What keeps us awake?

The role of the ascending arousal systems in the regulation of sleep and wakefulness mediated by the basal forebrain

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ACADEMIC DISSERTATION

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Aan mijn familie en vrienden

If people were meant to pop out of bed in the morning, we would all sleep in toasters

(Author unknown, attributed to Jim Davis, Garfield)

If you think you are too small to make a difference, try sleeping with a mosquito in the room

(Dalai Lama XIV)

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications

I Mäkelä KA*, Wigren H-K*, Zant JC, Sakurai T, Alhonen L, Kostin A, Porkka-Heiskanen T, Herzig KH (2010) "Characterization of sleep-wake patterns in a novel transgenic mouse line overexpressing human prepro-orexin/hypocretin", Acta Physiologica. 198(3): 237-249

II Zant JC, Leenaars CHC, Kostin A, Van Someren EJW, Porkka-Heiskanen T (2011) "Increases in extracellular serotonin and dopamine metabolite levels in the basal forebrain during sleep deprivation", Brain Research 1399: 40-48

III Zant JC, Rozov S, Wigren H-K, Panula, P, Porkka-Heiskanen T (2012) "Histamine release in the basal forebrain mediates cortical activation through cholinergic neurons", Journal of Neuroscience Sep 19;32(38):13244-54

IV Rozov SV, Zant JC, Karlstedt K, Porkka-Heiskanen T, Panula P "Periodic properties of the histaminergic system of the mouse brain", Submitted

The publications are referred to in the text by their roman numerals

* These Authors share equal contribution in the publication

PUBLICATIONS THAT WERE USED IN OTHER DISSERTATIONS

Study I was used in the thesis of Kari Antero Mäkelä "The roles of orexins on sleep/wakefulness, energy homeostasis and intestinal secretion." (Faculty of Medicine, University of Oulu, 2010)

AUTHOR CONTRIBUTION

In Study I Janneke Zant performed the analysis of the sleep data and participated in writing the manuscript, by providing her contribution to the material and methods, results, and discussion sections.

In Study II Janneke Zant planned the experiments, performed them, analyzed the data and wrote the manuscript.

In Study III Janneke Zant planned the experiments, performed them, analyzed the data and wrote the manuscript. In addition, she set up a new system to perform continuous microdialysis over several days.

In Study IV Janneke Zant set up a new system for *in vivo* microdialysis in mice and, together with the first author, planned and performed the microdialysis experiments; she also participated in writing the manuscript.

ABSTRACT

Sleep and wakefulness are regulated by an intricate interplay of multiple systems originating in the brainstem, hypothalamus, and basal forebrain (BF) that are under both circadian and homeostatic control. The circadian regulation is important for the timing of sleep, and the homeostatic regulation drives the intensity of sleep, based on the duration and quality of preceding wakefulness. The ascending arousal systems mediate their effects on wakefulness and cortical activity via a thalamic and an extrathalamic route. The extrathalamic route through the BF sends projections to the entire brain and could be involved in sleep homeostasis, because during prolonged wakefulness or sleep deprivation (SD), the extracellular build-up of adenosine specifically inhibits cholinergic neurons of the BF.

In this thesis I explored the role of the ascending arousal systems in the regulation of sleep, wakefulness, and cortical activation mediated by the BF. Polysomnographic recordings with or without combined *in vivo* microdialysis in freely behaving animals were used, to test the following hypotheses: During SD, the ascending arousal systems increase their activity to counteract the effects of increased sleep pressure. Increased activity of the ascending arousal systems increases BF activity during SD and results in a homeostatic sleep response. The actions of the ascending arousal systems are, in the BF, mediated by cholinergic neurons.

The results show that overexpression of orexin leads to minor changes in vigilance state distribution, and to increased slow wave intrusions during prolonged wakefulness. During SD, the extracellular levels of serotonin and dopamine metabolites increased gradually, reaching a plateau from the third hour of SD. In contrast, extracellular BF histamine levels increased immediately when SD started and remained at the same level throughout the six hour SD, returning back to baseline immediately afterwards. On the baseline day, extracellular histamine levels showed a strong correlation with the amount of wakefulness, during both the light and the dark period. Although during wakefulness all studied ascending arousal systems increased their transmitter release in the BF, not all of them seem to be involved in the regulation of sleep homeostasis. The orexinergic, serotonergic, and dopaminergic systems show signs that they are involved in the regulation of sleep pressure, or at least that they are affected by sleep homeostasis. The increased sleepiness of mice overexpressing orexin might indicate that the orexinergic system is capable of influencing sleep homeostasis. The gradual increase in dopamine and serotonin turnover in the BF during SD shows that those transmitter systems are affected by increased sleep pressure, and they might counteract sleep pressure or add to it. On the other hand, histamine release in the BF is not affected by sleep pressure, and histamine perfusion into the BF does not result in a homeostatic response, showing that the histaminergic system is

neither affected by nor able to manipulate sleep pressure in the BF, and thus shows no signs of being involved in sleep homeostasis mediated by the BF.

Histamine perfusion into the BF led to theta-enriched cortical arousal, which did not result in a homeostatic sleep response. Because perfusion of other excitatory transmitters into the BF did induce a homeostatic response, the type of activation of the BF neurons might be crucial to result in the build-up of sleep pressure, and this is most likely receptor and neurotransmitter specific.

Perfusing a histamine receptor 1 antagonist into the BF resulted in a dramatic decrease in wakefulness and cortical arousal, demonstrating that activation of the BF by histamine receptor 1 is essential in sustaining wakefulness and a high level of cortical activation.

Finally, a specific neurotoxin was used to lesion the cholinergic neurons of the BF, after which the effects of histamine and histamine receptor 1 antagonist perfusion into the BF were ablated, indicating the importance of the cholinergic BF in mediating the wake-promoting effects of histamine. A lack of activation of the cholinergic BF by histamine may be important in initiating and maintaining NREM sleep.

According to these results, the BF might be the main route for histamine to promote wakefulness, it might also be the main route for the other ascending arousal systems to promote wakefulness, however this should be further investigated. A better understanding of the mechanisms by which the BF is activated and mediates this activation to the cortex might help us to find better therapies or medications for those suffering from sleep or wake disorders.

ABBREVIATIONS

3-MT	3-methoxytyramine
5-HT	5-hydroxytryptamine, serotonin
$5\text{-HT}_{1\text{-}7}$	serotonin receptor 1-7
Ach	acetylcholine
aCSF	artificial cerebrospinal fluid
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BF	basal forebrain
cAMP	cyclic adenosine monophosphate
ChAT	choline acetyltransferase
COMT	catechol-O-methyltransferase
CSF	cerebrospinal fluid
$D_{1\text{-}5}$	dopamine receptor 1-5
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DRM/MRN	dorsal and median raphe nuclei
EEG	electroencephalogram
EMG	electromyogram
FFT	fast Fourier transform
GABA	gamma-aminobutyric acid
H1R H2R	histamine receptor 1 and 2
HDB	horizontal diagonal band of Broca
HDC	histidine decarboxylase
HPA	hypothalamic–pituitary–adrenal axis
HPLC	high-performance liquid chromatography
hPPO	human prepro-orexin
HVA	homovanillic acid
LC	locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine
LH	lateral hypothalamus
MCPO	magnocellular preoptic area
(M)MSLT	(murine) multiple sleep latency test
MnPO	median preoptic area
MOA-A, MAO-B	monoamine oxidase A, monoamine oxidase B
MS/DBB	medial septum/diagonal band of Broca

NMDA	N-methyl-D-aspartate
NREM sleep	non-rapid eye movement sleep
OX1R, OX2R	orexin-1 receptor, orexin-2 receptor
PKA	protein kinase A
POAH	preoptic anterior hypothalamus
PPT/LDT	pedunculopontine and laterodorsal tegmental nuclei
REM sleep	rapid eye movement sleep
SCN	suprachiasmatic nucleus
SD	sleep deprivation
SEM	standard error of mean
SERT	serotonin transporter
SI	substantia innominata
SN	substantia nigra
SSRI	selective serotonin re-uptake inhibitor
SWA	slow wave activity
TMN	tuberomammillary nucleus
VLPO	ventrolateral preoptic area
vPAG	ventral periaqueductal gray
VTA	ventral tegmental area

1 REVIEW OF THE LITERATURE

Sleep and wakefulness are two mutually exclusive vigilance states. Sleep is a naturally occurring quickly reversible behavioral state of quiescence characterized by a reduced responsiveness to external stimuli, usually accompanied by a species-specific timing of daily sleep periods and typical sleep postures. Wakefulness is characterized by consciousness, awareness and activity. In addition to defining the vigilance states on the basis of these criteria, in mammals and birds sleep and wakefulness are also defined by characteristic electroencephalogram (EEG) and electromyogram (EMG) patterns in polysomnographic recordings, in which the EEG describes the electrical activity of cortical neurons and the EMG describes the electrical activity of muscles.

1.1 Vigilance states and EEG correlates

Based on EEG and EMG recordings, three separate vigilance states can be identified: wakefulness, non-rapid eye movement (NREM) sleep (also referred to as slow wave sleep), and rapid eye movement (REM) sleep (also known as paradoxical sleep).

Wakefulness is characterized by low-amplitude fast activity in the EEG combined with a high muscle tone. At sleep onset the EEG waves will start slowing and their amplitude starts to increase. Sleep is divided into two distinct states: NREM and REM sleep. NREM sleep is defined by high-amplitude low-frequency EEG activity and decreased muscle tone. REM sleep is characterized by a low-amplitude desynchronized EEG and a loss of muscle tone. The REM sleep EEG is very similar to the EEG of wakefulness, and therefore the EMG is needed to distinguish the two (Fig. 1).

Rodent sleep is polyphasic, which means that their sleep consists of periods of NREM and REM sleep that are interspersed with wakefulness.

1.1.1 Wakefulness-specific EEG correlates

In rodents, during wakefulness, the high theta range (above 7 Hz) is associated with active exploratory behavior and attentive wakefulness (Kramis et al., 1975; Lancel, 1993), varying between 4-12 Hz depending on the animal, species, and recording method (Winson, 1972; van Lier et al., 2003). The beta (15-30 Hz) and gamma (30-60 Hz) ranges are also prominently present in the waking EEG (reviewed in Steriade, 2006). During quiet wakefulness, the slower EEG frequencies become more prevalent, the delta (0.5-4 HZ) and

low theta (4-7 Hz) ranges being associated with increased sleep pressure and drowsiness during wakefulness.

1.1.2 NREM sleep-specific EEG correlates

Typically, during NREM sleep three main EEG patterns are present: slow oscillations (0.5-1 Hz), slow waves, also termed delta waves (1-4 Hz), and sleep spindles (12-15 Hz). The amount and amplitude of the slow oscillations and slow waves is referred to as slow wave activity (SWA), and reflects sleep intensity. Sleep spindles are found during the lighter stages of NREM sleep in human sleep (Dijk, 2009), and occur shortly before the transition from NREM to REM sleep in rodents (Vyazovskiy et al., 2004).

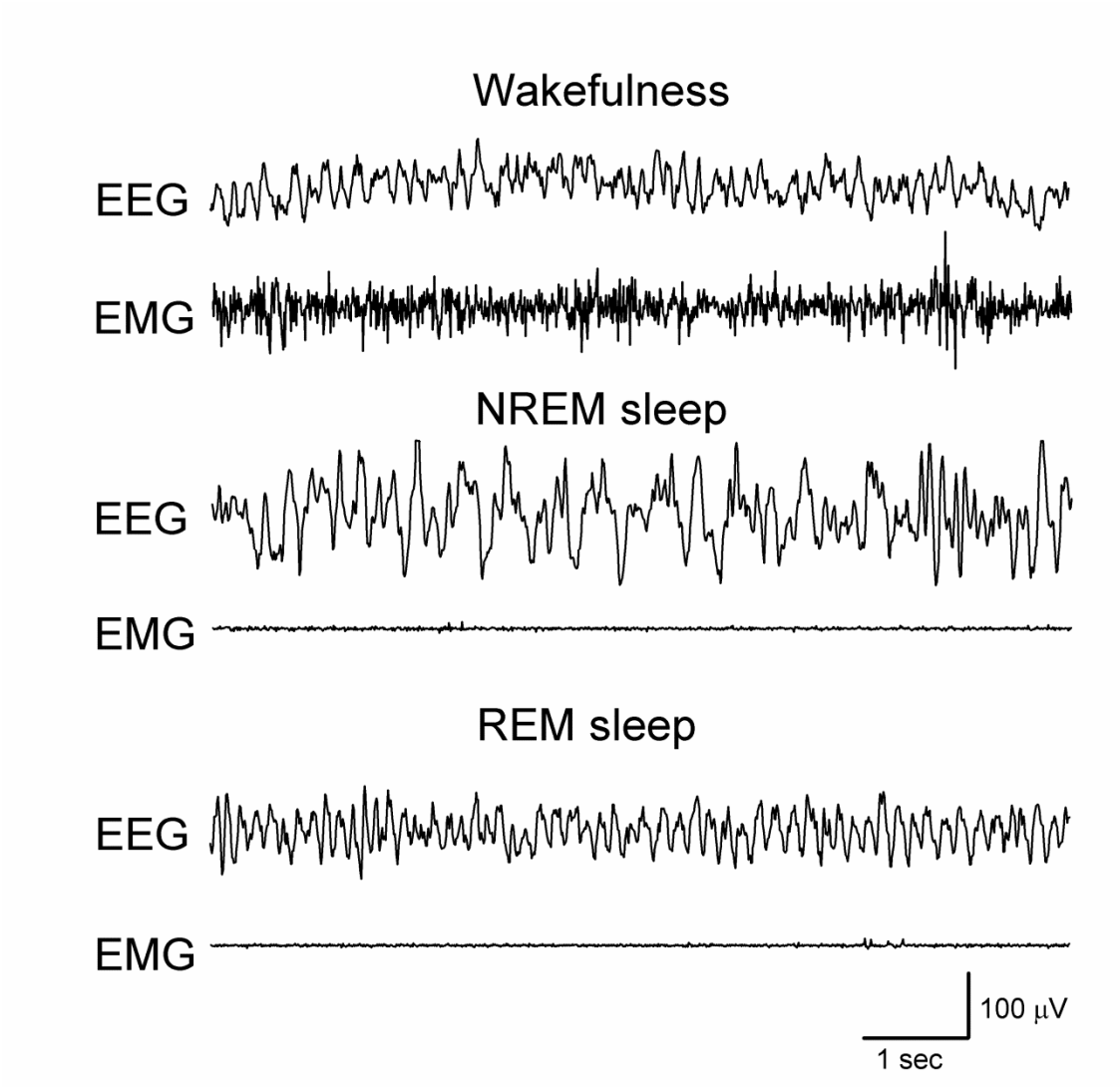

Figure 1. Vigilance state-specific polysomnographic recordings
Eight seconds of data from EEG and EMG recordings from Han-Wistar rats showing the specific characteristics of the three different vigilance states.

1.2 Regulation of wakefulness

1.2.1 Ascending arousal systems

Several collateral and overlapping neurotransmitter systems are involved in the promotion of wakefulness. Morruzi and Magoun (1949) were the first to describe the ascending reticular activating system, a net-like structure in the brainstem that is capable of inducing cortical activation. Subsequent studies showed that the neurons responsible for arousal were not part of the undifferentiated reticular formation, but consisted of monoaminergic and cholinergic neurons that reside in specific cell groups, together comprising the ascending arousal systems (Fig. 2; reviewed in Jones, 2003). The ascending arousal systems send widespread projections to the cortex via two main pathways, a dorsal pathway that is relayed in the thalamus, and an extrathalamic ventral route that extends through the hypothalamus and is conveyed to the cortex by the basal forebrain (BF; Starzl et al., 1951). These multiple alternative systems for promoting wakefulness are vastly interconnected and mutually excitatory to each other. This high level of redundancy may be necessary so that when one part of the system fails another can take over and maintain wakefulness.

The monoaminergic cell groups that project to the forebrain include the noradrenergic locus coeruleus (LC), the serotonergic dorsal and median raphe nuclei (DRN/MRN), dopaminergic neurons from the ventral tegmental area (VTA), substantia nigra (SN), and ventral periaqueductal gray (vPAG), and histaminergic neurons from the tuberomammillary nucleus (TMN; Dahlstrom and Fuxe, 1964; Panula et al., 1989; Kocsis et al., 2006; Lu et al., 2006). These cell groups send most of their projections to the cortex via the ventral pathway, while a smaller amount of projections is sent to the thalamus via the dorsal route. In general, neurons from these groups fire most actively during wakefulness, decrease their activity during NREM sleep, and stop firing during REM sleep (Aston-Jones and Bloom, 1981; Steininger et al., 1999; Kocsis et al., 2006; Takahashi et al., 2006).

Another part of the ascending arousal systems is a group of neurons found in the lateral hypothalamus (LH), containing orexin, also known as hypocretin (de Lecea et al., 1998; Sakurai et al., 1998). These neurons send both ascending and descending projections; they project to the entire cerebral cortex, as well as to the brainstem and BF, with particularly dense input to the TMN and the LC (Peyron et al., 1998), and fire predominantly during wakefulness (Lee et al., 2005a; Mileykovskiy et al., 2005).

A group of brainstem cholinergic neurons that project to the forebrain is found in the pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT). They mainly innervate the

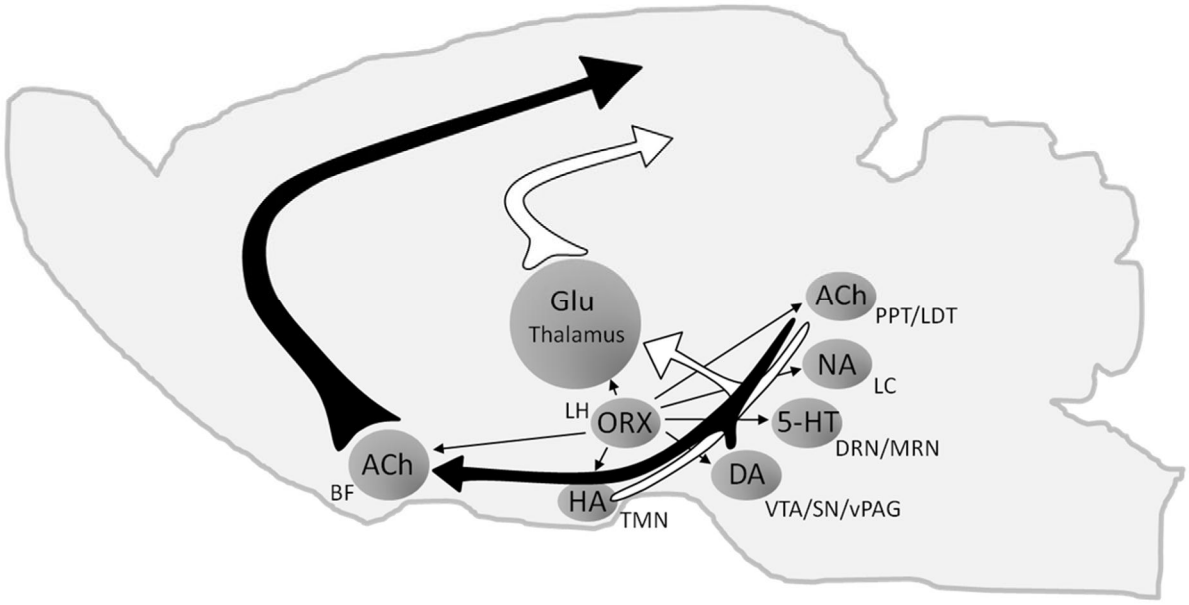

Figure 2. Ascending arousal systems and their pathways
Schematic sagittal representation of the major neuronal systems and their main pathways involved in promoting wakefulness. 5-HT, 5-hydroxytryptamine (serotonin); Ach, acetylcholine; BF, basal forebrain; DA, dopamine; DRN/MRN, dorsal and median raphe nucei; Glu, glutamate; HA, histamine; LC, locus coeruleus; LH, lateral hypothalamus; NA, noradrenalin; ORX, orexin; PPT/LDT, pedunculopontine and laterodorsal tegmental nuclei; SN, substantia nigra; TMN, tuberomammillary nucleus; vPAG, ventral periaqueductal gray; VTA, ventral tegmental area.

thalamus, but also the LH, BF, and prefrontal cortex (Satoh and Fibiger, 1986; Hallanger et al., 1987).The firing rate of PPT/LDT neurons is highest during wakefulness and REM sleep, and lowest during NREM sleep, suggesting that they contribute to cortical activation during both wakefulness and REM sleep (el Mansari et al., 1989; Steriade, 1993). Because their firing rate usually increases just before the transition from cortical slow waves to faster frequencies (Boucetta and Jones, 2009), these neurons might have an important role in the initiation of wakefulness and REM sleep.

Although all of these ascending arousal systems are important in regulating wakefulness, only those that were studied in the original manuscripts included in this thesis will be introduced in detail below (Section 1.4).

1.2.2 The basal forebrain

The BF consists of cholinergic, glutamatergic and GABAergic neurons that play a central role in regulating wakefulness and cortical arousal (Detari and Vanderwolf, 1987; Buzsaki et al., 1988). These neurons are located in an area that extends from the medial septum/diagonal band of Broca (MS/DBB) to the magnocellular preoptic area (MCPO), substantia innominata (SI), and horizontal diagonal band of Broca (HDB).

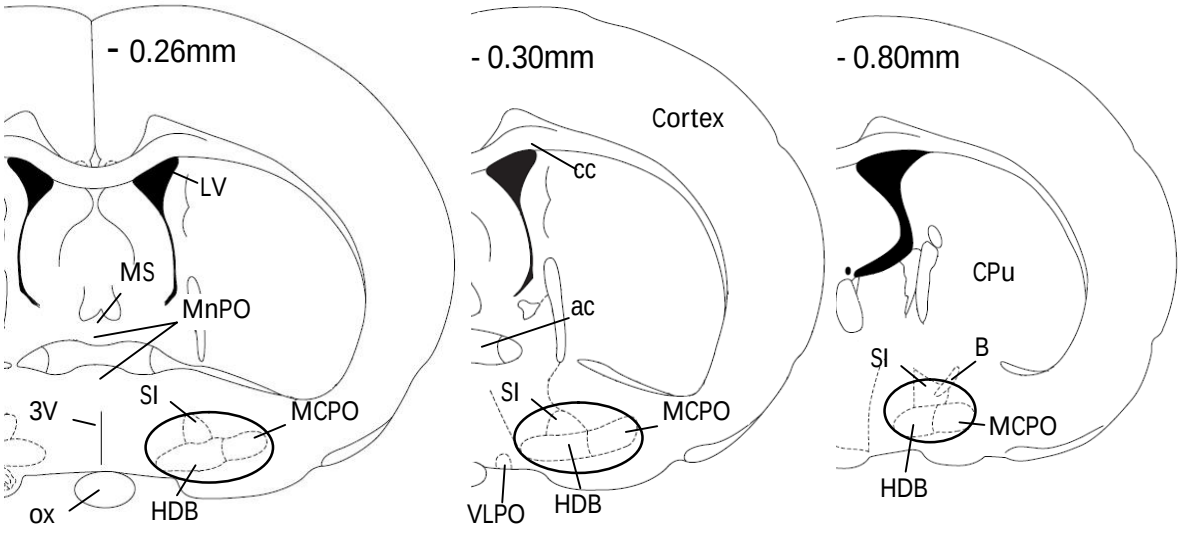

Figure 3. The basal forebrain area
Schematic coronal representation of the location of the basal forebrain (circled area). In this thesis the BF is defined as the cortically projecting area including the MCPO, SI, and HDB. Coordinates at the top of each drawing show the distance from bregma. 3V, third ventricle; ac, anterior commissure; B, basal nucleus (Meynert); cc, corpus callosum; CPu, caudate putamen; HDB, horizontal diagonal band of Broca; LV, lateral ventricle; MCPO, magnocellular preoptic area; MnPO, median preoptic area; MS, medial septum; ox, optic chiasm; SI, substantia innominata; VLPO, ventrolateral preoptic area.

The cholinergic neurons send widespread ascending projections to the cerebral cortex and limbic systems (Rye et al., 1984; Saper, 1984; Luiten et al., 1987); MS/DBB neurons project to the hippocampus, where they are involved in generating hippocampal theta (Winson, 1978), whereas the MCPO, SI, and HDB neurons innervate the cortex and are suggested to modulate cortical theta activity (Lee et al., 2005b; Jones, 2008). In the studies that are discussed in this thesis the BF is defined as the cortically projecting area including the MCPO, SI, and HDB (Fig. 3).

The BF receives input from all of the ascending arousal systems (Semba et al., 1988a; Semba et al., 1988b; Jones and Cuello, 1989; Panula et al., 1989; Szymusiak, 1995; Peyron et al., 1998; Jones, 2004). *In vitro* studies showed that cholinergic neurons are excited by glutamate, noradrenalin, histamine, dopamine and orexin (Fort et al., 1995; Khateb et al., 1995; Eggermann et al., 2001; Berlanga et al., 2005), and inhibited by serotonin (Khateb et al., 1993). In addition to ascending projections to the cortex, the BF neurons send descending projections back to the ascending arousal systems (Semba et al., 1989; Szymusiak, 1995).

Recordings of BF cholinergic neurons and measurements of cortical acetylcholine (Ach) release show the highest activity and maximal ACh release during wakefulness and REM sleep. During NREM sleep, the cholinergic neurons are least active, and ACh release is low (Detari et al., 1999; Szymusiak et al., 2000). Activation of BF neurons with glutamate agonists promotes wakefulness and ACh release (Lamour et al., 1986; Manfridi et al., 1999; Cape and Jones, 2000; Wigren et al., 2007). On the other hand, lesions of the cholinergic neurons can reduce wakefulness, whereas excitotoxic lesions that kill both cholinergic and non-cholinergic neurons cause increased EEG delta activity (Kaur et al., 2008). Even larger lesions that cover most of the BF clearly reduce wakefulness (Buzsaki et al., 1988).

A large additional population of cortically projecting BF neurons produce GABA, and a smaller number produce glutamate (Freund and Gulyas, 1991; Gritti et al., 1997; Hur and Zaborszky, 2005; Henny and Jones, 2008). GABAergic neurons, accounting for about one-third of the cortically projecting neurons, are co-distributed with the cholinergic population (Gritti et al., 1997). Two physiologically distinct groups of GABAergic neurons exist; one is active during wakefulness, and a second group is associated with NREM sleep (Duque et al., 2000; Manns et al., 2000; Modirrousta et al., 2004). GABAergic BF neurons innervate, and are thought to inhibit, cortical GABAergic interneurons and deep layer pyramidal cells (Freund and Meskenaite, 1992; Henny and Jones, 2008), which could result in the disinhibition of cortical circuits.

These results demonstrate the importance of the BF in promoting wakefulness and suggest that cholinergic and non-cholinergic neurons of the BF act together to produce wakefulness (Szymusiak et al., 2000; Jones, 2005).

1.3 Sleep regulation

Two groups of sleep-active neurons were identified in the preoptic anterior hypothalamus (POAH); they are located in the ventrolateral preoptic area (VLPO; Sherin et al., 1996) and the median preoptic area (MnPO; Suntsova et al., 2002; Gong et al., 2004; Sakai, 2011). Both the VLPO and MnPO send inhibitory GABAergic projections to the wake-promoting neurons of the ascending arousal systems, and in the VLPO neurons GABA is co-localized with galanin (Sherin et al., 1998; Steininger et al., 2001; Gaus et al., 2002; Suntsova et al., 2007; Uschakov et al., 2007). Thus, by inhibiting the ascending arousal systems, the VLPO and MnPO can promote NREM sleep.

1.3.1 The two process model of sleep regulation

Sleep is regulated by the interaction of two major biological factors that together dictate the timing and intensity of sleep. These factors are the homeostatic and the circadian process, as described in the two process model of sleep regulation (Fig. 4; Borbely, 1982; Achermann et al., 1993). In this model, the homeostatic process (process S) increases during wakefulness as sleep pressure accumulates, and decreases during sleep. The circadian process (process C) does not depend directly on prior sleep-wake amounts, and modulates the timing of sleep according to the intrinsic circadian rhythm of approximately 24 hours (reviewed in Takahashi et al., 2008). The intrinsic rhythm of humans is slightly longer and that of rodents is slightly shorter than 24 hours; it uses light to synchronize to the light-dark cycle of the environment. The circadian rhythm also controls many biological and physiological processes in the body, and is regulated at the molecular level by a transcriptional-translational feedback loop in the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972).

These two processes work together in regulating the timing and intensity of sleep. The circadian signal promotes wakefulness during the active period, resulting in increasing homeostatic sleep pressure, and when the homeostatic sleep pressure reaches a critical level in combination with a reduction of the circadian signal, sleep is initiated.

During prolonged wakefulness or sleep deprivation (SD) the animals are kept awake longer than usual, and because the sleep pressure keeps increasing during wakefulness this leads to additional sleep pressure, which results in a recovery sleep that is characterized by increased SWA and NREM sleep duration (Achermann and Borbely, 2003).

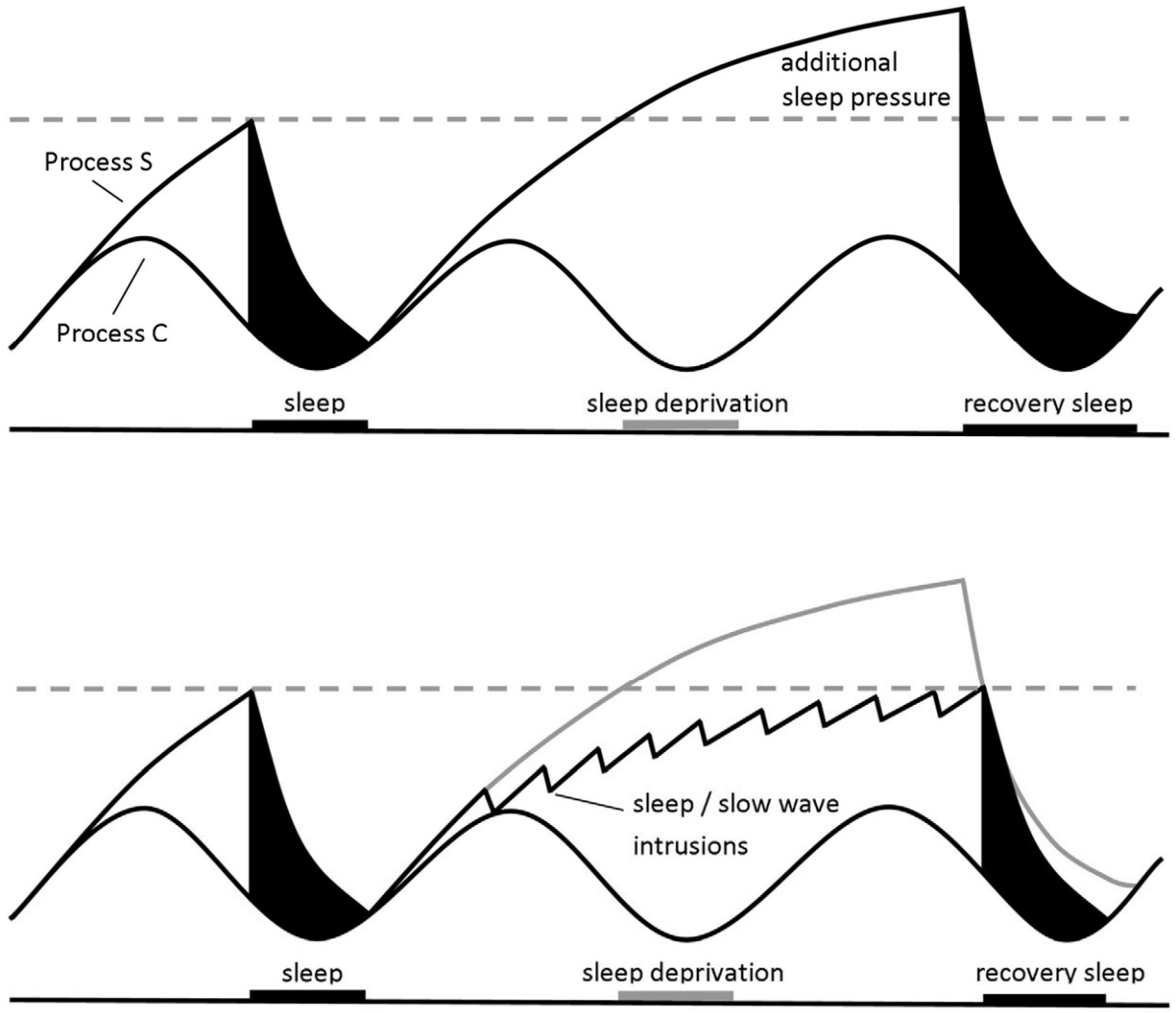

Figure 4. The two process model of sleep regulation (Borbely, 1982)
Sleep is regulated by an interaction of homeostatic sleep pressure (process S) and the circadian rhythm (process C), leading to alternating periods of wakefulness (white areas) and sleep (black areas). Upper panel: During wakefulness Process S increases in a saturating exponential way. Sleep is initiated when Process S is high and Process C is low; when sleep is initiated Process S decreases exponentially. SD induces additional sleep pressure which results in increased SWA and amount of NREM sleep during the subsequent recovery sleep. Lower panel: The amount and the quality of wakefulness are important factors in the increase of Process S; when wakefulness is interrupted by brief sleep or slow wave intrusions during SD, part of the sleep pressure can already be relieved and the build-up of Process S is thus attenuated.

During SD, characteristic EEG patterns of NREM sleep can leak into the waking EEG. These intrusions of slow waves into the waking EEG during SD are thought to reflect increased sleep pressure or sleepiness (Cajochen et al., 2002; Leemburg et al., 2010; Van Someren, 2010). Slow waves are usually only seen during NREM sleep, in fact they are the key characteristic of this state and thought to be responsible for the decline of sleep pressure, and thus process S. When slow waves intrude into the waking EEG they can also reduce sleep pressure (Leemburg et al., 2010). According to the two process model of sleep, the strongest reduction of sleep pressure occurs at the start of NREM sleep and declines exponentially (Achermann et al., 1993); therefore, although these intrusions are brief, they may still have a substantial effect on reducing sleep pressure.

In addition to the amount of preceding wakefulness, also the quality of wakefulness is an important factor in sleep pressure build-up. More exploratory behavior during wakefulness leads to increased SWA in the subsequent sleep period (Huber et al., 2007), and increased waking theta could be responsible for inducing the homeostatic response (Wigren et al., 2007).

1.3.2 Adenosine

Ever since Legendre and Piéron (1910) found that the injection of cerebrospinal fluid (CSF) from sleep-deprived dogs induced sleep in non-deprived animals, many studies have been performed to find the substance responsible for this sleep-inducing effect. This so-called endogenous sleep factor (factor S) should meet certain criteria: (1) it should induce and maintain sleep; (2) it should build up during wakefulness and prolonged wakefulness, and by doing so reflect sleep pressure; (3) it should increase SWA; (4) it should be eliminated during sleep (Borbely and Tobler, 1989).

Evidence points to the neuromodulator adenosine as a possible mediator of the homeostatic sleep response. Adenosine is the end-product of ATP metabolism; when energy demands are high, ATP consumption exceeds its synthesis and adenosine levels increase. According to Benington and Heller (1995), wakefulness is a state of high energy consumption that depletes brain energy stores, which in turn increases sleep pressure, and the concentration of adenosine. NREM sleep would be responsible for restoring the brain energy stores resulting in a decrease in adenosine levels.

Adenosine is a purine nucleoside that acts extracellularly on four types of G protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3; Fredholm, 2010). Adenosine inhibits the actions of wake-active neurons by binding to the A_1 receptor (Benington et al., 1995; Schwierin et al., 1996; Basheer et al., 2004; Arrigoni et al., 2006; Liu and Gao, 2007), and the activation of A_{2A} receptors is thought to stimulate or disinhibit the sleep-active neurons of the VLPO (Scammell et al., 2001; Morairty et al., 2004; Gallopin et al., 2005; Methippara et al., 2005).

The intra- and extracellular levels of adenosine are regulated by several enzymes. Adenosine is formed from AMP by 5'-nucleotidase, and adenosine kinase phosphorylates it back into AMP. Because the formation of adenosine depends on the amount of AMP, and AMP is formed when ATP is metabolized, the rates of ATP synthesis and its metabolism are crucial factors in the rate of adenosine increase. Adenosine can be cleared from the extracellular space by diffusion through equilibrative nucleoside transporters, by formation of AMP, or it is metabolized into inosine by adenosine deaminase (reviewed in Porkka-Heiskanen and Kalinchuk, 2011).

In addition to linking sleep regulation to neuronal activity and brain energy metabolism, adenosine also fulfills the criteria to be considered a sleep factor. Systemic or local administrations of adenosine or its receptor agonists increase NREM sleep and SWA (Feldberg and Sherwood, 1954; Haulica et al., 1973; Radulovacki, 1985; Ticho and Radulovacki, 1991; Benington et al., 1995; Satoh et al., 1996; Porkka-Heiskanen et al., 1997; Portas et al., 1997; Basheer et al., 1999; Porkka-Heiskanen et al., 2000), and extracellular adenosine levels increase during natural and prolonged wakefulness in the cholinergic BF and the cortex (Porkka-Heiskanen et al., 1997; Porkka-Heiskanen et al., 2000; Porkka-Heiskanen et al., 2002; McCarley, 2007). Furthermore, the most commonly used stimulant by humans, caffeine, promotes wakefulness by antagonizing the sleep-inducing actions of adenosine through the A_1 and A_{2A} receptors (Fredholm et al., 1999).

1.3.3 Basal forebrain and sleep homeostasis

In addition to its wake-promoting effects, the cholinergic BF plays an important role in the homeostatic sleep response to prolonged waking. During natural and prolonged wakefulness extracellular adenosine levels increase selectively in the wake-promoting BF area, and to a lesser extent in the cortex (Porkka-Heiskanen et al., 1997; Porkka-Heiskanen et al., 2000). Because adenosine levels increase due to energy consumption, which is high during wakefulness, and because adenosine levels increase specifically in the BF, the BF could have the unique possibility to keep track of the amount and the intensity of preceding wakefulness. And subsequently the BF could regulate the appropriate amount of recovery sleep.

Adenosine inhibits cholinergic neurons and possibly also other wake-promoting neurons in the BF via A_1 receptors (Thakkar et al., 2003; Gass et al., 2009). Selective lesioning of the BF cholinergic neurons with the immunotoxin 192IgG-saporin stops the increase in extracellular adenosine during SD (Blanco-Centurion et al., 2006; Kalinchuk et al., 2008), it reduces waking EEG theta (Kaur et al., 2008) and gamma (Berntson et al., 2002), and it decreases the homeostatic recovery sleep response (Kalinchuk et al., 2008). Unilateral perfusion of adenosine into the BF increased NREM sleep and delta power as well as c-Fos expression in both cholinergic and non-cholinergic neurons (Basheer et al., 1999). An increase in NREM

sleep and delta power (as seen during recovery sleep after SD) was also observed after perfusion of an adenosine transport inhibitor into the BF, but not when it was perfused into the thalamus, although it increased extracellular adenosine levels in both areas (Porkka-Heiskanen et al., 1997). These results demonstrate that the BF is particularly susceptible to the actions of adenosine and that sleep might be induced by inhibiting the wake-promoting cholinergic BF neurons.

What mechanisms underlie these site-specific actions of adenosine is unclear. Since SD does not affect the activity of enzymes that mediate adenosine metabolism (Alanko et al., 2003; Mackiewicz et al., 2003), increases in extracellular adenosine levels in the BF are most likely either the result of increased intracellular or extracellular ATP metabolism, or of decreased transporter activity. A recent study suggested that not the cholinergic neurons, but instead astrocytes might be the source of extracellular adenosine (Halassa et al., 2009). They found a decrease in SWA and in the amount of recovery sleep following SD in genetically engineered mice with inhibited gliotransmission, which prevents ATP release from astrocytes. Because antagonizing the A_1 receptor did not show any effect in these mice, they propose that ATP release from astrocytes modulates the accumulation of sleep pressure via a pathway that involves the A_1 receptor.

1.3.4 The "flip-flop" switch

The transitions between wakefulness and sleep are suggested to be controlled by the "flip-flop" switch (Saper et al., 2001; Saper et al., 2005). This model incorporates the fact that under normal physiological conditions transitions between vigilance states are fast and well-defined, and is based on the mutually inhibitory interactions between the sleep-promoting neurons of the VLPO and the wake-promoting monoaminergic transmitters of the ascending arousal systems (McGinty and Szymusiak, 2000; Saper et al., 2001).

Activity of the monoaminergic ascending arousal systems inhibits the VLPO and activates the cortex, which leads to wakefulness (Gallopin et al., 2000; Liu et al., 2010). During sleep the VLPO inhibits the monoaminergic ascending arousal systems by direct GABAergic and galaninergic projections (Gervasoni et al., 1998; Sherin et al., 1998). Thus, via reciprocal inhibition each group inhibits the activity of the other, removing the inhibition onto itself, and thereby indirectly reinforcing its own activity.

Orexin may be able to stabilize the flip-flop switch, because it influences both sides. During wakefulness orexin increases the activity of the monoaminergic neurons, and thus indirectly promotes inhibition of the VLPO. When orexin neurons become less active, and thus lift the inhibition on the VLPO, this results in a strong suppression of all the ascending arousal systems. The strongest evidence for orexin stabilizing sleep/wake states came from the observation that the orexinergic system is impaired in narcolepsy, a sleep disorder

characterized by behavioral state instability (McGinty and Szymusiak, 2000; Saper et al., 2001).

Under conditions of high sleep pressure, models of the flip-flop switch show signs of instability (Fulcher et al., 2010), which might explain why we observe micro-sleeps and slow wave intrusions in the EEG of animals and humans during SD.

1.4 The ascending arousal systems in detail

1.4.1 Orexin

Orexinergic system, projections, and innervation

Orexin was discovered simultaneously by two independent research groups (de Lecea et al., 1998; Sakurai et al., 1998). Because this newly discovered peptide increases food consumption in rats, one group named it orexin after the Greek word for appetite, orexis (Sakurai et al., 1998). The other group gave the peptide the name hypocretin, combining the names of the location where the peptide was synthesized (hypothalamus), and the hormone secretin, which structure the peptide resembled (de Lecea et al., 1998). The name orexin is used throughout this thesis.

Orexin is produced by orexinergic neurons, a rather small population of neuronal cells located in the perifornical area of the LH (de Lecea et al., 1998; Sakurai et al., 1998). Orexin neurons send ascending projections to the midline hypothalamic nuclei, the lateral preoptic area, the BF, and to the cortex. They also project to the LC, TMN, DRN/MRN, VTA, SI, and PPT/LDT (Peyron et al., 1998; Date et al., 1999; Saper et al., 2005).

Orexin neurons receive afferents from many components of sleep/wake related systems, including the BF, LC, DRN/MRN, VLPO, amygdala, infralimbic cortex, nucleus accumbens shell, preoptic area, lateral septum, and the bed nucleus of the stria terminalis (Sakurai et al., 2005; Yoshida et al., 2006).

Synthesis and metabolism of orexin

The precursor peptide prepro-orexin is proteolytically cleaved to form orexin A and orexin B. The peptides are packaged in dense core vesicles and are most likely synaptically released (Fig. 5; de Lecea et al., 1998; Sakurai et al., 1998).

Receptors

There are two known orexin receptors, the orexin-1 receptor (OX1R) and the orexin-2 receptor (OX2R), both of which are G protein-coupled receptors with excitatory effects.

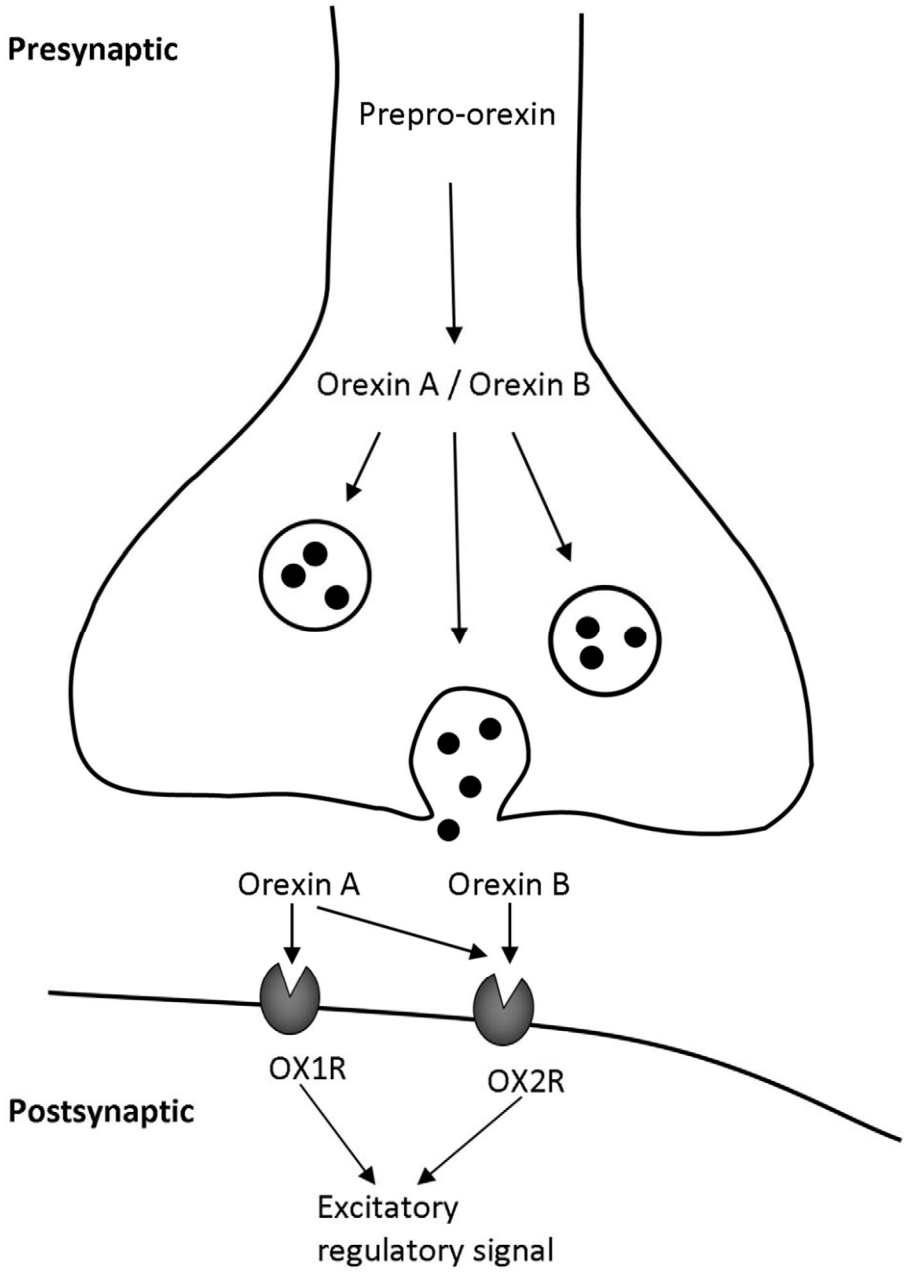

Figure 5. Orexinergic neurotransmission
The precursor peptide prepro-orexin is proteolytically cleaved to form orexin A and orexin B. They are packaged in dense core vesicles and most likely synaptically released into the synaptic cleft where they bind to OX1R or OX2R. OX1R, orexin-1 receptor; OX2R, orexin-2 receptor.

Although it is known that orexin receptor activation leads to a Ca^{2+} influx, the responsible mechanisms in neurons have not been well established (Sakurai et al., 1998). The OX1R shows a 10-fold higher affinity for binding orexin A, while the OX2R binds both isoforms with equal affinity. The orexinergic receptors show very distinct expression patterns. OX1R is found in the hippocampus, LC, and the anterior part of the hypothalamus near the SCN. The OX2R is mostly found in the TMN, LH, paraventricular nucleus, arcuate nucleus, amygdala, bed nucleus of the stria terminalis, and nucleus accumbens. Both receptors are expressed in the cortex, BF, DRN/MRN, VTA, and PPT/LDT (Trivedi et al., 1998; Lu et al., 2000; Marcus et al., 2001).

The orexinergic system in sleep-wake regulation

The first indication of the involvement of orexin in sleep-wake regulation was observed by Lin et al (1999), when they discovered that a mutation in the OX2R gene causes narcolepsy in dogs. Narcolepsy is a sleep disorder that is characterized by excessive daytime sleepiness and sleep fragmentation. Some patients suffer from cataplexy, a sudden weakening of the muscle tone that is usually triggered by emotional stimuli. Since this first discovery, many studies have found evidence which links the orexinergic system to narcolepsy (reviewed in Sakurai et al., 2010).

Orexin neurons are active during wakefulness and silent during NREM and REM sleep (Lee et al., 2005a; Mileykovskiy et al., 2005; Sakurai et al., 2010). Intracerebroventricular injection of orexin A or orexin B in rats increases the amount of wakefulness and decreases NREM and REM sleep (Hagan et al., 1999; Piper et al., 2000; Huang et al., 2001). Recently, optogenetic studies demonstrated that the orexin neurons are capable of inducing arousal from sleep (Adamantidis et al., 2007; Carter et al., 2009). Selective destruction of the orexin neurons induces symptoms of narcolepsy (Hara et al., 2001), similar as in orexin or orexin receptor knockouts (Chemelli et al., 1999). Compared to double knockouts, OX2R-knockout mice exhibit a less severe narcoleptic phenotype, while OX1R-knockout mice show only a mild fragmentation of sleep and wake states, without cataplexy (Willie et al., 2003; Sakurai et al., 2010). In addition, preliminary results from chronic overexpression of orexin in mice showed suppression of REM sleep during the light period and fragmented NREM sleep (Mieda et al., 2004).

Orexin is proposed to be involved in stabilizing the wake-state in the “flip-flop” switch (McGinty and Szymusiak, 2000; Saper et al., 2001). A recently described positive feedback loop shows that orexin neurons are able to activate themselves both directly and indirectly (via orexin-activated glutaminergic neurons) through OX2R. This mechanism might serve to maintain the activity of orexin neurons during wakefulness (Yamanaka et al., 2010), and enable them to activate the ascending arousal systems.

Orexin has an excitatory effect on cholinergic BF neurons, presumably through OX2R (Eggermann et al., 2001), and infusion of orexin into the BF increases wakefulness (Espana et al., 2001; Thakkar et al., 2001). The orexinergic system appears to be under the control of sleep homeostasis, because optogenetic stimulation after SD resulted less frequently in an arousal from NREM sleep (Carter et al., 2009).

1.4.2 Serotonin

Serotonergic system, projections, and innervation

Serotonergic projections innervate nearly all regions of the brain. Ascending pathways originate primarily in the DRN/MRN, while descending pathways innervating the spinal cord originate from the nucleus raphe magnus, pallidus, and obscurus (Dahlstrom and Fuxe, 1964).

The DRN/MRN send ascending projections to the BF, SN, VTA, MS/DBB, hypothalamus, and nucleus accumbens (Semba et al., 1988b; Tork, 1990). In addition, the DRN projects to the frontal cortex, striatum, and the ventral hippocampus, and the MRN projects to the cingulate cortex and hippocampus (Lorens and Guldberg, 1974; Geyer et al., 1976; Azmitia and Segal, 1978; McQuade and Sharp, 1997; Vertes et al., 1999; Kusljic and Van Den Buuse, 2012).

The DRN/MRN receive projections from the BF, LC, SN, vPAG, VTA, LH, and the medial prefrontal cortex (Marcinkiewicz et al., 1989; Behzadi et al., 1990; Hajos et al., 1998; Peyron et al., 1998).

Synthesis and metabolism of serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is synthesized from L-tryptophan. L-tryptophan is hydroxylated by tryptophan hydroxylase to form 5-hydroxy-tryptophan, which is subsequently decarboxylated by amino acid decarboxylase to form serotonin. Until it is released, serotonin is stored in synaptic vesicles, which protect the molecules from catabolizing enzymes. The activity of serotonin in the synapse is mainly terminated by its re-uptake into the presynaptic terminal by the serotonin transporter (SERT; Fig. 6).

Serotonin is metabolized by monoamine oxidase A (MAO-A) to 5-hydroxyindoleacetic acid (5-HIAA), which is the main metabolite of serotonin in the brain. The extracellular concentration of 5-HIAA is much higher than that of serotonin; in the rat brain about 1000 times. Two forms of MAO have been identified: MAO-A preferentially deaminates serotonin and norepinephrine, whereas MAO-B exhibits the highest affinity for β-phenylethylamine and dopamine (reviewed in Adell et al., 2002).

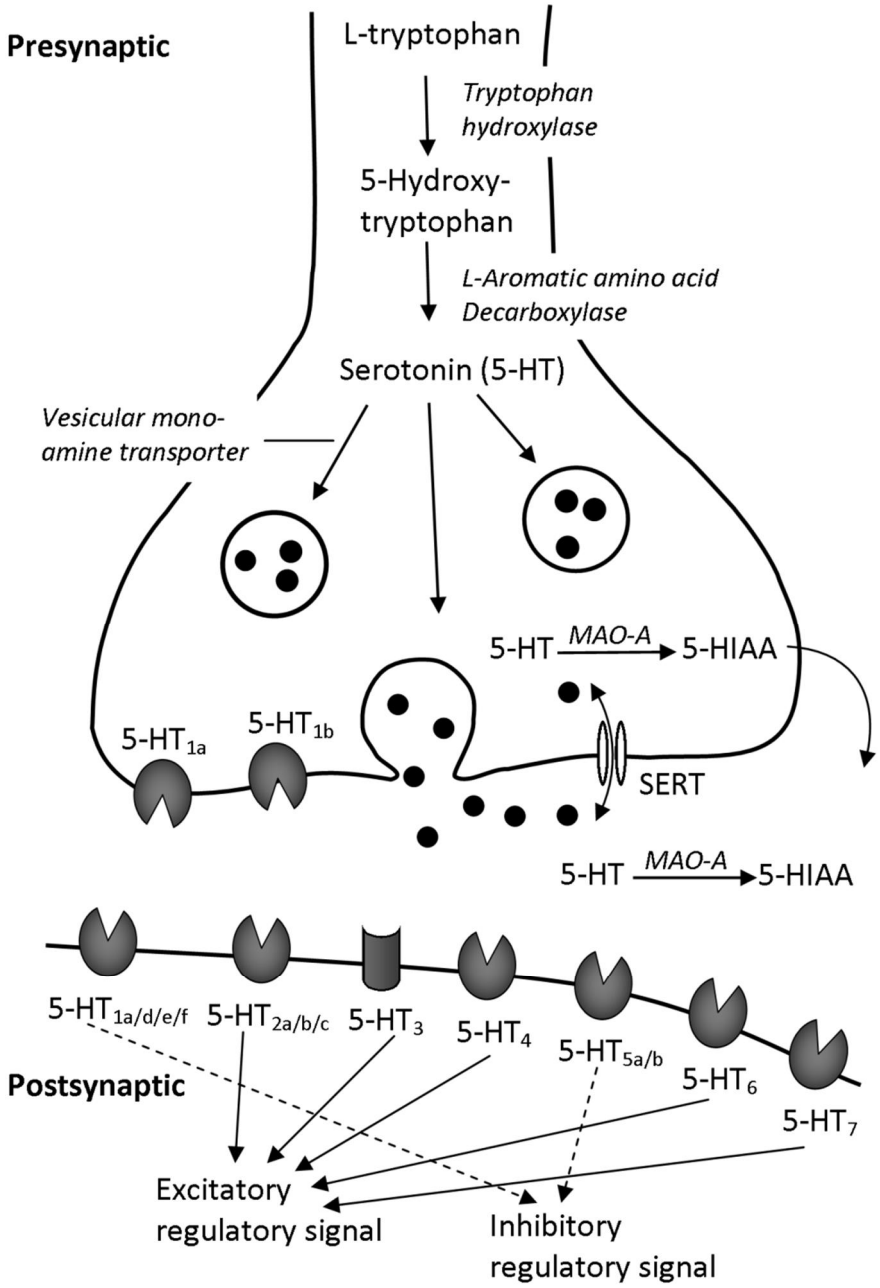

Figure 6. Serotonergic neurotransmission

Serotonin is synthesized in two steps from L-tryptophan and is stored in vesicles until released. It acts though seven main families of receptors and can be actively taken up by the serotonin transporter. Intra- or extracellularly serotonin is metabolized into 5-HIAA by MAO-A. 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; MAO-A, monoamine oxidase A; SERT, serotonin transporter.

Receptors

Seven main families of serotonin receptors (5-HT_{1-7}) have been identified, comprising in total 14 subtypes, with five subtypes of the 5-HT_1 receptor (a, b, d, e, f), three 5-HT_2 receptor subtypes (a-c), and two types of 5-HT_5 receptors (a and b).

Serotonin receptors mediate their effect through G protein-coupled second messenger systems, with the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel. The 5-HT_1 and 5-HT_5 receptor families have an inhibitory effect through G protein-coupled inhibition of adenylyl cyclase and subsequently cyclic adenosine monophosphate (cAMP). 5-HT_2 receptors are coupled to G proteins that increase the hydrolysis of inositol phosphates and elevate cytosolic Ca^{2+} levels, thus having an excitatory effect. The 5-HT_3 receptor triggers rapid depolarization due to a transient inward current, subsequent to the opening of nonselective cation channels (Na^+, Ca^{2+} influx). The response desensitizes rapidly. The 5-$HT_{4,6,7}$ receptor families all have excitatory effects through G protein-coupled stimulation of adenylyl cyclase and cAMP. cAMP causes activation of protein kinase A (PKA), which in turn activates other important signalling molecules. 5-HT_{1a} receptors are expressed throughout the brain. In the raphe nuclei, they are somatodendritic and act as autoreceptors to inhibit cell firing.

Postsynaptic 5-HT_{1a} receptors are located in a number of limbic structures, particularly the hippocampus. 5-HT_{1b} receptor expression is concentrated in the basal ganglia, striatum and frontal cortex, where they are thought to serve as autoreceptors. In addition, this receptor may act as a heteroreceptor controlling the release of other neurotransmitters, such as ACh, glutamate, dopamine, noradrenalin, and GABA. The level of expression of the 5-HT_{1d} receptor is very low, the presence of a 5-HT_{1d} autoreceptor in the DRN has been suggested, but not confirmed. 5-$HT_{1e,f}$ receptor mRNA has been identified in the brain, but little is known about the distribution and function, thus confirmation of a true physiological role for these receptor subtypes is still lacking. 5-HT_{2a} receptors are predominantly located throughout the cortex, caudate nucleus, nucleus accumbens, hippocampus, and basal ganglia. The expression of the 5-HT_{2b} receptor is restricted to a few brain regions, particularly cerebellum, lateral septum, hypothalamus, and medial amygdala. Due to the lack of a selective 5-HT_{2c} receptor ligand, the current knowledge concerning the distribution and function of this receptor is quite limited. 5-HT_3 receptors are present in numerous brain regions, including the CA1 pyramidal cell layer in the hippocampus, the dorsal motor nucleus of the solitary tract, and the area postrema. Less is known about the expression of the 5-HT_{4-7} receptors. 5-HT_6 receptor mRNA is located in the striatum, amygdala, nucleus accumbens, hippocampus, cortex, and olfactory tubercle. 5-HT_7 binding sites were found in the limbic system and thalamocortical regions (reviewed in Saudou and Hen, 1994; Adell et al., 2002; Hoyer et al., 2002; Hannon and Hoyer, 2008).

The serotonergic system in sleep-wake regulation

The discharge rate of serotonergic neurons in the DRN shows a distinct change in activity across the sleep-wake states. During wakefulness they fire at a regular tonic rate, which decreases during NREM sleep and practically disappears during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979). Extracellular serotonin levels are in correspondence with these changes in firing pattern (Portas and McCarley, 1994). Increases in serotonin levels or turnover in frontal cortex, hippocampus, hypothalamus, and brain stem are found during SD (Asikainen et al., 1997; Penalva et al., 2003).

There is also evidence for a role of serotonin in the promotion of sleep and sleep homeostasis. Serotonin inhibits cholinergic BF neurons *in vitro,* and when injected into the BF it attenuates cortical arousal *in vivo* (Khateb et al., 1993; Cape and Jones, 1998). Non-cholinergic neurons in the BF are also implicated in sleep–wake regulation (Hassani et al., 2009), and they can be hyperpolarized by serotonin (Fort et al., 1998). Administration of the serotonin synthesis inhibitor p-chlorophenylalanine during SD suppresses rebound NREM sleep during recovery sleep (Sallanon et al., 1983), indicating that serotonergic influences during wakefulness could prepare the brain for sleep, and leading to the assumption that serotonin serves an important function in sleep pressure (Jouvet, 1999).

The versatile role of serotonin in sleep-wake regulation is even more evident when we look at the effects of several receptor-specific manipulations on wakefulness, NREM, or REM sleep:

5-HT_{1a} and 5-HT_{1b} receptor knockout mice showed increased amounts of REM sleep, also seen after systemic injections of 5-HT_{1a} and 5-HT_{1b} receptor antagonists in wild-type mice, while injections of 5-HT_{1a} and 5-HT_{1b} receptor agonists in wild-type mice and rats increased wakefulness, and reduced NREM and REM sleep (Boutrel et al., 1999; Boutrel et al., 2002; Monti, 2010). The 5-HT_1 receptor family is inhibitory and, interestingly, manipulating the 5-HT_7 receptor, which is excitatory, shows the exact opposite effect on REM sleep. 5-HT_7 receptor knockout mice, as well as wild-type mice injected with a 5-HT_7 receptor antagonist either systemically or directly into the DRN, display reduced amounts of REM sleep (Hedlund et al., 2005; Monti, 2010). The 5-HT_2 receptor family may be more specifically involved in the regulation of NREM sleep. Ritanserin, a nonselective 5-HT_2 receptor antagonist, increased NREM sleep in humans (Idzikowski et al., 1986) and in rats (Dugovic et al., 1989). Antagonizing the 5-HT_{2a} receptor in mice increased NREM sleep, while 5-HT_{2a} receptor knockout mice showed an increased amount of wakefulness during baseline and also showed an attenuated NREM delta response to SD (Popa et al., 2005). In contrast, administration of a 5-HT_{2b} receptor antagonist increased wakefulness and decreased NREM and REM sleep (Kantor et al., 2004). In the case of the 5-HT_{2c} receptor, an early study found antagonists to promote NREM sleep (Sharpley et al., 1994), whereas more recent studies

showed that antagonizing the 5-HT_{2c} receptor increased wakefulness and decreased NREM sleep (Kantor et al., 2005), and that 5-HT_{2c} receptor knockout mice demonstrated increased amounts of wakefulness (Frank et al., 2002).

1.4.3 Dopamine

Dopaminergic system, projections, and innervation

The major ascending dopaminergic pathway comprises several groups of neurons located in the mesencephalic tegmentum. Dopamine-containing neurons from the SN project to the dorsal striatum, forming the nigrostriatal pathway. The VTA and SN jointly give rise to the mesolimbic and mesocortical pathway innervating the BF, nucleus accumbens, septum, amygdala, hippocampus, olfactory tubercles, and frontal cortex. The tuberoinfundibular pathway consists of a group of short neurons projecting from the arcuate nucleus of the hypothalamus to the median eminence and pituitary gland. This pathway regulates the function of the pituitary gland (Moore and Bloom, 1978). Lu et al (2006) recently discovered a group of wake active dopaminergic neurons in the vPAG matter, which projects to major components of the sleep–wake regulatory system including the BF cholinergic neurons.

Neurons from the brainstem, hypothalamus, and BF innervate the dopaminergic system. These include neurons from the DRN/MRN, LC, LH, TMN, PPT/LDT, and BF.

Synthesis and metabolism of dopamine

Dopamine is synthesized from the aromatic amino acid precursor, tyrosine, which is taken up through the blood brain barrier by a transporter into the dopaminergic neurons. Tyrosine is converted by tyrosine hydroxylase to L-3,4-dihydroxyphenylalanine (L-DOPA), in the cytoplasm DOPA decarboxylase transforms L-DOPA to dopamine, which is subsequently stored in synaptic vesicles where the molecules are protected from catabolizing enzymes. Dopamine is released from these vesicles by exocytosis into the synaptic cleft, upon induction of depolarization and Ca^{2+} influx. The action of dopamine in the synapse is terminated by active re-uptake into the presynaptic nerve terminal by the specific dopamine transporter (DAT), or by extracellular metabolism (Fig. 7; Elsworth and Roth, 1997).

Inside the neuron the main route of dopamine metabolism is oxidative deamination by MAO-B. MAO-B is the only metabolizing enzyme present in catecholaminergic neurons, although it is also found in glial cells and extracellularly (Agid et al., 1973; Elsworth and Roth, 1997). Intracellularly, dopamine is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) by activation of MAO-B. Extracellularly, DOPAC is then metabolized to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT). Extracellular dopamine metabolism follows a different route: it is first metabolized by COMT to 3-methoxytyramine (3-MT) and then oxidized by MAO-B to form HVA (Westerink, 1985; Wood and Altar, 1988). COMT is found

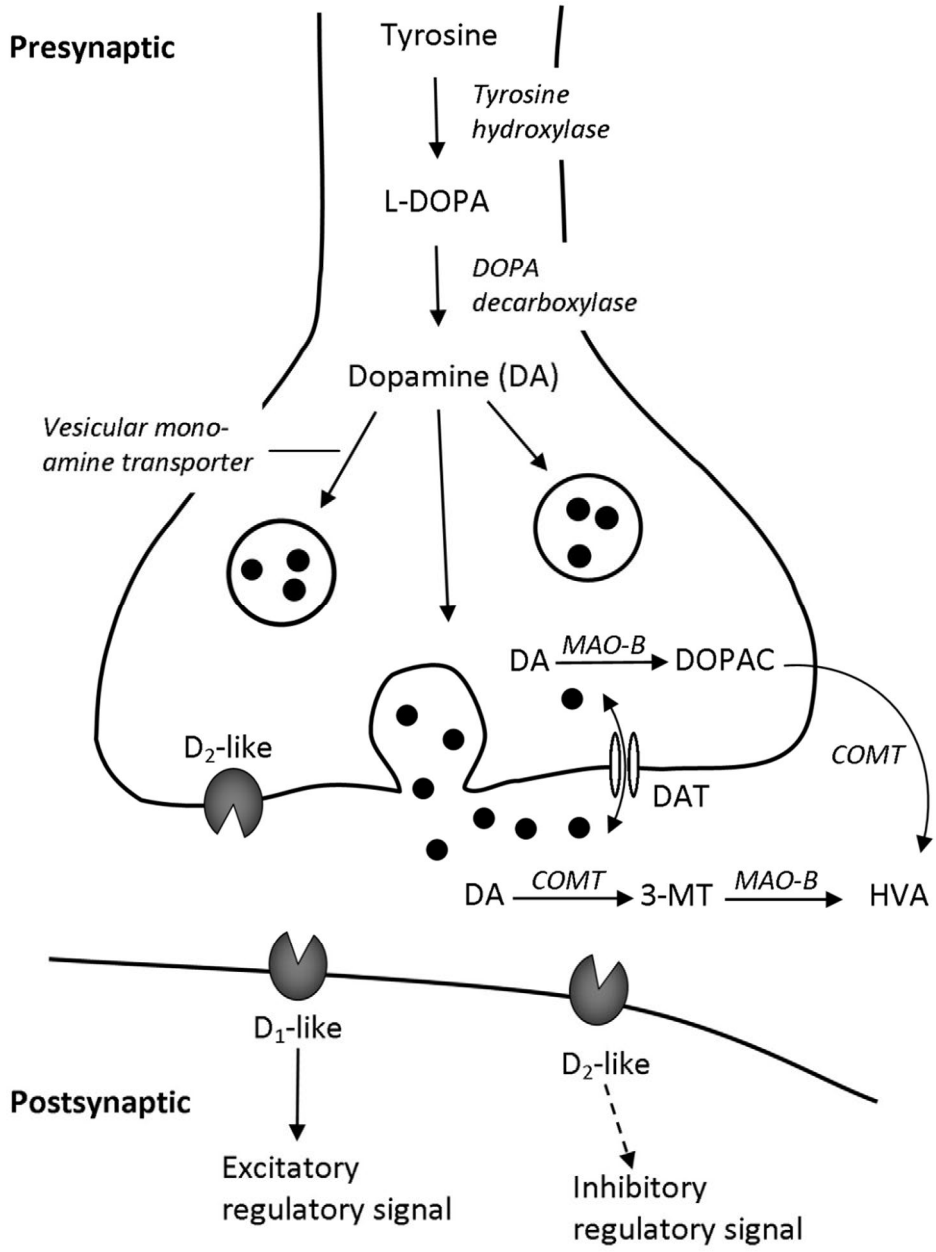

Figure 7. Dopaminergic neurotransmission

Tyrosine is converted to L-DOPA by tyrosine hydroxylase, which is transformed to dopamine by DOPA decarboxylase. Dopamine is stored in vesicles until released into the synaptic cleft, where it can bind to D_1-like or D_2-like receptors. The action of dopamine in the synaptic cleft can be terminated by active re-uptake by the DAT or by extracellular metabolism. Intracellular metabolism forms DOPAC via the monoamine oxidase route, extracellularly DOPAC is metabolized to HVA by COMT. Extracellular dopamine is first metabolized to 3-MT by COMT, and then oxidized by MAO-B to form HVA. 3-MT, 3-methoxytyramine; COMT, catechol-O-methyltransferase; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; L-DOPA, L-3,4-dihydroxyphenylalanine; MAO-B, monoamine oxidase B.

only outside dopaminergic neurons (Karhunen et al., 1995; Mannisto and Kaakkola, 1999), thus O-methylated metabolites provide markers of dopamine release. In the rat brain, DOPAC is the major metabolite, but the rate of HVA formation closely follows that of DOPAC (Westerink, 1979). In humans, however, the major brain metabolite is HVA (Elsworth and Roth, 1997).

3-MT has been suggested to be the best marker of dopamine release in the brain; because COMT does not exist inside the dopaminergic neurons, all 3-MT found in the living brain is the product of dopamine that is released from the nerve endings (Rivett et al., 1983; Kaakkola et al., 1987; Karhunen et al., 1995). However, the metabolism of dopamine by COMT is site-specific for the prefrontal cortex, where it accounts for 60% of dopamine metabolism against 15% in the rest of the brain (Karoum et al., 1994). The major role of COMT in prefrontal cortex dopamine metabolism may be related to lower expression of DAT in this area and the resulting decreased capacity of re-uptake (Sesack et al., 1998). In other brain regions, including the striatum and amygdala, DOPAC and HVA might be better indicators of dopamine turnover than 3-MT (Yavich et al., 2007)

Receptors

Dopamine mediates its effects through two families of G protein-coupled receptors, defined on the basis of their pharmacological profile, function, and homology, i.e. D_1- and D_2-like receptors (Kebabian and Calne, 1979). The D_1 and D_5 receptors belong to the D_1 family, and D_2, D_3 and D_4 to the D_2 family. D_1 receptors are coupled to stimulatory G proteins that activate adenylyl cyclase, leading to positive regulation of cAMP levels. Their stimulation results in the activation of the PKA cascade, which leads to an excitatory effect on the postsynaptic neuron. Conversely, the activation of the D_2 receptor family mostly inhibits G proteins and thereby inhibits the corresponding neuron, be it pre- or postsynaptic (reviewed in Missale et al., 1998).

The D_1 receptor is the most prevalent dopamine receptor in the brain; it is mainly expressed in the striatum, nucleus accumbens, and olfactory tubercle, and at lower levels in the cortex, limbic system, thalamus, and hypothalamus (Le Moine et al., 1991). Much less abundant is the expression of the D_5 receptor, which is found in the olfactory tubercle, hippocampus, and hypothalamus, it is, though, widely expressed on cholinergic cells in the cerebral cortex, striatum, BF, and diencephalon (Berlanga et al., 2005).

D_2 receptors are expressed in the striatum, olfactory tubercle, and nucleus accumbens on GABAergic neurons (Le Moine and Bloch, 1995). D_3 receptors are found primarily in limbic regions, i.e. the Isles of Calleja, nucleus accumbens, and olfactory tubercle. In the SN and VTA, D_2 and D_3 receptors are expressed by dopaminergic neurons, serving as autoreceptors (Meador-Woodruff et al., 1989; Weiner et al., 1991; Levant, 1997). The D_4 receptor shows the lowest expression of all dopamine receptors with some expression in the basal ganglia

and moderately more expression in the frontal cortex, amygdale, and hypothalamus (O'Malley et al., 1992).

The dopaminergic system in sleep-wake regulation

Dopaminergic neurons have a low basal firing rate that is similar across sleep-wake states. However, two distinct firing patterns can be distinguished: a slow tonic spiking pattern and a burst firing or phasic pattern (Grace and Bunney, 1984). The tonic spiking pattern is characterized by trains of spikes that discharge at steady, but irregular, intervals, and it is the typical firing pattern of the majority of dopamine cells encountered in untreated, anaesthetized rats. The phasic pattern consists of consecutive spikes in a burst, displaying progressively decreasing amplitude and increasing duration. The phasic burst spike pattern leads to an enhanced release of dopamine (Grace and Bunney, 1984) and may be related to waking (Rye and Jankovic, 2002; Dahan et al., 2007). During wakefulness the release of dopamine is higher than during NREM sleep (Trulson, 1985; Lena et al., 2005). Recently, a group of wake active dopaminergic neurons that project to major components of the sleep–wake regulatory system including the BF cholinergic neurons was indentified in the vPAG. After lesioning this region, a persistent increase in total sleep was observed (Lu et al., 2006)). Drugs that inhibit dopamine re-uptake or stimulate dopamine release are associated with arousal (Nishino et al., 1998); in addition, DAT knockout mice show increased wakefulness compared to their wild type littermates (Wisor et al., 2001). Lesions of dopaminergic nuclei in the cat induce a state of behavioral unresponsiveness and immobility (Jones et al., 1973). Studies using dopamine-receptor-deficient mice, or animals injected with an antisense vector demonstrate that dopamine D_1 and D_2 receptors facilitate behavioral arousal, while D_3 receptors mediate the opposite effect. D_1 and postsynaptic D_2 receptor agonists increase behavioral arousal and wakefulness, and decrease sleep (reviewed in Monti and Monti, 2007).

Dopamine modulates Ach levels in the cortex by binding to dopamine receptors that are located directly on cholinergic neurons. Activation of the dopamine D_1 receptor potentiates Ach release (Day and Fibiger, 1992; Day and Fibiger, 1993). A similar stimulatory effect on ACh release has been reported for the dopamine D_5 receptor, which was recently found to be expressed on cholinergic BF neurons (Hersi et al., 2000; Berlanga et al., 2005). In contrast, the D_2-like receptors attenuate ACh release (Stoof et al., 1987; Bertorelli and Consolo, 1990). In rat BF slices dopamine reduces inhibitory GABAergic transmission onto magnocellular BF neurons by activating presynaptic D1-like receptors (Momiyama and Sim, 1996).

1.4.4 Histamine

Histaminergic system, projections and innervation

Histamine neurons are found exclusively in a small region in the posterior hypothalamus, the TMN (Panula et al., 1984; Watanabe et al., 1984). They send projections that innervate practically the entire brain (Panula et al., 1989). The highest density of innervations is found in the hypothalamus, BF, septum, and olfactory tubercle. Structures innervated by the dorsal ascending pathway, through the thalamus, show a lower density of histaminergic projections. The cortex and hippocampus are moderately innervated. In addition to these ascending projections the histaminergic system also sends descending projections to all of the ascending arousal systems; in particular the SN and VTA are strongly innervated (Kohler et al., 1985; Inagaki et al., 1988; Panula et al., 1989).

The TMN receives input from the prefrontal/infralimbic cortex, septal regions, LC, DRN/MRN, LH, PPT/LDT, and VLPO (Ericson et al., 1989; Ericson et al., 1991; Chemelli et al., 1999).

Synthesis and metabolism of histamine

Histamine is synthesized in the brain from L-histidine by histidine decarboxylase (HDC), and is subsequently transported into vesicles by the vesicular monoamine transporter. When released the only means to terminate its action is through metabolism to *tele*-methylhistamine by the enzyme histamine N-methyltransferase, as no uptake system has been found (Fig. 8; reviewed in Haas et al., 2008).

Receptors

In the brain, histamine exerts its effect through three G protein-coupled receptors. Histamine receptors 1 and 2 (H1R and H2R) are found postsynaptically and induce an excitatory regulatory signal by activating the phospholipase C and the adenylyl cyclase pathways respectively. The histamine receptor 3 (H3R) inhibits the adenylyl cyclase pathway. It acts as an autoreceptor, reducing the release of histamine, while as a heteroreceptor it can regulate the release of several other neurotransmitters (Haas and Panula, 2003). H1R and H2R show a widespread expression in the brain; they are both expressed in the cortex, thalamus, VLPO, amygdala, septal nuclei, and cerebellum, and are colocalized in the hippocampus, LC, DRN/MRN, VTA, and SN. H1-receptors are also found in the BF, PPT/LDT, and nucleus accumbens (Bouthenet et al., 1988; Martinez-Mir et al., 1990; Traiffort et al., 1992; Vizuete et al., 1997). H3R are found in the cortex, nucleus accumbens, striatum, olfactory tubercles, and SN (Pollard et al., 1993).

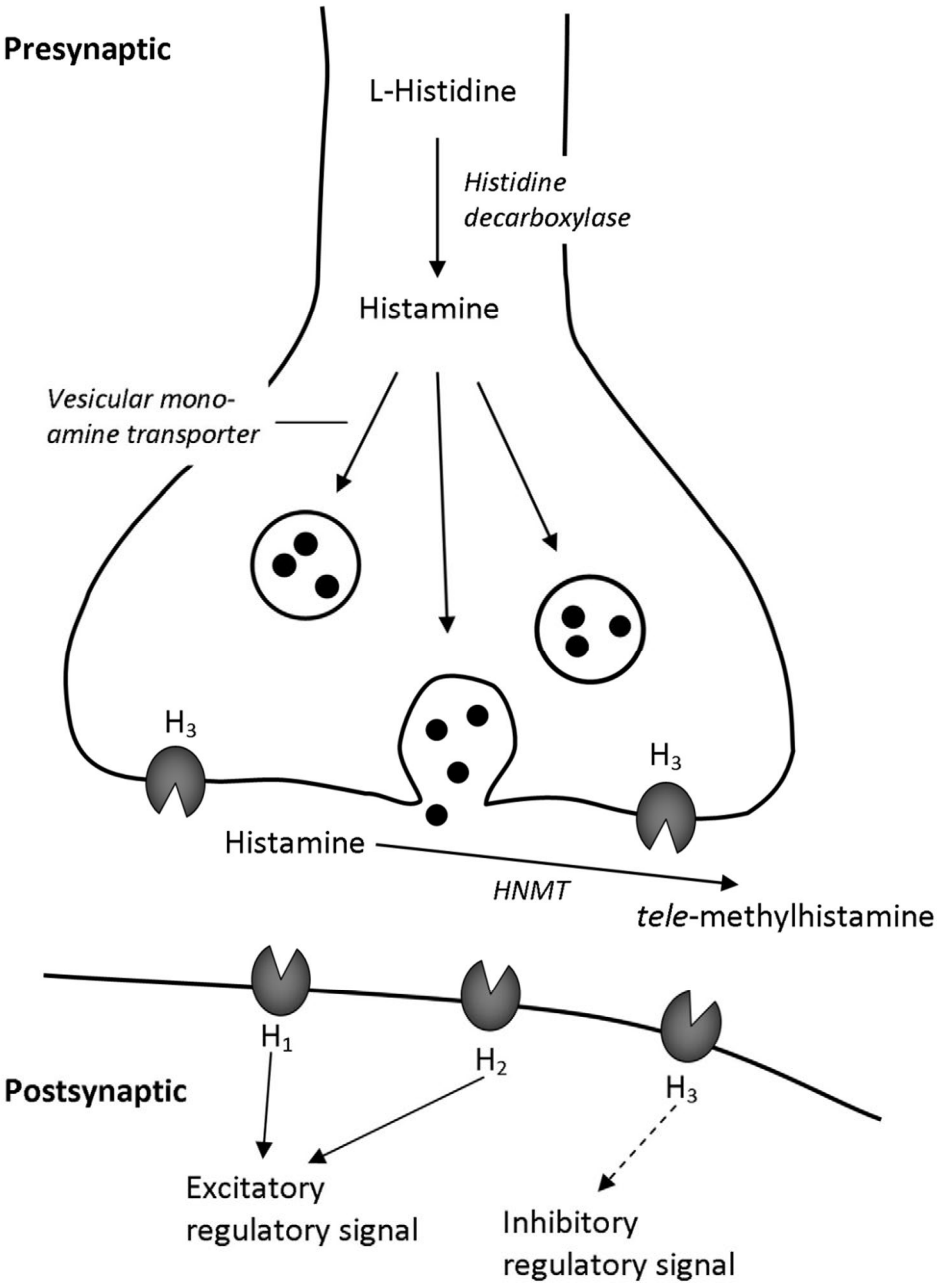

Figure 8. Histaminergic neurotransmission
L-histidine is converted by HDC to form histamine which is transported into vesicles by the vesicular monoamine transporter. When released, histamine can bind to H1, H2 or H3 receptors. Because no uptake system has been found, metabolism of histamine to *tele*-methylhistamine by the enzyme histamine N-methyltransferase is the only way to terminate its action.

The histaminergic system in sleep-wake regulation

The observation that classical antihistamines have sedative effects led to the hypothesis that histamine plays a role in sleep-wake regulation. These classical antihistamines exert their actions by blocking the H1R, and today the evidence still points towards the involvement of the H1R, but not the H2R, in mediating arousal (Monti et al., 1986; Monti et al., 1990).

Extracellular single-unit recordings of presumed histaminergic neurons which were performed in freely behaving cats (Sakai et al., 1990), and later in rats (Steininger et al., 1999), revealed that the discharge activity of these neurons is highest during wakefulness, that their firing rate slows down during NREM sleep, and disappears almost completely during REM sleep. Evidence that activity of the histaminergic system depends on wakefulness and does not follow a strict circadian pattern, came from a c-Fos staining experiment showing that histaminergic neurons are wake-active regardless of the time of day (Ko et al., 2003). More recently, identified histaminergic neurons were recorded in head-restrained non-anesthetized mice, showing that they are only active during wakefulness, showing the highest activity during attentive wakefulness, and are completely silent during NREM and REM sleep (Takahashi et al., 2006).

Histamine release that was measured from microdialysis, push-pull technique, or CSF samples, was highest during the active period in freely behaving rats (Mochizuki et al., 1992; Prast et al., 1992), in rhesus monkeys (Prell et al., 1989), and in human children (Kiviranta et al., 1994). Strecker et al. (2002) showed that histamine release in the POAH of cats was highest during wakefulness, lower during NREM sleep, and lowest during REM sleep. They did not observe any effect on extracellular histamine levels during a 6 h SD, suggesting that histamine does not relay information about sleep pressure to the sleep-promoting neurons of the VLPO. HDC knockout mice that are unable to synthesize neuronal histamine have difficulties in maintaining wakefulness in novel environments and show attenuated wakefulness due to shorter bout durations during the early part of the dark period (Parmentier et al., 2002).

In vitro studies demonstrated that histamine excites both the cholinergic (Khateb et al., 1995) and non-cholinergic neurons of the BF (Fort et al., 1998), predominantly via H1R. Infusions of histamine into the BF of freely moving rats showed that the BF indeed can play a role in mediating the arousal effect of histamine (Ramesh et al., 2004), it is, however, unclear what the effects on the EEG of freely moving rats are and what effect antagonizing the actions of histamine in the BF will have on the amount of wakefulness.

1.5 The ascending arousal systems and their possible role in the sleep and wake regulation mediated by the BF

During SD, the site-specific accumulation of adenosine inhibits cholinergic BF neurons, which results in an increased sleep pressure (Porkka-Heiskanen et al., 1997; Porkka-Heiskanen et al., 2000; Kalinchuk et al., 2008). This raises the question how the animals are able to stay awake during as much as a 6 h SD. In fact, during the first three hours of SD, the activity of both BF and cortical neurons is increased, which may be required to maintain vigilance (Kostin et al., 2010; Vyazovskiy et al., 2009). One explanation could be that the ascending arousal systems increase their activity to counteract the effect of sleep pressure. If this is the case, the BF, considering that it is an important ventral extrathalamic relay of the ascending arousal systems to the cortex, might play an important role in mediating the increased activity of the ascending arousal systems during SD.

All the ascending arousal systems have a higher discharge rate during wakefulness as compared to sleep and they all project to the BF. Even though some studies found increases in the level or turnover of serotonin in frontal cortex, hippocampus, hypothalamus, and brain stem during SD (Asikainen et al., 1997; Penalva et al., 2003), in human subjects brain dopamine levels were increased after SD (Volkow et al., 2008), and in the POAH of the cat histamine did not increase during SD (Strecker et al., 2002), nothing is known about the effects of SD on the levels of these monoamines in the BF. In addition, no studies have been performed to examine to what extent the ascending arousal systems are involved in sleep homeostasis mediated by the BF.

2 AIMS AND HYPOTHESES OF THE STUDY

The main aim of this thesis was to explore the role of the ascending arousal systems in the regulation of sleep, wakefulness, and cortical activation mediated by the BF. Do the ascending arousal systems increase their activity during SD? Does this increased activity counteract the sleep-promoting effects of SD? Does their activity during prolonged wakefulness induce a homeostatic sleep response? Are these actions mediated by cholinergic neurons?

Specific questions:

I OREXIN:

It is known that reduced levels of orexin lead to sleep fragmentation and narcolepsy, but does orexin overexpression lead to vigilance state consolidation, and what effect does it have on sleep homeostasis?

II SEROTONIN & DOPAMINE:

SD leads to a site-specific increase in extracellular adenosine that inhibits cholinergic BF neurons. During the first hours of SD the activity of BF neurons increases, suggesting increased activity of the ascending arousal systems to counteract this inhibitory effect. Are serotonin and dopamine systems affected by SD, and are their extracellular levels increased in the BF after SD?

III & IV HISTAMINE:

Many studies showed that histamine release is increased during the active/waking period. Is this because histamine release is under circadian control, or does it depend on vigilance state? Do histamine levels in the BF (study III), and/or in the TMN (study IV) correlate with behavioral state?

Does SD increase histamine release in the BF? And do elevated histamine levels in the BF induce wakefulness, associated with cortical high frequency theta activation, followed by increased sleep intensity (study III)?

To what extent do BF cholinergic neurons mediate the effects of histamine on cortical activation (study III)?

3 MATERIALS AND METHODS

Details of the materials and methods can be found in the original publications (I-IV). Only those procedures in which the author was personally involved are listed in this chapter.

The Ethics Committee for Animal Experiments at the University of Helsinki and the provincial government of Southern Finland approved all experiments, which were carried out in accordance with the laws of Finland and the European Union. We made every effort to minimize the number of animals used and their suffering.

3.1 Animals

Mice were used in the studies that required genetic manipulation; they were also used for microdialysis studies after the method had been set up for mice. Han-Wistar rats were used for microdialysis studies because of their docile nature, before the method had been modified to suit mice.

Mice

Study I

Five- to seven-month-old male Balb/c x DBA/2 (CD2) wild type or transgenic (overexpressing the hPPO gene) mice were individually housed in a 12-h light-dark cycle (n wild type = 11; n transgenic = 14). Standard rodent pellets and water were provided ad libitum. Before surgery, mice were habituated to handling for a 3- to 4-week period.

Study IV

Male C57BL/6J mice (10 weeks old; n = 5) were kept individually under a reversed 12-h light-dark cycle. Standard food pellets and water were available ad libitum. The mice were habituated to handling for 2 weeks before surgery.

Rats (Studies II & III)

Male Han-Wistar rats aged three to four months (300-400 g) were individually housed under constant temperature in a 12-h light-dark cycle (n study II = 16; n study III = 25). Food and water were provided ad libitum. The animals were habituated to handling beginning at least four days before surgery.

3.2 Surgery (Studies I, II, III, IV)

Under general anesthesia, the animals were placed in a stereotaxic device. Two gold-coated screws were fitted into the skull for frontoparietal epidural bipolar recording of the EEG. To record the EMG, two silver wire electrodes were inserted into the neck musculature.
In studies II & III a unilateral guide cannula for the microdialysis probe (CMA 11 Guide) was placed 3 mm above the BF area.
In study IV a unilateral guide cannula (CMA 7 Guide) was implanted into the posterior part of the hypothalamus 1 mm above the target site.
Finally, the guide cannula, screw electrodes and supporting screws were secured in place with acrylic dental cement. After recovery and adaptation to the recording conditions (at least one week), a two- or three-day recording was taken from each animal to ensure that they had fully adapted.

3.3 EEG/EMG recording and analysis (Studies I, II, III, and IV)

The EEG and EMG signals were amplified (gain 5 or 10 K), filtered (high pass: 0.3 Hz; low pass 100 Hz), and sampled at at least 200 Hz. EEG recordings were manually scored in two- or four-second epochs for wakefulness, NREM sleep and REM sleep in the Spike2 program using the script Sleepscore v1.01.
In order to examine the vigilance state distribution during our experiments, the vigilance states were scored according to standard criteria. Wakefulness was scored for low-amplitude desynchronized EEG activity in combination with activity in the EMG. NREM sleep was determined by high-amplitude delta (0.5-4 Hz) waves in the EEG and low-amplitude or absent EMG. REM sleep was identified by regular theta (5–9 Hz) activity in the EEG and low or absent EMG activity. In study II wakefulness and NREM were scored semi-automatically in 30 s epochs using custom-made scripts (Stenberg et al., 2003), while REM sleep was scored manually.
To score low and high EMG-activity wakefulness, the maximum EMG amplitude was determined for every 24-h file. Epochs were scored as low EMG-activity wakefulness when the amplitude of the EMG in the entire epoch did not rise above 25% of the maximum amplitude. High EMG-activity wakefulness was scored when the EMG amplitude rose above 25% of the maximum amplitude. Brief awakenings were scored for brief periods of wakefulness lasting no more than 16 s. Epochs containing artifacts were excluded from further analysis.
EEG power spectra were calculated within the 0.5-50 Hz frequency range by fast Fourier transform (FFT = 256, Hanning window, 0.5 or 1Hz resolution).

3.4 Video monitoring (Studies I and IV)

To check for possible symptoms of narcolepsy, such as cataplexy, in the orexin-overexpressing mice (study I), as has been reported for mice with a disrupted orexin system (Mochizuki et al., 2004), we continuously monitored the mice with standard miniature infrared surveillance video cameras throughout the third and fourth recording week. The video and EMG/EEG recordings showed no signs of sudden attacks of inactivity, or loss of muscle tone together with prominent EEG theta activity.
We also used video monitoring to quantify the motor activity of mice whilst measuring the extracellular histamine levels in the TMN and recording EEG/EMG (study IV).

3.5 Control day (Studies I, II, III, and IV)

The control day served as a reference to which subsequent EEG recordings and, if applicable, microdialysis samples, obtained during the SD days or during drug perfusion experiments (see below), were compared. This day also served to observe and, if needed, control for any diurnal effects. During the control day the animals were allowed to sleep and wake undisturbed. In the studies that included microdialysis the probes were continuously perfused with artificial CSF (aCSF) at a rate of 1 µl/min.

3.6 Sleep deprivation (Studies I, II, and III)

To study the effects of increased homeostatic sleep pressure, we deprived the animals of sleep by gentle handling (Franken et al., 1991), a method in which we introduced novel objects into the home cage whenever the animals appeared to be sleepy. This method stimulates active wakefulness and results in increased sleep pressure. The increased sleep pressure increases sleep propensity and leads to a recovery sleep, which is characterized by an increase in the amount, consolidation (as measured by brief awakenings or sleep fragmentation), and intensity (as defined by increased NREM delta (0.5 - 4 Hz) power) of the subsequent NREM sleep.

3.7 Murine multiple sleep latency test (MMSLT; Study I)

The MMSLT was adapted from the MSLT, which was developed in 1977 and is commonly used as a diagnostic tool to test sleepiness in humans (Carskadon and Dement, 1977). This test measures the sleep latency and is based on the idea that when sleep pressure is high the latency to fall asleep is low. The MMSLT protocol was developed by Veasey et al. (2004), and was slightly adapted for this study (I). Sleep latencies were measured during four

consecutive 20 min nap opportunities during baseline conditions and following 6 h of SD; prior to each nap opportunity the mice were kept awake for 10 minutes by gentle handling.

3.8 *In vivo* microdialysis and reversed microdialysis (Studies II, III, and IV)

We used *in vivo* microdialysis to examine the concentration of several neurotransmitters in the extracellular space of the BF of rats, and the TMN of mice. This technique is based on passive diffusion of small molecules across a semi-permeable membrane according to their concentration gradient. It allows us to continuously sample a specific brain area for multiple days in freely moving animals. When this is combined with EEG/EMG recording, we can correlate the concentrations of specific neurotransmitters to vigilance state. In addition, by using reversed microdialysis we can apply drugs to a very specific area and study the effects simultaneously.

Microdialysis probes (Rats: CMA 11, membrane length 2 mm; Mice: CMA 7, membrane length 1 mm) were inserted through their guide cannulae 18-24 h before the first experiment. Perfusion of aCSF by a microdialysis syringe pump (flow 1 µl/min), started either directly (study III & IV) or just after lights-on on the control or SD day (study II). Samples were collected at 20 or 30 min intervals continuously. In study II we stopped aCSF perfusion half an hour before lights-off, and disconnected the animals from the microdialysis tubing. For studies III and IV we adapted the system to keep the animals constantly connected to the microdialysis tubing, and consequently were able to continuously collect samples using an automated sample collection system over several days. We performed an *in vivo* recovery experiment to validate the stability of the recovery of the microdialysis probes over several days. We compared the maximum concentrations of histamine in 30-min samples on the control day and after five days of continuous perfusion and found no significant differences (data not shown). Samples were stored at −80 C until assayed. EEG and EMG were recorded continuously.

3.8.1 Reversed microdialysis (Study III)

In study III we used reversed microdialysis to perfuse either histamine at three different concentrations (100, 500 or 1000 µM), or pyrilamine (10 mM), a H1R antagonist, into the BF. The drug perfusions were carried out following the control day for up to five days after the control day. All drug solutions were prepared in aCSF. The three-hour drug perfusions were preceded by a three-hour baseline period of aCSF perfusion (same-day baseline).

To examine the arousal effect of histamine, histamine was perfused during the light period (sleep period for rats) between three and six hours after lights-on. During the dark period (active period for rats), the H1R antagonist was perfused from three to six hours after lights-off, to examine its sedative effects. After drug perfusion, the perfusion medium was

reverted back to aCSF while the perfusion continued non-stop. The perfusions of different drugs were performed at least 24 h apart in a counterbalanced design.

3.9 Unilateral local cholinergic lesion experiments (Study III)

In order to find out whether or not the cholinergic neurons in the BF are responsible for mediating histamine-induced cortical arousal we used the immunotoxin 192IgG-saporin to induce unilateral local cholinergic lesions (study III). This method was modified from Kalinchuk et al. (2008). 192IgG is a monoclonal antibody that binds to the p75 nerve growth factor receptor that is selectively expressed on cholinergic BF neurons. When linked to saporin, the newly formed 192IgG-saporin complex acts as a selective immunotoxin, destroying cholinergic BF neurons (Book et al., 1992; Heckers et al., 1994).
We used a modified microdialysis probe, with the membrane tip cut off and connected to a 10-µl Hamilton syringe, to inject the 192IgG-saporin (0.23 µg/µl) locally during the surgery for EEG/EMG electrode placement and microdialysis guide cannula implantation. To reach a total injection volume of 1 µl, 192IgG-saporin was injected into the HDB/SI/MCPO at a flow rate of 0.1 µl/min for 10 minutes. To minimize the chance of pulling out the injected solution while removing the modified probe, we left it in place for five minutes before taking it out.
Two weeks after the lesion, at which time local cholinergic lesions show optimal cell degeneration, the control day and drug perfusion experiments were repeated as described previously. For histamine perfusions, we used only the highest dose (i.e.1000 µM).

3.9.1 ChAT Immunohistochemistry (Study III)

After the experiments we verified the local cholinergic lesions by choline acetyltransferase (ChAT) immunohistochemistry. The ChAT antibody binds to ChAT, the enzyme responsible for the synthesis of Ach from acetyl-coenzyme A and choline, which is only expressed in cholinergic neurons.
We transcardially perfused the rats under deep anesthesia with a 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). We then removed the brains and postfixed them overnight in the same paraformaldehyde solution, after which they were submerged in a 30% sucrose solution at 4°C for four days for cryoprotection. After the brains sank, they were frozen and stored at -80°C. All the way through the BF area, coronal sections (40 µm) were cut on a freezing microtome and collected into PBS for immunohistochemical staining for ChAT. The sections were washed in PBS, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in PBS for 30 min at room temperature, after which the sections were treated with blocking solutions (0.5% Triton-X in PBS for 2 h; 3% donkey serum in PBS for 1 h) and incubated overnight at 4°C with a rabbit anti-ChAT primary antibody (1:1000). The next day, the sections were incubated with the secondary antibody (biotinylated donkey anti-rabbit IgG 1:300) for two

hours at room temperature and treated with avidin–biotin complex. Diaminobenzidine tetrahydrochloride was used for visualization. After staining, the sections were mounted on microscope slides and coverslipped with Eukitt quick-hardening mounting medium. We counted the ChAT-positive cells unilaterally in three sections (interval 200 µm) per rat using a light microscope at a 10x magnification; a 4x magnification was used to determine the outline of the BF. The cell counts of these three sections were averaged and compared between the lesion and control groups.

3.10 Histological verification of probe locations (Studies II, III, and IV)

After the experiments, the animals received a lethal dose of pentobarbital. To verify the probe locations, ink was injected through a modified microdialysis probe inserted into the guide cannula. The brains were then removed, frozen on dry ice, and stored at −80 °C.
Sections (20 µm) were cut on a freezing microtome, stained with Toluidine Blue, and visually inspected under a light microscope. Only data from animals with probe tips located within the limits of the BF region (studies II&III; HDB, MCPO, SI) or the posterior hypothalamus (study IV; TMN) were included in the analysis.

3.11 HPLC (Study II)

The concentrations of 5-HIAA, DOPAC and HVA were determined from the microdialysis samples using high-performance liquid chromatography (HPLC) combined with electrochemical detection. The system had a detection limit of approximately 5 nM. The HPLC setup consisted of a Waters 717Plus autosampler, a Waters 510 pump, a Symmetry C18 5µm column, and a Concorde electrochemical detector, set to a potential of 800mV using a Ag/AgCl reference electrode. Waters Millennium 32 software was used for system control and data collection/processing. The HPLC system was used in an isocratic mode at a 1 ml/min flow rate. The mobile phase consisted of 50 mM H_3PO_4, 50 mM citric acid, 0.15 mM EDTA, 2 mM octanesulphonic acid sodium salt, and 5% methanol, pH 2.5.

3.12 Statistical analyses

A global overview of the statistics used in studies I-IV is reported here, for more detailed information please consult the original publications.
All values are shown as mean ± standard error of mean (SEM). Statistical analyses were performed using SigmaStat 3.1. $P < 0.05$ was deemed significant.
For correlation analysis the Pearson Product Moment Correlation was used. When simple comparisons between two groups of animals were made we used unpaired t-tests, when comparisons were made between two time points within the same group of animals we

used paired t-tests. For more complicated analyses including several time-points and or multiple factors we used one-way or two-way repeated measures analysis of variance (ANOVA). When a control period was available, ANOVAs with multiple comparisons versus control group were used. All repeated-measures ANOVAs were followed by Holm-Sidak *post hoc* tests. In situations when the normality assumption failed, an ANOVA on ranks (a corresponding non-parametric test) was used, with Dunn's method as *post hoc* test.

4 RESULTS AND DISCUSSION

Table 1. Main results

Study	Main methods	Main results
I (mice)	Overexpression of the human prepro-orexin transgene (hPPO)	Increased orexin A levels and decreased OX2R expression in the hypothalamus of hPPO overexpressing mice
	EEG/EMG recording baseline	Reduced amount of REM sleep in hPPO overexpressing mice
	EEG/EMG recording 6 h sleep deprivation	Increased slow wave intrusion in the wakefulness-specific EEG of hPPO overexpressing mice
II (rats)	Microdialysis and EEG/EMG recording 6 h sleep deprivation	Gradual increase in extracellular serotonin and dopamine metabolite levels, reaching a plateau during the third hour of sleep deprivation Gradual decrease in metabolite levels during recovery sleep
III (rats)	Microdialysis and EEG/EMG recording baseline day 24 h in the BF	Extracellular histamine levels in the BF correlate with the amount of wakefulness independent of the time of day
	Microdialysis and EEG/EMG recording 6 h sleep deprivation	Immediate increase in extracellular histamine during sleep deprivation Immediate decrease during recovery sleep
	Histamine perfusion into the BF by reversed microdialysis	Increase in wakefulness and high theta EEG frequencies associated with attentive wakefulness Decrease in NREM sleep and delta EEG frequencies associated with sleep and drowsiness No NREM delta rebound after histamine perfusion
	Histamine receptor 1 antagonist perfusion into the BF by reversed microdialysis	Decrease in wakefulness and high theta frequencies in wakefulness-specific EEG Increase in light NREM sleep with slightly lower than normal delta power
	Histamine or histamine receptor 1 antagonist perfusion after lesion of the cholinergic neurons in the BF	No significant effects
IV (mice)	Microdialysis and EEG/EMG recording baseline day 24 h in the TMN	Extracellular histamine levels in the TMN correlate with the amount of wakefulness independent of the time of day Histamine release in the TMN correlates positively with high-theta and gamma EEG frequencies, associated with attentive wakefulness

4.1 The effect of overexpression of orexin on sleep-wake regulation (Study I)

In study I we characterized the sleep-wake regulation of transgenic mice overexpressing the human prepro-orexin (hPPO) transgene under control of its endogenous promoter.

4.1.1 hPPO overexpression reduced the amount of REM sleep during baseline recordings

We found increased levels of the hPPO transgene mRNA and orexin A in the hypothalamus of transgenic mice. Transgene overexpression decreased OX2R expression in their hypothalami, but we observed no effect on hypothalamic OX1R expression and OX1R/OX2R levels in the BF, cortex or hippocampus.

As expected, because cataplexy is a symptom of narcolepsy associated with decreased, and not increased, orexinergic transmission, the video recording in combination with EEG/EMG recording did not show any signs of cataplexy.

During the control day we did not find any statistically significant differences between the genotypes in the overall percentage of time spent in each vigilance state, in bout durations, the number of state transitions (NREM to wake or REM to wake transitions), or the number of brief awakenings (I; Table 1). Thus, we did not find evidence of vigilance state fragmentation or consolidation in the orexin-overexpressing mice. We did find minor but statistically significant differences in the percentage of time spent in each vigilance state per hour during the light-dark transition period. In addition, at several time points throughout the light-dark cycle transgenic mice spent less time in REM sleep (I; Fig.4)

Our observations with regard to the amount of REM sleep showed a slight decrease at several time points. These results are consistent with those of Mieda et al. (2004), who showed a decrease in the amount of REM sleep during the daytime in mice with a β-actin/ cytomegalovirus hybrid promoter to drive orexin overexpression. Both REM and NREM sleep were decreased in rodents after ICV injections of orexin into the lateral ventricle (Piper et al., 2000), or after its perfusion into the TMN during the light period (Huang et al., 2001). In contrast, orexin knockout mice showed increased amounts of REM sleep during the dark period (Chemelli et al., 1999; Willie et al., 2003). However, Mochizuki et al. (2004) recently reported that orexin knockout mice exhibit normal amounts of wakefulness, NREM, and REM sleep; they attribute the increased REM sleep observed in orexin knockout mice (Chemelli et al., 1999; Willie et al., 2003) to episodes of cataplexy, since the previous studies did not separate those from REM sleep (Mochizuki et al., 2004). It is obvious from the work of Willie et al. (2003) that the OX1R might be involved in the regulation of REM sleep. In our study, overexpression of orexin led to decreased REM sleep and, in contrast to the OX2R,

the OX1R expression was not affected, which points to the possibility that the observed decrease in REM sleep might be caused be increased activation of the OX1R by orexin A.

4.1.2 Increased slow wave intrusions in hPPO overexpressing mice during SD

When comparing transgenic and wild-type mice during SD, we found that SD was effective in keeping the animals of both genotypes awake, as mice were awake on average 93.7±1.7% of the time, although the transgenic mice did sleep a bit more (wild-type: 95.6±1.5%, n = 6; transgenic: 92.9±2.5%, n = 9). Compared to the wild-type, transgenic mice also showed a higher intrusion of slow waves during wakefulness as shown by an increase in low delta power (1 Hz). The fact that slow waves are more prominently present in the waking EEG during SD of orexin overexpressing mice might lead us to believe that the transgenic mice have a less stable EEG. However, the transgenic mice also showed slightly more sleep during the SD, and because there was also no increase in fragmentation of vigilance states it is also possible that the transgenic animals were sleepier than the wild-type controls. On the other hand, if the transgenic mice were indeed sleepier, we would have expected to find a shorter sleep latency in the MMSLT test, which was not the case.

During recovery sleep the amounts of NREM and REM sleep in both groups increased compared to baseline (Table 2), thus both groups demonstrated a recovery sleep response. Both genotypes showed the same amount of NREM sleep, number of brief awakenings, and latency to NREM sleep (as investigated by the MMSLT). The amount of REM sleep, though, was reduced in transgenic mice compared to wild-type controls at several time points during both the light and dark periods (I; Fig. 5e), as well as overall during the recovery sleep in the light period (I; Table 2). However, when recovery REM sleep during recovery sleep was normalized to the control day within each individual animal, no reduction in REM sleep remained, demonstrating that the transgenic mice have a normal REM sleep response to SD.

During recovery sleep, NREM sleep intensity, as defined by increased NREM EEG delta (1–4 Hz) power, was attenuated in the transgenic mice compared to wild-type controls. In the EEG power spectra after SD the NREM delta power increased in both genotypes (I; Fig. 5d); this increase, though, was attenuated in the low delta range (1-2 Hz) in the transgenic mice during the first two hours of recovery sleep. It is not surprising that the orexin-overexpressing mice exhibited an attenuation in the intensity of recovery sleep, because they showed a higher intrusion of slow delta waves in the waking EEG during SD; part of the sleep pressure had thus already been released during the SD, resulting in a lower sleep pressure at the start of recovery sleep.

4.1.3 Limitations of the hPPO overexpressing mouse strain

Characterization and quantification of the EEG of several inbred mouse strains revealed that EEG patterns demonstrate a high degree of heritability, and that distinct differences in NREM delta power exist between the Balb/c and DBA/2 strains (Franken et al., 1998). Additionally, BALB/c mice exhibit a weak diurnal rhythm (Valatx and Bugat, 1974). Consequently, it is possible that the mixed genetic background of the CD2 hybrid strain could at least partly be responsible for the differences in the distribution of vigilance states and in the EEG power found in this study (Franken et al., 2001).

4.2 Extracellular serotonin and dopamine metabolites increase in the BF during SD (study II)

In study II we measured the effect of increased sleep pressure on serotonin and dopamine turnover in the BF, as measured by their major metabolites using *in vivo* microdialysis in rats.

4.2.1 Increased serotonin metabolite levels in the BF during SD

During SD the extracellular levels of 5-HIAA in the BF progressively increased with every hour (II; Fig. 3a). After SD, the recovery sleep induced a steady decrease of the 5-HIAA concentration back towards baseline level. During the first 3 hours of the lights-on period on the control day, we observed a slight decrease in the BF 5-HIAA levels, after which the levels stabilized. Since extracellular 5-HIAA levels reflect serotonin turnover, at least when the re-uptake mechanism is intact (Rollema et al., 1996; Stenfors et al., 1999), the increase in 5-HIAA levels reflects a slow and steady increase of serotonin turnover in the BF during SD.

Serotonin is thought to stimulate wakefulness, because serotonergic neurons are most active and extracellular serotonin levels are highest during this vigilance state (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Portas and McCarley, 1994; Penalva et al., 2003). Indeed, on the control day we found higher levels of extracellular 5-HIAA during the parts of the light period corresponding to periods with higher amounts of wakefulness. In addition, we found that prolonged wakefulness induced by SD resulted in an increase in serotonin turnover.

On the other hand, there is also evidence for a role of serotonin in the promotion of sleep and in sleep homeostasis. Serotonin inhibits cholinergic BF neurons *in vitro,* and when injected into the BF it attenuates cortical arousal *in vivo* (Khateb et al., 1993; Cape and Jones, 1998). Non-cholinergic neurons in the BF are also implicated in sleep–wake regulation (Hassani et al., 2009), and they can be hyperpolarized by serotonin (Fort et al., 1998). Administration of the serotonin synthesis inhibitor p-chlorophenylalanine during SD suppresses rebound NREM sleep during recovery sleep (Sallanon et al., 1983), leading to the conclusion that serotonin serves an important function in sleep pressure (Jouvet, 1999).

The versatile role of serotonin in sleep-wake regulation is even more evident when we look at the effects of several receptor-specific manipulations on wakefulness, NREM, or REM sleep, which is addressed in the literature review.

Because of this multifaceted role of the serotonergic system, being more active during wakefulness and being able to stimulate both wakefulness and sleep, serotonin might be able to provide the brain with a signal for both the duration and the intensity of wakefulness, which would make the serotonergic system an important player in the homeostatic regulation of sleep. By selectively expressing certain receptors in specific areas of the brain, serotonin could stimulate wakefulness in the cortex, while it could attenuate cortical arousal by specifically inhibiting the BF. Indeed, the expression pattern of serotonergic receptors throughout the brain is quite diverse, with some receptors being widely expressed, whereas others have a very limited distribution (Saudou and Hen, 1994; Adell et al., 2002; Hoyer et al., 2002; Hannon and Hoyer, 2008).

In our results, the build-up of serotonin turnover during SD is slow and gradual; it resembles the way extracellular adenosine builds up during SD (Porkka-Heiskanen et al., 1997; Kalinchuk et al., 2011). Both adenosine and serotonin inhibit cholinergic neurons in the BF. Taken together with the previous findings we hypothesize that serotonin may be partly responsible for increases in sleep pressure by adding to the increase of homeostatic sleep pressure in the BF in a similar way as adenosine does.

4.2.2 Increased dopamine metabolite levels in the BF during SD

SD induced a gradual increase of extracellular DOPAC levels, reaching a plateau during the third hour. Until the second hour of recovery sleep the DOPAC levels did not decrease, but then quickly decreased towards baseline levels (II; Fig. 3b). Similarly to DOPAC levels, extracellular HVA levels increased gradually during SD, also reaching a plateau during the third hour of SD and lasting until the second hour of recovery sleep when a decrease towards the baseline level started (II; Fig. 3c). HVA levels did not completely return to baseline during the 3-h recovery period (II; Fig. 3c). The extracellular levels of both dopamine metabolites decreased during the first four hours on the control day, after which the levels stabilized.

Because dopamine is metabolized to DOPAC intracellularly or to 3-MT extracellularly, and both are transformed into HVA as the final metabolite (Ashcroft, 1969; Miyamoto et al., 1991), DOPAC and HVA are reliable indicators of dopamine turnover (Elsworth and Roth, 1997; Yavich et al., 2007).

Very few studies have looked at dopamine release in freely behaving animals. A study using voltametry showed an increase in dopamine release in the caudate nucleus of the cat during wakefulness (Trulson, 1985). Léna et al. (2005) showed that also in the nucleus accumbens

and in the medial prefrontal cortex of the rat dopamine release is higher during wakefulness as compared to NREM sleep. In our study we showed that in the BF extracellular dopamine metabolite levels were higher in the beginning of the control day, when the animals spent more time awake. In addition, our results showed an increase in dopamine turnover in the BF during SD.

The dopamine turnover during SD increased gradually, comparable to the way sleep pressure increases as reflected by the adenosine levels in the BF. The slow build-up of dopamine metabolites reaching a plateau in the third hour of SD also mimics the increase in neuronal firing observed during SD in the BF (Kostin et al., 2010) and the cortex (Vyazovskiy et al., 2009) of the rat. Because dopamine has an excitatory effect on cholinergic neurons, this suggests that increased dopamine turnover in the BF could assist in increasing the neuronal firing rate in both BF and cortex, promoting cortical arousal, and may counteract the inhibiting effects of sleep factors during SD.

4.2.3 Effects of SD on BF corticosterone levels

Because of a possible interaction between glucocorticoids and monoaminergic neurotransmission, and a possible occurrence of mild stress associated with SD that could lead to an activation of the hypothalamic–pituitary–adrenal (HPA) axis and thus increase corticosterone levels, we also measured corticosterone from the microdialysate. Extracellular corticosterone levels in the BF on the control day showed low levels during the morning and an increase at the end of the lights-on period, corresponding with the expected circadian peak (II; Fig. 4). No increase in serotonin and dopamine turnover was found on the control day, therefore an interaction between corticosterone increase and serotonin/dopamine turnover was not apparent.

The corticosterone levels remained at baseline level during the first three hours of SD, increasing only during the last three hours. Immediately from the start of recovery sleep the corticosterone levels returned back to the pre-SD baseline, hereby omitting the diurnal peak. In contrast, serotonin/dopamine turnover remained at or just below the maximum from the third hour of SD until the second hour of recovery sleep, again showing no influence of the corticosterone levels.

Comparing the individual maximum values of the corticosterone levels during the lights-on period on the control day to those of the SD period indicated that corticosterone concentrations during SD did not exceed the normal diurnal peak levels. Thus indicating that our method of SD by “gentle handling” did not induce substantial stress. Our results are in accordance with other studies that have shown SD to have only a mild or transient

activating effect on the HPA axis in rats (Tobler et al., 1983; Meerlo et al., 2002; Penalva et al., 2003).

The increase seen in corticosterone levels after the third hour of SD might reflect a need of the waking system for additional arousal-inducing substances to sustain vigilance, because dopamine turnover reaches its highest intensity at the third hour of SD, and at the same time also the neuronal activity has reached its maximum (seen in BF and cortical neurons induced by SD; Vyazovskiy et al., 2009; Kostin et al., 2010).

4.3 The role of histamine in sleep-wake regulation (Studies III&IV)

In studies III & IV we examined the role of histamine in sleep-wake regulation in the BF of rats (study III) and in the TMN of mice (study IV).

4.3.1 Histamine levels in the BF and the TMN correlate with wakefulness

To investigate whether histamine release in the BF correlates with vigilance state, we compared the histamine levels in microdialysis samples from the rat BF and the mouse TMN to the amount of wakefulness in the corresponding 30-min bin. We found a strong positive correlation between histamine release in the BF and wakefulness, which was unaffected by the light-dark cycle (III; Fig. 2B & C). In the mouse TMN we found that histamine release shows the highest correlation with wakefulness and that is correlates to a lesser extent with motor activity (IV; Table 3). A similar correlation between cortical histamine levels and wakefulness was previously found (Chu et al., 2004). In addition, Strecker et al. (2002) found that histamine release in the POAH of the cat is highest during wakefulness.

During the light period (sleep period for rodents) the histamine levels in the BF and the TMN were generally low and interspersed with increases during periods of waking (III; Fig. 2C, IV; Fig. 5A & B). This suggests that the potential circadian component in the regulation of brain histamine levels is overruled by vigilance state; in other words, during waking, histamine levels increase regardless of the time of day.

We found that histamine release positively correlated with high theta (7.5-9.5 Hz) and gamma (above 35 Hz) frequencies in the EEG recording, which are associated with active and attentive wakefulness. The extracellular histamine levels did not correlate with EEG markers of quiet wakefulness (4-7 Hz). During wakefulness, histamine release correlated inversely with the EEG delta range (1-4 Hz) that is associated with sleep (IV; Fig. 5C). These results are in line with previous findings showing that the activity of histaminergic neurons is highest during attentive wakefulness (Takahashi et al., 2006).

4.3.2 Histamine in the BF induces attentive wakefulness and increases cortical activity, but is not affected by increased sleep pressure, and does not induce a recovery sleep

In study III we found that histamine perfusion increased wakefulness and reduced NREM sleep (III; Fig. 3), as was already observed by Ramesh et al. (2004). When we examined the EEG power spectra, we found that histamine perfusion in the BF specifically increases attentive wakefulness. Histamine activates the cortex by increasing frequencies that associate with active and attentive wakefulness (high theta; 7-9 Hz), and decreasing frequencies that associate with quiet wakefulness (low theta; 4-7 Hz), drowsiness during wakefulness, or deep sleep during NREM sleep (delta; 0.5-4 Hz, III; Fig. 4). These results are very similar to the frequencies which we found to correlate with natural histamine release in study IV, and agree with the findings of Anaclet et al. (2009), who showed that histamine promotes wakefulness without increasing motor activity in a wheel running task. When we looked at the EEG power during histamine perfusion separately for low and high EMG-activity wakefulness, we found that in both cases the high theta frequency was enriched (III; Fig. 5A & B), and also that the distribution of low and high EMG-activity wakefulness during histamine perfusion did not change. Thus, histamine seems to specifically promote attentive wakefulness and not motor activity.

The theta-enriched cortical activation which is mediated by histamine in the BF does not lead to increased sleep pressure. Because the BF is a key site for the regulation of cortical activation and sleep homeostasis, and is also one of the main targets of histaminergic neurons (Panula et al., 1989), we expected to observe an effect of SD-induced increased sleep pressure on extracellular histamine levels in the BF. In addition, we expected histamine perfusion to lead to increased sleep pressure, due to increased activity of the BF. Contrary to our expectations, we did not observe a progressive increase in extracellular histamine concentrations during the 6-h SD period. Instead, the increase in histamine concentration started immediately at the initiation of the SD, and remained stable throughout the entire SD period (III; Fig. 2A). Because the build-up of sleep pressure during the SD period is gradual, the stable and immediate increase in the amount of extracellular histamine levels indicates that sleep pressure does not affect histamine release in the BF. It has already been shown that sleep pressure does not influence histamine levels in the POAH of the cat (Strecker et al., 2002). Together, these studies strongly indicate that histamine release is not regulated by sleep pressure, and does not transfer information about sleep pressure.

In addition, we did not find a NREM delta rebound after histamine perfusion. Considering the substantial increase in attentive wakefulness which is induced by histamine perfusion in the BF, and the extensive and long-lasting decrease in NREM delta power during the perfusion and its persistence during the first 3-h recovery period for the higher concentrations, this was rather surprising. We also did not find an increase in any other measures of sleep intensity after the histamine perfusions (III; Fig. 6), leading to the conclusion that histamine-induced prolonged wakefulness is not followed by the classic homeostatic response. Previous work also showed no increase in the amount of sleep after BF histamine perfusion (Ramesh et al., 2004) or after i.p. administration of H3R antagonists (Parmentier et al., 2007), but these studies did not examine the NREM EEG delta power, which is important because the increase of NREM EEG delta power is the key characteristic of rebound sleep.

Previous work from our lab shows that several glutamate agonists stimulate the BF and all induce prolonged wakefulness. Only NMDA perfusion increased high theta activity during this extended period of wakefulness, and only after NMDA perfusion a homeostatic response, measured by NREM delta power, was observed, suggesting an association between the homeostatic response and the high level of cortical activation (Wigren et al., 2007). In the present study we found that histamine perfusion led to increased high theta activity during wakefulness, but did not observe a homeostatic response. Histamine and NMDA activate the BF in different ways; histamine depolarizes BF cholinergic neurons and increases their tonic firing (Khateb et al., 1995), while NMDA promotes rhythmic bursting (Khateb et al., 1997). This different way of activating the BF could result in a different build-up of extracellular adenosine and sleep pressure and might be responsible for the lack of NREM delta rebound after histamine perfusion. It is also possible that the increase in theta power (20-30%) was not high enough, or that the increase in the amount of wakefulness was not sufficient to elicit a homeostatic response, although the increase in wakefulness was comparable to that seen after NMDA perfusion.

4.3.3 The cholinergic BF is a key site for histamine to promote cortical activation and wakefulness.

In order to study the importance of the BF in relaying the activity of the histaminergic system to the cortex we decided to use a H1R antagonist (pyrilamine). By using an antagonist we merely blocked the action of physiological histamine levels in the BF, and thus were able to get a better impression of the importance of histaminergic activation of the BF in cortical arousal under physiological conditions. When we antagonized the H1R in the BF, we found a radical decrease in wakefulness (III; Fig. 7a), which was even more surprising because the perfusion was performed unilaterally and reached only one of the

major brain regions that can activate the cortex. In addition, the high theta range in the wakefulness-specific EEG recording, which is associated with attentive wakefulness, was decreased after antagonist perfusion (III; Fig. 7b), showing the importance of histaminergic activation of the BF in maintaining a high level of cortical activation. Ramesh et al. (2004) found that histamine perfusion into the thalamus had no effect on wakefulness or NREM sleep. Together, these results suggest that of the several different ascending and descending pathways which histamine can employ to activate the cortex, the BF is the key site for histamine to promote cortical activation and wakefulness (III; Fig. 9).

The next question we addressed was whether indeed the cholinergic neurons of the BF were involved in mediating the arousal effects of histamine. We know that in guinea pig BF slices cholinergic neurons are activated mainly through H1R (Khateb et al., 1995), and that histamine perfusion in the BF of freely moving rats increased ACh release in the cortex (Cecchi et al., 2001). In contrast, the actions of histamine on GABAergic and glutamatergic neurons in the cortically projecting part of the BF are poorly characterized, and histamine can excite GABAergic neurons in the adjacent MS/DBB that projects to the hippocampus via both H1R and H2R (Xu et al., 2004). We therefore used a unilateral local BF cholinergic lesion approach (Kalinchuk et al., 2008) to investigate the effect of abolishing cholinergic BF neurons on histamine-induced cortical activation. Intraparenchymal injections of the immunotoxin 192IgG-saporin selectively lesion BF cholinergic neurons without affecting the number of parvalbumin- and GAD-immunopositive neurons (Pizzo et al., 1999; Kalinchuk et al., 2008; Kaur et al., 2008). Following the cholinergic lesions we repeated the histamine and H1R antagonist perfusions, and now these perfusions had no significant effect on vigilance state (III; Fig. 8).

Because the method spares the other neuronal types in the BF and because the lesion is local, thereby sparing, for instance, the septo-hippocampal cholinergic system (Berntson et al., 2002), the lack of significant effects on vigilance state after drug perfusion demonstrates that indeed the cholinergic BF neurons mediate histamine-induced cortical arousal.

5 CONCLUSIONS

The main conclusions of this thesis can be summarized as follows:

We found that, although during wakefulness all studied ascending arousal systems increase their transmitter release in the BF, not all of them seem to be involved in the regulation of sleep homeostasis. The orexinergic, serotonergic, and dopaminergic systems show signs that lead us to believe that they are involved in the regulation of sleep pressure, or at least that they are affected by sleep homeostasis. The orexinergic system appears capable of influencing sleep homeostasis, and the dopaminergic and serotonergic systems seem to either counteract or add to sleep pressure. On the other hand, the histaminergic system is neither affected by, nor able to manipulate sleep pressure in the BF and thus shows no signs of being involved in sleep homeostasis mediated by the BF.

In addition, we revealed that theta-enriched cortical arousal mediated by activation of the BF via the ascending arousal systems does not necessarily lead to a homeostatic sleep response. This response appears to depend on the type of activation that is induced in the BF, and is most likely receptor and thus neurotransmitter specific.

Finally, we established that histamine activates the cholinergic BF via histamine 1 receptors and that this activation is essential in sustaining a high level of cortical activation, indicating the importance of the BF in mediating the wake-promoting effects of histamine. Reduced activation of the cholinergic BF by histamine may be important in initiating and maintaining NREM sleep.

6 FUTURE DIRECTIONS

In this thesis the role of some of the ascending arousal systems in the regulation of sleep, wakefulness, and cortical activation mediated by the BF was elucidated. I have looked at certain aspects of several ascending arousal systems and have been able to answer some of the questions at hand; however, many questions remain to be clarified.

Are increased orexin levels indeed responsible for the increased slow delta wave intrusions into the waking EEG during SD, found in study I? Because the neural mechanisms behind these intrusions which could be seen as dissociative states with sleep leaking into wakefulness, are unknown, and because orexin is involved in vigilance state control by playing a crucial role in the "flip-flop" switch, orexin could be an excellent candidate to be involved in this sleep leakage.

We showed that some transmitter systems are affected by increased sleep pressure in the BF, but we still need to explore which of these are involved in increasing BF activity, and which type of neurons they activate most. Does this increased activity lead to increased adenosine concentrations in the BF, and a subsequent homeostatic sleep response? It also remains unclear what kind of activation is necessary to induce a NREM delta response mediated by the BF.

According to our results, the BF might be the main route for histamine to promote wakefulness; this, however, should be further investigated, for instance by stimulating the TMN whilst antagonizing the BF, or after inducing a cholinergic lesion.

The results from this thesis might contribute to the development of new sleep or wake medication. A histamine receptor 3 agonist could be a potent sleep inducing substance, because it can inhibit histaminergic transmission. Clinical trials are already underway. However, the results presented here indicate that inhibiting the histaminergic pathway results in light sleep, and might, therefore, not lead to the desired effect.

Histamine receptor 3 antagonists, that increase the histaminergic transmission, are tested for the treatment of excessive daytime sleepiness in narcolepsy patients and for treatment of ADHD. The results in this thesis show that histamine is very effective in increasing attentive wakefulness, which is beneficial for both groups of patients. Some caution should be taken, because the effect of histamine might last for many hours and thus could negatively influence the subsequent sleep.

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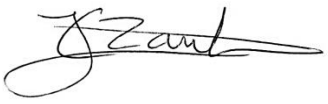

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