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Circadian and Histaminergic Regulation of the Sleep-Wakefulness Cycle

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ACADEMIC DISSERTATION

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1. List of original publications/author contribution

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Rozov S.V. conducted measurements of histamine, participated in reviewing and revising the manuscript

II. Rozov SV, Zant JC, Karlstedt K, Porkka-Heiskanen T, Panula P., Periodic properties of the histaminergic system of the mouse brain. Eur J Neurosci. 2014 Jan; 39(2):218-28.
Rozov S.V. planned and conducted the experiments, together with second author setup and maintained the system for a long-term microdialysis and performed the surgery, analyzed the data and wrote the manuscript

III. Rozov SV, Porkka-Heiskanen T, Panula P., On the role of histamine receptors in regulation of circadian rhythms. PLOS One 12/2015; 10(12):e0144694.
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Rozov S.V. planned and conducted the experiments, together with second author performed surgery and electroencephalographic study, performed the data analysis and wrote the manuscript.

2. Publications that were used in other dissertations

Zant JC, Rozov S, Wigren HK, Panula P, Porkka-Heiskanen T., Histamine release in the basal forebrain mediates cortical activation through cholinergic neurons. J Neurosci. 2012 Sep 19; 32(38):13244-54.

Rozov SV, Zant JC, Karlstedt K, Porkka-Heiskanen T, Panula P., Periodic properties of the histaminergic system of the mouse brain. Eur J Neurosci. 2014 Jan; 39(2):218-28.

3. Abstract

The hypothalamus has a unique position in the regulation of the sleep-wakefulness cycle, because it harbors key sleep-active and wakefulness-active neuronal populations, and the master circadian clock that is located in the suprachiasmatic nuclei. The hypothalamic areas, which are active specifically during wakefulness, include the hypocretinergic and histaminergic neurons that, in conjunction with monoaminergic nuclei of the brainstem and basal forebrain centers, constitute the ascending arousal system (AAS). The AAS in combination with the nuclei of preoptic area, and the circadian clock form a complex network that regulate the sleep-wakefulness cycle. Moreover, the maintenance of homeostasis that requires an adjustment of the amount of sleep depending on preceding wakefulness, physical or mental activity and health conditions, significantly contributes to the regulation of this cycle.

The current thesis project aimed at the assessment of the separate contributions of the circadian system and the histaminergic neurons in the regulation of the sleep-wakefulness cycle, and estimation of the degree of interaction between these systems.

The impact of the circadian process was examined by subjecting the animals to a short symmetric light-dark cycles that are close to or beyond the entrainment range of the mouse circadian system. We used electroencephalography in combination with behavioral analysis to quantify changes in spontaneous locomotion, wakefulness, non-rapid eye movement sleep and rapid eye movement sleep stages in response to an altered pacemaker rhythm.

We estimated the daily changes of the histaminergic system by using radioactive *in situ* hybridization, and by assaying activities of the key enzymes involved in the histamine metabolism, namely: histidine decarboxylase and histamine-N-methyltransferase. We also used *in vivo* microdialysis to assess diurnal profile of histamine release from the posterior hypothalamus.

In order to understand the possible role of the histaminergic regulation of circadian system functioning we utilized mouse strains that constitutively lack histamine 1 and 3 receptors.

We used *in vivo* microdialysis in combination with sleep deprivation to understand the histaminergic regulation of homeostatic sleep propensity.

We found that the shortening of the light-dark cycle significantly affected the distribution of sleep and wakefulness states, increased sleep propensity, diminished daily rhythms of power

of high θ-, and γ-waves and strengthened the phase-amplitude coupling between these frequencies. The 24-hour rhythms of production and release of histamine and its metabolite, 1-methylhistamine were detected, whereas activities of the enzymes had no detectable diurnal rhythm. In addition, histamine release was highly positively correlated with changes in the power of the θ-frequency range, which is characteristic of an active wakefulness state. Histamine release had significant negative correlation with the power of the δ- band, which is typical for drowsiness and non-rapid eye movement sleep.

The lack of histamine 1 receptor had no effects on the circadian rhythm of spontaneous locomotion, but the knockout of the histamine 3 receptor resulted in a substantial reduction of free-running activity rhythm amplitude. Remarkably, the expressions of the core clock genes, *Per1*, *Per2* and *Bmal1,* in the suprachiasmatic nuclei of the hypothalamus that house central circadian oscillator, the cerebral cortex and the striatum were not affected in these knockout models.

During 6-hours of sleep deprivation, the histamine release was constantly upregulated and comparable to its level during wakefulness, whereas when the sleep deprivation ceased, the release of histamine immediately dropped to the baseline level. Constant administration of histamine into the basal forebrain region caused a significant increase in the high θ- and γ-range power throughout infusion period, but the δ-wave activity during non-rapid eye movement slow-wave sleep remained similar to those of the control.

We conclude from our data that the circadian process may affect homeostatic regulation, thus indicating an interaction between rather than the mutual independence of these two processes. The circadian process also affects the histaminergic system albeit mainly at the level of histamine accumulation and release. The histaminergic regulation of the circadian system functioning remains elusive, and it needs further investigation especially in relation to histamine-3 receptor-mediated signaling. Finally, we could not find evidence of an involvement of the histaminergic system in homeostatic regulation of sleep-wakefulness cycle, since it neither affects, nor is affected by this process.

4. Abbreviations

-/-	homozygous knockout allele
+/+	homozygous wild type allele
AAS	ascending arousal system
ATP	Adenosine-5′-triphosphate
BF	Basal forebrain
BMAL1	Brain and Muscle ARNT-Like 1 basic helix-loop-helix/PAS protein
cAMP	cyclic adenosine monophosphate
CCF	cross-correlation function
CLOCK	Circadian Locomotor Output Cycles Kaput
CREB	Cyclic Adenosine Monophosphate Response Element Binding
CRY1	cryptochrome 1
CRY2	cryptochrome 2
DD	dark-dark schedule
DMH	dorsomedial nucleus of the hypothalamus
DR	dorsal raphe nucleus
EEG	electroencephalography
EMG	electromyography
GABA	γ-aminobutyric acid
$GABA_{A\gamma2}$	γ-aminobutyric acid Aγ2 receptor
$GABA_{B1}$	γ-aminobutyric acid B1 receptor
HCRTR1	hypocretin 1 receptor
HCRTR2	hypocretin 2 receptor
HDC	histidine decarboxylase
HNMT	histamine n-methyltransferase
HPLC	high pressure (performance) liquid chromatography
HRH1	histamine receptor 1
HRH2	histamine receptor 2
HRH3	histamine receptor 3
HRH4	histamine receptor 4
HT	hypothalamus
i.p.	intraperitoneal

LH	lateral hypothalamus
LC	locus coeruleus
LD	light-dark schedule
LDT	laterodorsal tegmental nucleus
MCH	melanin concentrating hormone
MnPO	median preoptic area of the hypothalamus
NMDA	N-methyl-D-aspartate
NPAS2	Neuronal PAS domain protein 2
NREM	non-rapid eye movement sleep
PAC	phase-amplitude coupling
PACAP	pituitary adenylate cyclase activated peptide
PER1	period 1
PER2	period 2
PPT	pedunculopontine tegmental nucleus
PSD	power spectral density
REM	rapid-eye movement sleep
REV-ERBα	nuclear receptor subfamily 1, group D, member 1 protein
RNA	ribonucleic acid
RORα	retinoic acid orphan receptor α
SCN	suprachiasmatic nucleus
SD	sleep deprivation
SN	substantia nigra
SPVZ	subparaventricular zone of the hypothalamus
s.c.	sub-cutaneous
SWA	slow wave activity
TMN	tuberomamillary nucleus
VLPO	ventrolateral preoptic nucleus
vPAG	ventral periaqueductal grey
VTA	ventral tegmental area
ZT	zeitgeber time

5. Literature review: sleep-wakefulness cycle

5.1 Overview

Early attempts at searching for the regulatory mechanisms of sleep revealed that the cerebrospinal fluid obtained from sleep-deprived dogs caused sleep in non-sleep deprived animals, which provided the first experimental evidence of the humoral regulation of sleep (Legendre & Pieron, 1913). A decade later the role of particular brain regions in the regulation of sleep and wakefulness was reported by von Economo, who found that patients with lesions of the preoptic area near the rostral end of the third ventricle suffered from severe insomnia (von Economo, 1926). His observations were later elaborated by Nauta (1946) who performed experimental lesions of different parts of the hypothalamus on rats and located a wakefulness-promoting center in the posterior hypothalamus and a sleep-promoting area in the anterior hypothalamus. Subsequent studies found the basal forebrain (BF), pontine tegmentum and the posterior hypothalamus to be the main wakefulness-active centers, whereas the preoptic area remained a principal sleep-active region. These centers in conjunction with the circadian and homeostatic regulation processes form a complex network to control sleep and wakefulness.

Although the regulatory mechanisms mentioned above play important and complementary roles in the maintenance of cortical wakefulness, a detailed description will be provided only for those studied in the original manuscripts included in this thesis, namely, the histaminergic and circadian regulation.

5.2 Sleep-active centers

Sleep is a collective term for a number of naturally occurring states that are characterized by largely inhibited sensory inputs, loss of consciousness, suppressed motor activity and, unlike hibernation or coma, rapid reversibility to wakefulness. The appearance of sleep is species-specific, but generally, it follows a circadian rhythm (Campbell & Tobler, 1984). Sleep can be divided into the non-rapid eye movement (NREM) and rapid eye movement (REM) phases, each of which is characterized by distinct regulatory mechanisms, that in turn lead to state-specific patterns of cortical activity, as can be recorded by methods such as electroencephalography (EEG; Lancel, 1993).

NREM sleep is characterized by a reduced muscle tone, high amplitude slow wave (1-4 Hz, δ-waves) activity (SWA) and presence of transient 12-15 Hz (α-waves) EEG oscillations, sleep

spindles. The complexity of an NREM state depends on the species: in the human and related primates it can be divided into three stages based on its depth whereas in rodents this subdivision is not typical (Dijk, 2009). The onset and the maintenance of NREM sleep is closely related to an elevation of activity in the ventrolateral preoptic (VLPO) and the median preoptic (MnPO) areas of the hypothalamus (Sherin *et al.*, 1996; Suntsova *et al.*, 2002). Lesions of the VLPO area cause permanent loss of about 50% of NREM and REM states, which thus shows the importance of this area for the regulation of sleep (Lu *et al.*, 2000).

Most of the neurons in the VLPO contain galanin and γ-aminobutyric acid (GABA) and have reciprocal inhibitory projections into the brain regions that are responsible for maintenance of the wakefulness state – the BF, the lateral hypothalamus (LH), the tuberomamillary region (TMN), the ventral tegmental area (VTA), the locus coeruleus (LC), the dorsal raphe nucleus (DR) and the pedunculopontine areas (Fig. 1B, Sherin *et al.*, 1996, 1998; Chou *et al.*, 2002). The MnPO neurons are GABA-ergic and mainly project into the VLPO-, LH-, DR- and LC-areas, but not into the TMN and the pedunculopontine tegmental nucleus (PPT) (Uschakov *et al.*, 2007). Several endogenous sleep-promoting substances, such as adenosine and prostaglandin D2 are known to have an excitatory effect on the VLPO (Ueno *et al.*, 1982; Morairty *et al.*, 2004; Gallopin *et al.*, 2005), which implicates the VLPO a site at which the homeostatic, circadian and ascending arousal system (AAS) efferent projections converge.

REM sleep is characterized by a complete muscle atonia, rapid eye movements, vivid dreams and a predominance of 7-9.5 Hz (high θ-waves), >30 Hz (γ-waves) and neuronal oscillations as monitored by EEG. During REM-sleep EEG activity resembles that of wakefulness, but most of the wakefulness-promoting neuronal clusters, except for groups of cholinergic neurons in the PPT and the BF remain completely inactive (Aserinsky & Kleitman, 1953; Hobson *et al.*, 1975; Steininger *et al.*, 1999).

5.3 Wakefulness-active centers (with focus on the histaminergic system)

Wakefulness is a vigilance state that is characterized by cortical activation (desynchronization) registered by EEG as low amplitude high frequency cortical oscillations (θ- and γ-waves), and activated sensory inputs, motor activity and consciousness (Lancel, 1993). Based on findings that the electrical stimulation of the rostral pons and the caudal midbrain causes an EEG desynchronization similar to wakefulness, Magoun and co-workers (Lindsley *et al.*, 1949; Moruzzi & Magoun, 1949) introduced the concept of the ascending reticular arousal system. Later studies identified distinct neuronal clusters within these brain areas that send extensive projections to the cerebral cortex via the thalamic and hypothalamic/BF relays. The activities of many of these clusters are coupled to wakefulness. Studies on the mechanisms of the

cortical activation revealed several sub-cortical areas directly involved in wakefulness: the monoaminergic nuclei of the brainstem, the thalamus, the medial and intralaminar nuclei; the BF and the posterior hypothalamus. These neuronal populations form a net-like structure and can be generalized as the AAS.

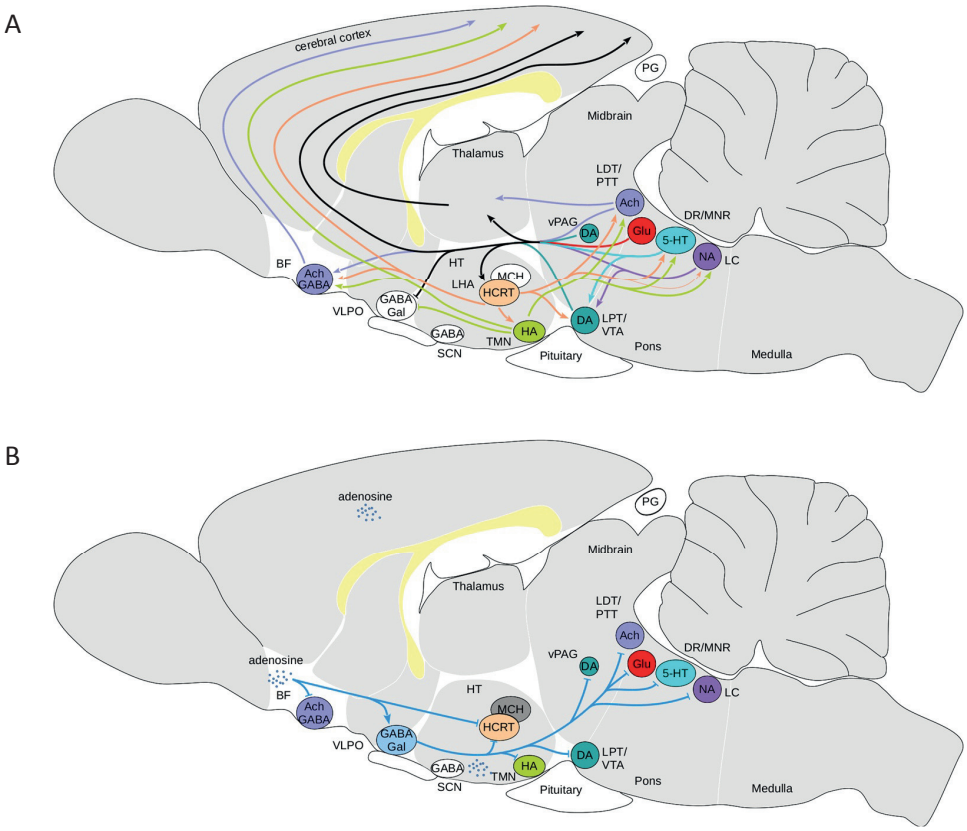

Fig. 1 Diagram of Mouse Brain showing main (A) wakefulness- and (B) NREM-sleep promoting pathways. Brain regions: BF - basal forebrain, DR - dorsal raphe nucleus, HT - hypothalamus, LDT - laterodorsal tegmental nucleus, LC - locus coeruleus, MNR - medial raphe nucleus, MCH - melanin concentrating hormone-containing neurons, HCRT – hypocretinergic neurons, PPT - pedunculopontine tegmental nucleus, PG - pineal gland, SN - substantia nigra, SCN - suprachiasmatic nucleus of hypothalamus, TMN - tuberomamillary nuclei of hypothalamus, vPAG - ventral periaqueductal grey matter, VTA - ventral tegmental area, VLPO - ventrolateral preoptic nucleus. Neurotransmitters: Ach - acetylcholine, GABA - γ-aminobutyric acid, GAL - galanin, GLU - glutamate, HA - histamine, 5-HT - 5-hydroxytryptamine, MCH - melanin concentrating hormone, NA – noradrenaline (figure modified from Richter *et al.*, 2014).

5.3.1 Midbrain/pons

The PPT and the laterodorsal tegmental nucleus (LDT) are heterogeneous cell groups that mainly consist of cholinergic neurons (Fig. 1A). They send efferent projections to the thalamic relay nuclei, the medial and intralaminar nuclei and thus constitute the dorsal pathway. A smaller bundle innervates the BF, and hence the PPT and LDT participate in the ventral pathway (Satoh & Fibiger, 1986; Hallanger *et al.*, 1987). These neurons are silent during NREM sleep, but start firing just before a transition from NREM to REM or wakefulness and stay active during the latter two stages (el Mansari *et al.*, 1989).

The noradrenergic neurons of the LC and the serotoninergic neurons of the DR and medial raphe nuclei are located adjacent to the cholinergic neuronal clusters but send their axons mainly through the ventral pathway (Fig. 1A). Both of these groups are silent during REM sleep and have decreased activity throughout NREM sleep and tonically discharge during wakefulness. Upon transition from NREM to wakefulness, the noradrenergic neurons become active before the onset of cortical activation (Aston-Jones & Bloom, 1981; Steininger *et al.*, 1999; Kocsis *et al.*, 2006; Takahashi *et al.*, 2010). Interestingly, a selective pharmacological lesion of the noradrenergic neurons of the LC did not cause any deficit in waking (Jones *et al.*, 1977). Likewise, a constitutive knockout of the noradrenaline-producing enzyme, dopamine β-hydroxylase, caused only a decreased latency of wakefulness-NREM transition after stressful stimuli, but the sleep-wakefulness cycle appears normal (Hunsley & Palmiter, 2003, 2004). Although the serotonergic neurons are the most active during wakefulness, the attempts to reveal their role in regulation of sleep-wakefulness cycle produced conflicting results. On the one hand, a lesion of the DR area or a pharmacological inhibition of serotonin production causes insomnia, which suggests either an indirect effect of serotonin on cortical activation or a role in the accumulation of sleep pressure (Petitjean *et al.*, 1985). Moreover, mice that lacked the brain serotonin-producing enzyme, tryptophan hydroxylase 2, had only minor alterations of their sleep-wakefulness cycle compared to their wild type counterparts (Savelieva *et al.*, 2008). On the other hand, depletion of tryptophan, a precursor of serotonin, caused either a complete disappearance (Nakamaru-Ogiso *et al.*, 2012) or an alteration (Kawai *et al.*, 1994) of the circadian cycle of sleep and wakefulness, although the total amount of sleep did not change.

The dopaminergic neurons are mainly located in the VTA, the substantia nigra (SN) and the ventral periaqueductal grey (vPAG) and the majority of them show equally low firing rates across the sleep and wakefulness states. Although all these cell groups project into the BF and further onwards into the cerebral cortex, only a subpopulation of dopaminergic neurons in

the vPAG was found to be specifically active during wakefulness. The lesion of the vPAG leads to a persistent increase of the total sleep time (Lu *et al.*, 2006) while retaining an intact circadian rhythm, whereas lesions of the VTA and the SN induce akinesia, but not a deficiency of the cortical activation (Jones *et al.*, 1973), which suggests differential roles of these neuronal groups in the maintenance of wakefulness.

5.3.2 Thalamus

The cortex receives the most abundant glutamatergic input from the thalamus and the latter is, in turn, innervated by all the structures involved in the maintenance of wakefulness (Jones & Leavitt, 1974). Therefore, the thalamus has been suggested as the main center involved in cortical activation (reviewed in Llinás & Steriade, 2006). However, lesions of some of the nuclei of this brain area in rats and cats had only transient effects on cortical activation (Buzsaki *et al.*, 1988; Vanderwolf & Stewart, 1988). Thus, the data regarding the role of the thalamus in the regulation of the sleep-wakefulness cycle are conflicting and require further investigation.

5.3.3 Basal forebrain

The BF (including the substantia innominata, the diagonal band of the Broca and the magnocellular preoptic area) plays a multimodal role in the regulation of sleep and wakefulness. It receives multiple excitatory inputs from the downstream AAS and serves as a relay between the AAS and the cerebral cortex (Semba *et al.*, 1988; Panula *et al.*, 1989; Peyron *et al.*, 1998). The BF consists of neurons that are active during NREM-, REM-, wakefulness-states or combinations of these (Szymusiak and McGinty, 1986; Takahashi *et al.*, 2009). These cells include partially overlapping populations of GABA-, acetylcholine- and glutamatergic neurons (Manns *et al.*, 2001). Cholinergic neurons send broad excitatory projections to the cerebral cortex and the limbic system (Rye *et al.*, 1984; Saper, 1984). These neurons are mainly active during wakefulness and REM-sleep during which they generate the θ-rhythm and are thought to modulate the cortical θ-activity (Lee *et al.*, 2005). GABA-ergic cells participate in cortical (Freund & Meskenaite, 1992; Henny & Jones, 2008) and thalamic projections (Asanuma & Porter, 1990) with a fraction of them being active during wakefulness whereas the other GABA-ergic cells are active during NREM sleep (Modirrousta *et al.*, 2004; Takahashi *et al.*, 2009). The glutamatergic cells of the BF primarily innervate the cortical interneurons (Henny & Jones, 2008). Although lesions of the BF with ibotenic acid cause an increase of SWA (Buzsaki *et al.*, 1988), a selective destruction of about 70% of cholinergic

neurons with saporin-Ig192 only transiently increased NREM sleep and had no effect on the power spectral density (PSD) distribution (Kaur *et al.*, 2008).

5.3.4 Hypothalamus

The hypothalamus has a unique position in the regulation of sleep-wakefulness cycle, as it harbors key sleep-active neuronal populations - the VLPO, the MnPO, the melanin concentrating hormone -positive cells of the LH; wakefulness-active cell groups including the hypocretinergic neurons (HCRT) of the LH, the histaminergic neurons of the TMN and the master circadian clock, located in the suprachiasmatic nuclei (SCN).

The HCRT-ergic neurons are predominantly wakefulness-active, though they start generating action potentials several seconds before wakefulness occurs and stay silent during sleep (Lee et al., 2005; Mileykovskiy *et al.*, 2005). The HCRT-ergic neurons receive projections from the preoptic area, the DR, LC in addition to the medial prefrontal cortex and the central nucleus of the amygdala (Yoshida *et al.*, 2006). The HCRT-ergic cells also contain dynorphin (Chou *et al.*, 2001) and glutamate, both of which are suggested to facilitate the excitatory action of the hypocretins (Schöne *et al.*, 2014). The effects of the hypocretins are mediated via the hypocretin receptors 1 and 2 (HCRTR1 and HCRTR2) that are found throughout the brain (Trivedi *et al.*, 1998; Marcus *et al.*, 2001). The pattern of *Hcrtr1* and *Hcrtr2* expression resembles that of the HCRT-ergic innervation, which includes the wakefulness-promoting centers, the TMN (specifically, histaminergic neurons), the LC and, to a lesser extent, the BF, the brain stem and the cortical regions (Peyron *et al.*, 1998). The notion that the HCRT-ergic neurons are involved in the regulation of sleep and wakefulness is based on their projections into the key wakefulness-promoting cell groups and this is supported by gain- and loss-of-function models. In the former case a local administration of HCRT-A to the posterior hypothalamus (Huang *et al.*, 2001; Yamanaka *et al.*, 2002) or a stimulation of the HCRT-ergic cells through artificially expressed channelrodopsin-2 using an optogenetic approach (Adamantidis *et al.*, 2007) were found to significantly increase the time spent awake and suppresses the NREM and REM sleep phases. In the latter case, subjects with an altered HCRT-ergic signalling, such as humans that lacked HCRT-ergic cells (Peyron *et al.*, 2000; Thannickal *et al.*, 2000), HCRT-deficient mice (Chemelli *et al.*, 1999) and HCRTR2- deficient dogs (Lin *et al.*, 1999) developed narcolepsy. This state is characterized by a permanent fragmentation of sleep and wakefulness including REM-sleep intrusions accompanied by a muscle atonia and a deficient waking under an emotional stress or motor challenge conditions. It is not, however, accompanied by overall alterations in total time spent awake, or disturbances in a circadian rhythms of sleep-wakefulness cycle (Mochizuki *et al.*, 2004).

These and other observations suggest that HCRT-ergic neurons play a central role in the stabilization of wakefulness state as discussed in a publication by Saper and colleagues (Saper *et al.*, 2010).

5.3.4.1 Histamine

The involvement of histamine in the maintenance of wakefulness was recognized in the 1960s, when it was found to induce cortical arousal (Bovet *et al.*, 1958; Monnier *et al.*, 1967) and the use of anti-allergic drugs, known to antagonize histamine's action by blocking the histamine H1 receptor (HRH1), was shown to cause somnolence (Jäättelä *et al.*, 1971; Bye *et al.*, 1974; Peck *et al.*, 1975). Neuroanatomical studies conducted in the 1980s located histaminergic neurons in the TMN of the hypothalamus (Panula *et al.*, 1984; Watanabe *et al.*, 1984). These neurons were found to be connected to most of the sleep and wakefulness-promoting brain areas and to the cerebral cortex (Panula *et al.*, 1989), which provides further evidence for its wakefulness-promoting role. In this respect, either the histamine deficiency (Kiyono *et al.*, 1985; Parmentier *et al.*, 2002) or its uncontrollable release or inactivation (Lin *et al.*, 1988; Yu *et al.*, 2015) lead to prominent alterations of wakefulness, which points on the necessity to understand regulatory mechanisms of histaminergic neurons.

5.3.4.1.1 Cytoarchitecture of the histaminergic system

The number of histaminergic neurons in the adult brain of vertebrates varies between species and ranges from only 40-45 in zebrafish (Sundvik & Panula, 2012) to about 64 000 in humans (Airaksinen *et al.*, 1991). Histaminergic neurons within the TMN can be roughly divided into five clusters (E1-E5) all of which are interconnected by scattered neurons (Inagaki *et al.*, 1990). In addition, a small group of the histamine-containing cells was found in the SCN. These cells do not produce L-histidine decarboxylase (HDC), which is the only enzyme in the nervous system that synthesize histamine, therefore, the origin of histamine in these neurons remains elusive (Michelsen *et al.*, 2005). Although histamine is the principal neurotransmitter synthesized by the histaminergic neurons, other substances that are known or supposed to participate in a signal transduction, including endomorphine-1 (Greco *et al.*, 2008), GABA (Vincent *et al.*, 1983; Senba *et al.*, 1985; Ericson, Köhler, *et al.*, 1991), galanin (Köhler *et al.*, 1986; Staines *et al.*, 1986), thyrotropin releasing hormone (Airaksinen *et al.*, 1992) and L-3,4-dihydroxyphenylalanine (Yanovsky *et al.*, 2011) can be found in histaminergic neurons. In addition to these markers, a set of receptors expressed by these neurons was characterized (Yang & Hatton, 1997; Sergeeva *et al.*, 2003; Uteshev & Knot, 2005; Yanovsky *et al.*, 2011). Sergeeva and colleagues (Sergeeva *et al.*, 2002) distinguished at least three distinct classes of histaminergic neurons based on the repertoire of the $GABA_A$ receptor subunits, expressed by

these cells. Thus, taking into account functional heterogeneity of the histaminergic cells (Miklós & Kovács, 2003; Giannoni *et al.*, 2009) and limited knowledge about their molecular phenotype, further division based on functional and biochemical properties cannot be ruled out.

5.3.4.1.2 Regulatory mechanisms

The histaminergic neurons generate periodic action potentials at a 1-4 Hz frequency in isolated tissue explants or in the absence of any excitatory stimuli in anesthetized animals (Reiner & McGeer, 1987; Haas & Reiner, 1988). These cells *in vivo* start the generation of the action potentials at about 400 ms after registration of cortical wakefulness, then exhibit a variety of firing patterns depending on the activity state and become completely silent just before the cortical synchronization occurs (Vanni-Mercier *et al.*, 2003; Takahashi *et al.*, 2006). In addition to the high frequency oscillations, *in vivo* microdialysis experiments on anesthetized or freely-moving animals revealed slow rhythms of histamine release that ranged from 1.6 h (Prast *et al.*, 1997) to 24 h (Mochizuki *et al.*, 1992). This suggests that several superimposed processes, such as afferent inputs from the other brain areas, hormonal and metabolic signals, e.g. CO_2 and glucose concentrations participate in the regulation of the physiological state of the histaminergic neurons. The histaminergic neurons were hitherto predominantly studied with respect to their role in histamine-mediated signaling, thus the emphasis in this dissertation will be mainly laid on the actual regulatory mechanisms of histamine metabolism.

The first rate-limiting step of histamine biosynthesis is the availability of its precursor, the essential amino acid L-histidine. It is taken up from the blood by active L-amino acid transporters (Yamakami *et al.*, 1998) and decarboxylated by the HDC (Waton, 1956) to form histamine. In the adult mammalian brain the HDC protein was found exclusively in the cytoplasm of the TMN neurons (Hayashi *et al.*, 1984). The promotor region of the *Hdc* gene contains binding sites for multiple transcription factors, which points to its complex regulation. These include an e-box element also known as E/E' box (Yu *et al.*, 2014), glucocorticoid receptor-like sequence (Zahnow *et al.*, 1998), Pituitary adenylate cyclase activated peptide (PACAP) -response element (McLaughlin *et al.*, 2004), GC-box, gastrin-response elements (Ai *et al.*, 2004) and myeloblastosis proto-oncogene - binding motif (Nakagawa *et al.*, 1997). However, the specific roles of these transcription factors in the regulation of the *Hdc* gene, apart from the e-box element, remain unknown. The brain and muscle ARNT-Like 1 basic helix-loop-helix/PAS protein (BMAL1) transcription factor is known to participate in the circadian regulation of the clock-controlled genes and interacts with e-

box sequence, which results in a periodic expression of the *Hdc* gene (Yu *et al.*, 2014). Studies that used a cell culture approach revealed that methylation might control the expression of the *Hdc* gene (Suzuki-Ishigaki *et al.*, 2000).

Upon synthesis the HDC protein is activated by a truncation of its C-terminus, which yields the functional enzyme (Fleming *et al.*, 2004). Pharmacological inhibition of the HDC enzyme leads to reduced wakefulness (Kiyono *et al.*, 1985) and suppresses the circadian rhythms of adrenocorticotropic hormone and corticosterone (Itowi *et al.*, 1989). The circadian rhythm of spontaneous locomotion is significantly longer in mice consituitively knockout for the *Hdc* gene than that of the wild type counterparts (Parmentier *et al.*, 2002). These knockout mice have an altered expression of the genes that constitute the circadian clock, *Per1* and *Per2* in the striatum and the cerebral cortex, although the expression of *Bmal1* remains intact (Abe *et al.*, 2004). The activity of the HDC protein in the rodent hypothalamus shows near-12 h changes (Tuomisto & Tuomisto, 1982) whereas the data in the literature regarding the total histamine level in different brain areas are controversial. Experiments performed on rats show peaks in the hypothalamic histamine levels during the inactivity period (Orr & Quay, 1975), whereas other studies found histamine levels to be either high in the activity period (Tuomisto & Eriksson, 1982) or constant throughout the day (Kobayashi & Kopin, 1974). Synthesized histamine is packed into the vesicles by vesicular monoamine transporter 2 (Merickel & Edwards, 1995) and stored primarily in the axonal varicosities prior to release.

The excitatory state of the histaminergic neurons is controlled by autoinhibitory inputs from the cell itself and from the neighboring histaminergic neurons (Arrang *et al.*, 1983; Prast *et al.*, 1994; Morisset, Rouleau, *et al.*, 2000). It is also modulated by afferent inputs from the cerebral cortex, and the lateral preoptic area (Ericson et al., 1991; Yang & Hatton, 1997). The glutamatergic excitatory inputs from the LH and the lateral preoptic area exert their effect via the NMDA and AMPA receptors (Yang & Hatton, 1997). The cholinergic neurons of the mesopontine tegmentum area activate the histaminergic neurons by means of α7-type nicotinic receptors (Uteshev & Knot, 2005). The serotonergic neurons of the DR also have an excitatory effect on the histaminergic neurons mediated by 5-HT_{2C} receptors (Eriksson *et al.*, 2001). The HCRT-ergic neurons of the LH send multimodal axons to the TMN, as described earlier. The extent of the cooperation between histaminergic and the HCRT-ergic neurons is currently the subject of debate. Several studies have shown that the effects of hypocretin A on wakefulness were completely blocked in animals that have a dysfunctional HRH1 receptor (Huang *et al.*, 2001; Yamanaka *et al.*, 2002), whereas other authors could not confirm it (Hondo *et al.*, 2010) or report the ability of the hypocretinergic neurons to maintain wakefulness without a functional HDC (Carter *et al.*, 2009).

The GABA-ergic projections come from the VLPO, which is considered the main inhibitory input (Steininger *et al.*, 2001) and the melanin concentrating hormone-positive cells of the LH (Jego *et al.*, 2013). The histaminergic cells express a number of $GABA_A$-receptor subunits (Sergeeva *et al.*, 2002). However, a recent study on mice with the histaminergic neuron-specific knockouts for $GABA_A\gamma_2$ or $GABA_{B1}$ subunits showed no alterations in sleep and wakefulness compared to their wild type counterparts, which suggest that GABA-inhibitory input to be dispensable in the regulation of wakefulness to sleep transition (Zecharia *et al.*, 2012).

Histamine is rapidly inactivated by histamine-N-methyltransferase (HNMT) upon release (Bowsher *et al.*, 1983; Hough *et al.*, 1986; Yoshikawa *et al.*, 2013) to yield 1-methylhistamine. The expression of the *Hnmt* gene is constant over 24 h and its activity is evenly distributed across the brain. 1-methylhistamine is further oxidized by monoamine oxidase B to produce *tele*-methylimidazole acetaldehyde, an unstable product that is subsequently catabolized by aldehyde dehydrogenase to form the end product *tele*-methylimidazole acid (Hough & Domino, 1979; Lin *et al.*, 1993).

5.3.4.1.3 Efferent projections and signaling

The efferent projections of the histaminergic neurons span the entire volume of the brain (Watanabe *et al.*, 1984; Panula *et al.*, 1989). They comprise processes to the forebrain through the dorsal and ventral ascending pathways and to the brain stem via the descending route. The density of the histaminergic fibers vary depending on the brain region. It is at the highest in the hypothalamus and at the lowest in the thalamus. The TMN itself has the densest network of the histaminergic processes, followed by the retrochiasmatic area, the supraoptic nucleus, the periventricular nucleus and the SCN of the hypothalamus, whereas the preoptic area, the paraventricular, dorsomedial and ventromedial nuclei receive moderate innervation (Panula *et al.*, 1989). Among extrahypothalamic targets, the BF, the SN and the VTA have high densities of the histaminergic fibers, and the olfactory bulb, the cerebral cortex (mainly the superficial layers), the hippocampus and the mesencephalic reticular areas receive moderate innervation. Interestingly, some of the processes of the histaminergic neurons are not myelinated (Hayashi *et al.*, 1984) and often do not form synapses (Takagi *et al.*, 1986), which suggests the involvement of histamine in volume transmission rather than the more traditional role of a neurotransmitter. Although the projections of the histaminergic neurons mostly overlap and early studies considered the TMN as a single functional unit (Ericson *et al.*, 1987), later reports pointed to their functional heterogeneity in response to stress stimuli or pharmacological treatments (Miklós & Kovács,

2003; Giannoni *et al.*, 2009), which, in combination with recent findings on the local effects of GABA (Yu *et al.*, 2015) and endomorphine-1 (Greco *et al.*, 2008), produced by the histaminergic cells, led to the hypothesis that different efferent projections may utilize a different subset of transmitters (reviewed in Blandina *et al.*, 2012).

The histamine-mediated signaling is carried out by four types of rhodopsin-like G-coupled receptors (Hill *et al.*, 1997), three of which (HRH1-3) are found in the central nervous system (Martinez-Mir *et al.*, 1990). The expression and distribution of HRH1 receptors in the brain is species-dependent (Chang *et al.*, 1979) and does not always match with the pattern of the histaminergic innervation. The distribution of HRH1 receptor in the rodent brain is high in the hypothalamus, the cortex, the BF, cholinergic and aminergic nuclei of the brain stem and the thalamus (Palacios *et al.*, 1981; Inoue *et al.*, 1996). The HRH1 is coupled to $G\alpha_{q/11}$ proteins, thus its signaling can be convergent with the other transmitter systems that utilize this type of G-proteins (Brown *et al.*, 2002). The signaling includes a phospholipase C - mediated stimulation of diacylglycerol and inositol-3-phosphate production, followed by an activation of Ca^{2+} release from the endoplasmic reticulum and activation of diacylglycerol - sensitive protein kinase C respectively (Leurs *et al.*, 1994). In addition to high expression in the regulatory centers of sleep and wakefulness and early reports on a sedative effect of HRH1 antagonists, subsequent pharmacological and the constitutive *Hrh1* gene knockout mouse studies added more support to the involvement of HRH1 in the regulation of sleep, wakefulness and circadian rhythms. Topical injection of the HRH1 agonist, 2-thiazolylethylamine, into the pars tuberalis of cats produced long-lasting arousal of the cerebral cortex, whereas the effect of a similar injection of histamine was attenuated by the HRH1 antagonist pyrilamine (Lin *et al.*, 1996). Excitatory action of histamine on the explants of the basal forebrain of rats was efficiently inhibited by pyrilamine (Khateb *et al.*, 1990). Systemic or topical injections of this compound into the lateral part of posterior hypothalamus caused induction of slow wave sleep (Lin *et al.*, 1988). Rats housed under 12 h light: 12 h dark schedule (LD12/12) and administered with pyrilamine had attenuated amplitude of the locomotor activity rhythm (Lozeva *et al.*, 2000) and changes in the feeding rhythm (Doi *et al.*, 1994). Several studies on *Hrh1*$^{-/-}$ mice reported an altered feeding rhythm (Masaki *et al.*, 2004) and a minor decrease in an overall locomotor activity (Inoue *et al.*, 1996; Huang *et al.*, 2001), whereas no changes were found by Yanai *et al.*, (1998).

The HRH2 receptor is mainly expressed in the cerebral cortex, amygdala, hippocampus, basal ganglia and the thalamus, whereas moderate to low expressions of the receptor are found in the areas associated with regulation of wakefulness and sleep, namely the pontine tegmentum, BF and the hypothalamus (Ruat *et al.*, 1990). The HRH2 is a constitutively active

receptor (Smit *et al.*, 1996), coupled to $G\alpha_S$-protein (Traiffort *et al.*, 1992). Its activation triggers the accumulation of cAMP that activates protein kinase A leading to phosphorylation of CREB and other transcription factors (Selbach *et al.*, 1997). Research findings about the involvement of the HRH2 receptor in the regulation of the sleep-wakefulness cycle in animal models are equivocal. The HRH2 agonist, impromidine, when topically injected into the preoptic area of a cat caused an increase of wakefulness (Lin *et al.*, 1994), whereas it had no effect if injected into the pontine tegmentum. Systemic injection of the brain-penetrating Hrh2 antagonist, zolantidine, had no effect on the light-induced phase shift (Eaton *et al.*, 1996) or a total amounts or distribution of NREM, REM and wakefulness states in rats (Monti *et al.*, 1990). Although the *Hrh2*-knockout mouse model is available (Kobayashi *et al.*, 2000), it is still poorly characterized in terms of the sleep-wakefulness cycle and the circadian rhythm.

The *in situ* hybridization and autoradiographic studies revealed a difference between the expression of Hrh3 gene and receptor binding patterns of HRH3 receptor in the rat brain. That is the *Hrh3* gene is highly expressed in layer 5 of the cerebral cortex, the thalamus, caudate putamen, nucleus accumbens, CA1 area of hippocampus, TMN, VMH, LC, DR and the SN, whereas little or no receptor binding was present in the layer 5 of the cerebral cortex, the thalamus, CA1 area of hippocampus, LC and the DR (Drutel *et al.*, 2001; Pillot *et al.*, 2002). These findings may be explained by the fact that HRH3 receptor can be located on pre- and post-synaptic membranes and serves as an autoreceptor, that regulates the release of histamine (Morisset, Rouleau, *et al.*, 2000) in addition to being a heteroreceptor that participates in the release of other transmitters (Brown & Reymann, 1996; Yamamoto *et al.*, 1997; Schlicker *et al.*, 1999). Several isoforms of the HRH3 receptor have been described, each of which has a specific expression pattern across the brain (Drutel *et al.*, 2001).

The HRH3 protein is a constitutively active receptor, coupled to $G_{i/o}$-proteins, which negatively links this receptor sub-type to P- and N-type of Ca^{2+} channels and to adenylyl cyclase (Moreno-Delgado *et al.*, 2006). Pharmacological studies carried out mainly in rodents revealed that treatment with either the imidazole- (Lin *et al.*, 1990; Monti *et al.*, 1991, 1996) or non-imidazole based HRH3-antagonists (Barbier *et al.*, 2004) dose-dependently promoted wakefulness, and sometimes made it more fragmented. The HRH3 agonists had the opposite effect (Lin *et al.*, 1990; Monti *et al.*, 1996, but see Monti *et al.*, 1991). As shown by Lin and co-workers (Lin *et al.*, 1990) the effect of the HRH3 antagonists can be blocked by the HRH1 receptor antagonist pyrilamine, which underscores the importance of HRH1 in this process. The spontaneous locomotion of the $Hrh3^{-/-}$ mice is reduced, especially during darkness (Takahashi *et al.*, 2002; Toyota *et al.*, 2002), which is accompanied by increases in sleep fragmentation (Gondard *et al.*, 2013).

Several studies on dissociated hippocampal cell cultures found the potentiation of NMDA-receptor mediated currents by histamine, which was presumably mediated through an interaction with the polyamine binding sites of the NMDA-receptors (Bekkers, 1993; Vorobjev *et al.*, 1993). Moreover the studies by Eaton et al. and Meyer et al. found that histamine causes phase shifts in hamster SCN explants (Eaton *et al.*, 1996; Meyer *et al.*, 1998). Glutamate is one of the main neurotransmitters of the retinohypothalamic tract (Hannibal, 2002, Gompf *et al.*, 2015), thus it is plausible that histamine may modulate the glutamatergic retinoreceptive input into the SCN (Meyer *et al.*, 1998), but this hypothesis is yet to be proven as no *in vivo* data have been published to date.

Histaminergic neurons along with the other wakefulness-active neuronal populations constitute the AAS. The components of the AAS are largely, positively coupled to each other and suppress the sleep-active centers, VLPO and MnPO, thus forming an interlocking inhibitory loop. In addition, the circadian and the homeostatic processes provide another layer of regulation of sleep and wakefulness.

5.4 The homeostatic process

The maintenance of homeostasis comprises the measures the organism takes to keep itself in a relatively stable condition needed for the survival. The efficient function of homeostat involves the recognition of changes in the process to which it must adjust in order to maintain a steady state, followed by a compensatory mechanism for these changes and a negative feedback loop between these parts. In relation to the sleep state, this negative feedback loop involves monitoring the "necessity for sleep" and adjustment of the amount of sleep accordingly. Multiple factors, such as wakefulness, an intensive physical or mental activity and many inflammatory diseases, are known to elevate the propensity for sleep. A search for neurochemical mechanisms involved in this homeostatic response resulted in the discovery of a number of endogenously produced substances that induce sleep. These substances accumulate during wakefulness, including during experimentally induced SD states and subsequently dissipate during sleep, which thus causes corresponding changes in SWA (Borbély & Tobler, 1989). These compounds include adenosine (Porkka-Heiskanen *et al.*, 1997), interleukins 1α and β (De Sarro *et al.*, 1997; Terao *et al.*, 1998; Manfridi *et al.*, 2003), prostaglandin D2 and nitric oxide (Ueno *et al.*, 1982; Inoué *et al.*, 1984).

Adenosine, which is one of the metabolites of ATP, received particular attention because of its association with sleep propensity on the one side and the metabolic state on the other. The elevation of turnover of ATP in response to a prolonged energy expenditure that occurs

during natural wakefulness or sleep deprivation (SD) is followed by the increase of adenosine concentration. The accumulation of adenosine in several brain areas such as the BF and the cerebral cortex is remarkably high (Porkka-Heiskanen *et al.*, 1997, 2000). Adenosine inhibits wakefulness-active neurons of the BF, TMN, LC and the HCRT-ergic neurons of the LH through its interaction with adenosine A1 receptors (Thakkar *et al.*, 2003). When administered by infusion into the BF, adenosine increases the amount of NREM sleep and SWA (Basheer *et al.*, 1999). Simultaneously, adenosine acts through the A2 receptors, and may either activate or disinhibit the VPLO, which promotes sleep (Morairty *et al.*, 2004; Gallopin *et al.*, 2005). Sleep and wakefulness centers mutually suppress each other, which enable the homeostatic regulators such as adenosine to shift equilibrium between the two states by suppressing the wakefulness-promoting neuronal groups. This disinhibits the sleep-promoting neuronal groups, which thus favors the the organism moving into a sleep state.

5.5 The circadian system

The circadian system is a mechanism of an adaptation that sets the timing for a variety of physiological processes. To serve its functions the oscillator should generate an endogenous rhythm even in the absence of external stimuli, entrain to certain exogenous rhythms, such as day-night cycles, but be relatively stable to compensate for the others such as temperature and the hormonal level and provide an external output. The circadian system of vertebrates has a hierarchical structure and comprises a master clock and peripheral oscillators that are synchronized to this clock. The master clock is located in the SCN of the anterior hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972), whereas most of the brain areas and other organs can be considered as peripheral oscillators (Yamazaki *et al.*, 2000; Yoo *et al.*, 2004). Some of these oscillators can generate their own rhythms even in the absesnce of the master clock (Yamazaki *et al.*, 2000), while the others lack periodicity with the SCN lesion (Abe *et al.*, 2002). The SCN receives a direct photic input from the retina via the retinohypothalamic tract (Moore et al., 1967, Pickard, 1980), that is formed by axons of the retinal intrinsically photosensitive ganglion cells (Gooley *et al.*, 2001; Berson *et al.*, 2002) and utilize PACAP and glutamate as transmitters (Hannibal *et al.*, 2002). An indirect photic input integrated with information about the locomotor activity comes from the neuropeptide Y-ergic projection of the intergeniculate leaflet of the thalamus (Janik & Mrosovsky, 1994). The SCN is innervated by the wakefulness-promoting centers, including the histaminergic neurons of the TMN (Panula *et al.*, 1989; Michelsen *et al.*, 2005) and the serotoninergic neurones from the DR (Abrahamson & Moore, 2001). The serotonergic afferent projection terminates mainly in the retinorecipient region (Barassin *et al.*, 2002; Versteeg *et al.*, 2015). The histaminergic immunoreactivity in the SCN of the mouse is highest in its ventromedial part and attributable

to fibers and to a sub-population of the SCN neurons (Michelsen *et al.*, 2005). The functional significance of histamine in these cells and its co-localization with the other transmitters remains to be studied.

The SCN neurons are active during photophase irrespective of whether the animal is diurnal or nocturnal. The master clock and peripheral oscillators are able to exhibit near 24-h activity even *in vitro* (Abe *et al.*, 2002; Yoo *et al.*, 2004). At the molecular level the clock's core consists of the transcription factors that form several interlocking positive (BMAL1, CLOCK) and negative (PER1, PER2, CRY1, CRY2, RORα, REV-ERBα) transcription and translation loops (Fig. 2) culminating in a 24 h long rhythm (Welsh *et al.*, 1995; Herzog *et al.*, 1997; Ye *et al.*, 2014). The post-translational modifications of PER and CRY proteins, including poly-phosphorylation of PER, and their proteasomal degradation are crucial to determine the periodicity of the clock (Lowrey *et al.*, 2000; Godinho *et al.*, 2007; Siepka *et al.*, 2007; Meng *et al.*, 2008).

The majority of the neurons in the SCN are GABA-ergic, but these neurons demonstrate diverse molecular phenotypes by producing a number of neuropeptides including arginine-vasopressin, vasoactive intestinal peptide, neuropeptide Y, gastrin-releasing peptide and others (Morin *et al.*, 2006).

The efferent projections of the SCN are limited to the areas adjacent to these nuclei, namely: the subparaventricular zone of the hypothalamus (SPVZ) and the paraventricular nucleus of the hypothalamus and to a lesser extent, the dorsomedial nucleus of the hypothalamus (DMH) and the medial preoptic area (Abrahamson & Moore, 2001). Although the SPVZ and the paraventricular nucleus of the hypothalamus do not directly participate in the regulation of sleep and wakefulness, these nuclei serve as relays to communicate circadian information further to the sleep- and wakefulness- promoting centers. This was shown in experiments where lesions of the SPVZ led to an ablation of the sleep-wakefulness cycle, temperature and the activity rhythms (Lu *et al.*, 2001). Dual tract-tracing studies revealed that the DMH, the medial preoptic area and the SPVZ extensively innervate the VLPO (Deurveilher *et al.*, 2002a), the MnPO (Deurveilher & Semba, 2003), LH, TMN, LC and the raphe nuclei (Deurveilher & Semba, 2005). The DMH serves as a second-order relay, because cell-specific lesions of the DMH completely eliminated rhythms of sleep and wakefulness, feeding, locomotor activity and corticosterone secretion (Chou *et al.*, 2003). Importantly, in both studies (Lu *et al.*, 2001; Chou *et al.*, 2003) the SCN remained intact which underlies the necessity of the DMH and the SPVZ in the conduction of circadian signals.

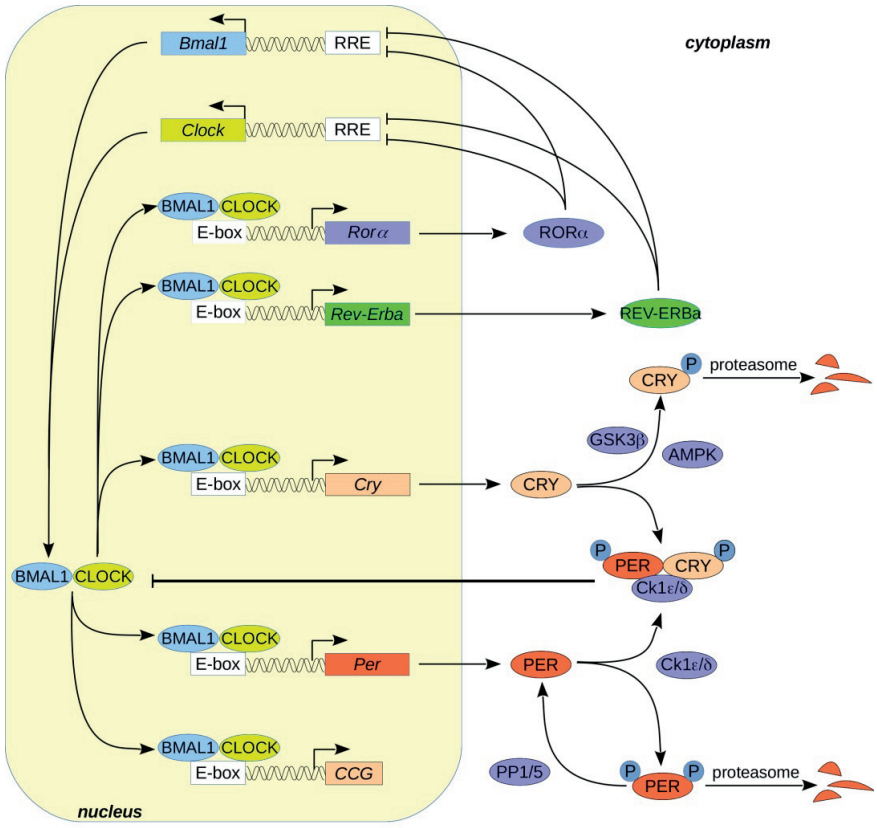

Fig. 2. Schematic representation of interaction of the core molecular components of the circadian clock. Proteins BMAL1 and CLOCK, or its homolog NPAS2, form a heterodimeric basic helix-loop-helix-PAS transcription factor that interact with E-box elements of *Cry*, *Per*, *Ror*α, *Rev-Erb*α and the clock-controlled genes and activate their expression. The stability of a newly produced PER and CRY proteins depends of their phosphorylation status, dynamically regulated by a set of kinases (CK1ε/δ, GSK3β, AMPK) and phosphatases (PP1/5). Once heterodimerized, PER-CRY complex is translocated to the nucleus and interacts with BMAL1-CLOCK complex to inhibit its function, including deactivation of its own expression. Once PER-CRY complex is degraded, the cycle is repeated. The role of a second negative feedback loop, formed by a RORα and REV-ERBα is mainly to delay the phase in CRY1 transcription. CCG - clock-controlled genes, PP1/5 - protein phosphatase 1 or 5, CK1ε/δ - casein kinase ε or δ, PER - Period 1 or 2, CRY - Cryptochrome 1 or 2, CLOCK - Clock, P - phosphate group, E-box - enhancer element, RORα -retinoid-related orphan receptor α, REV-ERBα - orphan nuclear receptor REV-ERBα, GSK3β - glycogen synthase kinase 3-beta, AMPK - adenosine monophosphate-activated protein kinase.

An SCN output to the paraventricular nucleus of hypothalamus controls the release of corticotropin-releasing factor (Vrang *et al.*, 1995). Another function of this projection is to relay the circadian signals onwards to the superior cervical ganglion, which controls the production of the neurohormone melatonin through the noradrenergic innervation of the pineal gland (Klein *et al.*, 1983; Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999). Melatonin synchronizes the circadian rhythms of the peripheral tissues with that of the SCN, upon its release from the pineal gland into the circulation, and thereby serves as a hormonal output of the master clock (Torres-Farfan *et al.*, 2006). Therefore, melatonin provides the SCN with a hormonal mechanism of interaction with peripheral oscillators, which complements neuronal efferent projections. In the absence of the SCN, some peripheral oscillators, including the cerebral cortex, the striatum and others, become arrhythmic (Abe *et al.*, 2002), although most of the "clock" proteins are expressed in these sites. Several studies have reported positive correlations between the expressions of the *Per1*/*Per2* genes in several parts of the brain and the alertness state during regular daily activity (Rath *et al.*, 2012), under SD conditions (Wisor *et al.*, 2008), chronic treatment with methamphetamine (Masubuchi *et al.*, 2000) or split rhythm in the CS mice (Abe *et al.*, 2001). This suggests the participation of these transcription factors in the regulation of processes other than the circadian clock. However, the regulatory networks in which they are involved are not fully understood.

As described above, the AAS, nuclei of preoptic area, homeostatic and circadian processes form a complex networks that regulate the sleep-wakefulness cycle. Several mechanisms of the sleep-wakefulness regulation were suggested as explanations of how these systems work in concert.

5.6 Fast sleep-wakefulness transitions and the interaction of homeostatic and circadian processes

The analyses of neuronal connections between the wakefulness- and sleep-promoting centers revealed their mutually inhibitory nature. The sleep-active neurons of the VLPO and the MnPO are mainly GABA-ergic and suppress their target neurons, whereas acetylcholine, serotonin, noradrenaline, dopamine and histamine are released by the wakefulness-promoting nuclei and efficiently inhibit the VLPO neurons firing as shown by a number of *in vitro* studies (Gallopin *et al.*, 2004; Liu *et al.*, 2010; Williams *et al.*, 2014). When either side of the switch gains an advantage over the other, a fast transition occurs because of a direct inhibition of a counterpart that in turn causes indirect dis-inhibition of itself. The role of HCRT-ergic neurons was proposed to stabilize the switch in either of these two states, and the instability of that switch causes narcolepsy (Chemelli *et al.*, 1999; Lin *et al.*, 1999; Peyron *et*

al., 2000). The transition between sleep and wakefulness takes only 1-2 seconds (rodents) to one minute (human), and is, probably, dependent on a size and complexity of a neuronal population involved. The mechanism described above is known as a "flip-flop" switch (Saper *et al.*, 2001). Since the focus of this model is on fast state transitions, it does not incorporate the circadian and homeostatic processes.

Several phenomenological models have been proposed including the two-oscillator model (Kronauer *et al.*, 1982), three-oscillator model (Kawato *et al.*, 1982) and the two-process model (Borbély, 1982) to explain long-term regulation of the sleep-wakefulness cycle. Recent advances in computational biology enabled to develop models that are based on anatomical and physiological data and gain support from several experimental paradigms (reviewed in Booth & Diniz Behn, 2014). Nevertheless, the two-process model is the most established and well recognized model and this will be referred to in the discussion. According to the two-process model, the homeostatic process increases during wakefulness and exponentially decays during sleep as a function of the prior waking time. The EEG correlate of this process is the SWA of NREM sleep. The circadian process *per se* does not depend on prior waking and is controlled by the circadian clock. During wakefulness, the homeostatic sleep pressure increases, but elevated activity of the circadian process counterbalances the sleep drive and thereby prevents sleep from taking place. The sleep propensity elevates over the course of the activity period and by the end of this period it reaches its maximum, whereas the circadian process decays and hence permits sleep to occur.

5.6.1 Methodologies used for investigating homeostatic and circadian processes

Since the time of the first formulation of concept of homeostatic and circadian processes the question has arisen as to what extent these two processes are interrelated. The individual assessment of either of these processes is complicated by their precise temporal coupling. Therefore several methodologies have been used to address this question:

Arrhythmicity aims to assess the homeostatic regulation in the absence of a functional circadian oscillator. This can be achieved by a stereotaxic lesion of the SCN (Coindet *et al.*, 1975; Mistlberger *et al.*, 1983; Easton *et al.*, 2004) or induced spontaneous arrhythmicity, including abrupt phase advances (hamsters; Ruby *et al.*, 1996; Steinlechner *et al.*, 2002), housing under constant light (rats: Eastman & Rechtschaffen, 1983; Ikeda *et al.*, 2000 and mice: Chen *et al.*, 2008) or short cycles, such as LD8/8 to LD10.5/10.5 (mice; Tribukait, 1956; Udo *et al.*, 2004).

Desynchronization utilizes light-dark cycles that are just beyond the entrainment range of the circadian oscillator and splits the daily rhythm into entrained and non-entrained components. The non-entrained component is believed to correspond to an endogenous periodicity of the circadian oscillator. This protocol has been used on human subjects (Dijk & Czeisler, 1995), rats (de la Iglesia *et al.*, 2004, Lee et al., 2009) and mice (Campuzano *et al.*, 1999) although the latter study produced controversial results.

Sleep deprivation exploits the property of the SWA to increase as a function of length of preceding wakefulness period. It is used to probe changes in the homeostatic regulation depending on experimental conditions, such as the pharmacological treatment, genetic knockout, etc.

Short light-dark or rest-activity cycles under constant darkness are used to stabilize the homeostatic process by alternating light and darkness or the rest and activity periods at high frequency, usually every 1 or 2 hours (Alföldi *et al.*, 1991; Yasenkov & Deboer, 2010, 2011).

Genetic manipulation. The knockout of one or several genes known to be involved in circadian clockwork. To date, mouse strains with dysfunctional *Per1*, *Per2*, *Clock*, *Bmal1*, *Npas2*, *Cry1*, *Cry2* genes alone or in combination were characterized in respect to the sleep-wakefulness cycle.

On the one hand, the application of some of these approaches may cause similar changes of the phenotype, i.e. both, genetic knockout (Wisor *et al.*, 2002; Laposky *et al.*, 2005) and constant light (Eastman & Rechtschaffen, 1983; Ikeda *et al.*, 2000) may induce behavioral arrhythmicity. On the other hand, different phenotypes can be observed by using experimental protocols in which the light-to-dark proportions are similar. A number of studies have shown that light-dark cycles with the length between 20 h and 23 h were able to induce either complete entrainment to a new cycle (Pittendrigh & Daan, 1976; Campuzano *et al.*, 1999; Scannapieco *et al.*, 2009; Casiraghi *et al.*, 2012), arrhythmicity (Tribukait, 1956; Udo *et al.*, 2004), relative co-ordination (Karatsoreos *et al.*, 2011), or desynchronation (Campuzano *et al.*, 1999). This variety of experimental protocols and animal species used produced a spectrum of hypotheses that ranged from complete independence of the circadian and homeostatic processes in regulation of the sleep-wakefulness cycle to highly co-ordinated interaction between them (Mistlberger, 2005)

The hypothesis that the circadian process neither determines the time of sleep nor interacts with the homeostatic process was mainly based on the experiments that used the rat model in which a stereotaxic lesion of the SCN did not cause any changes of total sleep time,

although the locomotor activity became completely aperiodic (Ibuka & Kawamura, 1975; Mistlberger *et al.*, 1983). When such rats were subjected to SD, an elevation of NREM SWA, during the recovery period had been found (Tobler *et al.*, 1983; Trachsel *et al.*, 1992) and was similar in amplitude to that in intact animals (Mistlberger *et al.*, 1983). Subsequent research done on monkeys (Edgar *et al.*, 1993) and mice (Easton *et al.*, 2004, but see Ibuka *et al.*, 1980) with the ablated SCN, and on spontaneously arrhythmic hamsters with the intact SCN (Larkin *et al.*, 2004) also suggested the independence of the circadian and homeostatic components. Yet, contrary to the above-mentioned studies, these reports found a significant elevation of total time spent asleep in response to aperiodicity induced by these methods.

The advent of the models, based on a genetic manipulation and the identification of a core "clock" genes led to a characterization of the sleep-wakefulness cycles of mouse strains, carrying knockouts of one or more of *Per1*/*Per2*, *Cry1*/*Cry2* (Wisor *et al.*, 2002), *Bmal1* (Laposky *et al.*, 2005) or *Clock* (Naylor *et al.*, 2000) genes. These animals were arrhythmic and manifested remarkable changes in the sleep homeostasis observed as elevated NREM, SWA and increased total sleep time. Despite this approach does not account for confounding factors such as a potential pleiotropy, the constitutive absence of the knockout gene product, the ablation of the gene in all the tissues and that some of these genes are also involved in other metabolic processes, the concept of interaction instead of a complete independence of the circadian and homeostatic regulatory mechanisms was supported by recent behavioral studies. The inverse correlation of a multiunit recording of the SCN activity and locomotion has been demonstrated previously (Deboer *et al.*, 2003; Nakamura *et al.*, 2008; Houben *et al.*, 2009). This is likely to be a mutually inhibitory process, as the suppression of the SCN neural activity by tetrodotoxin leads to the induction of locomotion (Houben *et al.*, 2014), whereas forced locomotion causes suppression of the activity of most of the neuronal sub-populations of the SCN (Deboer *et al.*, 2003; van Oosterhout *et al.*, 2012). In addition, SD is able to phase shift the circadian cycle (Antle & Mistlberger, 2000; Burgess, 2010). In line with these reports, Yasenkov and Deboer (2010, 2011) imposed ultrashort light-dark regime in mice and found a significant attenuation of a circadian amplitude of SWA during NREM sleep.

The brain areas that underlie the processes mentioned above are interconnected and hence may affect each other. Therefore, we examined the extent to which the combined circadian and histaminergic regulatory mechanisms interact alongside each other and also their individual effects on the sleep-wakefulness cycle.

6. Aims

The scope of the current work was to assess the interaction between the AAS, with the focus on the histaminergic neurons and the circadian system in the regulation of the sleep-wakefulness cycle. The individual contribution made by either of these systems was also studied. More specific goals were to study the following:

1. Estimate, how changes in length of a circadian period affect the sleep-wakefulness cycle, including the assessment of vigilance state distribution, fragmentation, their dynamic changes, and state-specific cross-frequency coupling.

2. Assess the periodic properties of the histaminergic system in mice, including diurnal expression, and activities of the enzymes involved in histamine metabolism. Determine the levels of histamine and its metabolite, 1-methylhistamine in several brain regions, dynamics of the histamine release and its correlation with the EEG markers of different vigilance states.

3. Understand the role histamine H1 and H3 receptors play in the regulation of the circadian system using constitutive knockout mouse models, monitor diurnal and free-running rhythms of locomotor activity, and monitor the expression of genes, that constitute the core of the circadian oscillator, *Per1*, *Per2* and *Bmal1* (*Arntl*), in several brain structures.

4. Understand the role of the histaminergic regulation in the homeostatic sleep propensity.

7. Materials and Methods

7.1 Animals

Seven to 10-week-old mice of either male (**II, IV**) or both sexes (**III**) of the following genotypes were used: CBA/J (**II**) and C57BL/6JCrl wild type, C57BL/6JCrl *Hrh1*$^{-/-}$ and C57BL/6JCrl *Hrh3*$^{-/-}$ (**II-IV**). The *Hrh1*$^{-/-}$ mice (Inoue *et al.*, 1996) were delivered from the Riken Research Center for Allergy and Immunology and maintained locally on the C57BL/6JCrl genetic background. The *Hrh3*$^{-/-}$ mice were supplied by Johnson and Johnson Pharmaceutical Research and Development, LLC, La Jolla, CA, bred and maintained by the Jackson Laboratory where they were selectively backcrossed with the C57BL/6J mice for at least 10 generations to show at least 99.5% identity to the C57BL/6J strain (Toyota *et al.*, 2002). The mice were then delivered to the animal facility of the University of Helsinki and maintained locally. CBA/J animals were acquired from Charles River Laboratories (Chatillon-sur-Chalaronne, France); the other strains were back-crossed with the background C57BL/6JCrl strain every 6-7 generations. The animals were taken from heterozygous non-brother-sister matings, thus littermates were used in this series of studies. Study (**I**) utilized male Han-Wistar rats aged 3–4 months (300–400 g), which were supplied by the animal facility of the University of Helsinki.

All the animals were housed in a plastic Macrolon cages with an open top in a soundproof room. The temperature was maintained at 22.5±1°C; standard food pellets (Scanbur, Sollentuna, Sweden) and water were provided *ad libitum*. The illumination at the bottom of the cages during the lights-on periods was 140±40 lux and complete darkness (< 0.5 lux) otherwise.

7.1.1 Design of the experiments

The rats in study **I** were housed individually and habituated for handling for at least 4 days before surgery. After the surgery, when the animals were implanted with electrodes for EEG, electromyography (EMG), and microdialysis probe, and recovery period, the animals were connected to EEG recording system three days before the experiment and to the microdialysis apparatus one day before the experiment. The animals were then allowed to behave freely during the next 24 h in order to provide the control data for the SD experiment. SD took place on the day after the control day. During the SD-day animals were gently handled for 6 h (Franken et al., 1991) starting 2.5 h after lights-on. The animals were also kept awake by introducing novel objects into the home cage.

The animals in study **II** (36 male 8-week-old C57BL/6J mice and 30 male 8-week-old CBA/J mice) intended to be used in the enzyme activity, histamine and 1-methylhistamine, and the *in situ* hybridization assays were housed in groups of three, under the LD12/12 for 2 weeks before the experiment.

The mice in study **III** were kept individually under LD12/12 for one week, then for two weeks under DD and afterwards kept under LD12/12 for one week.

The mice in study **IV** were kept individually for two weeks before surgery. Before the experiments, we implanted the animals with electrodes for EEG, EMG and microdialysis probe. At baseline, mice were maintained at a reversed symmetric 12-h light–12-h dark (LD 12/12) cycle (lights off at 8:30, lights on at 20:30). During the experiment, animals were sequentially subjected to LD 11/11, DD, LD 10.5/10.5, and LD 10/10 regimes for 14, 7, 14 and 14 astronomic days respectively.

All the experiments were performed in compliance with the conditions and regulations stipulated in the Finnish Act on the Use of Animals for Experimental Purposes, European Communities Council Directive of 24 November 1986 (86/609/EEC), and the Guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures. The experimental protocols were approved by the Animal Experiment Committee of the State Provincial Office of Southern Finland (licenses: ESLH-2008-01695_Ym-23; ESLH-2009-02832/Ym-23; ESAVI-2010-09651/Ym-23). The total number of animals used in the four studies was 213: i.e. 25 in study **I**, 116 in study **II**, 62 in study **III**, and 10 in study **IV**.

7.2 Chemicals

The chemical reagents used in the studies were as follows: Ketalar, Domitor, and Antisedane (Pfizer Animal Health, New York, USA); dental cement (Candulor, Wangen, Germany); buprenorphine (Temgesic; Reckitt Benckiser, Slough, UK); 3-methylhistamine, $CaCl_2$, NaCl, KCl, KH_2PO_4, and methanol (Merck, Whitehouse Station, NJ, USA); deoxyadenosine 5'-triphosphate, [α-^{33}P] (NEG312H; NEN Research Products, PerkinElmer, Waltham, MS, USA); 1-methylhistamine, aminoguanidine hydrochloride, citric acid, Denhardt's solution, dithiothreitol, histamine dihydrochloride, H_3PO_4, L-histidine, NaH_2PO_4, NaN_3, NaOH, N-lauroylsarcosine, *o*-phthalaldehyde, polyethylene glycol 300, pyridoxal phosphate, pargyline hydrochloride, $HClO_4$, β-mercaptoethanol, phenylmethanesulfonyl fluoride, S-adenosyl-methionine, and sodium salt of octanesulphonic acid (Sigma, St Louis, MO, USA); $MgCl_2$ and H_3BO_3 (Riedel-deHaën, Seelze, Germany); formamide (Amresco, Solon, OH, USA); tRNA (Roche, Basel, Switzerland); and dextran sulphate (Amersham, Amersham, UK).

7.3 Combined analysis of histamine, 1-methylhistamine, HDC and HNMT activities (II)

The current assay is a combination of the methods for a simultaneous determination of histamine and 1-methylhistamine (Miyamoto *et al.*, 2004), HDC activity (Niimi *et al.*, 1997), and HNMT activity (Scott *et al.*, 1991). The mice were euthanized at zeitgeber time (ZT; hours, passed after lights on) 4, 8, 12 (lights on), 16, 20 and 24 h (lights off) by decapitation, and the following brain structures were immediately collected: medulla oblongata, pons, cerebellum, midbrain, hypothalamus, thalamus, hippocampus, striatum, and the cortex, all according to the mouse brain stereotaxic atlas (Paxinos & Franklin, 2004). The brain areas were dissected on ice, the samples were snap-frozen in liquid nitrogen and stored at −80°C prior to the analysis.

Upon analysis the samples were homogenized with a Vibracell sonicator (Sonics, Newtown, CT, USA) on ice in 10 volumes of the homogenization solution. The homogenization solution consisted of 115 µM phenylmethanesulfonyl fluoride, 100 µM dithiothreitol and 100 nM 3-methylhistamine (internal standard) in a 0.1 M potassium phosphate buffer (pH 7.0). Sixty microlitres of the homogenate were immediately mixed with $HClO_4$ (final concentration 0.2M) to prevent degradation of histamine and 1-methylhistamine, and precipitated and non-precipitated homogenates were then centrifuged for 30 min at 15 000 g and at 4°C. Supernatants of precipitated samples were used for the analysis of histamine and 1-methylhistamine as described below. The supernatant (100 µL) from the non-precipitated samples was transferred to Amicon ultra 10K analytical filters (Millipore, Carrightwohill, Ireland), and centrifuged for 30 min at 14 000 g. Then, 200 µL of the homogenization solution was added to the concentrated samples and centrifuged twice (14 000 g, 30 min) to remove residual histamine and 1-methylhistamine before enzyme activity assays were performed. The volume of concentrated samples was adjusted to 200 µL by homogenization solution, and gently mixed. These samples were divided into 100 µL aliquots for either HDC or HNMT assays.

7.3.1 HDC and HNMT activity assays

The 100 µL sample prepared for either the HDC or HNMT assays as described above, was further divided into two halves: one part served as a negative control (without the substrate), and the other part was mixed with 50 µL of the HDC-reaction mixture, which consisted of (final concentrations) 5 µM aminoguanidine, 10 µM pyridoxal phosphate, 1% polyethylene glycol 300, and 1 mM histidine, diluted in the homogenization solution to initiate the reaction.

After incubation at 37°C for 60 min, the reaction was terminated by addition of 10 µL of 2M $HClO_4$ and then centrifuged at 15 000 g for 5 min. The concentration of histamine in the supernatant was determined by a high-performance liquid chromatography (HPLC). The pellet was used for the protein determination.

The procedure for HNMT enzyme activity measurement was analogous to that of the HDC activity assay, with a few exceptions. The HNMT reaction mixture consisted of (final concentrations) 100 µM pargyline, 25 µM S-adenosyl-methionine and 20 µM histamine diluted in the homogenization solution to initiate the reaction and incubated for 30 min at 37°C. After the reaction had been terminated by the addition of 10 µL of 2M $HClO_4$, the concentration of 1-methylhistamine in the supernatant was analyzed by the HPLC. The pellet was used for the protein determination.

7.3.2 HPLC analysis of histamine and 1-methylhistamine

The method was based on post-column *o*-phthalaldehyde/β-mercaptoethanol derivatization as reported in detail in Miyamoto *et al.*, (2004). Briefly, the supernatants of precipitated samples collected as described above were neutralized with 10 volumes of 0.4M borate buffer. They were then subjected to solid-phase extraction with BondElut CBA 100-mg cartridges (Varian, Palo Alto, CA, USA) that were equilibrated with 1 mL of CH_3OH, 1 mL of 0.01 M HCl, and 3 mL of H_2O. The cartridges were washed with 1 mL of water, and histamine, 1-methylhistamine and 3-methylhistamine were then eluted with 500 µL of 0.1 M HCl containing 1 mM ethylenediaminetetraacetic acid. Then, 50 µL of the eluate was analyzed by HPLC.

Histamine and 1-methylhistamine were separated on a 4.6 × 150-mm, 5-µm C18 Phenomenex Gemini column equipped with a SecurityGuard C18 4 × 3-mm pre-column cartridge (Phenomenex, USA) with the HPLC system described above. The mobile phase consisted of methanol/0.15 M KH_2PO_4 (4:96, v/v) containing 200 mg/L sodium salt of octanesulphonic acid (flow rate of 0.6 mL/min). The eluent line was connected by a T-piece to a reagent line that mixed a 0.05% *o*-phthalaldehyde/0.2% β-mercaptoethanol solution and 0.5 M NaOH in a short reaction coil. The analytical column and the reaction coil were kept at 42°C by a HIS25 heating oven (Grant Institute, Edinburgh, UK). Fluorescence was measured at λ_{Ex} 360 nm, λ_{Em} 450 nm.

7.4 HPLC analysis of histamine (I, II)

The analytical HPLC system consisted of four Shimadzu LC20AD pumps, a Shimadzu SIL-20AC autosampler, a Shimadzu RF-10Axl fluorescence detector, a Shimazdu CBM-20A controller, and lCSolution 1.21 software (Shimadzu, Kyoto, Japan). The dialysis samples were analyzed without any prior purification.

The histamine analysis method was based on the online post-column derivatization with *o*-phthalaldehyde, as described originally in Yamatodani *et al.*, (1985). Briefly, the samples were separated on a 4.6 × 50-mm, 5-µm SCX Phenomenex Luna column equipped with a SecurityGuard SCX 4 × 3-mm pre-column cartridge (Phenomenex, Torrance, CA, USA), with a mobile phase consisting of 0.25M KH_2PO_4 and 0.75 mM NaN_3 (flow rate of 0.6 mL/min). The samples were then automatically derivatized by online mixing with 0.1% *o*-phthalaldehyde, 2M NaOH and 0.2M H_3BO_3 in a reaction coil, incubated at 45°C, and finally stabilized with 3M H_3PO_4. Fluorescence was measured at λ_{Ex} 360 nm; λ_{Em} 450 nm.

7.5 Protein measurement (I, II)

Protein pellets were re-suspended in 0.1 M phosphate buffer (pH 7.0) by sonication. The total protein concentration was measured with the bicinchoninic acid protein assay, according to the manufacturer's instructions (ThermoFisher, Waltham, MS, USA). The activity was expressed as mole of product per hour per milligram of protein.

7.6 Radioactive *in situ* hybridization (II, III)

The animals (36 male 8-week-old C57BL/6J mice) were kept in groups of three (100 lux at the cage bottom) for 2 weeks before the experiment. The mice were euthanized by decapitation at the following time points: ZT 4, 8, 12 (lights on), 16, 20 and 24 (lights off). The brains were removed, rapidly frozen by immersion into cooled isopentane (-45°C), and then subsequently stored at −80°C. The frozen brains were covered with M1 embedding matrix (ThermoScientific, Calamazoo, MI, USA) before sectioning, then 20-µm sections were cut along the coronal plane using a Leica CM3050S cryostat (Leica, Wetzlar, Germany), and mounted onto SuperFrost slides (ThermoScientific, Portsmouth, USA). The section levels that corresponded to either the SCN or the TMN regions were determined according to the mouse brain stereotaxic atlas (Paxinos & Franklin, 2004). The sections were stored at −20°C prior to analysis.

The expression of *Hdc* mRNA was analyzed in subpopulations of the histaminergic neurons E2/E3 and E4/E5 neuron groups of the TMN (Inagaki *et al.*, 1990). The expressions of *Per1*, *Per2* and *Bmal1* mRNA were assessed in the striatum, the cingulate, motor and the somatosensory cortical areas and in the SCN.

DNA oligoprobes used for radioactive in situ hybridization:

gene	sequence
Hdc (**II**)*	5′-TGCTTGTATGGCTGCTCTGACTGCTGCGGGTGATGCTGGCTGAGG-3′
Per1 (**III**)**	5′-GCTCCTTCAGGGTCCTTATCAGTTCTTTGTGTGCGTCAGCTTTGG-3′
Per2 (**III**)**	5′-CCGTGTCTGACATGTGCTTGAAGATTCTTCACCCCGAAGGACCGAATCAC-3′
Bmal1 (**III**)**	5′-GCCATTGCTGCCTCATCGTTACTGGGACTACTTGATCCTTGGTCG-3′

DNA oligoprobes, were the same as in: * - Karlstedt *et al.*, 2001; **- Abe *et al.*, 2004.

The *in situ* hybridization protocol has been described previously (Dagerlind *et al.*, 1992), and was used with minor modifications according to Karlstedt *et al.*, (2001). The oligoprobes were labelled with deoxyadenosine 5′-triphosphate, [α-^{33}P] at their 3′-ends by using terminal deoxynucleotide transferase (Promega, Madison, WI, USA), according to the manufacturer's instructions. Non-incorporated nucleotides were removed by the purification with Sephadex G-50 QuickSpin cartridges (Roche). The brain sections, eight per mouse, were hybridized with the probe at 45°C for 24 h. After a series of high-stringency washes that removed the non-hybridized probe, Kodak BioMax films were exposed to the sections together with the ^{14}C-standards (Amersham, UK) for 10 days. Quantitative analysis of the autoradiograms was performed by using the MCID 6.0 platform (Imaging Research, St. Catharines, Canada). A film background (in gray levels) was measured adjacent to every tissue autoradiogram and the standards. The mean intensity values (in gray levels) were acquired from tissue autoradiograms that correspond to the striatum and the areas of cerebral cortex and linearized using the standards. The TMN and the SCN regions were defined based on the histology of the brain sections. The lower threshold of the intensity window was determined as two standard deviations from the mean pixel intensity of the background area. Only the pixels with the intensities that exceeded the lower threshold were included in the analysis. Then optical densities of acquired samples and standards were computed as:

$$OD = log_{10}(I_O/I_T)\text{ , whereby}$$

I_o - background intensity, in gray levels; I_T - signal intensity, in gray levels. Therefore the *OD* values represent the opacity of the film in response to exposure to a radioligand corrected for

film background. *OD* values of standards were fitted with a one phase exponential association function:

$$y = y_{max}(1 - e^{-kx}) \text{ , whereby}$$

y_{max} – maximum $\log_{10}$-intensity, k – coefficient of exponential slope. The intensity values of each autoradiogram converted to corresponding µCi/g values with equation (2). The resulting values were averaged for each subject and processed further as described below.

7.7 Implantation of EEG electrodes and microdialysis probes (I, II, IV)

Twelve- to 16-week-old male rats (**I**) or 10-week- old C57BL/6J mice (**II, IV**) were kept individually before surgery. The animals were operated under general anaesthesia: mice received ketamine intraperitoneally (i.p.; 75 mg/kg) in combination with medetomidine (i.p.; 1 mg/kg); rats received diazepam (i.p.; 2.5 mg/kg), medetomidine (i.p.; 0.4 mg/kg), ketamine (i.p.; 60 mg/kg). After exposition, the skull bones were cleaned and disinfected. The TMN in mice was targeted by implanting the guide cannula (CMA7, CMA/Microdialysis, Solna, Sweden) into the posterior part of the hypothalamus, 1 mm above the TMN at a stereotaxic co-ordinates (relative to bregma): anterior, −2.5 mm; lateral, +0.5 mm; and vertical, −4.4 mm (**II**). The BF area in rats were targeted by placing the guide cannula CMA11, 3 mm above the BF area, including the horizontal diagonal band of Broca, the substantia innominata, and the magnocellular preoptic area at a co-ordinates: anterior, −0.3 mm; lateral, 2.0 mm; vertical, −5.5 mm (**I**). All the stereotaxic co-ordinates were taken from (Paxinos & Franklin, 2004). The electrodes for electromyography were placed in the neck musculature. Two gold-coated screws were installed into the skull for frontoparietal epidural EEG recording. The electrodes, guide cannula and supporting screws were secured to the skull with dental cement. The animals were injected with Antisedane subcutaneously (s.c.; 0.5 mg/kg) and given the analgesic buprenorphine (s.c.; 0.1 mg/kg,) to expedite the recovery from anesthesia. The animals were left undisturbed for a recovery period of 1 week.

7.8 EEG/EMG recording and scoring (I, II, IV)

Electroencephlographic (EEG) and elctromyographic (EMG) recordings were started 6–7 days after surgery and continued throughout the course of the experiment. The EEG and EMG signals were amplified (gain, 10 000), filtered (high pass, 0.3 Hz; band stop: 50 Hz, low pass, 100 Hz), and sampled at 200 Hz. Low-frequency artefacts were filtered out by digitally processing the EEG signal through a high-pass filter (0.5 Hz) with Spike2 software (version 6; Cambridge Electronic Devices, Cambridge, UK). The EEG recordings were scored semi-

automatically using the algorithm developed by Rytkönen *et al.*, (2011) and further manually verified for specificity and the absence of artefacts. The scoring was performed at 4-s epochs for wakefulness, NREM sleep, and REM sleep, which were distinguished as follows: wakefulness was characterized by low-amplitude desynchronized EEG activity and a high-amplitude EMG activity; NREM sleep was characterized by high-amplitude δ-wave (1–4 Hz) EEG activity and a low-amplitude or absent EMG activity; REM sleep was characterized by regular high θ-wave EEG activity and decreased or absent EMG activity. Brief awakenings were scored for short periods of wakefulness that lasted for no more than 16 s. Epochs that contained artifacts or more than one vigilance state were excluded from any further analysis. The resulting data were used to compute the average amounts and number of episodes of wakefulness, NREM and REM sleep, in addition to state-specific PSD spectra. PSD specta values were computed using the fast Fourier transform with the following parameters: fast Fourier transform size - 512; epoch size - 4s; window - Hann; frequency range - 0-100 Hz. PSD of total EEG were averaged across the entire frequency range. PSD spectra were normalized by division of PSD values at each of vigilance states per average total PSD for the corresponding day. The normalized values were averaged across two days of a corresponding stage of experiment separately for every animal and used for a statistical evaluation.

7.8.1 Phase-amplitude coupling analysis (IV)

The phase-amplitude coupling (PAC) was analyzed by using non-processed EEG data from the last two days of each phase of the experiment. These data were exported from Spike2 to the Matlab format and filtered with a set of finite-response band-pass filters (step: 1 Hz; window width: 1 Hz; filter length: 1500 time points) that ranged from 2 to 100 Hz using MatLab 7.5 (MathWorks, Nattick, USA). Filtered data were then subjected to a Hilbert transform to obtain instantaneous phase and power values. Phase-amplitude coupling (PAC) was computed over 24 h in 1-min bins, separately for wakefulness, NREM, and REM sleep states using the following equation (Canolty et al., 2006)

$$PAC = \left| \frac{\sum_{t=1}^{n} a^t e^{i\varphi t}}{n} \right| , \text{where}$$

t - time point, *a* - power of modulated frequency at time point *t*, *i* - imaginary operator, *φ* - phase angle in radians of a carrier frequency at time point *t*, and *n* - total number of time points.

The normalized PAC values (PACz) were computed from the empirical distribution of PAC values created by a random re-ordering power time series relative to a phase time series followed by the calculation of the re-sampled PAC (Cohen, 2014). Next, we computed the matrices of mean PACz values frequency pair-wise for light and dark periods separately. We used paired t-tests to compare PACz values between different conditions. We thresholded resulting matrices of t-values to retain only those values that were less than p=0.01 to correct for multiple comparisons and we excluded the remainder from further analysis. Integrated t-values were computed for any contiguous array (cluster) of suprathreshold values. We tested these values for significance against the distribution of the integrated t-values of clusters acquired through the permutation-based generation of sets of matrices of t-values, followed by thresholding as described above. We considered clusters that were less than p=0.05 to be statistically significant. A detailed description of this procedure can be found in Cohen (2014).

7.9 Microdialysis (I, II)

One day before the sample collection, microdialysis probes (CMA 7 (1-mm membrane, **II**) or CMA11 (2 mm membrane, **I**); CMA/Microdialysis) were inserted through the guide cannula. The probes were connected to a sample collection system, and continuous perfusion (1 µL/min) with artificial cerebrospinal fluid (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$) started. Sample collection began 1 day after the probe implantation, and samples were taken at 30-min intervals throughout the experiment. The probe positions were verified after the experiment, when the animals were euthanized by decapitation and the brains were removed and sectioned with a cryostat along the coronal plane according to the stereotaxic atlas (Paxinos & Franklin, 2004).

7.10 Assessment of spontaneous locomotion (II, III, IV)

Each cage was equipped with a CAMZWMBLAH2N video camera combined with an infrared light source (Velleman, Gavere, Belgium). The video stream was captured and recorded continuously using GeoVision surveillance software (GeoVision, Taiwan) from 5 days before surgery until the end of the experiment. The recorded video data were converted and prepared for tracking with Virtualdub 1.9.2 (www.virtualdub.org), and tracking was then performed with the Ethovision 3.1 software package (Noldus Software, Wageningen, The Netherlands). The distance moved in a cage was calculated in 1 to 30-min time bins.

7.11 Data analyses

The χ^2-periodograms (**III**, **IV**; Sokolove & Bushell, 1978) were computed using the ChronosFit software (Viktor Persson, http://www.chronopharmacology.de), consequent multiple Student's t-tests; single cosinor analysis (Bingham *et al.*, 1982), and preparation data for further analyses were performed using the LibreOffice Calc (**II**, **III**, **IV**; The Document Foundation) or by the Microsoft Excel (**I**; Microsoft, USA) software. Cosinor and Lomb-Scargle periodograms (Ruf, 1999) were computed with the Cosinor and the LSP (Lomb-Scargle periodogram) software (**II**; Refinetti *et al.*, 2007). Cross-correlation analysis was performed with the SPSS 15.0 (**II**; SPSS, Armonk, NY, USA). Mixed model analyses were performed using the *nlme* library (**III**, **IV**; *nlme: Linear and Nonlinear Mixed Effects Models: R software*, 2013) for the R software (R Development Core Team, 2013). The EEG data processing, Individual/group cosinor and PAC analyses were performed by the MatLab 7.5 (**IV**; MathWorks, Nattick, USA) software. The fitting of two-process and logistic models was performed using the Prism software (GraphPad Inc., USA). One-way analysis of variance (ANOVA) followed by Holm–Sidak or Dunn's post hoc tests and the Pearson product moment correlation tests (**I**) were performed using the SigmaStat 3.1 program (SPSS Science Software).

8. Results

8.1 The effect of shortened circadian cycle on sleep and wakefulness (IV)

Spontaneous locomotion

The analysis of spontaneous locomotion revealed a stable entrainment for the LD12/12, LD11/11 and LD10.5/10.5 cycles (Fig. 1A, B) in all the animals tested. Under the LD10/10 regime locomotion was largely suppressed (Fig. 1B) and re-distributed between the day and night making the activity period less prominent compared to the other regimes (Fig.1B). The shortening of the LD-period was accompanied by a gradual linear increase of a mean distance moved during the day time (Fig. 1C, left panel) and a slight increase followed by a significant linear decrease in this parameter during the night (Fig. 1C, right panel).

Distribution of sleep and wakefulness across the circadian cycle

Changes in the animals' activities towards an equal distribution across the cycle were mirrored by a rearrangement of a sleep-wakefulness architecture for which both the length (Fig. 2D-F) and number of fragments (Fig. 2A-C) of all vigilance states showed a similar tendency.

Power spectrum analysis

The comparative analysis of a frequency spectrum revealed that during the wakefulness state, the power of the high θ- frequency gradually reduced whereas the power of the γ-frequency band was increased (Fig. 3A, B). During NREM sleep the power of the δ-, α- and β- frequency ranges increased compared to the LD12/12 regime (Fig. 3C, D). During REM sleep, the power of α- and β- frequency ranges increased compared to that of the LD12/12 regime (Fig. 3E, F). We performed a state-specific analysis of periodograms of the frequency bands mentioned above to scrutinize the details and characteristics of the differences.

The assessment of the temporal dynamics of the high θ- and γ-frequency bands' power during wakefulness shows a disappearance of their diurnal changes under the LD10.5/10.5 period (Fig. 4E, F). This disappearance was accompanied by a profound reduction of amplitude of the high θ-band, whereas the power of the γ-band changed in the opposite direction, thus confirming the results of the spectral analysis. Under the LD10.5/10.5 schedule, the within-group variation of a phase angle of the γ-band was higher compared to the LD12/12 regime (Fig. 4F).

The analysis of the concomitant changes in spontaneous locomotion and the high θ-band dynamics for over seven days started from a switch from the constant darkness (DD), when all the animals showed free-running periodicity that was characteristic for C57BL/6J mouse strain, to LD10.5/10.5 regime (Fig. 5). The results show that within five days after the switch the diurnal periodicity of high θ-band gradually disappeared, whereas the diurnal period of spontaneous locomotion remained entrained to the new light-dark cycle.

The power and decay rate of the NREM δ-wave were significantly higher under short cycles compared with those of the LD12/12 condition (Fig. 6A, B), which suggest an elevated sleep pressure under the short cycles. This was further supported in the analysis of a buildup rate of the δ-wave after the wakefulness-to-NREM transition, when under the LD11/11 and LD10.5/10.5 regime the power of SWA, reached in 1 min was significantly higher compared to the LD12/12 schedule (Fig. 6 C, D). In addition, power of α- and β- frequency bands increased upon a shortening of a diurnal period.

PAC analysis

We conducted an exploratory analysis of PAC across 1-50 Hz frequency range for each vigilance state separately and found a significant progressive elevation of PAC between γ- and high θ-frequency ranges during wakefulness upon the shortening of the diurnal light-dark period (Fig. 7). This effect appeared during both the scotophase and photophase. No other changes in PAC were significant, albeit the measure of PAC we used was likely to underestimate the true differences as described in (van Driel *et al.*, 2015).

8.2 Periodic properties of histaminergic system (II)

Expression of the *Hdc* gene

We assessed the temporal pattern of *Hdc* gene expression at 4-h intervals over a period of 24 h in C57BL/6J mice using quantitative radioactive *in situ* hybridization. The expression of the *Hdc* gene in adult mice takes place exclusively in the TMN. Therefore it was measured in the E2/E3 and E4/E5 subpopulations of the histaminergic neurons. No significant periodicity in the mRNA quantity was found in either group (Fig. 1).

HDC and HNMT enzyme activities

The histaminergic neurons have a wide arborisation across the brain, thus the activities of key enzymes of the histamine metabolism, HDC and HNMT, may differ between the brain areas. Therefore, activities of HDC and HNMT were measured in the hypothalamic, striatal and cerebral cortex samples of C57BL/6J mice. The enzymatic activity of HDC showed no 24-h periodicity in any of the structures analyzed, but it was approximately three-fold higher in the hypothalamic samples than in the other regions (Table 1). Surprisingly, the activity of this enzyme in the hypothalamic samples had a significant 12-h periodicity with an estimated acrophase at 5.48 h, as shown by a cosinor analysis (Fig. 2A, C, E).

HNMT had almost the same activity in all brain structures at all the times examined, with the exception that in the hypothalamus HNMT had near 12-h oscillations in activity (Table 1), with an estimated acrophase at 11.64 h, which was in anti-phase to that of HDC (Fig. 2 B, D, F).

Histamine and 1-methylhistamine levels

We studied the diurnal profile of the total tissue histamine levels in several brain areas in two mouse strains: melatonin-deficient (C57BL/6J) mouse and CBA/J melatonin sufficient mouse. We found that the histamine level showed significant 24-h rhythmicity, with an acrophase at 22.72 h only in the hypothalamic samples of the CBA/J mice (Table 2A, Fig. 3). No such rhythm was found in the cortical or striatal structures sampled in this mouse model. In contrast, the C57BL/6J mice did not show any changes in the histamine content in any of the structures examined. The mean levels of histamine in the CBA/J mice were significantly lower than those in C57BL/6J mice (Table 2A, Fig. 3).

Analysis of the 1-methylhistamine content in the hypothalamus, cerebral cortex and striatum revealed clear-cut diurnal changes over a 24-h-period and a calculated maximum ZT close to

20.5 h for both mouse strains. The CBA/J mice showed significantly lower levels of 1-methylhistamine than the C57BL/6J mice (Table 2B, Fig.4).

Histamine release, wakefulness, and spontaneous locomotion

We measured the release of histamine in the posterior hypothalamus over several days. The posterior hypothalamus was chosen as it has the densest network of histaminergic fibers. In addition, we recorded locomotor activity and EEG of the animals in an attempt to correlate the release of histamine with behavioral or physiological markers, such as different vigilance states. Cosinor analysis revealed 24-h and overlaid 8-h periodicities in the histamine release, with an orthophase at 17.63 h (Table 3). Cross-correlation analysis revealed that the highest correlation of the histamine release occurred with the percentage wakefulness (CCF 0.783-0.876) and somewhat lower correlations with motor activity (CCF 0.649-0.672) with a time lag 0. In order to test for a relationship between histamine release and the occurrence of specific frequencies in the EEG activity, the histamine levels in the dialysates were correlated with the EEG power spectra over the 1–45-Hz frequency range (0.5-Hz bins) calculated for wakefulness in 30-min epochs. The strongest positive correlation between the histamine release and the EEG power spectra were found in the high θ-range (7.5–9.5 Hz) and the γ-range (>35 Hz), both of which are indicative of active and attentive wakefulness in rodents (Spearman's correlation *(rho):* ρ=0.83 ± 0.22). No correlation was found with the low θ-range (4–7 Hz), which indicates quiet wakefulness. A strong negative correlation was observed with the δ-range (1–4 Hz), which is associated with sleep pressure/sleepiness during the awake state (Spearman's correlation: ρ=−0.83 ± 0.3).

8.3 The effect of *Hrh1* and *Hrh3* knockout on daily rhythms (III)

Spontaneous locomotion

Under the LD12/12 regime *Hrh1*$^{-/-}$ mice had a prominent diurnal activity rhythm (Fig. 1B), with a main period of 24±0.07 h (Fig. 1C, upper panel) and they were completely entrained to the LD 12/12 cycle. Under conditions of DD, 11 out of 13 *Hrh1*$^{-/-}$ mice manifested a free-running activity with a mean main period 23.71±0.09 h (Fig. 1C, lower panel), which was indistinguishable from the wild-type mice that had a period of 23.67±0.18 h, whereas two *Hrh1*$^{-/-}$ animals had no statistically significant periodicity. We were not able to find any differences between the acrophases and amplitudes of locomotor activity of wild type and *Hrh1*$^{-/-}$ mice under the LD12/12 regime (Fig. 1D, upper panel) or DD (Fig. 1D, lower panel) regime. Linear mixed model analysis of the effects of genotype, daytime and illumination regime on fragmentation of locomotor activity revealed strong effects of interaction between a phase of LD-cycle and the genotype, whereas neither interaction nor the main effects of genotype were significant (Fig. 1E).

The *Hrh3*$^{-/-}$ strain was completely entrained to a LD12/12 cycle with a main period 23.98±0.06 h (Fig. 2B). When housed under DD, five out of 19 *Hrh3*$^{-/-}$ mice had prominent free-running circadian activity period, whereas it was significantly dampened in the remaining 14 mice. The amplitude and mesor of spontaneous locomotion of the wild type group under the LD12/12 schedule were not significantly different from their knockout counterparts (Fig. 2D, upper panel) whereas both variables were dramatically reduced in *Hrh3*$^{-/-}$ animals under DD (Fig. 2D, lower panel). Linear mixed model analysis of the effects of genotype, daytime and the illumination regime on the fragmentation of locomotor activity revealed strong interaction between daytime and the regime whereas interactions and the main effects of genotype were not statistically significant (Fig. 2E).

Expression of *Per1*, *Per2* and *Bmal1* genes

The expression of *Per1*, *Per2* and *Bmal1* genes in the *Hrh1*$^{-/-}$ (Fig. 3), *Hrh3*$^{-/-}$ (Fig. 4) and wild type mice was determined at ZT6 h and ZT14 h for the cerebral cortex, the striatum and the suprachiasmatic nuclei by means of radioactive *in situ* hybridization. Linear mixed-model analysis followed by the leave-one-out test revealed no statistically significant interactions between genotype, ZT or brain structure on expression of these genes between knockout and wild type mice.

8.4 Histaminergic regulation of basal forebrain under sleep deprivation conditions (I)

Histamine release under normal conditions

We also investigated whether histamine release in the BF of rats correlates with the percentage of wakefulness similar to monitoring the release of histamine in mice. The microdialysis samples were collected every 30 min over 24 h and histamine concentrations were measured with HPLC. We found a strong positive correlation (Pearson's correlation: ρ=0.84) between extracellular histamine levels in the BF and the percentage of wakefulness in the corresponding time bins (Fig. 2B, C) irrespective of the time of day.

Histamine release under sleep deprivation

We assessed the release of histamine from the basal forebrain during SD and compared it with baseline. Histamine release elevated within 1 hour after the start of SD, then remained constantly high throughout the rest of the SD session (for 6 h) and then rapidly decreased to a baseline upon release to recovery sleep (Fig. 2A). We found no detectable elevation of histamine levels over the course of the SD period (Fig. 2A).

9. Discussion

The regulatory mechanisms of the sleep-wakefulness cycle are commonly described in terms of either slow processes such as homeostatic, circadian and sometimes ultradian, or fast processes that are involved in the transitions between vigilance states.

9.1 Circadian regulation

The estimation of the impact of the circadian regulation on the sleep-wakefulness cycle is complicated by several factors, including its synchronization with the homeostatic process and indirect effects caused by an interaction between the circadian oscillator with wakefulness-promoting and sleep-promoting centers. In addition, the ability of external time cues such as light and foraging to affect rest and wakefulness states *per se,* requires careful interpretation of results based solely on behavioral data.

9.1.1 Interaction with homeostatic processes (IV)

Study (**IV**), showed that short (LD11/11 and LD10.5/10.5) circadian cycles caused significantly more consolidated NREM sleep, which was accompanied by elevations of the SWA during the photophase compared to what was obtained under the LD12/12 cycle. The fact, that the circadian regulation was affected by this light-dark regime was supported by the disappearance of diurnal periodicity of the high θ- and γ- waves during wakefulness under the LD10.5/10.5 cycle and also the progressive rearrangement of sleep and wakefulness bouts towards an aperiodic state. Therefore, taking into account methodological considerations (as discussed later), our data show that the disturbance of the circadian oscillator may produce alterations in the homeostatic process.

The effects on the sleep-wakefulness cycle notwithstanding, the LD10.5/10.5 regime significantly strengthened the PAC between the high θ- and γ- waves. The primary source of the θ-activity in the rodent brain is the hippocampus, which has been extensively reviewed by Buzsáki (2002), although several studies reported the θ-activity generation by the enthorinal (Alonso & García-Austt, 1987) and cingulate areas of the cerebral cortex (Leung & Borst, 1987). The cerebral cortex γ-oscillations are known to be strongly affected by the hippocampal θ-activity (Buzsáki, 2002; Sirota *et al.*, 2008) and this modulation in rodents is likely to occur via a volume conduction as demonstrated by Sirota and colleagues (2008). An elevation of the θ-γ phase-amplitude coupling has also been associated with active learning and memory retrieval in humans and in mice (Canolty *et al.*, 2006; Tort *et al.*, 2009). On the

other hand, the animals kept under short cycle regimes performed memory tests significantly worse than animals under normal conditions (Karatsoreos *et al.*, 2011). Moreover, lesions of the SCN restored the performance of hamsters that had a previously induced aperiodicity (Fernandez *et al.*, 2014). In that respect, our finding is surprising and whether the observed changes in PAC is a consequence of an altered connectivity remains to be investigated.

9.1.2 Interaction with sleep and wakefulness-promoting centers with an emphasis on the histaminergic system (I)

The second issue is any indirect effects that the circadian oscillator may have on the sleep-wakefulness cycle, which may be caused by circadian modulation of the key sleep and wakefulness-promoting centers, including VLPO, MnPO, LC, TMN, BF, PTT, DR and others. Although the SCN has a very limited number of direct projections into these brain regions, several relay areas such as the SPVZ, the paraventricular nucleus of the hypothalamus, the DMH and the medial preoptic area conduct downstream circadian signals to these centers (Deurveilher *et al.*, 2002b; Deurveilher & Semba, 2003, 2005).

The VLPO receives strong GABA-ergic afferents from the DMH (Chou *et al.*, 2003), in addition to a scarce innervation from the SCN itself (Sun *et al.*, 2001; Chou *et al.*, 2002). This is accompanied by daily fluctuations in the *c-Fos* expression in this nucleus (Novak & Nunez, 1998). However, bilateral lesions of the VLPO did not cause significant changes in the circadian rhythms of sleep-wakefulness cycle or core body temperature (Lu *et al.*, 2000), although producing 50-60% loss of NREM sleep, an increased fragmentation of sleep-wakefulness cycle and a reduction of SWA during NREM sleep.

The activities of neurons in the LC and the DR have a clear circadian component, that persists under DD (Semba *et al.*, 1984; Cagampang *et al.*, 1993) and, at least in the case of the LC, is dependent on the intact DMH, which is a first-order and second-order effector of the SCN (Aston-Jones *et al.*, 2001). Likewise, a release of acetylcholine into the cerebral cortex, as was assessed by *in vivo* microdialysis is also circadian (Kametani & Kawamura, 1991). In contrast, the dopamine content in the VTA and SN does not change throughout the circadian cycle. Neither the lesions of these structures, nor the high mesencephalic transection that disconnected them from the SCN had any significant effects on the circadian rhythm of sleep and wakefulness (Mouret & Coindet, 1980; Hanada & Kawamura, 1981; Lu *et al.*, 2006, Blum *et al.*, 2014).

Several studies demonstrated the circadian regulation of the HCRT-ergic neurons, because the production of HCRT mRNA (Taheri *et al.*, 2000) and proteins (Yoshida *et al.*, 2001) undergo

diurnal changes that disappear when a lesion of the SCN is made (Zhang *et al.*, 2004). Yet, *Hcrt2*$^{-/-}$ mice have normal circadian rhythms of sleep-wakefulness cycles, although wakefulness was severely fragmented (Mochizuki *et al.*, 2004, 2011). The observation that the activity of the hypocretinergic neurons is highly correlated with locomotion at any circadian phase (Estabrooke *et al.*, 2001; Zeitzer *et al.*, 2003) may however resolve this apparent controversy.

We found that the diurnal variation of the histamine level in the CBA/J mice had a maximum during the scotophase, which is in line with previous findings (Tuomisto & Tuomisto, 1982; Michelsen *et al.*, 2005; Yu *et al.*, 2014). The histamine rhythm was strain-dependent and restricted only to the hypothalamus, and no rhythm was found in the C57BL/6J strain. In contrast to the findings reported by Yu *et al.*, 2014, we were unable to detect any 24 h periodicity of the *Hdc* gene expression and activity. Instead, the 12 h activity period was significant, which is in line with that reported by Tuomisto and Tuomisto (1982). The activity of the primary histamine breakdown enzyme, HNMT had no 24-h-period, which agrees with that reported by Tuomisto and Tuomisto (1982) and Yu and colleagues (2014). We observed a diurnal periodicity in the histamine release using *in vivo* microdialysis. The release was highly positively correlated with spontaneous locomotion and attentive wakefulness and this is further addressed later in this discussion (section 9.2.2).

The histamine release in mice and rats eventually reached the nocturnal level upon wake up for foraging during the photophase. Notably, the release was high irrespective of the phase of the circadian oscillator and similar findings were reported on the histamine release in narcoleptic patients (John *et al.*, 2004). Therefore, although the histamine release had a clear 24-h periodic component, it remains to be investigated, whether this periodicity was caused by the circadian regulation or a masking effect of light-dark cycle. One approach to this could be to use microdialysis in conjunction with EEG, performed under a short light-dark cycle (LD10.5/10.5). Challenging the oscillator with such an exotic light-dark regime would enable the assessment of whether the periodic release of histamine follows the intrinsic circadian rhythm or rhythm of spontaneous locomotion, which is entrained to a short cycle.

Another question is if the diurnal changes of the histaminergic neurons are caused by the circadian regulation, whether these cells are capable of generating this rhythm themselves or otherwise, which neuronal pathway could conduct it? Although the TMN was not tested explicitly, the majority of the brain regions after being isolated from the SCN rapidly became arrhythmic (Abe *et al.*, 2002). The indirect connections include areas involved in the regulation of sleep and wakefulness, such as the preoptic area (Wouterlood & Gaykema,

1988), VLPO (Chou *et al.*, 2002), the HCRT-ergic neurons (Abrahamson *et al.*, 2001), and the DMH (Deurveilher & Semba, 2005). The VLPO and preoptic areas utilize GABA as the main transmitter and inhibit the TMN neurons mainly through the $GABA_A$ receptor (Yang & Hatton, 1997). The HCRT-ergic neurons excite the TMN neurons through the actions of HCRTR2, NMDA and the AMPA receptors (Yang & Hatton, 1997). Recent studies on mice that lack either $GABA_A\gamma_2$ or $GABA_{B1}$ receptors selectively in the TMN cells (Zecharia *et al.*, 2012) or *Hcrt*$^{-/-}$ or *HcrtR2*$^{-/-}$ mice (Mochizuki *et al.*, 2011) show that the periodic component of the sleep–wakefulness cycle was indistinguishable from that of the wild-type animals. The results from a lesion study on the DMH (Chou *et al.*, 2003) suggest that the DMH is a plausible candidate as a relay site that conducts a circadian information to the TMN.

9.2 Histaminergic regulation

9.2.1 The interaction with the circadian process (III)

As mentioned in the literature review section, pharmacological (Doi *et al.*, 1994) or genetic (Abe *et al.*, 2004) manipulations that target the histaminergic system may affect the circadian rhythms of sleep and wakefulness in addition to feeding and drinking. This can be achieved by either a direct modulation of the SCN by histamine (Stehle, 1991; Kim *et al.*, 2015) or by joint action of both systems that presumably take place in the regulation of the hypothalamo-pituitary axis (Kjaer *et al.*, 1994). Therefore, we attempted to find which histamine receptor subtype would potentially mediate these changes.

The participation of HRH1 receptor in the transduction of the circadian information in mammals gained support from findings of the intense [^{3}H]-pyrilamine binding in the SCN area of mice (Abe *et al.*, 2004) and rats (Lozeva *et al.*, 2000). Rats housed under the LD12/12 cycle and chronically administered with the HRH1 receptor antagonist, pyrilamine, had an attenuated amplitude of spontaneous locomotion (Lozeva *et al.*, 1998). In addition, several studies on *Hrh1*$^{-/-}$ mice reported an altered feeding rhythm (Masaki *et al.*, 2004) and a minor decrease in overall locomotor activity (Inoue *et al.*, 1996; Huang *et al.*, 2001), whereas Yanai et al., showed marked reduction in locomotor activity during the dark phase of the light-dark cycle (Yanai *et al.*, 1998). Notably, none of these studies assessed the parameters in question under the DD conditions, which suggests that even if the HRH1 receptor participated in the mediation of circadian information, this effect was masked by light. We found that *Hrh1*$^{-/-}$ mice housed under DD had a free-running locomotor activity period similar to that found in wild type animals, and daily changes in the transcription of *Per1, Per2* and *Bmal1* genes in all examined brain structures were similar as compared to the wild type siblings.

The role of HRH2 in the circadian regulation was assessed by pharmacological inhibition of this receptor by cimetidine, which led to an occasional potentiation of the effect of histamine on the SCN neurons in the rat brain slice preparations (Stehle, 1991) However, HRH2 did not cause any phase shifts when administered to hamsters (Eaton *et al.*, 1996). Similarly, the brain-penetrating HRH2 antagonist, zolantidine, had no effect on the light-induced phase shift in hamsters or on the amount or distribution of NREM, REM and wakefulness states in rats (Monti *et al.*, 1990).

Several studies on *Hrh3*$^{-/-}$ mice found significantly diminished spontaneous locomotion (Takahashi *et al.*, 2002; Toyota *et al.*, 2002), less voluntary movement, lower body temperature (Toyota *et al.*, 2002) and less time spent awake (Gondard *et al.*, 2013) during the scotophase, but these were not apparent during the photophase when compared to their wild type controls. These changes emerged as an overall reduction of amplitude, but not as a disappearance of these daily rhythms. Under DD conditions the voluntary activity of *Hrh3*$^{-/-}$ mice as assessed by a running wheel test was reduced by 25%, although the circadian rhythm was also preserved (Toyota *et al.*, 2002). This finding is similar to that found in *Hdc*$^{-/-}$ mice whose wheel running activity rhythm was significantly blunted under both LD and DD conditions (Abe *et al.*, 2004). This could be explained by the histamine-dependent involvement of HRH3 in voluntary activities and/or an overall reduction of locomotion during the scotophase, which is caused by the low histamine levels typical for *Hrh3*$^{-/-}$ mice (Toyota *et al.*, 2002).

In our study the spontaneous locomotion of *Hrh3*$^{-/-}$ mice under a DD regime was monitored and we found it significantly decreased (**III**). This finding combined with wheel-running activity data reported by Toyota and colleagues can be interpreted as indication that not only voluntary but also involuntary activities are affected by the absence of functional HRH3 (Toyota *et al.*, 2002). It is also important to note, that the HRH3 receptor is constitutively active (Morisset, Traiffort, *et al.*, 2000) and can serve as both a presynaptic and a postsynaptic heteroreceptor, which participates in the control of the release and action of the other neurotransmitters (Blandina *et al.*, 1996; Yamamoto *et al.*, 1997). Thus, whether the observed phenotype was caused by an altered histamine-mediated signaling or an impaired modulation of other neurotransmitter systems remains to be investigated.

At the same time the period length of the spontaneous locomotion under DD and the expression of *Per1, Per2* and *Bmal1* genes in *Hrh3*$^{-/-}$ mice were similar to those of their wild type counterparts, which suggests that circadian regulation was not affected by the absence of the HRH3. This is in contrast to *Hdc* knockout mice that have abnormally long free-running

activity rhythm and a lack periodic expression of *Per1* and *Per2* genes in the cerebral cortex and the striatum (Abe *et al.*, 2004). The discrepancies between histamine-deficient and histamine-receptor-deficient phenotypes raise the possibility that histamine may exert its effects not only through its canonical receptors but also through other mechanisms. For example, an NMDA-mediated histamine signaling has been proposed (Bekkers, 1993; Vorobjev *et al.*, 1993). This hypothesis was further supported by studies, where histamine caused phase shifts in hamster SCN explants, which suggests it is involved in a modulatory potential of a glutamatergic retinoreceptive input to the SCN (Eaton *et al.*, 1996; Meyer *et al.*, 1998). Nevertheless, this hypothesis is yet to be proven as no *in vivo* data have been published to date.

9.2.2 The interaction with the homeostatic process (II and III)

The firing rate of the histaminergic neurons is low and might not be detected at all during sleep time but it is high during wakefulness (Takahashi *et al.*, 2006). Histamine release as measured by *in vivo* microdialysis shows a similar pattern in the prefrontal cortex (Chu *et al.*, 2004), the preoptic area of the hypothalamus (Strecker *et al.*, 2002), the anterior hypothalamus (Mochizuki *et al.*, 1992) and the posterior hypothalamus (Prast *et al.*, 1992). The use of *in vivo* microdialysis in combination with behavioral tracking and EEG enabled us to confirm that the elevation of histamine release in the TMN of mice and the BF of rats reflects the increase of spontaneous locomotion. We also found that the histamine release from the TMN region had almost no correlation with quiet wakefulness (low θ-range 4–7 Hz), but was highly positively correlated with the high θ-range (7.5–9.5 Hz) and the γ-range (> 35 Hz) frequencies, which are associated with active wakefulness and alertness. Conversely, the release was negatively correlated with the δ-range (1–4 Hz), a sign of sleepiness and a sleep pressure during wakefulness, which is in a good agreement with Prast *et al.*, (1997). An apparent discrepancy between our data and those of Prast *et al.*, (1997) regarding the γ-waves is likely because of different consciousness states under which the measurements were performed.

The basal forebrain is known to be involved in the regulation of sleep homeostasis and arousal of the cerebral cortex. Therefore, we investigated whether the histamine release in this area reflects the increase in the sleep propensity under SD conditions. Throughout 6 h of SD the histamine release reached its wakefulness level and was constantly high. When the SD session ceased, histamine release immediately dropped to a baseline, which is in line with the findings reported by Strecker and colleagues (2002), who obtained similar results on the preoptic area of cats.

We tested if histamine *per se* can affect the homeostatic sleep drive in the experiments reported in (**I**). Histamine was continuously administered into the BF and a significant increase of the high θ- and γ-range power throughout infusion period was found. Surprisingly, the NREM SWA during the consequent recovery period of sleep remained similar to a control. In agreement with these data Yu *et al.* (2014) recently found that mice with constantly high histamine levels, caused by a deletion of the *Bmal1* gene targeted in the histaminergic neurons, had an elevated overall locomotion, but significantly attenuated SWA after SD compared to their wild type counterparts.

Therefore the inability of endogenously synthesized or exogenously infused histamine to increase NREM SWA during the recovery period, in combination with its steadily high release rate under SD suggest that histamine is likely neither affected by nor a carrier of the homeostatic sleep pressure.

9.3 Methodological considerations

The term "circadian" is used only for the experiments conducted under DD conditions, whereas the term "diurnal" and "daily" is applied to changes under periodic conditions, which in this series of studies were LD12/12, LD11/11 and LD10.5/10.5. The terms "periodic" or "cyclic" describe any detectable periodicity irrespective of any changes in housing conditions.

II: During the enzyme activity studies in which histamine assays, 1-methylhistamine assays, and the *in situ* hybridization assay were carried out, the mice were housed in groups of three. This housing arrangement may have potentially confounded the results due to activity cues being given from on individual to another. Although this issue has not been addressed specifically, we interpreted data reported by Mistlberger & Skene (2004) and Paul & Schwartz (2007) to mean that, the effects of social cues are likely to be relatively small in the presence of light since this is a strong time cue. Importantly, the housing conditions were identical for all the mice in the analysis.

III The gene expression was examined under the LD12/12 regime, therefore the potential masking effect of light may have confounded it. Although only two sampling times, ZT6 and ZT14 were chosen, they were based on previous studies that identified minima and maxima of expressions for *Per1, Per2* and *Bmal1* genes in the brain areas of the SCN, the prefrontal/cerebral cortex and in the striatum (Shigeyoshi *et al.*, 1997; Sun *et al.*, 1997; Abe *et al.*, 1998, 2004; Shearman *et al.*, 1999). Thus, except for the expression of the *Per2* gene in the SCN, the two sampling times chosen were close to optimal.

We used the constitutive knockout models, for which the gene modification is present through the development of the animal. Therefore, we cannot rule out the possibility of compensatory changes that may have confounded the measured parameters. The use of inducible knockouts could circumvent this problem.

The mice were housed individually in a soundproof room in this study, though they could see and hear each other. This may cause an entrainment of the rhythms by audial disturbance, which may mask the original rhythm. This issue was addressed previously.

IV: The order the animals were subjected to the short cycles was not randomized, thus an "order effect" might have taken place.

A carryover effect that is attributable to a single subject design: i.e. the previous condition, may affect the following treatment. To overcome any carryover effect we introduced 1 week of washout period (DD) between the LD11/11 and LD10.5/10.5 and the free-running period was essentially similar to what normal C57BL/6J animals have. Thus, there are only two confounders: effect of the LD12/12 on LD11/11 (which is a common practice in such studies) and the effect of the LD10.5/10.5 on LD10/10.

10. Conclusions

- The shortening of the circadian rhythm significantly affects the distribution of sleep-wakefulness states, increases sleep propensity, diminishes daily rhythms the high θ- and γ-waves and causes an elevation of the PAC. These findings suggest an interaction between the circadian rhythm and the homeostatic regulation, rather than autonomous functioning of these processes.
- The histaminergic system displays daily rhythms of production and release of histamine and its metabolite, 1-methylhistamine, whereas activities of the enzymes HDC and HNMT exhibited no detectable diurnal rhythm. Whereas the lack of HRH3 receptors was associated with a significant attenuation of circadian amplitude of spontaneous locomotion, neither this nor *Hrh1*$^{-/-}$ had an effect on the diurnal changes in the expression of *Per1*, *Per2* and *Bmal1* genes.
- Finally, we could not find any evidence for the involvement of the histaminergic system in the homeostatic regulation of the sleep-wakefulness cycle, since it neither affects, nor is affected by this process.

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