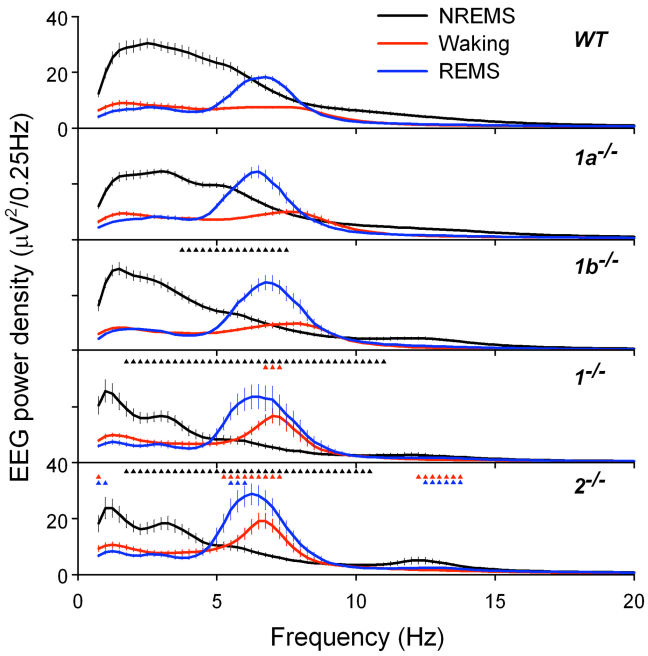


Figure 7.3: Average EEG power spectra (±SEM) for NREMS, REMS, and waking during baseline.

For clarity, only the frequency range for which major genotype differences were observed is shown (0.75-20 Hz at 0.25Hz bins). Genotype affected the EEG spectra of the three behavioral states (two-way ANOVA for each state for factors 'genotype', bin, and their interaction, $p<0.0001$). Colored triangles above each set of spectra indicate frequency bins for which power density differed from WT mice (Dunnett's two-tailed *t* test, $p<0.05$; black, NREMS; blue, REMS; red, waking; color coding of lines and triangles match).

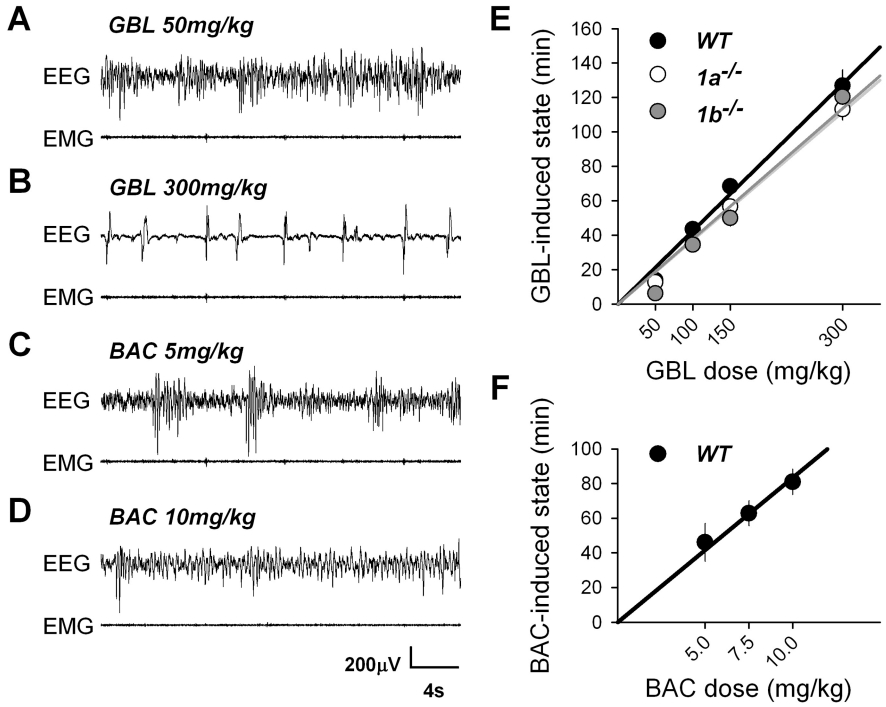


Figure 7.4: EEG and EMG effects.

A–***D***, Representative traces illustrating the effects of GBL and BAC on the EEG and EMG in WT animals after 50 (***A***) and 300 (***B***) mg/kg of GBL and after 5 (***C***) and 10 (***D***) mg/kg of BAC. Similar EEG and EMG patterns after GBL were found in $1a^{-/-}$ and $1b^{-/-}$ mice. GBL did not affect behavior or EEG in $1^{-/-}$ or $2^{-/-}$ mice (data not shown). ***E***, Length of GBL-induced state in $1a^{-/-}$, $1b^{-/-}$, and WT mice. The length of GBL-induced state increased linearly and dose-dependently (linear regression; WT, n=8, R^2=0.99; $1a^{-/-}$, n=8, R^2=0.99; $1b^{-/-}$, n=9, R^2=0.96). The length of GBL-induced state varied with dose and genotype and was, in general, longer in WT mice (two-way ANOVA for factor 'genotype' p<0.0011, 'dose' p<0.0001, and their interaction p=0.42; genotype, $1a^{-/-}$=$1b^{-/-}$<WT; Tukey's test, p<0.05; dose:,50<100<150<300 mg/kg; Tukey's test, p <0.05). ***F***, Length of BAC-induced state after each dose of BAC in WT mice. The length of BAC-induced state increased linearly and dose-dependently within this dosage range (linear regression, WT, n=8, R^2=0.96; one-way ANOVA for factor 'dose', p=0.033; 5=7.5<7.5=10 mg/kg; Tukey's test, p<0.05).

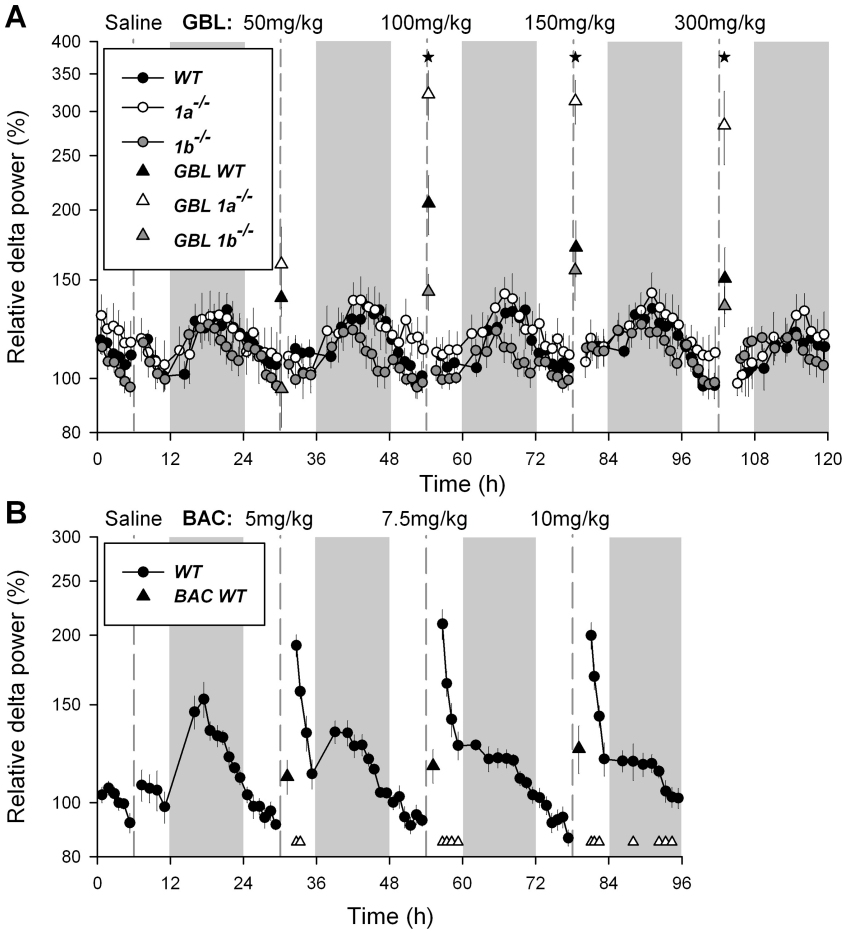


Figure 7.5: EEG delta power (1-4 Hz) during the GBL- and BAC-induced state and its time course during subsequent NREMS (mean±SEM).

A, Delta power during GBL-induced state (triangles) increased from 50 to 100 mg/kg, where it reached a plateau (50 mg/kg, <3 highest doses in *1a*$^{-/-}$ and *1b*$^{-/-}$ mice; one-way ANOVA, Tukey's test, $p<0.05$). Plateau levels reached were around twofold higher in *1a*$^{-/-}$ mice than in *1b*$^{-/-}$ and WT mice (one-way ANOVAs, $p<0.0001$; Tukey's tests, $p<0.05$, stars). For NREMS delta power (circles), a comparison among genotype (*1a*$^{-/-}$, *1b*$^{-/-}$, WT), day (1-5), and time (18 intervals per day) was performed (three-way ANOVA for factors 'genotype' $p<0.0001$, 'time' $p<0.0001$, 'day' $p=0.0003$ and their interactions: 'genotype' x 'day' $p=0.0020$, 'genotype' x 'time' $p=0.040$, 'time' x 'day' $p=0.10$, 'genotype' x 'day' x 'time' $p=0.10$). Although the time course of NREMS delta power did not differ among the three genotypes, the overall dynamic range was smaller in *1b*$^{-/-}$ and larger in *1a*$^{-/-}$ mice compared with WT mice (Tukey's tests, $p<0.05$). For NREMS delta power, differences among drug days were observed, but not in a dose-dependent manner (Tukey's test, $p<0.05$: 150=saline=50=100>50=100=300 mg/kg). ***B***, Delta power during BAC-induced state (black triangles) did not increase with dose in WT mice (one-way ANOVA, $p=0.64$). BAC affected the time course of delta power in NREMS (circles; two-way ANOVA for factor 'day' (1-4) $p= 0.079$, 'time' (18 intervals per day) $p<0.0001$ and their interaction $p<0.0001$). A large increase in NREMS delta power occurred after the BAC-induced state, followed by a decrease below saline levels during the subsequent dark period (white triangles mark significant differences from saline; Dunnett's two-tailed *t* test, $p<0.05$). Note the dose-dependent decrease in delta power during the dark period (one-way ANOVA for factor 'day' $p<0.0001$: saline>5=7.5>7.5=10 mg/kg; Tukey's test, $p<0.05$).

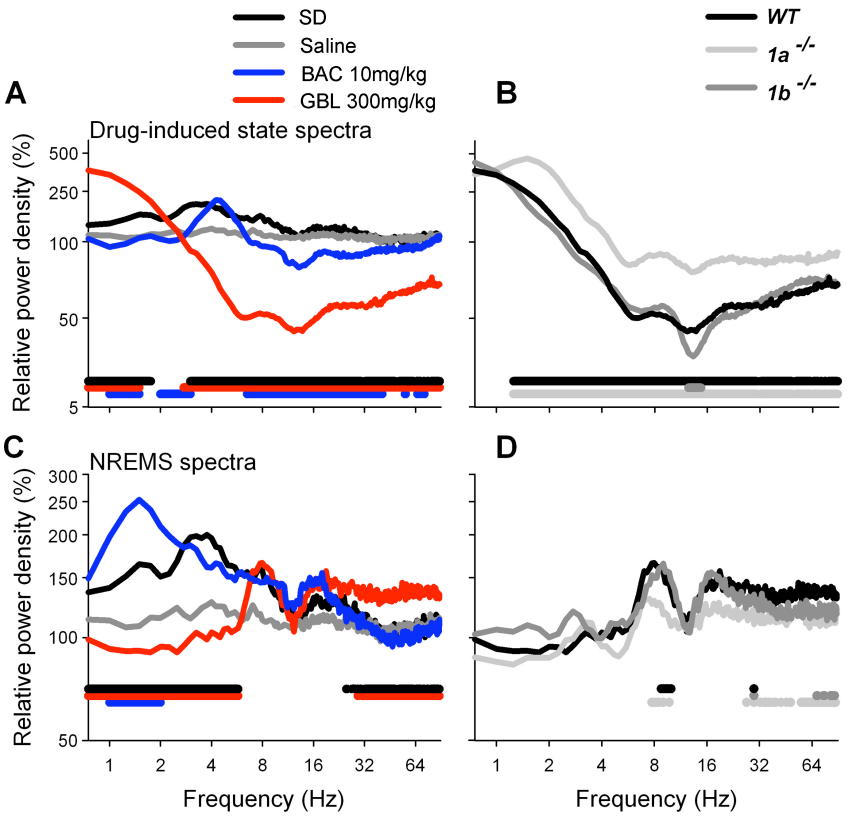


Figure 7.6: EEG spectra during and after the drug-induced state for the highest doses of BAC (10 mg/kg) and GBL (300 mg/kg).

All spectra (0.75-90 Hz; at 0.25Hz bins) were expressed as a percentage of the NREMS EEG spectrum averaged over the last 4 h of the baseline rest period, thereby allowing direct comparison among genotypes, drugs, and conditions. ***A***, BAC- and GBL-induced state EEG spectra in WT mice (blue and red lines, respectively). For comparison, EEG spectra during the first 20 min of NREMS after 6 h sleep deprivation (SD; black) and after saline administration (gray line) were included. Spectra significantly differed among conditions (two-way ANOVA for factors 'condition', 'bin', and their interaction $p<0.0001$). Horizontal colored lines indicate frequency bins in which EEG power significantly differed (GBL vs SD, red; BAC vs SD, blue; GBL vs BAC, black; Tukey's test, $p<0.05$). The GBL-induced state EEG spectrum differed strongly from that of the BAC-induced state, especially in the low delta (0.75–1.75 Hz) frequencies and for frequencies>3 Hz. ***B***, GBL-induced state spectra in $1a^{-/-}$, $1b^{-/-}$, and WT mice (light gray, dark gray, and black lines, respectively; WT same as in ***A***). Spectra significantly differed among genotypes (two-way ANOVA for factors 'genotype', 'bin', and their interaction, $p<0.0001$). Horizontal colored lines mark frequency bins in which genotypes differed ($1a^{-/-}$ vs $1b^{-/-}$, black; $1b^{-/-}$ vs WT, dark gray; $1a^{-/-}$ vs WT, light gray; Tukey's test, $p<0.05$). EEG changes in $1b^{-/-}$ mice closely resembled those of WT. ***C***, EEG spectra during the first 20 min of NREMS after GBL- (red) and BAC- (blue) induced state and after SD (black) and saline (gray line) in WT mice. SD and saline spectra same as in ***A***. Spectra were affected by condition (two-way ANOVA for factors 'condition', 'bin', and their interaction, $p<0.0001$; Tukey's test for genotype, $p<0.05$), largely due to the low spectral values reached after GBL in frequencies<7 Hz. Statistics and color coding as in ***A***. ***D***, EEG spectra during the first 20 min of NREMS after GBL-induced state in $1a^{-/-}$, $1b^{-/-}$, and WT mice (color coding as in ***B***). Spectra significantly differed among genotypes (two-way ANOVA for factors 'genotype', 'bin', and their interaction, $p<0.0001$; statistics and color coding as in ***B***).

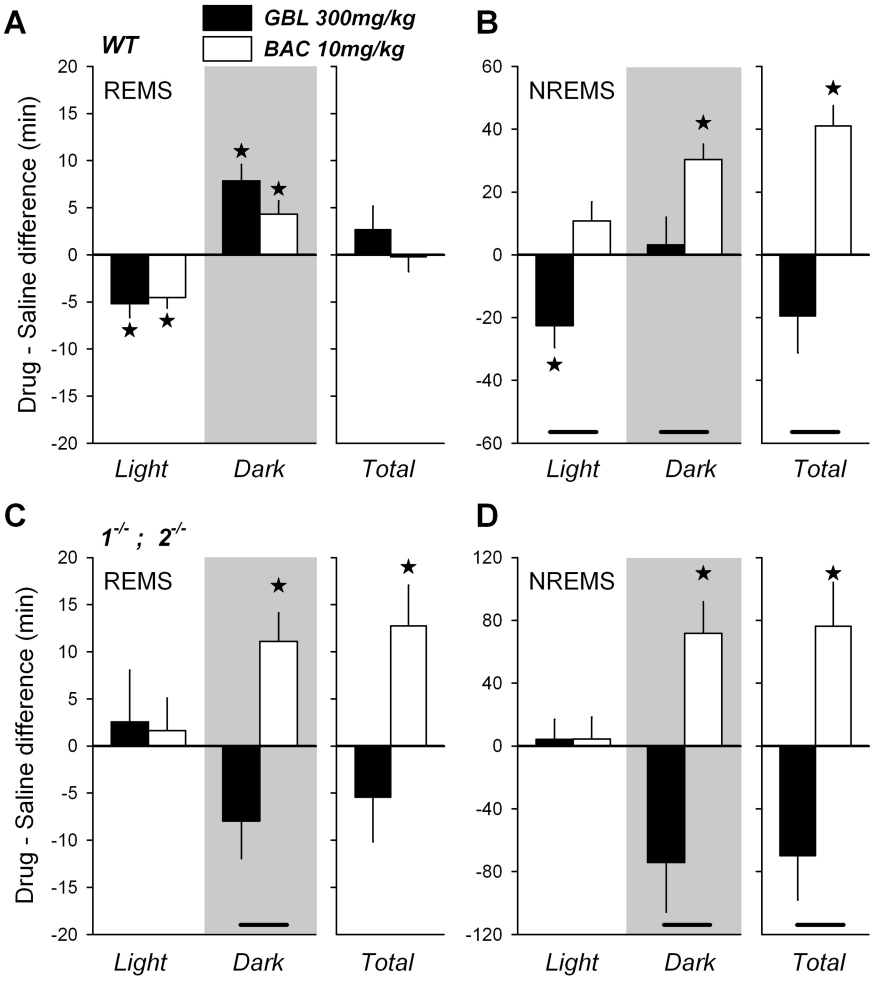
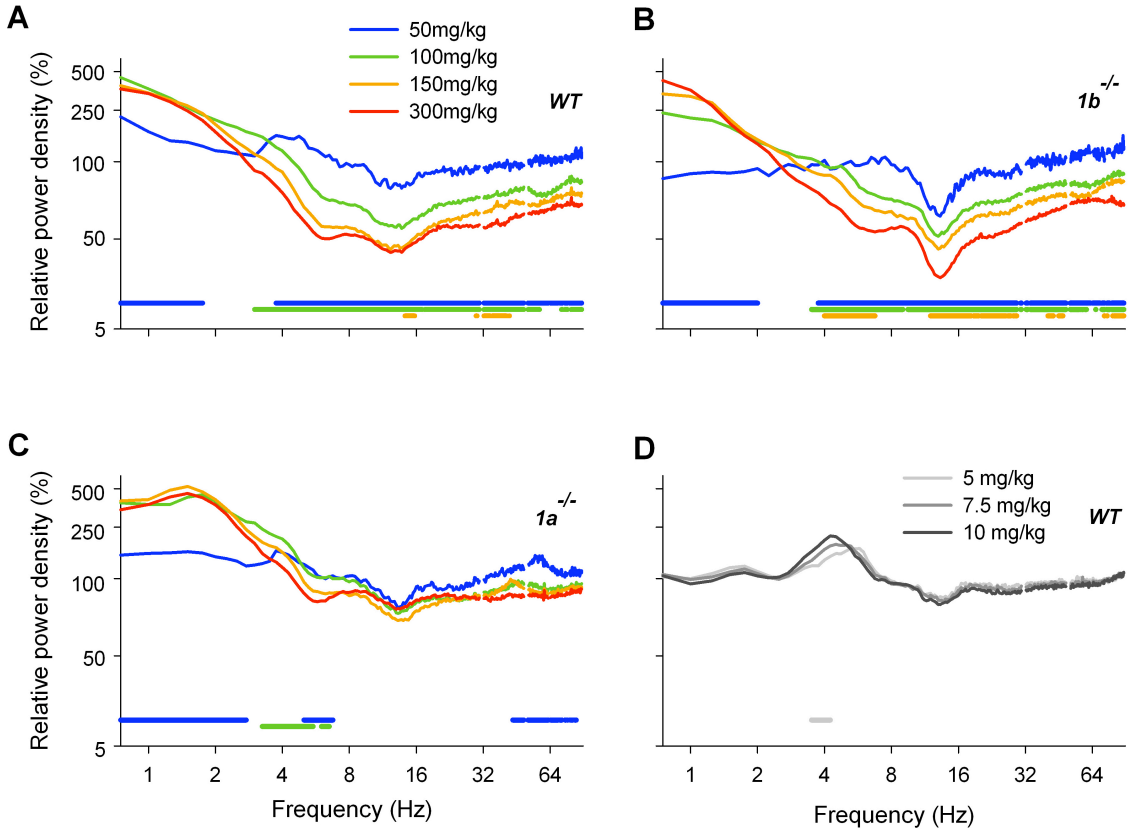


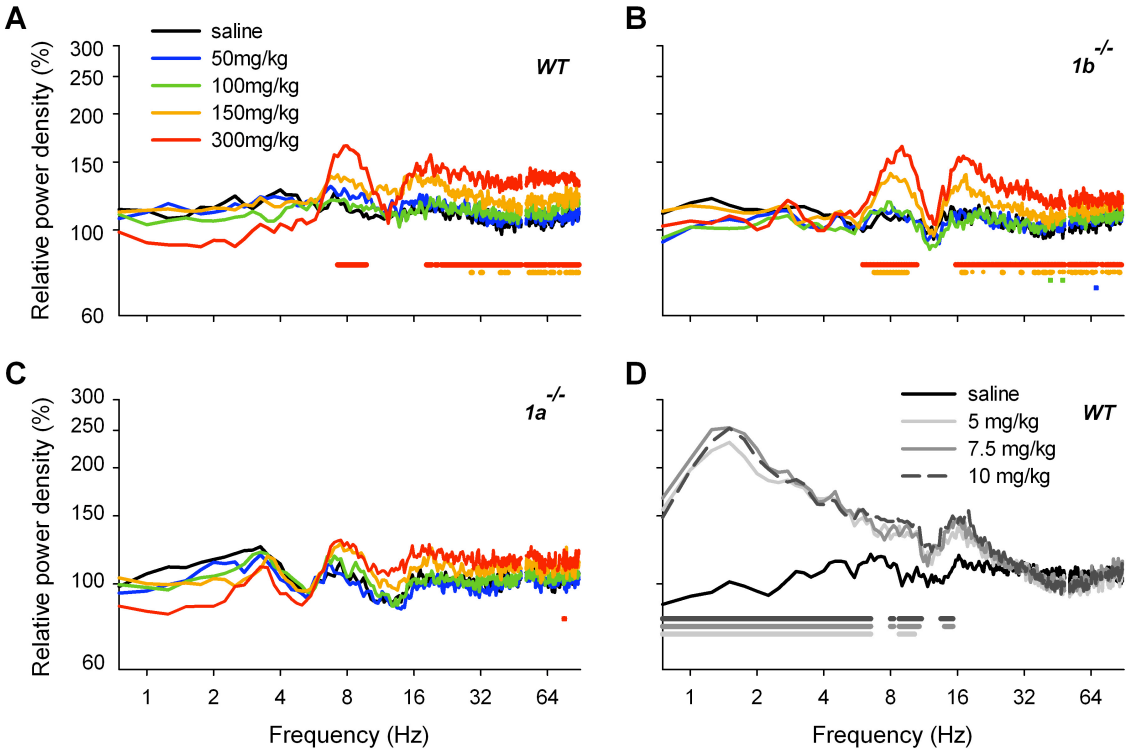
Figure 7.7: Effects of GHB and BAC on the amount of NREMS and REMS.

A–***D***, Drug–saline differences in NREMS and REMS length (mean±SEM), counted from the end of drug-induced state in WT mice (***A***, ***B***) or from the time of injection in $1^{-/-}$ and $2^{-/-}$ mice (***C***, ***D***), to the end of the following dark period. Drug effects are shown only for the highest dose of GBL (300 mg/kg) and BAC (10 mg/kg). ***A***, Both drugs decreased REMS during the remainder of the light period (Light) in WT mice (n=8), a decrease that was compensated for during the subsequent dark period (Dark; gray area), resulting in no overall difference (Total). ***B***, During the light period, NREMS amount significantly decreased only after GBL. In the subsequent dark period NREMS, BAC increased NREMS compared with saline, resulting in a large overall increase (hypersomnia). ***C***, ***D***, Although neither drug affected REMS (***C***) or NREMS (***D***) during the light period in $1^{-/-}$ and $2^{-/-}$ mice, in the subsequent dark period, BAC surprisingly increased both sleep states whereas GBL tended to decrease sleep. Over the 18 h following drug injection (Total), BAC strongly increased REMS and NREMS. Note that results from $1^{-/-}$ and $2^{-/-}$ mice were pooled (n=3 per genotype per group), as no genotype differences were observed. Stars mark statistical differences from saline (one-way ANOVA, p<0.05; paired t test, p<0.05). Significant differences between drugs are shown by connecting lines (one-way ANOVA, p<0.05; Tukey's test, p<0.05).



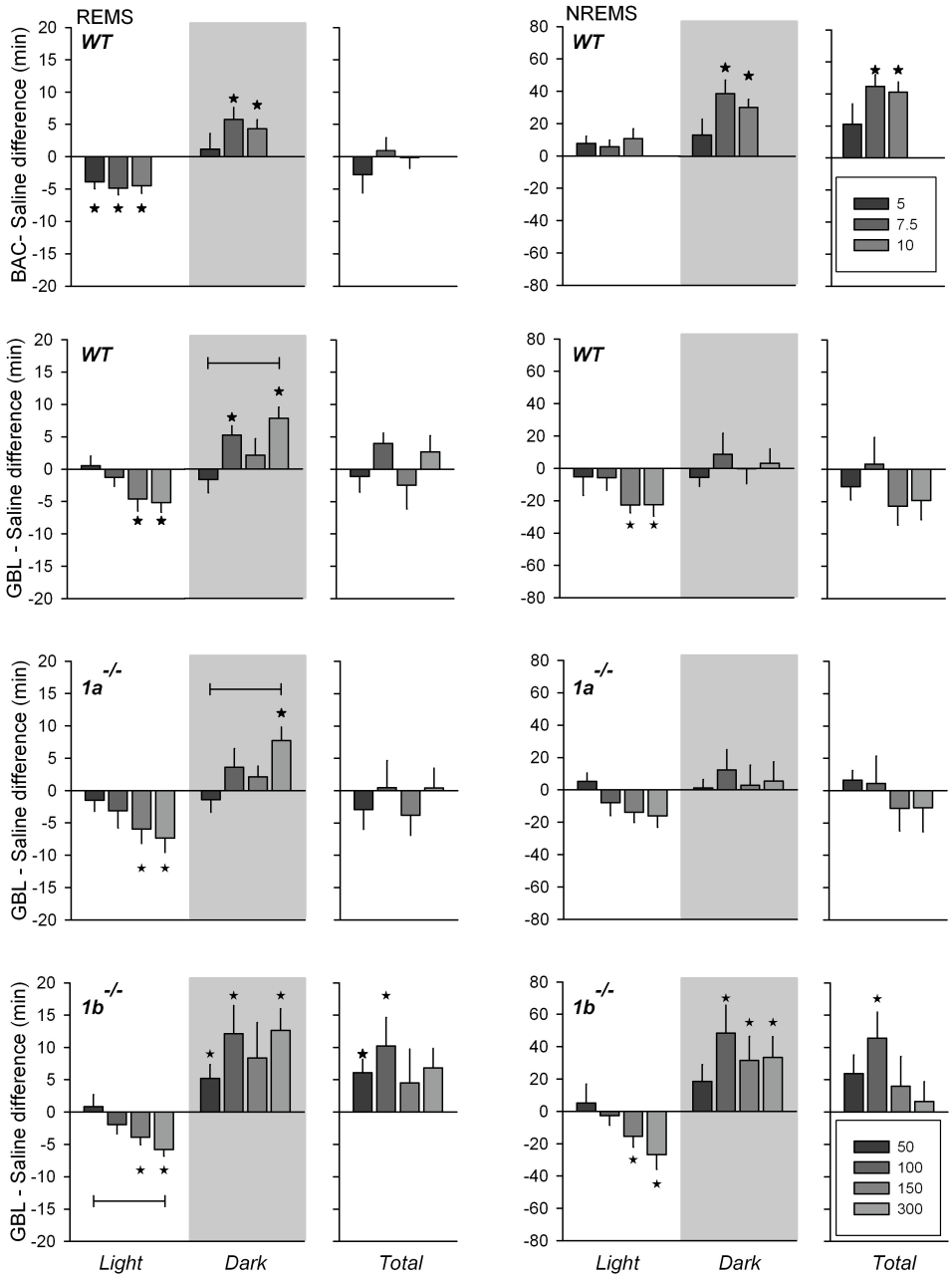
Suppl 7.1: Drug-induced state EEG spectra for each dose of GBL in $1a^{-/-}$, $1b^{-/-}$, and WT mice and BAC-induced state in WT mice.

Average EEG spectra (from 0.75-90 Hz at 0.25Hz resolution) were expressed as a percentage of the mean EEG spectrum during NREMS averaged over the last 4 hours of the baseline rest period (see Methods and Results). Logarithmic scales were used for both relative power density and frequency. GBL-induced state EEG spectra varied according to genotype (WT, $1b^{-/-}$ and $1a^{-/-}$ mice), dose (saline and the 4 GBL doses) and frequency (0.25Hz bins) (three-way ANOVA for factors 'genotype' , 'dose' , 'bin', $p<0.0001$, and their interactions: 'genotype' x 'dose' , 'genotype' x 'bin', and 'dose x bin' $p<0.0001$; 'genotype' x 'dose' x 'bin' $p=1.0$). ***A,*** WT mice showed a dose-dependant increase in low delta (0.75-1.75 Hz) and a progressive decrease from 3 to 90 Hz (one-way ANOVA for factor 'dose' $p<0.0001$, Dunnett's two-tailed t test; contrasted to 300 mg/kg for each frequency bin; $p<0.05$, color of horizontal lines match dose; e.g., blue = 50 vs 300 mg/kg). ***B,*** GBL-induced state EEG spectra in $1b^{-/-}$ mice displayed a similar pattern as WT mice with an even more accentuated decrease in sigma activity (10-15 Hz) for each dose (one-way ANOVA for factor 'dose' $p<0.0001$, Dunnett's two-tailed t test (control = 300 mg/kg) every 0.25 Hz, $p<0.05$). ***C,*** In $1a^{-/-}$ mice, although GBL-induced state EEG spectra changed with dose similarly as in WT and $1b^{-/-}$ mice (one-way ANOVA, $p<0.0001$), this effect was strongly attenuated in the 4-90Hz range (Dunnett's two-tailed t test; contrasted to 300 mg/kg, $p<0.05$). ***D,*** BAC-induced state EEG spectra after each dose of BAC showed an increase in power density at around 4 Hz and a decrease in sigma activity (10-15 Hz) in WT mice. Although spectral changes in the EEG of the BAC-induced state significantly varied with dose (two-way ANOVA for factors 'dose' $p<0.0001$, 'bin' $p<0.0001$, and their interaction $p=1.0000$), the dose-dependent changes were exclusively localized at 4 Hz (Dunnett's two-tailed t test, contrasted to 10 mg/kg, $p<0.05$).



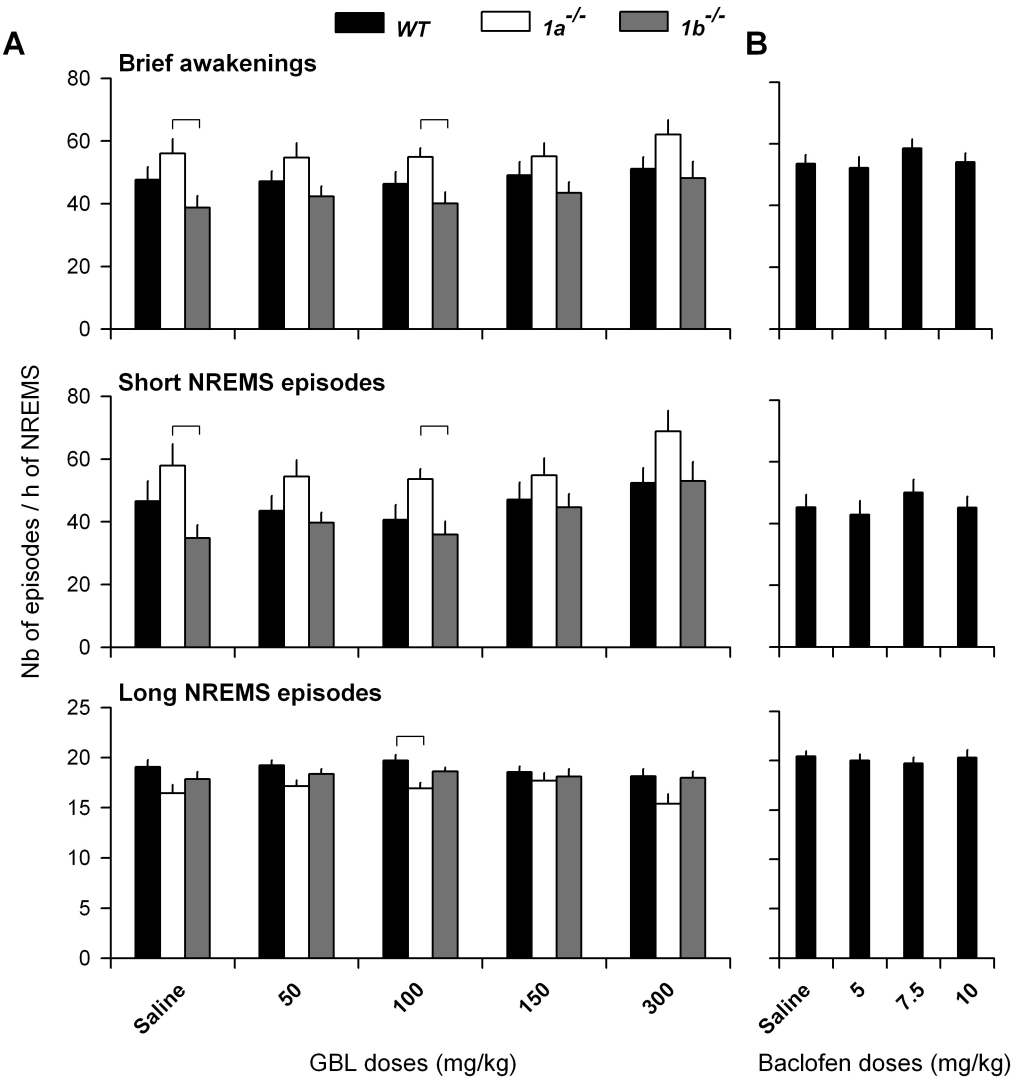
Suppl 7.2: EEG spectra during the first 20 min of NREMS following drug-induced state for each dose of GBL in $1a^{-/-}$, $1b^{-/-}$, and WT mice and for each dose of BAC in WT mice.

Average EEG spectra (0.75 – 90 Hz; 0.25Hz resolution) were expressed as a percentage of the mean EEG spectrum during NREMS averaged over the last 4 hours of the baseline rest period (see Methods and Results) allowing direct comparison with EEG spectra during drug-induced state (see Suppl 7.1 for details). EEG spectra following GBL injections were affected by genotype (WT, $1b^{-/-}$, and $1a^{-/-}$ mice), dose (saline and the 4 GBL doses) and frequency bin (0.25Hz: bin; three-way ANOVA for factors 'genotype', 'dose', 'bin' $p<0.0001$, and their interactions: 'genotype' x 'dose', 'genotype' x 'bin', and 'dose' x 'bin' $p<0.0001$; 'genotype' x 'dose' x 'bin' $p=1.0$). ***A,*** WT mice showed a dose-dependant increase in theta (around 8 Hz) and gamma (>20 Hz) activity (one-way ANOVA for factor dose $p<0.0001$, Dunnett's two-tailed t test; contrasted to saline, $p<0.05$). Horizontal lines indicate differences from saline color-coded according to dose. ***B,*** EEG spectra in $1b^{-/-}$ mice displayed a similar pattern of change as WT mice with an even more accentuated difference between saline and GBL doses (one-way ANOVA for factor 'dose' $p<0.0001$, Dunnett's two-tailed t test; contrasted to saline, $p<0.05$). ***C,*** In $1a^{-/-}$ mice, although the pattern of the dose-dependent changes in EEG spectra was similar to WT and $1b^{-/-}$ mice (one-way ANOVA for factor 'dose' $p<0.0001$), differences from saline did not reach significance levels (Dunnett's two-tailed t test; contrasted to saline, $p<0.05$). ***D,*** EEG spectra during NREMS after each dose of BAC showed a strong increase in power density in the delta and theta frequency ranges compared to saline, and in the high sigma range (13-15 Hz) for the two higher doses (one-way ANOVA for factor 'day' $p<0.0001$, Dunnett's two-tailed t test; contrasted to saline, $p<0.05$). Horizontal lines indicate differences from saline with gray-scales matching dose. EEG spectra after all doses of BAC were different from saline and the lowest dose was different to those of the two other (one-way ANOVA for factor 'dose' $p<0.0001$, Tukey's test, $p<0.05$, saline < 5 < 7.5 = 10 mg/kg).



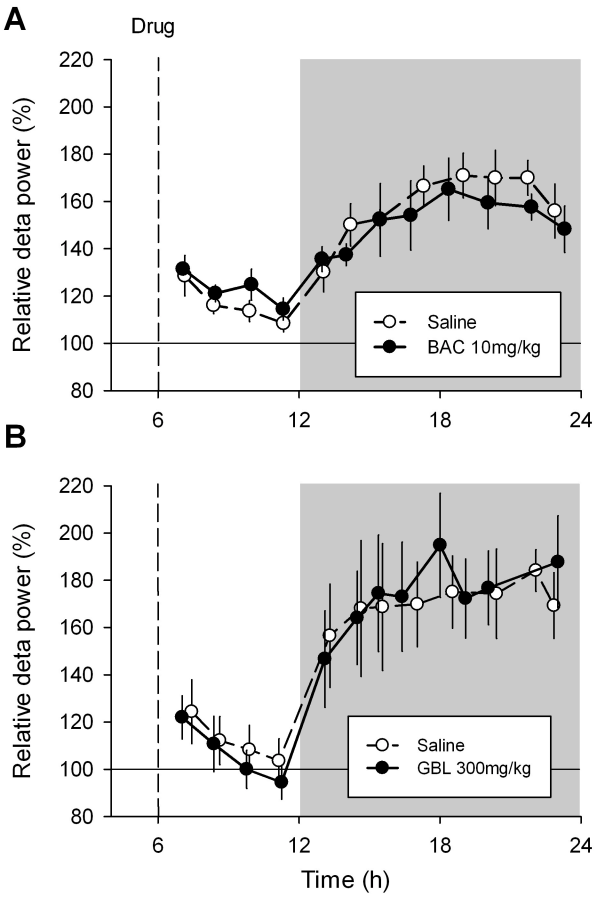
Suppl 7.3: GBL-saline and BAC-saline differences in NREMS and REMS amounts from the end of drug-induced state to the end of the following dark period in the various genotypes.

Mean differences (±SEM) were shown after each dose of GBL (50, 100, 150, and 300 mg/kg) in *1a*$^{-/-}$ and *1b*$^{-/-}$ and WT mice and each dose of BAC (5, 7.5, and 10 mg/kg) in WT mice. GBL and BAC were administrated the middle of the light period. "Light" and "dark" areas represent drug - saline differences in REMS and NREMS amounts from the end of drug-induced state to the end of the light period and for the 12h dark period, respectively. Overall drug - saline differences for REMS or NREMS (from the end of the drug induced-state to the end of the following dark period) were summarized in "Total" areas. Stars indicate significant changes in REMS (left) and NREMS (right panels) after drug administration compared to saline conditions (one-way ANOVA, $p<0.05$; paired t test, $p<0.05$). Horizontal lines connect doses for which a significant difference was obtained (one-way ANOVA, $p<0.05$; Tukey's test, $p<0.05$).



Suppl 7.4: Effects of GBL (left) and BAC (right panels) on sleep fragmentation.

Brief awakenings (upper) and the number of short (middle) and long (lower panel) NREMS episodes were calculated as in Figure 7.1. Averaged over the 24h of each day, the number of brief awakenings and the number of short and long NREMS episodes did not vary with drug dose (one-way ANOVA for factor 'dose' $p>0.1$). Over the entire GBL experiment, $1a^{-/-}$ mice had more brief awakening and shorter NREMS episodes and fewer long NREMS episodes than $1b^{-/-}$ and WT mice (one-way ANOVA factor 'genotype' p <0.0001 for the 5 recording days) consistent with the observation under baseline conditions (Figure 7.1). Horizontal lines connect genotypes for which significant differences were observed within each day (Tukey's test, $p<0.05$). In WT mice, all three markers of sleep consolidation were similar in GBL and BAC experiment (two-way ANOVA for factors 'experiment' and 'day': $p>0.4$).



Suppl 7.5: EEG delta power (1-4 Hz) of NREMS after saline, GBL, BAC administration in mice deficient for functional $GABA_B$ receptors.

Time course of the EEG delta power following the injection of saline and either the highest dose of BAC (10 mg/kg) (***A***) or GBL (300 mg/kg; n=6/treatment) (***B***) in $1^{-/-}$ and $2^{-/-}$ mice. No differences were found between the time course of delta power following saline injection and that following drug injections (Two-way rANOVA for factor 'treatment' p=0.7749 for BAC and p=0.9913 for GBL).

8. Human study:

The effects of sodium oxybate and baclofen on EEG, sleep, vigilance and memory

8.1. Abstract

Sodium oxybate (SO), the sodium salt of γ-hydroxybutyric acid (GHB), has been shown to increase EEG slow-wave (delta) activity in non-rapid eye movement sleep (NREMS). Delta activity is an index of sleep pressure, which decreases during sleep and increases with increasing duration of wakefulness. To investigate whether SO affects the homeostatic process of sleep and thus induces physiological deep sleep, we administrated SO before an afternoon nap and before the subsequent nighttime sleep in healthy volunteers. Because it is known that SO acts as a low-affinity agonist of $GABA_B$ receptors, we also compared its effects with those of baclofen (BAC), a high-affinity agonist of $GABA_B$ receptors. In addition, memory and neurobehavioral performance were assessed. We found that both SO and BAC counteracted the nap effects on the subsequent sleep by decreasing sleep latency and increasing total sleep time, deep sleep during the first NREMS episode and EEG delta and theta power during NREMS. However, SO also increased EEG delta and theta power during REMS and a nap under SO, with high level of delta power, did not affect the following nighttime sleep. This suggests that even if SO induces EEG slow waves, these are not involved in the homeostatic regulation of sleep. Thus, GHB seems to not produce physiological sleep. BAC showed very similar effects on sleep and EEG, but with a delayed action. This different BAC dynamics did not allow us to determine if BAC affects or not the homeostatic process of sleep. Although we found differential effects of BAC and SO on REMS, the EEG similarities induced by these two drugs suggest that SO might primary act through

$GABA_B$ receptors without completely excluding the involvement of other receptors. Finally, overall, a nap under SO and BAC did not affect psychomotor performance and subjective sleepiness as well as memory consolidation.

8.2. Introduction

Sodium oxybate (SO) is the sodium salt of γ-hydroxybutyric acid (GHB), an endogenous fatty acid synthesized in the brain, recently accepted as a treatment for the sleep disorder narcolepsy (Xyrem®). GHB has been demonstrated to increase slow-wave sleep (SWS) and/or slow-wave activity, also called EEG delta activity (0.75-4.5 Hz), in a dose-dependent fashion in healthy subjects[145,237,283] and in patients with narcolepsy and fibromyalgia[158,290283]. The increase of slow-wave sleep (SWS) and EEG delta activity has been hypothesized to represent cortical recovery from prior wakefulness and a time of neurophysiologic restoration or recuperation[66,291]. Like other restorative behaviors, sleep is homeostatically regulated. On one hand, sleep loss produces proportional increases in the tendency to fall asleep (sleep drive)[292] and in EEG delta activity during the recovery NREMS[95]. On the other hand, an afternoon/evening nap reduces the amount of SWS and EEG delta activity during the subsequent nocturnal sleep[293]. Thus, EEG delta activity, as a marker of sleep need/pressure, increases proportionally with increasing duration of prior wakefulness and decreases over the course of a sleep period in humans as well as in all animals so far studied[93,96,100].

We recently reported that the increase of EEG delta activity produced by GBL, a precursor of GHB, did not affect physiological sleep regulation in mice[294]. This finding is also supported by other studies describing paradoxical EEG delta waves induced by GHB and its precursors in awake humans[145,236] and animals[200,238]. Together, this challenges the claimed physiological sleep-promoting effects of GHB. The first aim of this study was to investigate whether pharmacological enhancement of EEG delta activity with GHB is involved in the homeostatic

regulation of sleep in humans, which would support the capacity of GHB/SO to induce normal physiological sleep. To this end, we used an afternoon nap protocol to decrease sleep pressure during the subsequent nighttime sleep and investigated how slow-wave activity, induced by GHB, could modulate the homeostatic regulation of sleep (sleep pressure) in healthy volunteers.

Previous animal reports suggest that GHB acts through $GABA_B$ receptors to affect the EEG and sleep[222,241,294]. In order to determine the role of $GABA_B$ receptors in GHB response in humans, baclofen (BAC), a high-affinity agonist of $GABA_B$ receptors, was also administrated and its effects compared with those of GHB.

One potential adverse effect of GHB is sedation[295,296] and napping is known to increase alertness[297]. Thus, we assessed GHB effects on sustained vigilant attention by a psychomotor vigilance task (PVT) and subjective alertness by Karolinska sleepiness scale (KSS).

Finally, growing evidence continues to demonstrate that, following learning, additional 'offline' memory improvements develop during sleep[298,299,300]. Consolidation and encoding of both procedural and declarative memory have shown to be sleep-dependent[301,302]. Interestingly, a positive correlation between performance and SWS and/or delta power artificially induced (transcranial direct current stimulation (tDCS)) was found[303,304,305]. Thus, because GHB increases EEG delta power and because another potential adverse effect of GHB is memory impairment[306,307,308,309], we investigated the effect of GHB on declarative memory with two tasks (a two-dimentional (2-D) object-recognition task and a unrelated word-pair associate learning task), and procedural memory with a finger sequence tapping task.

8.3. Materials and Methods

Subjects and Procedures

Participants were healthy, of European origin and right-handed males (*n*=13 ; mean age: 23.5 ± 1.6 years old; age range: 20-26 years old) with a body mass index between 18.5 and 24.8 kg/m^2 recruited by a public ad at the University of Lausanne (Switzerland) and Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne, Switzerland). They were paid for participation in this study. They reported having no personal or family history of neurologic, psychiatric, or sleep disorders, being in good health, not having recent stressful life events or transmeridian flight and not taking any medication or having consumed illicit drugs at least 2 months before the study. All were non-smokers or soft smokers (max 5 cigarettes per day), GHB-naive and reported no excessive consumption of alcoholic beverages and stimulant drinks (coffee, tea, cola, red-bull, etc; they had to be able to stop drinking any of these beverages during several days without any problem). Their sleep, anxiety and depression questionnaires revealed that they were good sleepers with regular bedtimes (11-12 p.m.), no subjective sleep disturbances, no anxiety and depression (normal score at: Epworth sleepiness scale, Horne and Ostberg questionnaire (neutral type), and Beck anxiety and depression inventories). Upon reception of their written informed consent, they were screened by brief anamnesis, physical examination, blood test and wrist actimetry during two weeks and also for chronic or acute cardiovascular, respiratory, hepatic or renal diseases. They performed an assessment session where they spent two nights and a day in the sleep laboratory for diagnostic polysomnography to exclude sleep disorders like sleep apnea and/or periodic limb movements in sleep (PLMS), but also to verify if they were able to sleep during an afternoon nap at 3 p.m. Subjects with a sleep apnea index and/or a PLMS index of 5 or more per hour of sleep, sleep efficiency lower than 85%, disturbances in sleep stage architecture, or unable to sleep more than 30 min during the afternoon nap were excluded.

The local ethics committee for research on human subjects and the Swissmedic approved the study protocol, which was carried out in accordance with the declaration of Helsinki. The study included five sessions of three consecutive nights separated by one week. In each session, the first and the second night served as an adaptation and a baseline night, respectively. The day following the baseline night, subjects stayed in the lab and took a nap at 3 p.m. The third night was the last night of the session, called experimental night. For each night bedtime was scheduled from 11 p.m. to 7 a.m. The nap lasted maximum 2 h but was stopped after one NREMS episode at the first appearance of REMS. If REM sleep appeared before sleep stages 3 and 4, the nap was not interrupted. If the nap lasted less than 30 min and/or did show stage 3 and 4, the nap and the subsequent night were excluded. During each night and nap EEG, EMG, electrooculogram (EOG), electrocardiogram (ECG) and core body temperature were recorded. Subjects received a single drug (baclofen (BAC) or sodium oxybate (SO)) per session either before the nap or before the third night and their vigilance and memory were also assessed (Figure 8.1).

Drugs

During the entire study, a dose of 30 mg/kg of sodium oxybate (Xyrem®, oral solution, 500mg/ml, USB-Pharma SA, Bulle, Switzerland) and a dose of 0.35 mg/kg of baclofen (oral suspension prepared from pills of Lioresal® 10 mg, Novartis-Pharma, Basel, Switzerland) were given once before the nap and once before the third night according to the randomized, placebo-controlled, double-blind, crossover design. Thus, subjects received per session either one drug and three placebos or four placebos. Subjects took a solution (placebo or sodium oxybate) and a suspension (placebo or baclofen) 2 min before the nap and 2 min before the third night of each session (Figure 8.1).

In order to avoid side effects including drowsiness, sleepiness and nausea during wakefulness after the nap, we chose relatively low doses known to affect sleep according to

the literature[237,251]. In healthy adults, the half-life and the median T_{max} of BAC are 3.8-4 h[282] and 1.8 h[310], respectively, and for SO, 30-50 min and 30-60 min[311], respectively.

Vigilance assessment

Psychomotor vigilance task (PVT) is a simple visual reaction time task with no learning and virtually independent of aptitude[312]. Ten-minute PVT (PVT-192 Psychomotor Vigilance Task Monitor, Ardsley, NY) were performed 15 min before baseline night, every 2 hours during the following day starting at 9 a.m. and around 1 h after the third night wake-up time. Before and after a PVT, subjects carried out a Karolinska sleepiness scale (KSS). The KSS is a 9-point rating scale which provides a subjective and momentary measurement of alertness/sleepiness (1 = very alert, 9 = very sleepy)[313]. Therefore, subjective alertness and objective vigilance of the subjects were tested.

Memory assessment

An unrelated word-pair associate learning task and a 2-D object-location memory task were used to assess declarative memory, while a finger sequence tapping task evaluated procedural memory.

The unrelated word-pair associate learning task seems to benefit particularly from SWS [314,315,316] and emotion would modulate memory consolidation[302]. Five sets of 36 different French word-pairs (12 positives, 12 negatives and 12 neutral word-pairs), one by session, were chosen randomly from a list of 866 words showing a medium concreteness and imagery [317]. Words consist of 4–10 letters and pairs are of low semantic relatedness. They were presented on a 15 inches flat computer screen.

At learning testing at 2 p.m., subjects were asked to learn 36 word-pairs by forming a mental association/image of both objects. Each pair was presented once for 4 s with an inter-stimulus interval of 100 ms. Immediately after the first run, subjects performed a cued recall,

i.e. the first word of each pair was presented for 10 s and they were instructed to type the second word using the computer keyboard. During these 10 s, subjects could see the result of their typing. Visual feedback was given in each case by presenting the correct second word for 2 s independent of whether the response was correct or not, to enable re-encoding of the correct word-pair. The first word of the next pair was showed after an inter-stimulus interval of 3 s. At retrieval testing at 8 p.m., the same cued recall procedure was used as during the learning phase. To indicate memory consolidation, we used the difference in the number of correctly recalled words at retrieval and minus that at learning.

The 2-D object-location memory task was based on a previous study [315]. Performance on this type of task relies on temporal lobe structures including the hippocampus [318,319] and benefits from SWS[315]. It consists of 10 card-pairs showing different images which are part of the Karolinska Directed Emotional Faces System (KDEF; [320]). These images are standardized facial expressions of emotions, presented by amateur actors. Five sets of 10 different card-pairs (3 happy faces, 3 angry faces and 4 neutral faces) were chosen randomly from KDEF, one for each session. Throughout the task, all 20 possible spatial locations are shown as grey squares on a 15 inches flat screen ("the back of the cards"). The locations are geometrically ordered in a checkerboard-like fashion (4 x 5 matrix). The 5 sets of card-pairs use different locations.

At learning testing at 2 p.m., subjects were instructed to memorize the two locations associated with each image. The first card of each card-pair was presented alone for 1 s followed by the presentation of both cards for 4 s. After an inter-stimulus interval of 1 s, the next card-pair was presented in the same way. The whole set of card-pairs was presented once. Immediately after the first run, recall of the spatial locations was tested using a cued recall procedure, i.e., the first card of each pair was presented and the subject had to indicate the location of the second card with a computer mouse. Visual feedback was given in each case by presenting the second card at the correct location for 2 s independent of whether

the response was correct or not, to enable re-encoding of the correct location of the card-pair. After presenting a card-pair both cards were replaced by grey squares again, so that guessing probability remained the same throughout the run. Feedback was given about the number of correctly recalled card-pairs. At retrieval testing at 8 p.m., the same cued recall procedure was used as during the learning phase. To indicate memory consolidation, we used the difference in the number of correctly recalled card locations at retrieval minus that at learning.

The finger sequence tapping task was adapted from previous studies indicating a robust sleep-dependent improvement in skill on this task, especially REMS and stage 2[321,322,323]. It requires the subject to press repeatedly one of five 5-element sequences ('1-2-4-3-1', '2-1-3-4-2', '3-4-2-1-3', '4-1-3-2-4' or '2-3-1-4-2') on a keyboard with the fingers of the non-dominant hand as fast and as accurately as possible for 30-s epochs interrupted by 30-s breaks. The numeric sequence was displayed on the screen at all times to keep working memory demands at a minimum. Each 30-s trial was scored for speed (number of correctly completed sequences) and error rate (number of errors relative to total number of tapped sequences).

At learning, subjects performed on twelve 30-s blocks. The average score for the last three of these blocks was used to indicate learning performance. At retrieval, subjects were tested on another three blocks. Performance and accuracy are given as the absolute difference in averaged numbers of correct sequences or errors on the three blocks at retrieval minus the average of the last three blocks at learning, respectively.

The order of memory tasks at leaning and retrieval as well as the different sets of word-pairs, card-pairs and tapping sequences was balanced across subjects and sessions. E-Prime software (Psychology Software Tools, Pittsburgh, PA) was used to design and run word-pair memory task and object-location memory task, while the finger-tapping task was made using MATLAB® R2007a (The MathWorks Inc, Natick, MA).

Polysomnographic, temperature and actigraphic recordings

Six EEG channels (F3, C3, O1 and F4, C4, O2 referenced against linked mastoids A2 and A1 respectively), two electrooculograms (EOG; one to each outer cantus), two surface submental electromyogram (EMG) electrodes, and one electrocardiogram (ECG) signal were recorded throughout each night and nap of each session in individual bedrooms using Embla® N7000 recording system (Embla Systems, Broomfield, CO). Only data from C3-A2 EEG derivation are reported here. Signals were filtered by a high-pass filter (EEG and EOG: −3 dB at 0.5 Hz; EMG: 10 Hz; ECG: 1 Hz), a low-pass filter (EEG: −3 dB at 35 Hz, EMG: 70 Hz), and a notch filter at 50 Hz. Data were sampled at a frequency of 100 Hz (EEG, EOG and ECG) and 200 Hz (EMG). Infrared video was also simultaneously recorded. The raw signals were stored on-line on a computer hard drive and off-line on DVDs and a hard disk. Sleep stages during nights and naps were visually scored by a registered PSG technologist on a 20-s epoch basis (Somnologica® Software, Embla systems, Broomfield, CO) according to standard criteria [324]. The EEG power spectra of consecutive 20-s epochs (average of five 4-s epochs, fast Fourier transform routine, Hamming window, frequency resolution 0.25 Hz) were calculated using MATLAB R2007a (The MathWorks, Natick, MA) and matched with the sleep scores. Movement- and arousal-related artifacts were visually identified and excluded. If more than 50% of a 20s epoch contained artifacts, the entire epoch was removed from the spectral analysis.

The NREMS–REMS cycles were defined according to criteria of Feinberg and Floyd[325]. For the completion of the first and the last cycle, no minimal criterion for the REMS duration was applied. Sleep-onset REMS period (SOREMP) was defined as at least one 20-s epoch of REMS occurring in the first 18 min of sleep (NREMS stage 1, 2, 3, 4 and REMS). The SOREMP did not contribute to sleep cycle length, i.e., when a SOREMP was present, the first cycle started after

the SOREMP according to above-mentioned criteria (succession of a NREMS episode and a REMS episode). At least four NREMS–REMS cycles were completed in all recordings.

Core body temperature data was sampled once per minute and stored via a portable device (Mini-Logger®, series 2000, Mini-Mitter, Bend, OR) connected to disposable rectal probe (Steri-probe, Cincinnati Sub-Zero Products, Inc., Cincinnati, OH). Temperature was recorded during each night and each laboratory day during the five sessions of the study.

At least, ten days before the assessment session and seven days before each session of the study, an actiwatch was worn by subjects to their left wrist and a sleep agenda was filled out in order to control their sleep schedule (23:00-07:00), their sleep quality and their activity. Actiwatchs (Mini-Mitter, Bend, OR) sampled activity once per 30 s.

Data Analyses and Statistics

The effect of treatments and nap on sleep variables, the EEG in REMS and NREMS (stages 2 to 4), sustained vigilant attention (PVT), subjective alertness (KSS) and memory were analyzed in 13 subjects. Of the 13 subjects included, one subject took anti-histamine medication for a rash provoked by a soap allergy. The two affected sessions of this subject were excluded (sessions: PL-SO and BAC-PL, for study design see Figure 8.1). Because of insufficient sleep during naps (<30min), two sessions of another subject were not taken into account for analysis except sleep and EEG data for the two baseline nights (sessions: PL-BAC and PL-PL). Finally, the last night of one subject was excluded due to adverse effects provoked by SO (dizziness and anxiety) in the beginning of the night (session: PL-SO). Note that after an unremarkable general clinical exam, the subject slept and reported a good night (monitored.) Thus, for the following analysis, we included n=13, 12, 11, 12 and 13 for SO-PL, BAC-PL, PL-SO, PL-BAC, and PL-PL, respectively, and for comparison before the experimental (EXP) night, n =13, 12 and 33 for SO, BAC and PL, respectively. In addition, for all variables tested below, the baseline (BLN) nights of the five treatments (PL-PL, BAC-PL, SO-PL, PL-BAC,

and PL-SO) did not significantly differ ($p>0.05$). The same was true for the three placebos administrated before the nap (PL-PL, PL-SO, and PL-BAC), which allowed us to regroup them under the term 'PL'. Spectral analysis was performed on C3-A2 derivation. Due to large extent of artifact on A2 for one subject, his C3-A2 trace was not included in the following analysis reducing the number of subjects for spectral analysis to 12.

To approximate a normal distribution, absolute power densities were log-transformed before statistical tests. The SAS 9.1 statistical software (SAS Institute, Cary, NC) was used. The effects of nap and treatments on sleep variables and the EEG were assessed by comparing EXP night with BLN night: two-way mixed-model analyses of variance (ANOVA) with the within-subject factors 'treatment' (PL-PL, BAC-PL, SO-PL, PL-BAC, and PL-SO) and 'night'. Sleep cycle and treatment effects on SWS and REMS duration, and EEG frequency ranges were estimated by performing a two-way mixed-model ANOVA with the within-subject factors 'treatment' and 'cycle' (1^{st}-3^{rd}) on BLN and EXP night or on the ratio EXP/BLN (see results). One-way mixed-model ANOVA with the within-subject factor 'treatment' (PL, BAC, and SO) served to evaluate effects of treatment on sleep variables and the EEG during the nap and on memory tasks. To assess the emotional factor on the word-pair associate learning task performance, a two-way mixed-model ANOVA with the within-subject factors 'treatment' and 'emotion' was carried out. Finally, to estimate treatment effects on sustained vigilant attention and subjective alertness, a two-way mixed-model ANOVA for the within-subject factors 'treatment' (PL, BAC and SO) and 'time' (time points where tasks were performed) and a one-way mixed-model ANOVA with the within-subject factors 'treatment' at specific time points were used. The significance level was set at $\alpha < 0.05$. To localize differences within subjects paired 2-tailed *t*-tests, Tukey-Kramer's tests or Dunnett-Hsu's tests (control= PL-PL or PL) were only performed if main effects or interactions of the ANOVA were significant. The majority of the statistics is indicated in the figure legends. EEG power

was computed for consecutive 0.25-Hz bins and for specific frequency bands. The frequency bins and bands are indicated by the encompassing frequency ranges (e.g., 0.75-4.5 Hz band denotes 0.625-4.625 Hz).

8.4. Results

Nap

During the scheduled nap, subjects had a sleep episode between 42 and 116 min with at least 7 min of SWS. In PL conditions (PL-PL, PL-SO and PL-BAC; Figure 8.1), sleep efficiency was below BLN night (one-way mixed-model ANOVA for factor 'condition' (BLN or NAP) $p<0.0001$) as expected [293], but not sleep latency ($p=0.3718$) (Table 8.1, Table 8.2, and Figure 8.2A). Compared to PL conditions, naps under SO showed an increase in total sleep time (TST), sleep efficiency and REMS, as well as a decrease in REMS latency, movement time (MT) and stage1. Although BAC, as PL, did show significantly lower TST and REMS compared to SO, for several sleep variables, BAC was intermediate between SO and PL (sleep efficiency, REMS latency, MT as well as combined arousal variables and light sleep, see Table 8.1 and Figure 8.2A,B). The duration of SWS was significantly increased after SO compared to PL, but not compared to BAC, which was again intermediate between the two (Figure 8.3C). However, when expressed as a percentage of TST, the significant difference between SWS in SO condition and SWS in PL condition was lost (Table 8.1).

Except for four naps, subjects were awakened after having completed one NREMS episode. During these four naps, subjects under SO were awakened at the end of the 2-h-opportunity nap without completely terminated NREMS episode. These naps had a sleep latency between 7 and 8 min, a sleep onset REMS period (SOREMP; i.e. REMS latency shorter than 18 min) lasting between 30 and 50 min, a SWS amount between 36 and 73 min, and a sleep efficiency above 92% suggesting that subjects slept sufficiently and SO affected the classical

duration and structure of sleep (90-min sleep cycle with NREMS episode followed by REMS episode). The fact that naps under SO was significantly longer than under BAC or PL, also supported SO-induced changes in sleep duration (see 'time in bed' (TIB) in Table 8.1). Interestingly, several other cases of SOREMP were shown under SO (8/13 naps (62%)), as well as under BAC (5/12 naps (42%)) and under PL (13/33 naps (39%)). The subjects exhibiting a SOREMP were allowed to sleep until the beginning of the following REMS episode or until the end of the 2-h sleep opportunity.

Number and duration of SOREMPs were higher under SO compared to PL and BAC, although the difference between SO and BAC was only a tendency, probably due to high variability of SOREMP appearance under each treatment and the low number of subjects (Tukey-Kramer's test for SOREMP number: SO vs PL p=0.0248, SO vs BAC p=0.1884, and BAC vs PL p=0.8463; Figure 8.2C,D and Table 8.1). Similarly, REMS latency was lower with SO than with PL, but with only a tendency for BAC (Tukey-Kramer's test: SO vs BAC p=0.1259). REMS duration during these naps reflected the duration of SOREMPs because naps were stopped at the end of the first NREMS episode (Figure 8.3D).

Experimental night vs Baseline night

All subjects displayed a good sleep quality during BLN nights. Typically for young healthy subjects, they fell asleep within the normal time range (12 min), exhibited little intermittent wakefulness and considerable amount of SWS, particularly during the first half of the night.

In placebo (PL-PL) conditions, an afternoon nap decreased TST, sleep efficiency, stage 4 and SWS, but increased sleep latency, wakefulness after sleep onset (WASO) and light sleep (stage 1) of the following nighttime sleep (EXP night; Table 8.2). However, drug treatments influenced several sleep variables. Although SO administrated before the EXP night and BAC before the nap increased sleep latency during the EXP night compare to the BLN night, this increase was significantly lower than that of the three other treatments (Tukey-Kramer's test,

$p<0.05$; Figure 8.2A). TST during the EXP night in BAC-PL condition was unchanged compared to the BLN night and significantly higher to all other treatments during the EXP night except PL-SO (Tukey-Kramer's test, $p<0.05$; Table 8.2). The decrease of SWS found in placebo condition was not present in PL-SO and BAC-PL. The same was also found for stage 4 in PL-SO treatment. Moreover, BAC administrated before the nap (BAC-PL) and SO administrated before the EXP night (PL-SO) affected SWS exclusively during the first cycle of the EXP night (Figure 8.3A). Together, this suggests that the two conditions (BAC-PL and PL-SO) not only counteract the effect of the nap on sleep latency but also on SWS and TST.

Interestingly, BAC administrated before the nap and before the EXP night (BAC-PL and PL-BAC) increased significantly REMS during the entire EXP night compared to BLN night, while PL-SO significantly decreased REMS specifically in the beginning of the EXP night (cycle 1 and 2) compared to the BLN night, although the overall mean REMS amount in PL-SO condition during the EXP night did not differ (for statistics and illustration see Figure 8.3B, Table 8.2). BAC-PL and SO-PL significantly induced SOREMPs in the beginning of the EXP night compared to the BLN night (paired *t*-test: $p<0.05$). Moreover, in the EXP night, BAC-PL and PL-SO increased significantly the number and the duration of SOREMPs compared to PL-PL, respectively (Figure 8.2C,D and Table 8.2). This increase of SOREMPs after PL-SO and BAC-PL treatments was also reflected through the decrease of REMS latency (Table 8.2). Although the averaged number of SOREMPs for SO-PL, PL-BAC and PL-PL did not differ from zero (paired *t*-test: $p>0.05$), a total absence of SOREMPs was found only for SO-PL (number of SOREMPs by treatment: SO-PL, 0/13 (0%); PL-PL, 2/12 (17%); PL-BAC, 2/12 (17%); PL-SO, 6/11 (55%); BAC-PL, 8/12 (67%); for statistics see Figure 8.2). Compared to EXP night, 3 out of 65 BLN nights exhibited a short SOREMP (2 -11min; SO-PL (1/13 (8%)), PL-PL (1/12 (8%)), and BAC-PL (1/12 (8%)), but overall the number of SOREMPs did not differ from zero (paired *t*-test: $p>0.05$).

BAC seems to consolidate sleep during the EXP night, because combined arousal variables and light sleep (stage1+WASO+MT) together were significantly lower in PL-BAC and BAC-PL compared to the other three treatments and compared to the BLN night (Tukey-Kramer's test $p<0.05$; Figure 8.2B and Table 8.2.). Moreover, number of transitions from one state to another was significantly lower for PL-BAC and BAC-PL compared to the three other treatments (data not shown).

Note that for all sleep variables tested SO-PL did not significantly differ from PL-PL, meaning that the strong effects on sleep induced by SO when administrated before the nap did not affect subsequent nocturnal sleep.

NREMS EEG

To characterize the effect of the nap and treatment on sleep quality and on the homeostatic regulation of sleep, the spectral composition of the EEG in NREMS (stage 2-4) was quantified. After placebo intake, a nap reduced EEG power in delta and theta frequency ranges (0.75-7.25 Hz) and enhanced 12.5 Hz and overall beta frequency range (>16 Hz) during the subsequent night (Figure 8.4A,B). The largest differences were present in the first NREMS episode for delta, theta and sigma power (cycle1: Figure 8.6A,B.C). According to the homeostatic process of sleep[293], delta but also theta power decreased within the course of sleep for both the BLN and EXP nights. Conversely, sigma slightly increased from the first and second NREMS episodes to the third NREMS episode during BLN night, but decreased from the first to the second NREMS episode, then re-increased in the third episode during the EXP night (two-way mixed model ANOVA see Figure 8.6; Tukey-Kramer's test, $p<0.05$).

To determinate the effect of SO and BAC on the EEG, all drug treatments were compared to placebo (PL-PL) treatment (ratio EXP/BLN of each drug treatment was expressed as a percentage of the ratio EXP/BLN of PL-PL treatment; Figure 8.4C). Neither relative NREMS

spectra of PL-PL and SO-PL nor those of PL-SO and PL-BAC differed between them (two-way mixed-model ANOVA see Figure 8.4C; Tukey-Kramer's test, $p<0.05$).

Compared to PL-PL treatment, PL-SO, BAC-PL, and PL-BAC in EXP night increased delta and theta frequency ranges (0.75-7.25, 0.75-9.25, and 1.5-8.25, respectively), and BAC-PL also decreased sigma frequency range (13.5-13.75 Hz).

Delta, theta and sigma power were analyzed during the three first NREMS episodes. The largest effects of drugs were present in the first episode of NREMS. BAC-PL and PL-SO enhanced significantly delta and theta power, while they reduced sigma power during the first cycle ($p<0.05$; Figure 8.7A,B,C). Already in the second NREMS episode, none of the treatments differed from the PL-PL treatment. However, it is interesting to note that PL-BAC tended to increase delta power in the second cycle without reaching the significance due to a high variability, suggesting that BAC took longer time to affect the EEG than SO, which is consistent with the pharmacokinetics of both drugs (Figure 8.7A). During the nap, SO, but not BAC, differed from PL; SO enhanced delta and theta frequency ranges (0.75-10 Hz) and reduced sigma frequency range (13.75-14.75 Hz) (Dunnett-Hsu's test $p<0.05$; Figure 8.4D). Interestingly, SO administrated before the nap affected the EEG during the nap, but no difference with PL-PL were found in the EXP night, which is consistent with results obtained with sleep variables.

REMS EEG

Similar to NREMS, a spectral analysis was performed for REMS. In PL-PL treatment, a nap did not affect REMS spectrum (Figure 8.5A,B). EEG delta power decreased across cycles, and the BLN and EXP nights tented to differ even if the significance level was not reached (two-way mixed-model ANOVA for factors 'night' $p=0.0536$, 'cycle' $p=0.0026$ and their interaction $p=0.1097$). However, by taking each cycle separately, delta power in the EXP night was significantly lower than in the BLN night during the first REMS episode only (one-way mixed-

model ANOVA for factor 'night' by cycle, $p<0.05$; paired t-test, $p<0.05$; Figure 8.6D, statistics not shown on the figure), and difference among cycles was only found for the BLN night, which showed a lower delta power in the third cycle compared to the first and second cycles (one-way mixed-model ANOVA for factor 'cycle' by night, $p<0.05$; Tukey-Kramer's test, $p<0.05$); Figure 8.6D). Theta power differed by cycle and night (two-way mixed-model ANOVA see Figure 8.6E). Like delta power, theta power was reduced, but significantly, during the EXP night compared to the BLN night exclusively during the first cycle and only the BLN night showed significant differences between cycles (cycle 1>3). Note that SOREMPs were not included in this analysis.

As for NREMS, to determinate the effect of SO and BAC on the EEG, relative REMS spectra of all drug treatments were compared to placebo (PL-PL). PL-SO, BAC-PL, and PL-BAC in the EXP night increased delta and theta frequency ranges (2.5-6.75, 0.75, 1.25-8.25, and 1.25-8.25, respectively) compared to PL-PL (Dunnett-Hsu's test $p<0.05$; Figure 8.5C). During the first cycle, delta power was enhanced in PL-SO, while during the second REMS episode delta power was reduced in PL-BAC compared to placebo (Dunnett-Hsu's test $p<0.05$ and see Figure 8.7D). PL-SO and BAC-PL increased theta power, while PL-BAC and BAC-PL started or continued to increase it, respectively, indicating a delayed and longer duration effect (Figure 8.7E).

During the nap, six subjects showed a SOREMP in BAC, SO and PL conditions. Thus, in these six subjects, EEG spectral analysis of SOREMPs was performed to determinate whether a SOREMP under drug had a different EEG fingerprint compared to PL. Neither SO nor BAC differed from PL (two-way mixed-model ANOVA for factors 'treatment' and 'bin', see Figure 8.5D).

Vigilant attention and subjective alertness

Laboratory experiments have indicated that afternoon naps improve subjective alertness and cognitive performance in young adults[326]. Thus, to quantify the effects of the nap with or without drugs on vigilant attention and alertness, we compared the trial performed just before BLN night with that just before EXP night (Figure 8.8A,C and Figure 8.1). Mean and the 10% fastest reaction times as well as the KSS scores were increased during the trial performed just before EXP night compared to the trial performed just before BLN night independent of the treatment (two-way ANOVA for factors 'trial' $p \leq 0.023$ and 'treatment' $p \geq 0.6298$ and their interaction $p \geq 0.1659$, see Figure 8.8A,C). By comparing each treatment separately, this increase of alertness and attention just before EXP night was significant for the 10% fastest reaction time in all treatments and for the KSS scores only for BAC treatment. Therefore, generally the nap increased vigilant attention and subjective alertness late in the evening independent of the treatment during the nap.

To evaluate the effects of a nap under SO or BAC on vigilant attention and alertness compared to those of a nap under PL, we analyzed the time course of mean, the 10% slowest and the 10% fastest reaction times on PVT and KSS scores starting with the trial performed just before the nap and ending with the trail performed just before EXP night (Figure 8.8B,D and Figure 8.1). All variables quantifying attention and subjective alertness differed by trial but not by treatment (two-way ANOVA). Overall, data described a fast increase of attention and alertness just after the nap compared to just before the nap followed by a plateau and then a slow and slight decrease until the end of the evening. Interestingly, by comparing the three treatments administrated before the nap (PL, BAC and SO) at each trial separately, only one trial, just after the nap, differed significantly between the three treatments for the following variables: mean and the 10% slowest reaction times as well as the KSS scores (one-way ANOVA for factor 'trial' by treatment: $p<0.05$ see Figure 8.8B,D). Unlike BAC and PL, SO

did not show the increase of vigilant attention and subjective alertness during this trial (Tukey-Kramer's test $p<0.05$, but note that for KSS score only PL differed significantly from SO). Two hours later this difference has already disappeared suggesting that SO slightly and temporarily affected sustained vigilant attention and the evaluation of subjective alertness compared to PL and BAC. Because naps under SO were significantly longer than naps under BAC and PL (see Table 8.1), sleep inertia (transitional state of lowered arousal occurring immediately after awakening from sleep and producing a temporary decrement in subsequent performance) may be the reason why subjects under SO showed lower cognitive performance and subjective alertness than subjects under BAC or PL [327], even if the trial just after the nap was performed at least 15 min after awakening. This hypothesis was tested by doing a correlation between TST and the 10% slowest reaction times (the most affected variable) resulting in a non-significant correlation for the three treatments (SO: $R^2=0.0068$, $p=0.3206$; BAC: $R^2=0.2752$, $p=0.0795$; PL: $R^2= 0.0724$, $p=0.1238$).

Memory tasks

Growing evidence demonstrates that sleep, and its varied stages, play an important role in the consolidation of both procedural and declarative memories [328,329]. Not only nocturnal sleep but also daytime naps can improve memory performance [321,330,331]. In this study, we used a finger sequence tapping task to assess procedural memory, while declarative memory was tested by a verbal and a non-verbal tasks (word-pair associate learning task and 2-D object-recognition memory task, respectively). On average, for each task, subjects reached ~60% of correct responses at learning and there was no significant learning difference among treatments (BAC, SO, and PL; Table 8.3 with statistics).

Performance on the unrelated word-pair associate task and the finger sequence tapping task was increased similarly for all treatments at retrieval compared to learning ($p<0.05$; Figure 8.9A,B), while for the 2-D object-recognition task, subjects did not show any improvement at

retrieval (Figure 8.9C). Accuracy (error rate) of the finger-tapping task was similar at learning and retrieval (Figure 8.9A, right panel). Interestingly, a nap under SO or BAC did not differently affect any tested memory variables compared to a nap under PL.

Numerous behavioral studies have demonstrated that sleep benefits the consolidation of emotional relative to neutral memories compared with wakefulness [302,332,333,334,335,336]. Thus, we explored the consolidation of neutral, positive, and negative emotional memories using the word-pair associate learning task (Figure 8.9B, left panel). Subjects remembered similarly negative, positive as well as neutral words and performance was higher at retrieval for all three emotional categories compared to learning, except negative words in SO condition for which performance was similar at learning and at retrieval. However, note that performance for negative, positive and neutral words in SO condition did not differ. Together, these results suggest that neither the emotional burden of the words nor treatment during the nap affected memory.

8.5. Discussion

These findings provide a substantial evidence that SO and BAC decreased sleep latency in low sleep pressure condition, produced SOREMPs and strongly increased EEG delta and theta power in NREMS as well as in REMS. Both counteracted the homeostatic effect of an afternoon nap on subsequent nocturnal sleep. The increase in the EEG delta power during the nap induced by SO, did not affect the following sleep suggesting that the delta activity produced by SO does not interfere with the homeostatic regulation of sleep. Thus, SO-induced slow waves seem different from those produced during physiological sleep. The differential dynamics of sleep and EEG effects of BAC did not allow us to conclude whether or not BAC affects the homeostatic process of sleep.

Overall, memory, neurobehavioral performance and subjective alertness were not affected by SO and BAC. However, SO induced a slight and temporary decline of vigilance after the nap (potentially independent of sleep inertia).

BAC reduced WASO and NREMS stage 1 suggesting a more consolidated sleep than SO and placebo during EXP night. The lack of increased consolidated sleep by SO during a long sleep (EXP night) might be due to its short action, because during a short sleep (nap) SO did show a decrease in stage 1 and MT. Thus, although the effect of BAC and SO on EEG and sleep were similar for several variables, their differential dynamics of action and affinity for $GABA_B$ receptors may explain the differences observed between them.

The effects of a nap on sleep and EEG

All 13 subjects succeeded in falling asleep during the afternoon nap. Consistent with a previous study[293], sleep latencies and sleep efficiencies during the nap were similar and lower compared to BLN nights, respectively. Moreover, in BLN conditions, mean values of sleep variables were coherent with results reported in healthy volunteers[337]. In placebo (PL-PL) conditions, an afternoon nap decreased TST, sleep efficiency and increased sleep latency during the postnap (EXP) sleep compared to BLN sleep. SWS was reduced in the entire sleep episode with a marked decrease during the first cycle, while the enhancement of REMS in the first sleep cycle did not significantly affect the mean REMS duration of the night.

It has been proposed that NREMS exerts a sleep-dependent progressive disinhibition of REMS in the course of sleep episode [338,339]. The increase in the duration of the first REMS episode from 12.9 (BLN) to 24.3 min (EXP) and the occurrence of a SOREMP (2/12 in EXP night) is further evidence for a disinhibition of REMS by the reduced NREMS pressure. This effect was present, however, only in the first sleep cycle and did not result in a significant increase in REMS in the entire sleep episode.

At the level of EEG, REMS EEG spectra of the entire night did not significantly differ between BLN night and EXP night (postnap night). However, by looking at the three first sleep cycles of the night, we found that the EEG theta power slightly decreased over the cycles in BLN condition and theta power in the first REMS episode of EXP night was significantly reduced compared to BLN night. The same tendency was found for delta power in REMS. To our knowledge this is the first study showing that the time course of theta and potentially delta power are affected by low sleep pressure and are decreased across sleep episodes suggesting that the EEG theta power and eventually the EEG delta power might represent potential markers of the homeostatic regulation of REMS. However, previous experiments with a sleep desynchrony protocol and a REMS-deprivation protocol did not find theta (4.5-8 Hz) but alpha (8.25-11 Hz) power as a potential marker of REMS propensity[340]. Further investigations on the dynamics of the EEG theta and delta power during REMS under low and high sleep pressure are needed to better understand their involvement in the homeostatic regulation of REMS.

The effects of a nap on the NREMS EEG spectra were not limited to the delta frequency range but extended to the theta frequency range. It has been observed that activity in the delta and theta frequency ranges decreases in the course of a sleep episode and is enhanced after sleep deprivation and reduced after an afternoon/evening nap[92,293,341,342]. Thus, our results are in good agreement with the expected reduction of sleep pressure induced by an afternoon nap. Moreover, activity in the sigma frequency range (spindle band) typically shows a small increase in the course of sleep [342,343,344] and a reduction after sleep deprivation[345]. In the present study, sigma frequency range changed in the expected direction, which was opposite to that induced by sleep deprivation. The effects of the nap on these three frequency ranges were particularly obvious in the first sleep cycle.

Taken together, in placebo condition, the changes in the NREMS EEG spectrum and sleep variables, dependent on the duration of prior wakefulness and sleep history, illustrate perfectly the homeostatic component of sleep regulation and are perfectly consistent with previous studies[293].

The effects of BAC and SO on sleep and the EEG

To evaluate the effects of a nap under SO or BAC and the effects of SO and BAC on EXP night (postnap night) on sleep and its homeostatic regulation, we compared sleep variables and the EEG in NREMS and REMS of the different drug conditions with placebo condition.

Naps under SO were longer than naps under PL. The increase of sleep efficiency and the decrease of combined arousal variables with light sleep (stage1+WASO+MT) of naps under SO suggest that these naps showed more consolidated sleep than naps under PL. SO increased slightly SWS duration compared to PL, however the significance was lost when SWS was expressed as a percentage of TST. Together these findings are consistent with previous studies regarding the dose used, the study design and the subjects (healthy volunteers). For example, Lapierre and colleagues showed that, with a dose of GHB similar to ours, healthy volunteers during a morning nap showed a decrease in WASO compared to PL, but identical SWS duration and sleep latency[237]. A significant increase in SWS duration during nocturnal and daytime sleep were found in healthy volunteers and in patients with sleep disturbances, only at higher doses[145,236,283,288,346].

Administration of SO before EXP night counteracted the effects of the nap by strongly decreasing sleep latency and increasing TST, sleep efficiency and SWS in the first NREMS episode. These findings are very similar to the results obtained during the nap under SO except for sleep latency, which was not reduced during the nap compared to placebo and BAC treatment. This is probably due to a ceiling threshold of sleep latency that cannot be further shortened with drugs. During BLN night and nap in our healthy subjects, all

treatments including placebo would be around this ceiling level, whereas during postnap sleep, the long sleep latency in placebo condition (induced by napping) could be reduced by the drugs.

At the EEG level, SO administrated before the nap or before EXP night had similar effects. It increased EEG delta and theta power in NREMS. This result on NREMS spectra is consistent with a recent report[346] which showed that even under high sleep pressure a dose of 3.5g of SO increased delta and theta power compared to placebo. Importantly, these effects are not exclusive to NREMS because also present in REMS during EXP night. This suggests a pharmacological EEG effect of SO rather than an induction of physiological SWS with a high prevalence of delta and theta waves restricted to NREMS. Moreover, a nap under SO did not affect the postnap sleep. Thus, it seems that SO acutely modifies sleep but its effects are not involved in homeostatic regulation of sleep, i.e. changing physiological sleep need and pressure. This is also consistent with what we recently reported in mice[294]. However, our results both in mice and humans, contrast with a recent study testing the effects of SO on sleep loss, which found that administration of SO during sleep restriction affected the rebound of delta power during recovery night (lower rebound) leading to the conclusion that SO seems to be involved in the homeostatic process of sleep.

A common effect shared by BAC and SO was the increased occurrence of SOREMPs. First, although intriguing for healthy volunteers to show SOREMPs even under placebo treatment, others have also reported similar phenomenon during naps and nocturnal sleep[293,347,348,349,350]. Incidence of SOREMPs is increased in sleep-deprived subjects and in patients suffering from narcolepsy, depression, obstructive sleep apnea syndrome and periodic leg movement disorder. It is highly unlikely that our subjects included were in any of these conditions due to the careful screening and monitoring performed (continuous activity assessment,

habituation night before each session, and see also Materials and Methods). It is also interesting to note that SOREMPs appeared in placebo conditions especially under low sleep pressure (5% for BLN night vs 17% for EXP night (PL-PL)) and mostly during the nap (39% (PL)). As already mentioned, it has been proposed that NREMS pressure may inhibit REMS which supports our findings. In addition, circadian REMS propensity may probably play a role[349], as well as the young age of our subjects (23 years old). It would be, therefore, interesting to further investigate which factor influence manifestation of SOREMPs in healthy populations, a topic poorly studied[348].

During the nap, the duration of SOREMPs was much longer with SO treatment compared to BAC and PL treatments, and the number of SOREMPs significantly higher than that in PL treatment. During EXP night, overall, both BAC-PL and PL-SO treatments showed higher number of SOREMPs with a longer duration compared to other treatments and BLN nights. REMS latency was also reduced with SO treatment during the nap and for PL-SO and BAC-PL during EXP night, mainly due to SOREMPs. This again pinpoints a delayed effect of BAC and the similitude between PL-SO and BAC-PL conditions. In addition, BAC and SO seems to induce SOREMPs which have never been described to our knowledge for BAC. Although rarely, SOREMPs under GHB has been already reported [236,283]. To investigate whether SOREMPs under SO and BAC were different at the EEG level to SOREMPs under placebo, we analyzed SOREMP spectra during naps where we found the highest number of SOREMPs in each conditions. Although, SOREMP duration was longer with SO than with BAC and PL, no significant difference was found between SOREMP spectrum in SO, BAC, and PL. This suggests that SOREMP induced pharmacologically did not differ from 'physiological' SOREMP at the EEG level, that was not the case for the REMS spectrum which showed a large increase in EEG theta and delta power under SO and BAC during EXP night. However, note

that SOREMP spectrum analysis was performed in only 6 subjects who showed a SOREMP in the three conditions.

BAC shares many common sleep and EEG effects with SO, but with a delayed action. During the nap, BAC did not differ from PL, but for several sleep variables, it was intermediate between SO and PL. BAC tended to increase sleep efficiency and to decrease MT and combined arousal variables with light sleep. However, the effects of BAC administrated before the nap was much stronger and visible during the subsequent nighttime sleep, where it counteracted the effects of the nap by strongly decreasing sleep latency and increasing TST, sleep efficiency and SWS in the first NREMS episode, like SO administrated immediately before the nighttime sleep. The delayed effect of BAC was also confirmed at the EEG level. BAC administrated before the nap slightly affected NREMS spectrum during the nap (i.e. it is intermediate between PL and SO), but had strong and similar effects on subsequent sleep than those exerted by SO-PL treatment on nap and SO-PL treatment on EXP night. During the first part of EXP night, BAC-PL, like SO-PL, increased NREMS and REMS delta and theta power and decreased NREMS sigma power. In agreements with the delayed sleep effect of BAC, the EEG delta and theta powers were also increased in PL-BAC condition but tented to be more pronounced from the 2^{nd} cycle of the sleep episode, and TST and sleep latency were less affected than BAC-PL. Although, BAC, like SO, counteracts sleep and EEG effects of the nap and increased delta and theta power in NREMS and REMS suggesting induction of a "pharmacological" sleep rather that physiological sleep, we cannot conclude whether or not BAC affects the homeostatic process of sleep due to its delayed action. Hence, further investigations are needed to better understand the involvement of BAC in homeostatic regulation in humans. One interesting experiment could be to administrate BAC at least 2 hours before a night sleep (T_{max} of BAC : 1.8 h, half-life: 4 h). A nap protocol is probably not

ideal because of the prolonged action of BAC on sleep and the EEG. Note that we chose to administrate the dose of BAC just before sleep, like SO, because in mice the EEG effects of BAC appeared after ~ 15 min[294] and to avoid any interaction with learning during vigilance and memory tasks.

Although we found a high level of similarity between BAC and SO, several sleep and EEG aspects are different including WASO, stage 1 and REMS duration, as well as SOREMPs. Both PL-BAC and BAC-PL treatments increased REMS duration during EXP night compared to placebo, while PL-SO did not increase REMS duration during the entire sleep episode and even showed a decrease in the first and in the second REMS episode compared to placebo and BLN night, respectively. This decrease of REMS with SO in healthy volunteers is consistent with previous reports[145,346], but see also ref.[237]. Importantly, SOREMPs were not taken into account in the first REMS episode. Thus, the fact that both PL-SO and BAC-PL did show significant occurrence of relatively long SOREMPs explains probably at some extent why REMS duration for the whole night in PL-SO condition where similar to placebo and why in BAC-PL the overall duration of REMS was increased. However, this BAC-related increase in REMS seems to be SOREMP-independent, because PL-BAC, which did not produce significantly SOREMPs (2/12 like placebo), also showed a significant increase of REMS compared to placebo. We also found REMS EEG differences between BAC and SO during the EXP night. Theta power and, in a lesser extent, delta power was increased specifically during the first REMS episode for PL-SO compared to placebo, while for BAC-PL, this increase was still present during the second and the third cycle. PL-BAC increased also the theta power during REMS but from the second cycle, underscoring again the delayed effect of BAC on sleep and the EEG. Also, unlike SO, BAC decreased WASO and NREMS stage 1 during the EXP night, suggesting induction of a more consolidated sleep and longer action compared to SO.

Therefore, SO and BAC showed some similar effects on sleep and EEG in humans, although evident differences were observed suggesting distinct mechanisms of action. This is consistent with a previous study in narcoleptic teenagers, which showed that BAC and SO increased TST and EEG delta power, but only SO decrease excessive daytime sleepiness and attacks of cataplexy[252]. Previous animal reports also showed differential effects for BAC and GHB [246,286]. It is known that SO and BAC are low- and high-affinity agonists of $GABA_B$ receptors[287] and we recently showed that the acute effects on sleep and the EEG in mice are mediated exclusively through $GABA_B$ receptors[294]. Although involvement of other receptors could not be completely excluded[186,208], the discrepancy between BAC- and SO-effects may probably be explained by their differential pharmacokinetics and affinity for $GABA_B$ receptors. Indeed, BAC has a longer half-life than SO (4h vs 30-50 min) and it was shown that low-affinity agonists can have very different or even opposite effects compared to high-affinity agonists[234,246]. Furthermore, potassium channels tetramerization domain-containing proteins that function as auxiliary subunits of $GABA_B$ receptors are most likely involved in the modulation of these differences[235].

Concerning the involvement of other receptors, selective $GABA_A$ agonists, such as gaboxadol (THIP), and the GABA uptake inhibitor tiagabine, showed increased SWS duration, EEG delta and theta power during a night sleep compared to placebo[123,351]. Gaboxadol also decreased the EEG sigma frequency band in NREMS and increased EEG delta and theta frequency bands in REMS[352]. In addition, Mathias and al. showed that gaboxadol counteracts the disrupting effects of a nap on subsequent sleep by promoting SWS, EEG delta and theta power in NREMS and by decreasing sleep latency[353]. These effects closely match those evoked by both SO and BAC suggesting, that as SO and BAC, gaboxadol exhibits significant hypnotic actions under conditions in which sleep pressure is experimentally reduced and in part mimics sleep

and EEG modifications seen during recovery sleep after sleep deprivation (i.e. increase of SWS, EEG delta and theta activity and decrease in EEG sigma activity in NREMS). In contrast, benzodiazepines as well as zopidem and zolpiclone, agonistic $GABA_A$ modulators, decrease EEG slow (delta) waves and increase EEG spindle (sigma) frequency band in NREMS, even in high sleep pressure conditions[354,355,356,357,358]. Although it was shown that BAC and GHB did not bind to $GABA_A$ receptors[202,359], they may share effects on neuronal processes underlying the generation/synchronization of slow waves.

Vigilance

A recent study showed that the enhancement of SWS by SO results in a reduced response to sleep loss on measures of alertness and attention[346]. In the present study, in low sleep pressure condition, SO and BAC did not affect the nap-dependent increase of subjective alertness in late evening compared to placebo, even if sleepiness was considered as a frequent side effect of BAC[360] and SO showed increased EEG delta activity during the afternoon nap. Furthermore, temporary, just after the nap, SO suppressed the increase of vigilant attention and alertness seen in BAC and placebo conditions. This effect could be due to either the direct pharmacological effect of SO on vigilance or the effect of the long nap induced by SO. We did not find a correlation between TST and neurobehavioral performance, but it does not excluded the effect of sleep inertia. Subjects performed vigilance tasks at least 15 min after awakening. In a non-sleep deprived situation, sleep inertia should last only a few minutes especially at this time of day (high circadian influence) and if subjects were awakened in light sleep (stage 1,2 or REMS). In SO condition, two subjects were awakened in SWS at the end of the nap, however, their performance 15 min later was not worse than the other subjects. A recent study in healthy volunteers showed that GHB dose of 2.4 g/70 kg still showed sedative-like side effects 2 h after administration, but then disappeared quickly[295]. However, they did not find changes in psychomotor

performance assessed by Digit Symbol Substitution Task. Together, this suggests that SO and BAC do not affect the benefit of a nap on attention and subjective alertness in the late evening and are comparable to placebo across the afternoon except SO which showed a slight and temporary reduction of performance after the nap.

Memory

As expected[330], we found an improvement of word-pairs correctly recalled after a nap but neither SO nor BAC affected this performance. Thus, the 'extra' delta power induced by SO during the nap did not increase performance of this declarative memory task compared to placebo. Moreover, it was shown that emotion facilitates memory encoding[302,332,361]. However, we did not find any superior or inferior retention levels for positive or negative word-pairs compared to neutral word-pairs, although there is a tendency after a nap under SO to remember more positive rather than negative word-pairs.

For the 2-D object-recognition task, the other declarative memory task, learning and retrieval did not differ. Maybe a nap was not sufficient to increase performance as observed with a similar task after 8 h of nocturnal sleep[315]. Another plausible explanation would be the high inter-individual variability of performance found at learning for this task especially. Indeed, at learning, subjects were tested only once and, although the averaged mean performance was around 60%, the range of objects correctly localized was between 20-90%. A previous study showed that only subjects who had most strongly acquired a declarative memory task during the training session showed a sleep-dependent performance benefit after a nap[362]. Therefore, to potentially improve the sensitivity of the task, each subject should have fulfilled a criterion of 60% of performance at leaning and the number of card-pairs should have been increased to avoid a too high level of performance after the first test. The result obtained with this task may not be as relevant as that with word-pairs for which the inter-individual variability was lower at learning.

As for the word-pairs associate learning task, we found an increased performance for the finger sequence tapping task independent of the treatment administrated before the nap. This improvement of procedural memory is in agreement with previous reports[321,331].

Therefore, although SO- and BAC-induced memory impairment were reported in animals [195,363,364,365,366] and/or in humans [306,367,368], and although SWS and delta power was associated with increased performance in declarative memory task, we did not find any difference in memory consolidation with either SO or BAC compared to placebo.

Conclusion

We found that both BAC and SO counteracts the effects of a nap on the subsequent sleep by decreasing sleep latency and increasing TST and particularly SWS during the first NREMS episode and EEG delta and theta powers during NREMS. However, SO also increased EEG delta and theta power during REMS and a nap under SO with high level of delta power did not affect the following night sleep. This suggests that SO is not involved in the homeostatic regulation of sleep and thus, do probably not induce a physiological sleep. BAC showed very similar effects on sleep and EEG compared to SO, but with a delayed action. This different BAC dynamics did not allow us to determine if BAC affects or not the homeostatic process of sleep. Both BAC and SO increased the occurrence of SOREMPs during the nap and during the subsequent sleep, but their effects on REMS differed with an overall increase for BAC and a tendency to decrease particularly in the beginning of the night for SO. The strong similarities of BAC and SO effects on the EEG suggest that SO act through $GABA_B$ receptors, but the difference of the dynamics of action and on REMS may suggest different $GABA_B$ receptor modulation. Involvement of other receptors is not excluded. Finally, overall, a nap under SO and BAC does not affect psychomotor performance and subjective sleepiness neither memory consolidation compared to placebo.

8.6. Tables and Figures

Table 8.1: Visually scored variables during the nap after placebo (PL), baclofen (BAC), or sodium oxybate (SO) intake.

Variables	PL	BAC	SO
TIB (min)	74.5 ± 2.5[a]	71.7 ± 3.1[a]	95.5 ± 7.0[b]
TST (min)	58.6 ± 2.1[a]	60.4 ± 2.8[a]	84.1 ± 7.7[b]
SE (%)	79.9 ± 2.1[a]	84.6 ± 2.6[ab]	86.9 ± 3.0[b]
SL (min)	10.6 ± 0.9	8.6 ± 1.2	9.1 ± 1.2
REMSL (min)	32.7 ± 4.7[a]	31.6 ± 8.2[ab]	18.9 ± 5.8[b]
WASO (%)	12.0 ± 6.1	4.7 ± 2.7	4.7 ± 3.6
S1 (%)	16.3 ± 1.2[a]	13.7 ± 2.0[a]	9.2 ± 2.3[b]
S2 (%)	36.4 ± 2.3	32.6 ± 3.2	30.0 ± 4.8
S3 (%)	5.8 ± 0.4	5.1 ± 0.9	4.7 ± 0.6
S4 (%)	40.4 ± 2.2	43.7 ± 3.7	38.5 ± 4.6
SWS (%)	46.2 ± 2.2	48.9 ± 3.7	43.2 ± 4.5
REMS (%)	9.5 ± 1.3[a]	11.8 ± 2.4[a]	22.3 ± 4.0[b]
MT (%)	0.6 ± 0.0[a]	0.6 ± 0.0[ab]	0.4 ± 0.1[b]
WASO+MT+S1(%)	28.5 ± 6.8[a]	18.6 ± 4.4[ab]	14.1 ± 5.6[b]
SOREMP (#)	0.4 ± 0.1[a]	0.4 ± 0.1[ab]	0.6 ± 0.1[b]
SOREMP (min)	4.5 ± 1.1[a]	5.1 ± 1.8[a]	19.0 ± 5.5[b]

Mean values (±SEM) from lights off to lights on (time in bed (TIB)). The nap was stopped after one cycle from the first appearance of REMS, except if REMS appeared before stage 3 and 4 present in all naps analyzed. Total sleep time (TST), sleep latency (SL; first epoch of S2 or REMS from lights off), REMS latency (REMSL), and duration of sleep onset REMS period (SOREMP) were expressed in min. Wakefulness after sleep onset (WASO), Stage 1 to 4 (S1-S4), slow-wave sleep (SWS; Stage 3+4), REMS, movement time (MT) and combined arousal variables and light sleep (WASO+MT+S1) were expressed as a percentage of TST. Sleep efficiency (SE) was calculated by dividing TST by TIB (%). *a-b*: variables for which mean values significantly differed do not share the same character (one-way mixed-model ANOVA for factor 'treatment'; Tukey-Kramer's test, $p<0.05$).

Table 8.2: Visually scored variables in baseline (BLN) and experimental (EXP) night for the 5 different treatments.

	BLN night	EXP night				
Variables	**All**	**PL-PL**	**BAC-PL**	**SO-PL**	**PL-BAC**	**PL-SO**
TIB (min)	480.0	480.0	480.0	480.0	480.0	480.0
TST (min)	453.6 ± 2.1	414.4 ± 7.2†	451.4 ± 3.4‡	412.6 ± 7.5†	427.6 ± 5.9†	437.5 ± 5.0†
SE (%)	94.5 ± 0.4	86.3 ± 1.5†	94.1 ± 0.7 ‡	86.0 ± 1.6†	89.1 ± 1.2†	91.2 ± 1.1†
SL (min)	12.1 ± 1.0	40.0 ± 5.4†	19.8 ± 3.6†‡	39.3 ± 5.2†	40.6 ± 5.9†	17.8 ± 2.3†‡
REMSL (min)	58.8 ± 2.2	49.4 ± 7.5	19.9 ± 8.4†‡	64.7 ± 4.8	49.1 ± 6.3	25.4 ± 8.7†
WASO (%)	3.3 ± 0.5	6.4 ± 1.5†	1.9 ± 0.3†	7.2 ± 2.2	2.8 ± 0.8	5.8 ± 1.3†
S1 (%)	7.8 ± 0.4	10.0 ± 1.0†	5.1 ± 0.5†‡	10.7 ± 1.3	6.1 ± 0.6†‡	10.6 ± 1.5
S2 (%)	43.9 ± 0.8	44.7 ± 1.8	44.1 ± 1.3	48.1 ± 1.8	42.8 ± 2.2	42.4 ± 1.7
S3 (%)	4.1 ± 0.2	3.9 ± 0.4	4.0 ± 0.4	4.0 ± 0.5	4.7 ± 0.4	3.1 ± 0.3
S4 (%)	17.7 ± 0.5	13.6 ± 1.0†	15.1 ± 1.2†	12.3 ± 1.2†	15.6 ± 1.4†	17.0 ± 1.1
SWS (%)	21.8 ± 0.6	17.5 ± 1.3†	19.1 ± 1.5	16.3 ± 1.4†	20.4 ± 1.7†	20.1 ± 1.1
REMS (%)	27.0 ± 0.4	28.9 ± 1.3	31.9 ± 1.1†	25.8 ± 0.9	31.7 ± 1.5†	27.5 ± 1.0
MT (%)	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
WASO+MT+S1(%)	11.5 ± 0.9	16.8 ± 2.0†	7.6 ± 0.6†‡	18.2 ± 3.2	9.4 ± 1.1†‡	16.7 ± 2.5
SOREMP (#)	0.0 ± 0.0	0.2 ± 0.1	0.7 ± 0.1†‡	0.0 ± 0.0	0.2 ± 0.1	0.5 ± 0.2†
SOREMP (min)	0.3 ± 3.2	0.1 ± 0.1	10.6 ± 4.1†	0.0 ± 0.0	3.5 ± 3.3	18.6 ± 5.8†‡

Mean values (±SEM) from lights off to lights on (time in bed (TIB)). BLN night: for clarity, BLN night of the 5 sessions was averaged, although for statistical analysis each subject's EXP night was compared to its corresponding BLN night. EXP night: treatment intake before the nap and before EXP night (5 possibilities: placebo (PL) then PL, baclofen (BAC) then PL, sodium oxybate (SO) then PL, PL then BAC, and PL then SO). For variable definition see Table 8.2.

EXP night significantly different from BLN night : † (p<0.05). Treatment significantly different from PL-PL : ‡ (p<0.05) .

Table 8.3: Memory performance

			PL Mean ± SEM	SO Mean ± SEM	BAC Mean ± SEM	*p*
Finger sequence tapping task	***Speed***	***learning***	20.81 ± 0.95	21.15 ± 1.57	20.47 ± 1.46	NA
		change	+2.70 ± 0.42	+2.26 ± 0.46	+3.33 ± 0.48	NA
	Error rate	***learning***	0.51 ± 0.09	0.43 ± 0.09	0.49 ± 0.10	NA
		change	-0.06 ± 0.08	-0.05 ± 0.11	-0.12 ± 0.10	NA
Word-pair associated task		***learning***	22.57 ± 1.19	20.69 ± 1.97	23.50 ± 1.60	NA
		change	+4.64 ± 0.80	+5.23 ± 1.06	+4.25 ± 0.99	NA
2D object-location task		***learning***	5.66 ± 0.30	5.62 ± 0.67	5.67 ± 0.73	NA
		change	-0.08 ± 0.35	-0.77 ± 0.68	-0.67 ± 0.54	NA

Memory performance for the administration of PL, SO or BAC after training. For the procedural finger sequence tapping task, performance during learning is indicated as speed (number of correctly tapped sequences) and error rate (errors per sequence). For the declarative word-pair learning task and the declarative 2D object-location task, performance during learning is indicated as number of correctly recalled word-pairs and correctly located card-pairs, respectively. For both declarative tasks, performance changes are calculated as absolute difference between memory performance during learning and retrieval (retrieval minus learning). Data are mean values (±SEM), and *p*-values for one-way mixed-model ANOVA for factor "treatment" PL, BAC, and SO). $P > 0.05$ is NA. (see also Figure 8.9)

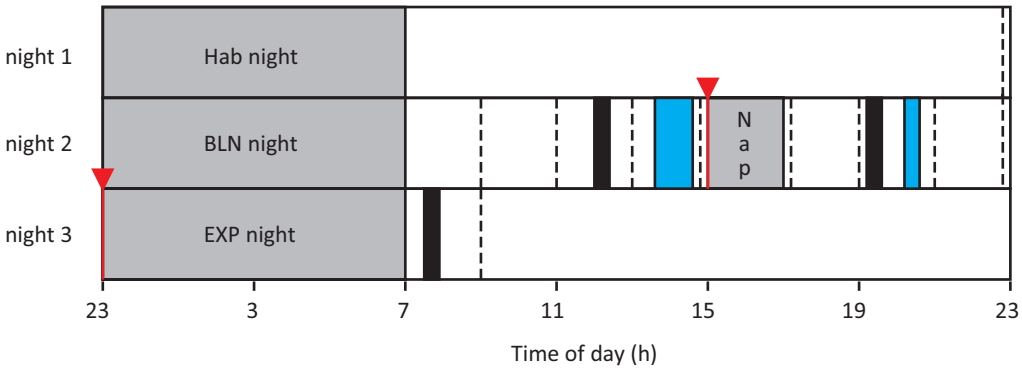


Figure 8.1: Schedule of a typical study session.

Each subject performed 5 similar sessions, which differed only by the treatment that they received. One week separated each session. Subjects started a session with a first 8h habituation (Hab) night, spent the day outside the lab and came back to carry out a 8h baseline (BLN) night. The following day, they stayed in the lab and performed vigilance tasks (PVT and KSS) every 2 h and three memory tasks before and after a 2h opportunity nap starting at 3 p.m. Finally, they spent a last 8h experimental (EXP) night and left the lab in the morning after having performed last vigilance tasks. Grey bars indicate times of nighttime and daytime sleep periods, and black bars depict mealtimes. Test times for the 10min PVT, preceded and followed by the KSS, are illustrated by the dashed lines. Blue Bars represent the time when memory tasks were performed. Before the nap and before the EXP night, subjects received a placebo (PL) and either sodium oxybate (SO), baclofen (BAC) or placebo (PL) leading to the 5 possibilities: PL-SO, SO-PL, PL-BAC, BAC-PL and PL-PL (red triangles and lines).

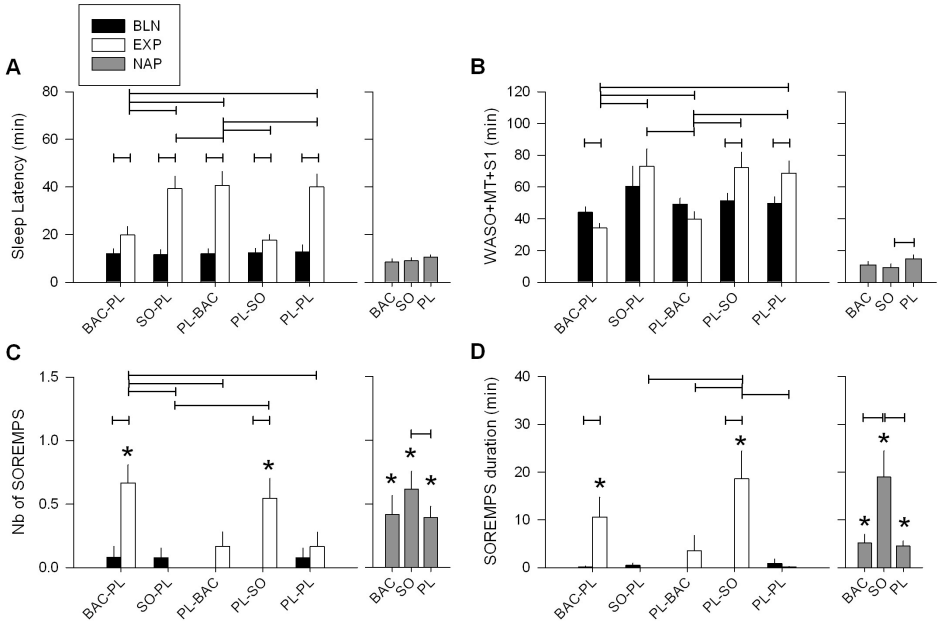


Figure 8.2: Condition and treatment effects on sleep parameters.

A, The afternoon nap increased sleep latency (first 20s epoch of stage 2 or REMS) during the subsequent night (EXP night) compared to BLN night in all treatments (right panel: two-way mixed model ANOVA for factors 'night' $p<0.0001$, 'treatment' $p<0.0001$ and their interaction $p<0.0001$; connected lines: Tukey-Kramer's test by treatment, $p<0.05$). However, this increase was strongly reduced when BAC was administrated before the nap or SO before EXP night (connected lines: Tukey-Kramer's test by night, $p<0.05$). This difference amongst treatment was not seen during the nap (left panel: one-way mixed-model ANOVA for factor 'treatment' $p=0.1156$)). ***B,*** During nighttime sleep, combined arousal variables and light sleep (wakefulness after sleep onset/latency (WASO) + Movement time (MT) + Stage 1 (S1)) differed among treatments (right panel: two-way mixed model ANOVA for factors 'night' $p=0.1070$, 'treatment' $p<0.0001$ and their interaction $p=0.0307$). While PL and SO increased combined arousal variables and light sleep during EXP night compared to BLN night (not significant in SO-PL treatment due to the high variability during BLN night), BAC before the EXP night did not affect them and BAC before the nap even decreased them (Tukey-Kramer's test by night for factor 'treatment', $p<0.05$). During the nap, SO decreased significantly the combined WASO+MT+S1 compared to PL (left panel: one-way mixed-model ANOVA for factor 'treatment': Tukey-Kramer's test, $p<0.05$). ***C,D,*** During nights, number and duration of sleep onset REMS periods (SOREMPs) were affected by both treatments and nights (right panel: two-way mixed model ANOVA for factors 'night' $p<0.0001$, 'treatment' $p<0.0006$ and their interaction $p<0.0003$). Only BAC administrated before the nap and SO given before the EXP night showed significant appearance and increased duration of SOREMPs during the EXP night (paired t-tests $p<0.05$, star). Moreover, they showed respectively a significant increased number of SOREMPs and a longer duration of SOREMPs compared to PL-PL as well as PL-BAC and SO-PL treatments (Tukey-Kramer's test, $p<0.05$). During the nap, number and duration of SOREMPs were increased in all treatments (left panel: one-way mixed-model ANOVA: factor 'treatment'; Tukey-Kramer test, $p<0.05$)). SO significantly augmented SOREMP number and duration compared to PL and, PL and BAC, respectively. For all panels, bars depict the mean values of each variable (mean±SEM), connected lines result from Tukey-Kramer's test, $p<0.05$ and BLN night of each treatment did not differ significantly for each variable.

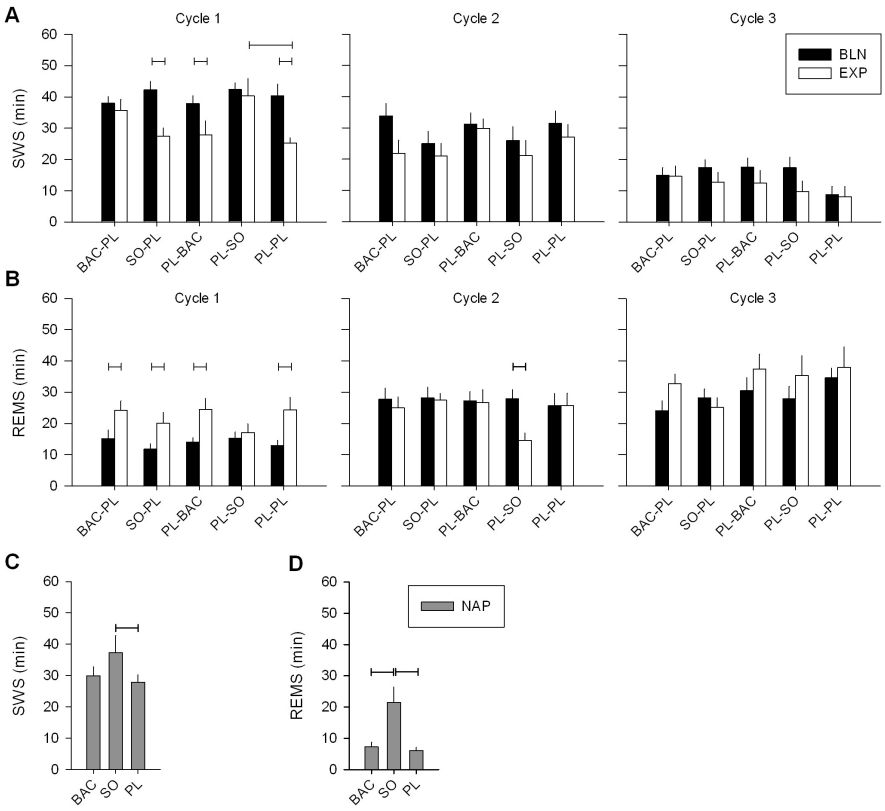


Figure 8.3: SWS (stage 3 and 4) and REMS during the three first sleep cycles of the nighttime sleep and during the afternoon nap.

A, During the first cycle of EXP night, SWS was decreased in PL-PL, SO-PL and PL-BAC treatments, but did not differ from BLN night in BAC-PL and PL-SO treatments (left panel: two-way mixed model ANOVA for factors 'night' p=0.0001, 'treatment' p=0.0256 and their interaction p=0.0542; connected lines: Tukey-Kramer's test by treatment for factor 'night' p<0.05). Moreover, SWS was significantly higher when SO was given before the EXP night compared to PL. Overall, during the second and the third cycle, SWS was significantly lower in EXP night compared to BLN night (two-way mixed model ANOVA for factors 'night' p<0.05, 'treatment' p>0.1 and their interaction p>0.6; paired t-test: factor 'night' p<0.05). ***B,*** REMS was significantly higher in EXP night compared to BLN night for the first cycle only (left panel (1st cycle): two-way mixed model ANOVA for factors 'night' p<0.0001, 'treatment' p=0.4538 and their interaction p=0.3926). Interestingly, PL-SO treatment was the unique treatment which did not show an significant increase in REMS during EXP night compared to BLN night (Tukey-Kramer test by treatment for factor 'night' p<0.05). Moreover, in the second cycle, this same treatment exhibited a shorter duration of REMS compared to BLN night (one-way mixed-model ANOVA for PL-SO treatment: factor 'night' p=0.0024). ***C,*** During the nap, SO increased SWS compared to PL but not compared to BAC (one-way mixed-model ANOVA for factor 'treatment' p=0.0268; Tukey-Kramer test p<0.05). ***D,*** Naps were stopped when REMS was visually identified except if it was a SOREMP (see Materials and Methods and Figure 8.2). The three treatments showed significant increased in REMS duration (paired t-tests p<0.05) due to SOREMPs. SO induced a longer duration of REMS than BAC and PL (one-way mixed-model ANOVA for factor 'treatment' p<0.0001, Tukey-Kramer's test p<0.05). For all panels, bars depict the mean values of each variable (mean±SEM; n=13).

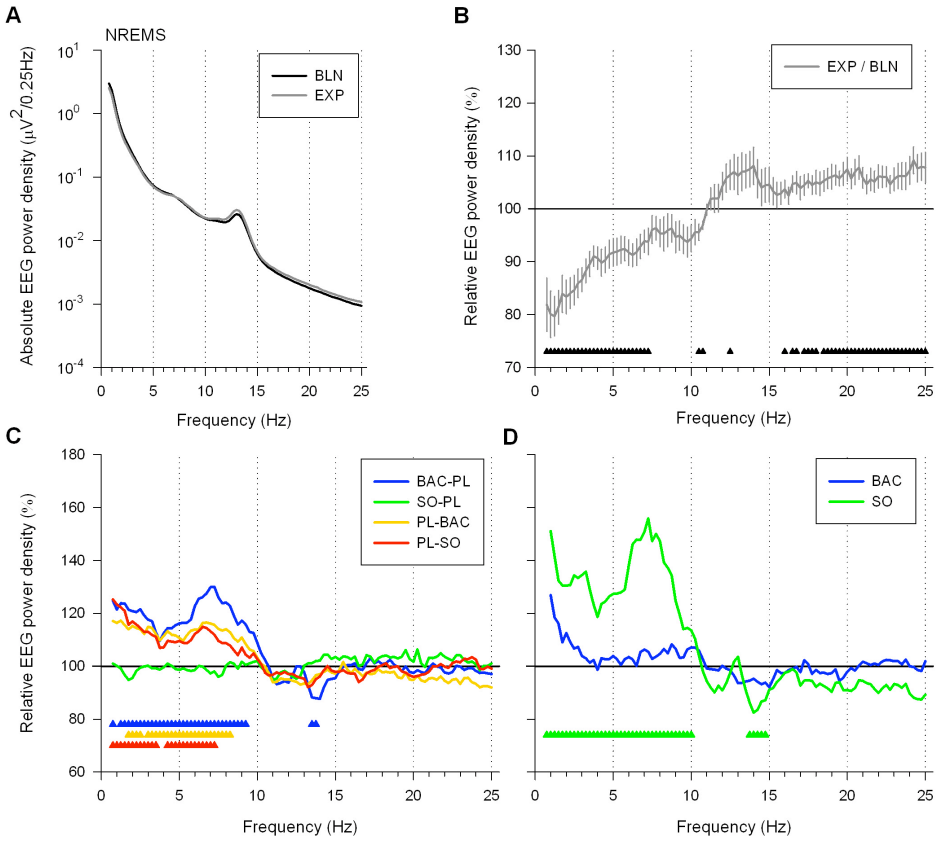


Figure 8.4: EEG power spectra of NREMS during nighttime sleep and during the nap.

A, Absolute NREMS spectra of BLN night and EXP night for PL-PL treatment (0.75-25 Hz, at 0.25Hz bins; note the logarithmic scale of absolute EEG power density). ***B,*** The ratio between these two spectra (EXP/BLN) was performed leading to a relative NREMS spectrum where 100% represents absolute NREMS spectrum of BLN night. NREMS spectrum differed significantly between nights and frequency bins (two-way mixed model ANOVA for factors 'night' p=0.0214, 'bins' p<0.0001 and their interaction p=0.9982. Low frequency bins (0.75-7.25 Hz), were significantly lower and a bin from the sigma band (12.5Hz) as well as overall high frequency bins (16-25 Hz) were significantly higher during EXP night compared to BLN night (black triangles: one-way mixed model ANOVA by bin for factor 'night' p<0.05) ***C,*** Relative NREMS spectrum of each drug treatment (EXP/BLN) were expressed as a percentage of relative NREMS spectrum of PL-PL treatment depicted in ***B***. Relative NREMS spectra during EXP night was affected by treatment and by bin (two-way mixed model ANOVA: factors 'treatment' p<0.0001, 'bin' p<0.0001 and their interaction p<0.0001). Overall, BAC-PL, PL-BAC and PL-SO treatments differed significantly from PL-PL, while SO-PL did not (Dunnett-Hsu's test (control=PL-PL) p<0.05). Colored triangles depict bins for which power differed significantly from PL-PL (Dunnett-Hsu's test p<0.05, blue: BAC-PL, yellow: PL-BAC and red: PL-SO). ***D,*** During the nap relative NREMS spectra after SO and BAC treatments were expressed as a percentage of PL treatment. Overall, relative NREMS spectrum after SO was different from that after PL, while that after BAC did not differed from the two others (two-way mixed model ANOVA for factors 'treatment' p<0.0001, 'bin' p<0.0001 and their interaction p=0.1923; Dunnett-Hsu's test p<0.05). Colored triangles illustrate bins for which power differed significantly from PL. (Dunnett-Hsu's test p<0.05, green: SO, blue: BAC). For each panel, lines depict the mean values (±SEM; n=12).

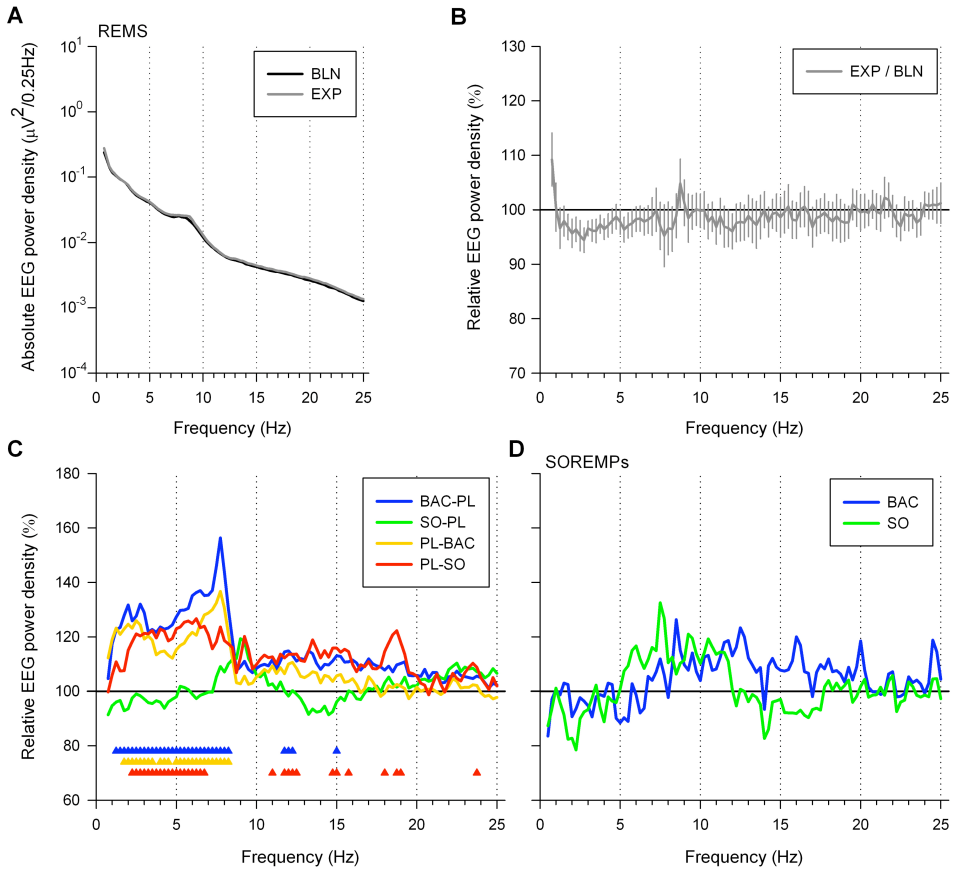


Figure 8.5: EEG power spectra of REMS during nighttime sleep and during the nap.

A, Absolute REMS spectra of BLN night and EXP night for PL-PL treatment (0.75-25 Hz, at 0.25Hz bins; note the logarithmic scale of absolute EEG power density). ***B,*** The ratio between these two spectra (EXP/BLN) was calculated leading to a relative REMS spectrum where 100% represents absolute REMS spectrum of BLN night. REMS spectrum did not differ significantly between nights (two-way mixed model ANOVA for factors 'night' p=0.0999, 'bins' p<0.0001 and their interaction p=0.1). ***C,*** To illustrate the comparison of each treatment to the PL-PL treatment, relative REMS spectrum of each drug treatment (EXP/BLN) was expressed as a percentage of relative REMS spectrum of PL-PL treatment depicted in ***B***. Relative REMS spectra during EXP night were affected by treatment and by bin (two-way mixed model ANOVA for factors 'treatment' p<0.0001, 'bin' p<0.0001 and their interaction p<0.0001). Overall, BAC-PL, PL-BAC and PL-SO treatments differed significantly from PL-PL, while SO-PL did not (Dunnett-Hsu's test (control=PL-PL) p<0.05). Colored triangles depict bins for which power differed significantly from PL-PL (Dunnett-Hsu's test for each bin: p<0.05, blue: BAC-PL, yellow: PL-BAC and red: PL-SO). ***D,*** During the nap, relative SOREMP spectra after SO and BAC treatments were expressed as a percentage of PL treatment. Relative SOREMP spectra did not differ among treatments (two-way mixed model ANOVA: factors 'treatment' p=0.2732, 'bin' p<0.0001 and their interaction p=1.0; note that n=6). For all panels, lines depict the mean values (±SEM; For ***A, B, C***: n=12).

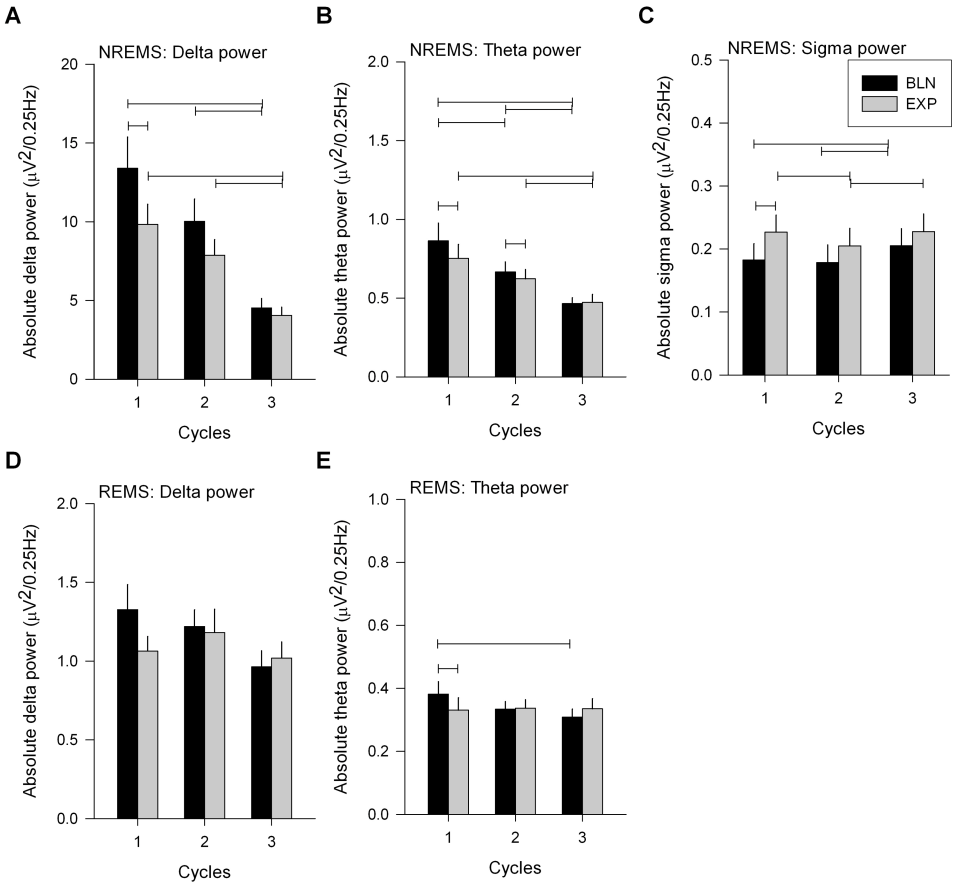


Figure 8.6: Absolute EEG delta, theta and sigma power of NREMS and EEG delta and theta power of REMS during the three first cycles of nighttime sleep in PL-PL treatment.

Absolute power derived from the average of 0.25Hz bins included in the specific frequency range, i.e. delta (0.75-4.5 Hz), theta (4.75-8 Hz) and sigma (12-15 Hz) (mean±SEM: n=12). ***A,*** Absolute delta power of NREMS during the three first cycles of BLN night and EXP night for PL-PL treatment differed significantly between nights and cycles (two-way mixed model ANOVA: factors 'night' p=0.0041, 'cycle' p<0.0001 and their interaction p<0.5899). Moreover, delta power showed an overall cycle-dependent decrease (cycle 1 > 2 > 3: Tukey-Kramer's test p<0.05). ***B,*** As delta, absolute theta power of NREMS differed significantly between nights and cycles (two-way mixed model ANOVA for factors 'night' p=0.0069, 'cycle' p<0.0001 and their interaction p<0.4093) and showed an overall cycle-dependent decrease (statistics see ***B***). ***C,*** Although absolute sigma power of NREMS during the three first cycles of BLN night and EXP night for PL-PL treatment differed significantly between nights and cycles as delta and theta power (two-way mixed model ANOVA for factors 'night' p=0.0001, 'cycle' p<0.0001 and their interaction p<0.1453), it exhibited a lower level in cycle 2 compared to cycle 1 and 3 (Tukey-Kramer's test: p<0.05). ***D,E,*** In REMS, absolute delta power differed only between cycles (two-way mixed model ANOVA for factors 'night' p=0.0536, 'cycle' p=0.0026 and their interaction p=0.1097); cycle 1 = 2 > 3: Tukey-Kramer's test: p<0.05), while absolute theta power differed between cycles and nights (two-way mixed model ANOVA for factors 'night' p=0.0081, 'cycle' p=0.0485 and their interaction p<0.0049; cycle 1 =2 > 2 = 3: Tukey-Kramer test: p<0.05). For each panel, connected lines depict night and cycle differences by cycle and by night, respectively (Tukey-Kramer's test p<0.05).

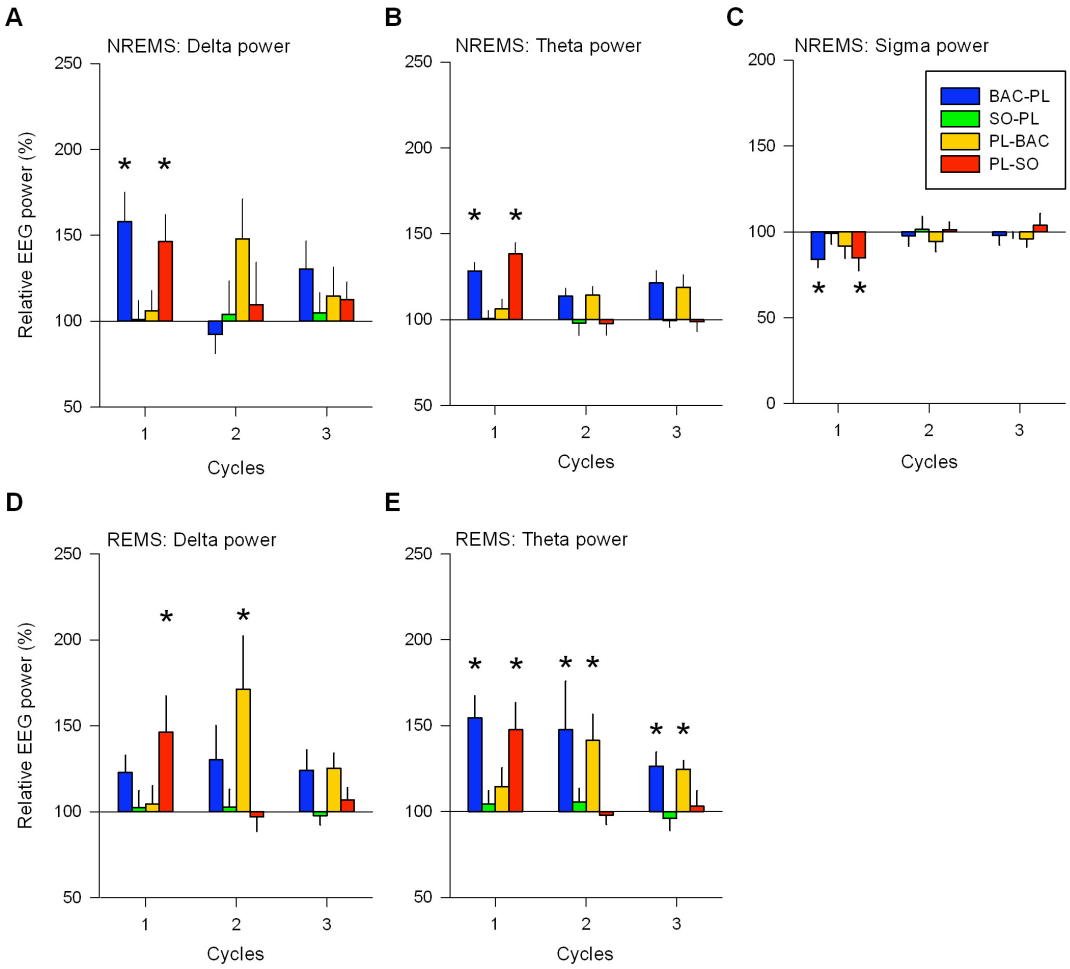


Figure 8.7: EEG delta, theta and sigma power of NREMS and EEG delta and theta power of REMS during the first three sleep cycles.

Relative EEG delta (0.75-4.5 Hz), theta (4.75-8 Hz) and sigma (12-15 Hz) power (mean±SEM; n=12) correspond to the EXP/BLN night ratio of each drug treatment expressed as a percentage of the EXP/BLN night ratio of the PL-PL treatment in the three first cycles of nighttime sleep. This illustrates the difference between drug treatments and PL-PL treatment for specific frequency ranges. ***A,B,C,*** Only in the first cycle, BAC-PL and PL-SO treatments showed increased relative delta and theta power and decreased relative sigma power compared to PL-PL treatment (one-way mixed model ANOVA by cycle for factor 'treatment' p<0.05; Dunnett-Hsu's test (control=PL-PL) p<0.05: star). ***D,*** In REMS, compared to PL-PL treatment, relative delta power for PL-SO treatment increased during the first cycle, while it decreased for BAC-PL treatment during the second cycle (one-way mixed model ANOVA by cycle for factor 'treatment' p<0.05; Dunnett-Hsu's test (control=PL-PL) p<0.05: star). ***E,*** Relative theta power in REMS increased for BAC-PL and PL-SO treatment during the first cycle and increased for BAC-PL and PL-BAC treatments during the second and third cycles (for statistical tests see ***D***).

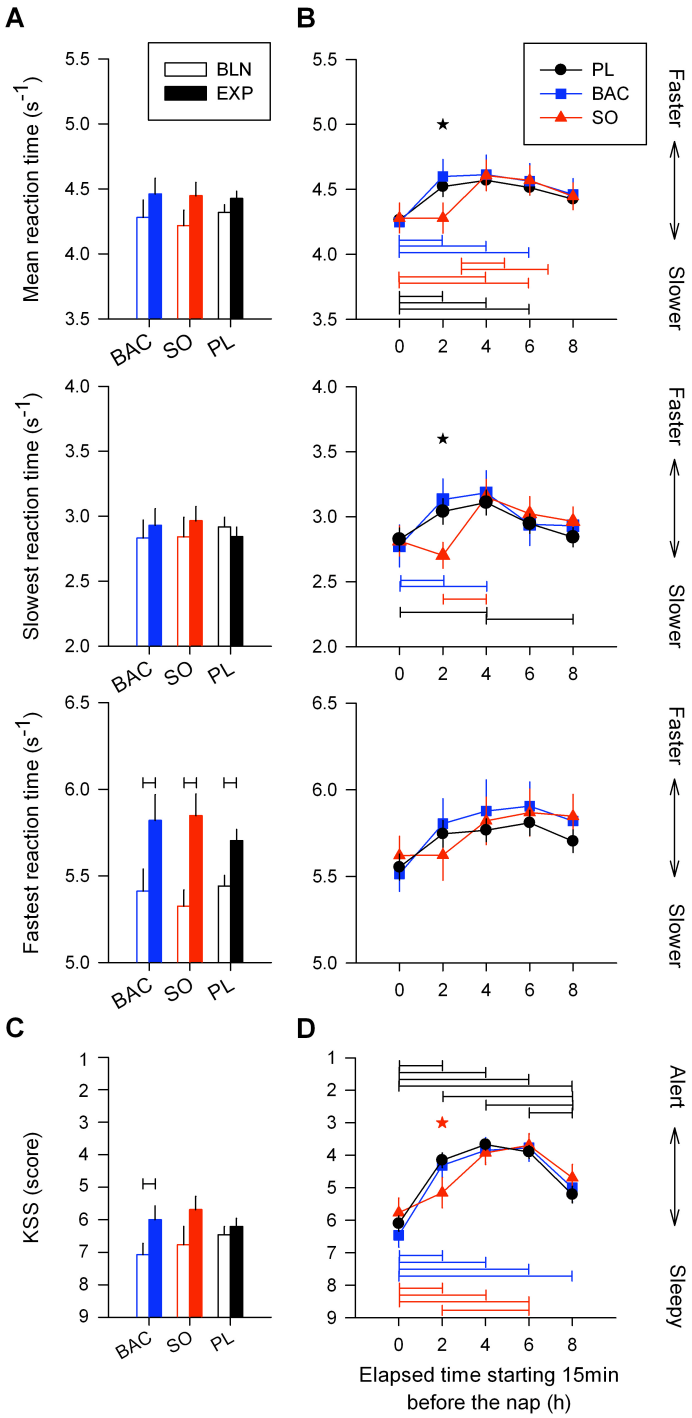


Figure 8.8: Effects of nap with or without drug on sustained vigilant attention and subjective alertness.

Objective measurement of cognitive performance was assessed by 10min psychomotor vigilance task (PVT) and subjective alertness by Karolinska Sleepiness Scale (KSS). ***A.*** Mean, the 10% slowest and the 10% fastest reaction times (RT) expressed as speed (1/RT) are plotted for the trial performed 15 min before bedtime for the BLN and EXP nights (upper, middle and lower panel, respectively). Overall, mean and the 10% fastest speed (upper and lower panel) were affected by the nap but not by treatment (two-way mixed model ANOVA for factors 'time' $p \leq 0.0023$, 'treatment' $p \leq 0.7599$ and their interaction $p \geq 0.1728$). Black connected lines depict speed difference before BLN and EXP nights (one-way mixed-model ANOVA for factor 'time' by treatment; Paired t-test $p<0.05$). ***B.*** Mean, the 10% slowest and the 10% fastest speed were plotted at 5 consecutive trials with the first trial performing just before the nap (hour 0) and the last trial just before EXP night (hour 8). Generally, sustained vigilant attention was not affected by treatment, but by the time when trial was carried out (for the 3 panels: two-way mixed model ANOVA for factors 'time' $p \leq 0.0003$, 'treatment' $p \geq 0.2205$ and their interaction $p \geq 0.2197$). However, by analyzing each trial separately, SO treatment differed significantly from the two other treatments only at the trial just after the nap (one-way mixed-model ANOVA for factor 'treatment' by trials; Tukey-Kramer test $p<0.05$: black star). Colored connected lines show speeds, which are significantly different within the same treatment (Tukey-Kramer test $p<0.05$): black: PL, Blue: BAC and red: SO). ***C,*** Although independently of the treatment, the KSS score was increased during the trial performed just before EXP night compared to the trial performed just before BLN night (two-way ANOVA for factors 'trial' $p=0.0010$, 'treatment' $p=0.6298$ and their interaction $p=0.1659$), analysis done by treatment separately showed that only

BAC treatment exhibited a significant increase of subjective alertness (black connected lines: one-way mixed-model ANOVA for factor 'time' by treatment; Paired t-test $p<0.05$). ***D,*** Similar to results obtained with PVT, subjective alertness obtained by KSS was not generally affected by treatment, but by trial time (two-way mixed model ANOVA for factors 'time' $p<0.0001$, 'treatment' $p=0.1695$ and their interaction $p=0.9041$). Moreover, alertness after the nap was also temporarily altered by SO compared to PL (red star: one-way mixed-model ANOVA for factor 'treatment' by trial; Tukey-Kramer's test $p<0.05$). However, this was not significantly different between SO and BAC treatments $p=0.1547$. For all panels, bars depict the mean values of each variable (mean±SEM; $n=13$).

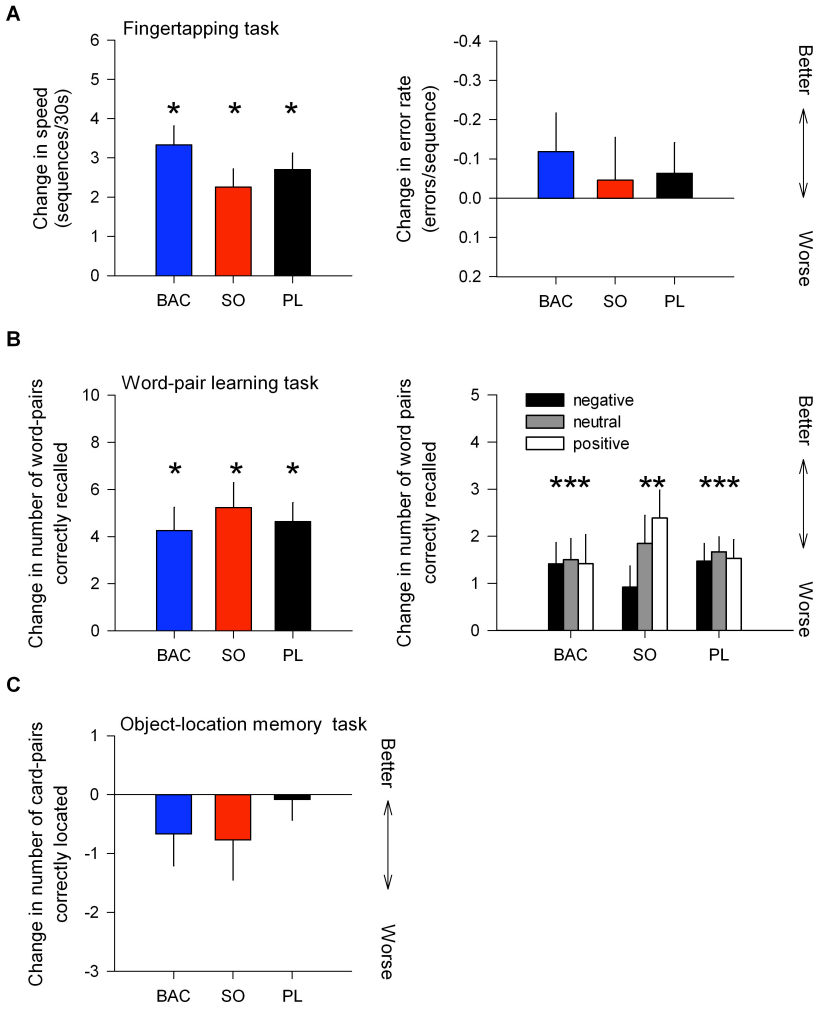


Figure 8.9: SO and BAC do not affect memory

A, Performance to the finger sequence tapping task is illustrated by the difference of the number of correctly completed sequences between retrieval and learning (change in speed; left panel) and the accuracy by the difference of the number of errors relative to total number of tapped sequences between retrieval and learning (error rate; right panel). ***B,*** The gain of performance to the unrelated word-pair associate learning task is shown by the difference of the number of word-pairs correctly recalled at retrieval minus those at learning (left panel). Out of 36 word-pairs, 9 were emotionally negative, 9 emotionally neutral, and 9 emotionally positive (right panel). ***C,*** Performance to the 2-D object-recognition task is evaluated by comparing the number of card-pairs correctly located at retrieval compared to this at learning (difference: retrieval-learning). Similarly for all treatments, performance of fingertapping task and of the word-pair associate learning task was increased (star: paired t-test $p<0.05$); one-way mixed-model ANOVA for 'treatment' $p>0.7683$). The emotionality of the words and drugs did not influence the performance (two-way mixed model for factors 'treatment' $p=0.8027$, 'emotion' $p=0.3886$ and their interaction $p=0.5765$). At retrieval, error rate during the fingertapping task and performance to the object-location task did not differ from learning, and treatment has no effect (paired t-test $p>0.05$); one-way mixed-model ANOVA for 'treatment' $p>0.5208$). For all panels, bars depict the mean values of each variable (mean±SEM; $n=13$).

9. Conclusions and perspectives

GHB does not induce physiological sleep

We showed that GHB in mice and in healthy volunteers induced EEG delta activity, a marker of sleep propensity. However, this increase is not involved in the homeostatic regulation of sleep, suggesting that GHB does not produce physiological deep sleep. In mice, behavior and EEG pattern induced by GHB suggest rather an anesthetic-like state particularly at high doses. Alertness as well as neurobehavioral and memory performance were not further improved by a nap under GHB, rich in EEG delta activity, compared to a nap under placebo. With GHB treatment, we even found a temporary and slight suppression of the increase in subjective alertness and vigilant attention shown after a nap with placebo treatment. This is additional evidence suggesting no restorative effects of EEG delta activity induced by GHB.

A very recent study by Walsh and colleagues[346], however, challenges our findings. They found that under high sleep pressure, vigilant attention and subjective alertness benefit from a short sleep under SO/GHB compared to a short sleep under placebo. They also showed with their sleep restriction protocol that EEG delta power in NREMS during recovery night sleep was slightly lower in healthy volunteers having prior naps under GHB than those having prior naps under placebo. Thus, they concluded that pharmacological enhancement of SWS/EEG delta power with GHB resulted in a reduced response to sleep loss on measures of alertness and attention, and SWS and NREMS EEG delta power enhancement during sleep restriction appears to result in a reduced homeostatic response to sleep loss. Sleep under GHB could have restorative effects only in high sleep pressure conditions, which does not contradict our results. However, the reduction of homeostatic response after naps with GHB remains unexpected compared to our findings. This discrepancy might be due to spectral analysis (they did not normalize EEG power density, while we did) or to subjects (they

included males and females, Caucasians and African Americans, we included only male Caucasians). Apart from this study, several other observations support the fact that GHB does not induce physiological sleep. First, GHB was used as an anesthetic agent and as far as we know no anesthetic induces physiological sleep, although the vast majority produce EEG slow (delta) waves[369]. Second, EEG hypersynchrony was not specific to NREMS but found also after GHB administration in awake animals[200,238,370] and humans[145,236,371]. Finally, according to our results in healthy volunteers and a study in narcoleptics[283], the increase of EEG delta activity is not exclusive to NREMS, but also found in REMS. This support the view of an overall pharmacological increase of slow-wave activity independent of sleep states. In conclusion, as far as we know we are the first to show concretely that GHB does not induce physiological sleep. Nevertheless, further investigations to support this finding and to better understand the role of increased EEG slow-wave activity by GHB in behavior are needed.

Our results also bring new questions about how GHB improves symptoms of narcolepsy. The hypothesis suggesting that GHB induction of a deep and restorative nighttime sleep would explain the decrease of excessive daytime sleepiness in narcoleptics is not supported by our results in healthy volunteers. Therefore, the mechanism by which GHB decreases both excessive daytime sleepiness and cataplexy attacks remains elusive and needs further investigation.

GHB mechanisms of action

By using functional $GABA_B$ receptor deficient mice, we showed that all acute effects of GHB on sleep and the EEG are due to the stimulation of $GABA_B$ receptors. Hence, these receptors seem to exclusively mediate the behavioral and EEG effects of GHB, at least in mice. By comparing the sleep and EEG effects of BAC, a specific and high-affinity agonist of $GABA_B$

receptors, to those of GHB, a low-affinity agonist of $GABA_B$ receptors, we demonstrated that the two drugs do not have identical effects (even if closely related). In contrast to GHB, BAC induced a delayed hypersomnia and lower EEG delta power during BAC-induced state in mice and, in humans, BAC increased REMS and decreased WASO. We also clearly established that BAC and GHB have distinct dynamics of action with BAC having a delayed effect. This discrepancy could be due to their pharmacokinetics with a longer half-life for BAC and to their differential affinity for $GABA_B$ receptors. For example, Curz and colleagues[246] showed bi-directional effects of BAC and GHB on the mesolimbic dopaminergic system. They found that the coupling efficacy (EC_{50}) of G protein-gated inwardly rectifying potassium (GIRK, Kir3) channels to $GABA_B$ receptor was much lower in dopamine neurons than in GABA neurons of the VTA, depending on the differential expression of GIRK subunits. Consequently, in rodent VTA slices, a low concentration of BAC caused increased activity, whereas higher doses inhibited dopamine neurons. At behaviorally relevant doses, BAC activated GIRK channels in both cell types, but GHB activated GIRK channels only in GABAergic neurons. This may explain the opposing effects on the reward pathway exerted by BAC and GHB and similar mechanisms might be found in regions of the brain involved in sleep. Another example is the differential role of glutamate in $GABA_B$ receptor-mediated effects of GHB and BAC in mice. Indeed, Koek and colleagues[286] found that NMDA receptor antagonists enhance the cataleptic effects of GHB but not those of BAC. Thus, differential interactions of glutamate with the $GABA_B$ receptor mechanisms mediating the effects of GHB and BAC may explain sleep and EEG differences found between BAC and GHB. Moreover, a recent study brought a new promising explanation for the pharmacology and kinetics of the $GABA_B$-receptor response. This study showed that $GABA_B$ receptors in the brain are high-molecular-mass complexes of $GABA_{B1}$, $GABA_{B2}$ and members of a subfamily of the KCTD proteins. KCTD proteins 8, 12, 12b and 16 show distinct expression profiles in the brain and associate with

$GABA_{B2}$ as tetramers. This co-assembly changes the properties of the $GABA_{B1,2}$ core receptor by modifying agonist potency and altering the G-protein signaling. As for the study of Cruz and colleagues previously described, the differential affinity of GHB and BAC of $GABA_B$ receptors may therefore lead to distinct effects in specific brain areas, which affect behavior. Taken together, this means that differential effects of BAC and GHB on sleep and the EEG do not exclude the fact that they both act exclusively on $GABA_B$ receptors.

Interestingly, our mice study challenges the specificity of BAC for $GABA_B$ receptors due to the presence of BAC-induced delayed hypersomnia in $GABA_B$-receptor-deficient mice. It was shown that BAC did not bind to $GABA_A$ receptors[372], but to our knowledge no experiment was done to see potential action of BAC on other receptors. However, it is worth noting that in our experiment, a relatively low number of $GABA_B$-receptor-deficient mice (*n*=6) were used and the presence of seizure could have affected sleep. Thus, it would be interesting to characterize further the mechanism by which BAC induces delayed hypersomnia.

Others studies, in mice and in humans, showed that gaboxadol, a $GABA_A$ agonist, and tiagabine, a GABA uptake inhibitor, had similar sleep and EEG effects than GHB. Both drugs increase EEG delta and theta power in NREMS and REMS, and enhance SWS in humans and in rodents[124,352,373,374,375,376]. Although it was shown that GHB did not directly bind to $GABA_A$ receptors, it is possible that GHB after having metabolized into GABA may indirectly act through $GABA_A$ receptors. This transformation into GABA is not possible for BAC. A future interesting experiment would be to administer GHB to mice deficient in the delta subunit of $GABA_A$ receptor. Indeed, it was shown that these mice did not respond to gaboxadol[377].

Concerning the contribution of other receptors, theoretically, GHB receptors could be involved in the GHB response. However, it seems unlikely because our mouse experiment showed no effect of GBL/GHB in mice lacking $GABA_B$ receptors and a mouse report showed that the sedative/hypnotic effect of GHB and BAC were not reproduced by analogues of GHB which are not able to bind $GABA_B$ receptors[188].

Altogether, this suggests that GHB and BAC act most probably and only through $GABA_B$ receptors to affect sleep and the EEG (except for the delayed hypersomnia induced by BAC). However, these two drugs could share mechanisms involving in slow wave generation with agonists of other receptors such as $GABA_A$ ones. Interconnection between $GABA_A$ and $GABA_B$ function was already described for other effects[378].

Role of $GABA_B$ receptors and their subunits in sleep and EEG

In baseline conditions, a lack of one $GABA_B$ receptor subunit altered the sleep/wake distribution over the 24h day in mice, although the total amount of sleep and wakefulness were not modified. For example, mice deficient for the $GABA_{B1}$ and $GABA_{B2}$ subunits, which do not express functional $GABA_B$ receptors, started their major sleep period 6 hours after WT mice. Therefore, $GABA_B$ receptors seem to play a crucial role in circadian distribution of sleep/wake. We would like to better understand this phenomenon by performing a circadian experiment with these mice. Because $GABA_B$ is also known to be involved in light signaling and is expressed in SCN, we are planning to see how the circadian phase of these animals could be shifted by light[279,379]. Because of the spontaneous epileptiform activity of $GABA_{B1}^{-/-}$ and $GABA_{B2}^{-/-}$ mice, this experiment will also give us new insights of the effects of seizures in the endogenous circadian clock, a topic still poorly studied.

We also found that a lack of the $GABA_{B1a}$ subunit in mice is sufficient to develop spontaneous epileptiform activity. This underlines the differential role of the two isoforms of the $GABA_{B1}$ subunit on the EEG and brain excitability. Although the mechanism by which a lack of $GABA_{B1a}$ subunit leads to an epileptiform phenotype remains to be discovered, the differential neuronal subcellular localization of the two isoforms is probably the cause. Indeed, $GABA_{B1a}$ assembles heteroreceptors inhibiting glutamate release, while predominantly $GABA_{B1b}$ mediates postsynaptic inhibition[220]. Since glutamate is the major excitatory neurotransmitter in the brain, a loss of inhibition of its release may lead to a hyperexcitability neuronal, a characteristic of seizures.

$GABA_{B1}$ and $GABA_{B2}$ mice and to a lesser extent $GABA_{B1b}$ mice, showed decreased theta and high delta activity in their EEG NREMS spectrum. Interestingly, we found that GHB and BAC, which are agonists of $GABA_B$, increased delta and theta activity in humans and particularly at low dose in mice. This emphasizes the role of $GABA_B$ receptors in the generation of low frequency oscillations originating from thalamocortical networks[250,380,381].

Temperature, EEG topography and memory

During each session of our human study, core body temperature was recorded. We are planning to analyze these data soon. Both BAC and GHB are known to induced hypothermia in rodents and in humans. Moreover, temperature decreases during NREMS and is negatively correlated with EEG delta power in NREMS[382,383,384,385]. Thus, temperature analysis would inform us on the potential correlation between EEG effects of the drugs and temperature.

In this work, only results for the central scalp EEG derivation (C3-A2) were presented, although frontal and occipital EEG derivations were also recorded. We would like to analyze

other derivations and especially the frontal one. Indeed, there are sleep state-related and frequency-specific regional changes of EEG frequency bands [386,387]. For example, in young adults, it was reported that delta power was higher in frontal derivations than in more posterior derivations during the initial part of sleep. Moreover, frontal areas of the cortex seem to be important for cognitive skills and particularly responsive to changes in sleep propensity[388]. Thus, a putative restorative process of NREMS may be most intense over the frontal cortex and the effects of GHB and BAC even more pronounced.

Sleep is implicated in memory consolidation. As expected, we found an improvement of declarative and procedural memory performance after a nap. However, neither GHB nor BAC affected memory consolidation despite their EEG modifications. It was shown that distinct sleep stages was involved in the consolidation of different types of memories[389]. For example, NREMS stage 2 and spindle density have been correlated with improvement of procedural memory[321,390], whereas declarative memory would benefit from SWS[314]. Thus, a future interesting analysis on our data would be to confirm the correlation between sleep variables and memory performance and particularly to see how GHB and BAC affect these correlations.

Final conclusion

Although the mechanisms by which GHB improves narcolepsy symptoms remain to be developed further, this present work has provided strong evidence that GHB does not induce physiological sleep as well as the unique role of $GABA_B$ receptors in its response. It has underlined differences and similarities shared by BAC and GHB in humans and in mice. It has demonstrated that the 'extra' EEG delta power produced by GHB did not generally affect subjective alertness, neurophysiological and memory performance. It has described in

details the importance of the $GABA_B$ receptors and its subunits on sleep and the EEG. Finally, it has brought new perspectives on the role of $GABA_{B1a}$ subunit in spontaneous seizure generation and on the circadian contribution of $GABA_B$ receptors on sleep/wake distribution.

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11. Abbreviations

5-HT	Serotonin
1,4-BD	1,4-butanediol
AC	Adenylyl cyclase
Ach	Acetylcholine
AD	Adenosine
BAC	Baclofen
BF	Basal forebrain
BLN	Baseline night
BZD	Benzodiazepine
cAMP	Cyclic adenosine-3',5'-monophosphate
CREB2	cAMP responsive element binding protein-2
DA	Dopamine
DRN	Dorsal Raphé nucleus
EEG	Electroencephalogram
EMG	Electromyogram
EOG	Electrooculogram
ERK1/2	Extracellular signal-regulated kinase 1
EXP	Experimental (night)= postnap (night)
FFT	Fast fourier transformation
GABA	Gamma-aminobutyric acid

Gal	Galanin
GAT-1	GABA transporter 1
GBL	Gamma-butyrolactone
GHB	Gamma-hydroxybutyrate
GIRK,Kir3	G-protein-gated inwardly rectifying potassium
Glu	Glutamate
Gly	Glycine
GPCR	G-protein coupled receptors
H1,2	Histamine 1,2 receptors
Hab	Habituation night
Hcrt	Hypcretin = orexin
His	Histamine
KCTD	Potassium channel tetramerization domain-containing
KDEF	Karolinska Directed Emotional Faces System
KSS	Karolinska sleepiness scale
LC	Locus coeruleus
LDT	Laterodorsal tegmentum
LTP	Long term potentiation
MCH	Melanin-concentrating hormone
MT	Movement time
NA	Noradenaline
NREMS	Non-rapid eye movement sleep (stages 2-4 in humans)
Orx	Orexin =Hypocretin

peri-Lcα	Peri-locus coeruleus α
PFP	pontine reticular formation
PL	Placebo
PPT	Pendonculopontine tegmentum
PVT	Psychomotor vigilance task
REC	Recovery
REM	Rapid eye movement
REMS	Rapid eye movement sleep
REMSL	REMS latency
RGS	G-protein signaling
RT	Reaction time
S1	NREMS Stage 1
S2	NREMS Stage 2
S2	NREMS Stage 3
S4	NREMS Stage 4
SCN	Suprachiasmatic nucleus
SD	Sleep deprivation
SDs	sushi domains
SE	Sleep efficiency (TST/TIB)
SL	sleep latency
SO	Sodium oxybate
SOREM	Sleep onset REMS
SOREMP	Sleep onset REMS periods

SWS	Slow-wave sleep= NREMS stage 3+4
THIP	Gaboxadol
TIB	Time in bed
TMN	Tuberomammillary nucleus
TST	Total sleep time
VLPO	Ventrolateral preoptic nucleus
vPAG	Ventral periaqueductal gray matter
VTA	Ventral tegmental area
WASO	Wakefulness after sleep onset