The involvement of central corticotropin-releasing hormone and its receptors in sleep-wake regulation of mice

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(Christoph P N Romanowski)

Meiner Familie

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I Abstract

The corticotropin-releasing hormone (CRH) is widely recognised as the major activator of the hypothalamic-pituitary-adrenocortical (HPA) axis, thereby mediating neuroendocrine, autonomic, and behavioural responses to stress. Dysregulation of the release of stress hormones, caused by excessive CRH secretion from the hypothalamus, is frequently observed in patients with affective disorders such as depression. One of the cardinal symptoms of major depression is a severe impairment of sleep (e.g. reduced sleep intensity, disinhibition of rapid eye movement sleep (REMS), and early morning awakenings). Consequently, besides a role of CRH in stress-induced arousal, its additional contribution to spontaneous sleep-wake regulation was suggested in literature. Due to the lack of highly specific CRH receptor antagonists and adequate CRH receptor knockout animal models, the mechanism and pathways by which CRH communicates its arousal function remained indistinct. Up to now it is unclear whether CRH interferes with sleep by a direct central action, or if the activation of the HPA axis and the subsequent release of peripheral stress hormones are mandatory.
The present study with conditional CNS-specific CRH receptor type 1 (CRH-R1 CKO) and conventional CRH receptor type 2 knockout mice (CRH-R2 KO), allows assessment of CRH effects on wakefulness and sleep separately from a functional HPA axis together with various levels of CRH receptor system functionality. In addition, challenging sleep homeostasis in these mouse lines by sleep deprivation allows investigating the involvement of CRH and its receptor system in basic sleep-wake regulatory processes.

Besides slight dissimilarities between the baseline sleep profiles in the various genotypes, CRH-R1 CKO displayed a markedly different response to intracerebroventricular (i.c.v.) CRH injections. The dose-dependent increases in wakefulness and decreases in non-REM sleep (NREMS), which could be observed in all other mouse lines, were almost totally absent in CRH-R1 CKO. The dose-dependent REMS suppression on the other hand persisted in all, even CRH-R1 CKO, animals. This suggests that the centrally expressed CRH receptor type 1 (CRH-R1) but not the CRH receptor type 2 (CRH-R2), mediates the crucial effects of CRH on wake induction and NREMS suppression. Since REMS inhibition by CRH still occurred in CRH-R1 CKO animals pretreated with a highly specific CRH-R2 antagonist, the clear role of central CRH and both receptors in REMS suppression remains elusive.

Sleep deprivation induced significant increases in plasma corticosterone levels in all mouse lines, demonstrating HPA axis activation and suggesting that all mice perceived sleep loss as a stressor. After termination of sleep deprivation, all animals responded with a significant

increase of slow wave activity (SWA), an indicator of sleep intensity, followed by a rebound of NREMS. With the exception of CRH-R1 CKO mice, all mice furthermore similarly displayed REMS rebound. Another difference in response to sleep deprivation constitutes the course of SWA in CRH-R1 CKO which was significantly increased over baseline levels for a longer period as compared to all other mouse lines. Accordingly CRH-R1 CKO animals presumably sleep more intensely or efficiently than mice of the other breeding lines. These results suggest that CRH mediates the effects, at least the stressful component, of sleep loss, and moreover that CRH-R1 is essentially involved in sleep homeostasis.

This study is the first to show considerable evidence for a crucial involvement of central CRH and CRH-R1 in arousal and the suppression of NREMS. It could further be shown that activation of the HPA axis is not a prerequisite of these effects. Additionally, the action of central CRH, mediated by CRH-R1 seems to influence sleep quality. The role of CRH-R2 has to be regarded as of a minor nature. The impact of CRH on REMS regulation demands further investigation.

II Introduction

2.1 Ways to define sleep

Although a wide range of resting states can be observed within the animal kingdom (e.g. hibernation and torpor), all these states are clearly distinguishable from what we call sleep. In lower vertebrates and invertebrates, small size or, for latter, absence of anatomical requirements for electroencephalogram (EEG) recordings (e.g. a developed cortex) complicate the issue of defining sleep. As a scaffold for scientific approaches to investigate sleep in such simple organisms, a behavioural definition of sleep has been proposed. For example, the sleep state (resting state) should be rapidly reversible, there should be a place preference or a specific position while sleeping (resting), during this period arousal thresholds should be increased (i.e. decreased responsiveness to sensorial stimulation), and the process should be subject to homeostatic (recovery needed after deprivation) and also circadian regulation (Campbell and Tobler, 1984;Siegel, 2008). Therefore the presence of a resting period in many animals is indisputable. Even organisms like plants and neurospora show daily cycles of activity, but the definition of “sleep” has not yet been extended to *Archaeplastida* and the subkingdom of *Dikarya*. In mammals, however, which have a developed cortex or neocortex, polysomnography is a reliable tool to distinguish different vigilance states. Hence a combination of EEG, electromyogram (EMG), and electrooculogram (EOG) recordings can be applied to classify the level of waking and the different stages of sleep.

2.1.1 Sleep in invertebrates

Many invertebrates show periods of quiescence (e.g. *Apis spec*. (Kaiser and Steiner-Kaiser, 1983); *Periplaneta spec*. (Tobler and Neuner-Jehle, 1992); *Heterometrus* and *Pandinus spec*. (Tobler and Stalder, 1988)), though only the fewest display a resting behaviour satisfying all previously defined behavioural criteria. So far only *Drosophila melanogaster* expresses an ensemble of all behaviours that qualify its resting as behavioural sleep (increased arousal threshold, rebound after deprivation etc.) (Greenspan et al., 2001;Hendricks et al., 2000;Shaw et al., 2000). However no claims on the appearance of rapid eye movement sleep (REMS) in invertebrates have been made so far. Further studies have been conducted in *Caenorhabditis elegans* demonstrating sleep-like resting periods (i.e. lethargus) (Bessereau, 2008;Raizen et al., 2008).

2.1.2 Sleep in vertebrates

A larger number of sleep studies has been conducted in vertebrates as compared to invertebrates. In fish (e.g. *Danio rerio*) (Yokogawa et al., 2007a, b;Zhdanova et al., 2001) as well as in amphibians (*Hyla septentrionalis*) (Hobson et al., 1968) sleep-like resting states have been described. A clearer picture emerges from studies on reptile sleep, where EEG analysis could detect sings of sleep concomitant with behavioural quiescence. Although early studies reported signs of a REMS-like vigilance state in reptiles, no recent study could reproduce these data so far (Siegel, 2008). However, EEG studies in birds documented REMS and non-REMS (NREMS) (Rattenborg et al., 2004a, b). The majority of studies were done in mammals (e.g. humans, cats, mice, rats) where REMS and NREMS can be clearly identified behaviourally as well as by EEG analysis and are distinct from waking or periods of quiet wakefulness. According to the well received criteria by Rechtschaffen and Kales (Rechtschaffen and Kales, 1968) (very recently slightly modified by the American Academy of Sleep Medicine; Iber et al., 2007), sleep or cognitive levels can be classified further in higher mammals (i.e. humans) which, up to a certain degree, is also possible in lower mammals (i.e. rodents). Human vigilance is divided into wakefulness, four different NREMS stages (stages 1-4), and tonic or phasic REMS (Carskadon and Dement, 2005;Carskadon and Rechtschaffen, 2005) (Figure 1, page 5).

Wakefulness is characterised by a low-amplitude, desynchronised cortical EEG with fast oscillations (activated EEG) in the range of 20-60Hz (beta and gamma range) and the presence of muscle tone in the EMG. Thresholds for sensory responses are lowest, voluntary movements occur, and thoughts are progressive and logical (high consciousness). In transition to a drowsy state, alpha waves (8-13Hz) occur.

Stage 1 sleep is initially dominated by alpha waves and progressively by low-voltage (<20µV) mixed frequency (2-7Hz; theta range) activity and vertex sharp waves in synchronising EEG signals. Typically slow eye movements can be detected during stage 1 sleep.

Stage 2 sleep is characterised by occasional slow oscillating (<4Hz) waveforms with an amplitude too low as to fulfil the criteria for stage 3 or 4 sleep. Typically distinctive sleep spindles (sequence of at least 6-7 cycles of 12-14Hz waves lasting 0.5-1.0 seconds) and K-complexes (negative sharp waves followed immediately by a slower positive component frequently accompanied by subsequent sleep spindles) can be detected in the EEG. Furthermore theta activity (5-8Hz) occasionally mingles with other frequencies of the recorded signal.

Stage 3 sleep is characterised by an increased presence (20-50% of recording time) of high-voltage (>75μV) slow wave oscillations (<4Hz; delta waves) in the EEG.

Stage 4 sleep is characterised by the same criteria as stage 3 sleep, however the EEG signal is dominated by delta waves (>50% of recording time).

REMS is defined by the occurrence of low-voltage, mixed-frequency activity (6-9Hz; hippocampal theta waves; activated EEG) in the EEG with the presence of no muscle tone (atonia) in the EMG and characteristic singlets or clustered rapid eye movements in the EOG. Further REMS-specific physiological signs are myoclonic twitches (facial and distal limb musculature), pronounced fluctuations in cardio-respiratory rhythms and core body temperature as well as penile erections or clitoral tumescence.

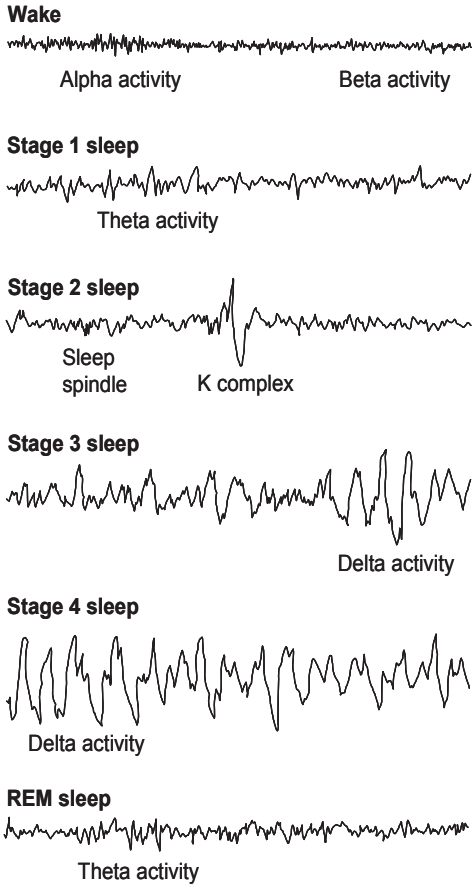

Figure 1: Human EEG signals of different vigilance stages (adapted from Horne, 1988).

Wakefulness is characterised by a low-amplitude, desynchronised cortical EEG with fast oscillations (20-60Hz; beta and gamma activity). While progressing to sleep alpha waves occur. Stage 1 sleep is initially dominated by alpha waves (8-13Hz; alpha activity) and subsequently by low-voltage waves of mixed frequencies (2-7Hz; theta range). A typical feature of Stage 2 sleep is the occurrence of slow oscillating waves (<4Hz), sleep spindles (12-14Hz), and K-complexes. Stage 3 and 4 are determined by the steadily increasing appearance of high amplitude slow wave oscillations (<4Hz; delta waves). REMS is defined by low-voltage, mixed-frequency activity (6-9Hz; theta waves) in the EEG, no muscle tone (atonia) in the EMG, and rapid eye movements in the EOG.

These basic electrophysiological signs for vigilance states are similarly seen in all mammals. In those animals (and additionally in some avian species) sleep is subdivided in NREMS and REMS. Sometimes NREMS is divided into light and deep sleep or accordingly into slow wave sleep I (SWS I; presence of sleep spindles in cortical EEG) and SWS II (presence of high-voltage low frequency waves [<4Hz] in cortical EEG). Light sleep resembles stage 1 and 2 of human NREMS, while deep sleep corresponds with stages 3 and 4. In some larger animals (e.g. rats) REMS can be divided into phasic and tonic REMS according to the detected eye movements occurring in the EOG during this vigilance state.

Box 1: Cornerstones of sleep research over the last 100 years.

1913: Postulation of the concept of humoral sleep regulation. (Legendre and Piéron, 1913)
1929: Discovery of the human electroencephalogram (EEG). (Berger, 1929)
1930: Proposition of an arousal pathway actively maintaining wakefulness. (Economo, 1930)
1935: Distinction of waking, sleep, and dreaming EEG in humans. (Loomis et al., 1935a, b)
1937: Utilisation of an animal model (i.e. cats) in sleep research. (Klaue, 1937)
1946: Indication of hypothalamic sleep regulation in rats. (Nauta, 1946)
1949: Wake and sleep are maintained actively not passively. (Moruzzi and Magoun, 1949)
1953: Description of REMS in humans. (Aserinsky and Kleitman, 1953a, b)
1958: Objective identification of REMS in cats. (Dement, 1958)
1959: Description of REMS as “paradoxical sleep”. (Jouvet and Michel, 1959)
1962: Allocation of REMS regulation to the brainstem. (Jouvet, 1962)
1972: Postulation of the monoamine theory of sleep-wake regulation. (Jouvet, 1972)
1973: Identification of a sleep-promoting humoral factor (“delta”). (Monnier and Schoenenberger, 1973)
1975: Postulation of the reciprocal interaction model of REMS regulation. (McCarley and Hobson, 1975)
1982: Postulation of the two process model of sleep regulation. (Borbély, 1982a)
2006: Postulation of the flip-flop switch model of REMS regulation. (Lu et al., 2006)

2.2 The regulation of sleep and wakefulness in mammals

2.2.1 The two process model

The suggested model comprises of a sleep-wake dependent homeostatic (Process S) and a sleep-wake independent circadian (Process C) component, which by interacting explain the timing of sleep and waking (Borbély, 1982a) (Figure 2). Although it was initially proposed to account for sleep regulation in animals (i.e. rats) (Borbély, 1980;Borbély, 1982b) its validity has been extended to human sleep (Daan et al., 1984;Daan and Beersma, 1984). Comprehending a physiological basis and mathematical simplicity, the two process model is well accepted in the field of sleep research and provides a basis for many models of fatigue and performance regulation (Achermann, 2004;Akerstedt et al., 2004;Belyavin and Spencer, 2004).

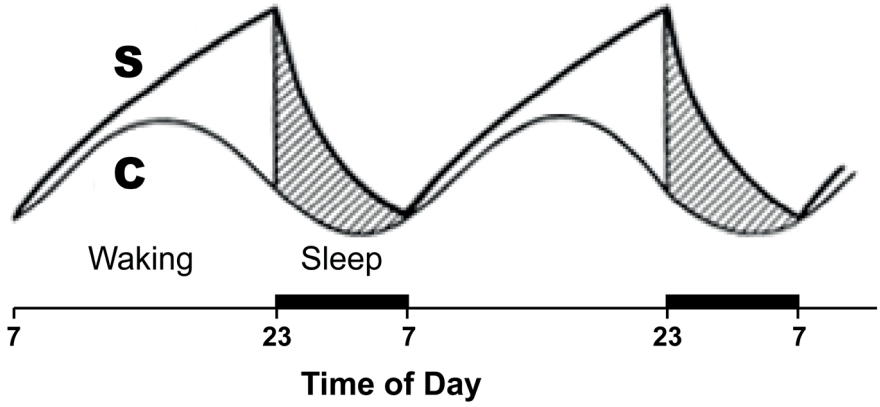

Figure 2: The two process model (adapted from Borbély and Achermann, 2000).

The scheme illustrates the time course of the homeostatic (S; upper curve) and the circadian process (C; lower curve) over two days (x-axis indicates time; black bars represent the sleeping period). Sleep propensity, as indicated by Process S, increases during waking and decreases during sleep. Process C, representing wake drive, is displayed as a sine function with a maximum and minimum in the middle of the waking and sleeping period, respectively. Sleep occurs when Process S peaks and Process C abates. At the end of the sleeping period, sleep propensity is the lowest and a little rise of wake drive suffices to wake up.

2.2.1.1 Homeostatic control of sleep (Process S)

As early as 1937, it could be shown that “sleep need” is high during the first part of the sleep episode and progressively declines with sleep duration. Similarly, both the arousal threshold and predominance of slow wave activity (SWA; equivalent to delta activity which encompasses EEG components in the frequency range of 0.5-4Hz as obtained by spectral analysis) are high at the onset of sleep and decrease gradually (Blake and Gerard,

1937;Loomis et al., 1937). Since the decrease in arousal thresholds during the course of sleep is independent of sleep stages (Rechtschaffen et al., 1966) but correlates to the decrease of SWA, sleep intensity seems to be reflected by SWA rather than by the different sleep stages (Borbély, 1982a). A large number of studies have demonstrated an increase of SWA during the subsequent sleeping period following partial or total sleep deprivation (SD) in mice (Huber et al., 2000), rats (Franken et al., 1991;Tobler and Borbély, 1990), cats (Tobler and Scherschlicht, 1990), rabbits (Opp et al., 1997;Tobler et al., 1990), and humans (Borbély et al., 1981). Therefore SWA represents an ideal indicator correlating with sleep pressure, whose initial level is dependent on the duration of prior waking (Borbély, 1982a, b). Consequently it constitutes an ideal quantitative marker to track the dynamics of the homeostatic Process S. With prolonged waking, the homeostatic sleep pressure increases and ultimately initiates sleep by interacting with the circadian component (Process C). Even in the absence of external circadian clues (light-dark conditions) the sleep onset occurs after a period of extended waking. Amongst other sleep-promoting factors, e.g. brain adenosine levels have been implicated as a homeostatic indicator of sleep need (Basheer et al., 2004;Radulovacki et al., 1984;Strecker et al., 2000).

2.2.1.2 Circadian control of sleep (Process C)

Whereas humans are diurnally active, some mammals (e.g. laboratory rodents) are nocturnal animals. Further humans are monophasic sleepers, possessing one main sleep consolidating phase with four to six recurrent, ca. 90-min cycles (shallow to deep NREMS followed by REMS and very short awakenings; Figure 3, page 9). On the other hand, many animals are polyphasic sleepers (multiple, interrupted sleep phases). The sleep-wake distribution is species-specific and commonly (e.g. mammals) appears predictably within a 24 hour cycle. Lesion studies in rodents and non-human primates have shown that the suprachiasmatic nucleus (SCN) of the hypothalamus, pacemaker of the internal clock in mammals (Dibner et al., 2010), is a crucial brain area for maintenance of the 24 hour circadian rhythm in sleep drive (Edgar et al., 1993;Mendelson et al., 2003;Mistlberger, 2005). Bilateral lesions of the SCN or homologous regions severely disrupt the circadian sleep-wake pattern in rats (Tobler et al., 1983), squirrel monkeys (Edgar et al., 1993), and humans (Cohen and Albers, 1991). The internal clock is synchronised to the external day-night cycle by signals consisting of light inputs from the retina during day and melatonin release from the pineal gland during night (Cassone et al., 1986;Gillette and McArthur, 1995). These signals are reflected on a molecular basis by expression of various clock-related genes (e.g. *Period*, *Chryptochrome* and *Clock*) that influence activity of the SCN ultimately leading to the generation of the circadian rhythm (Mistlberger, 2005). Although the main efferent projections

from the SCN are intra-hypothalamic, most of the major sleep-wake regulating brain areas receive direct inputs from this nucleus (Deurveilher et al., 2002;Deurveilher and Semba, 2003;Deurveilher and Semba, 2005;Jones, 2003;Jones, 2005). In this respect sleep timing (onset and offset) is rigorously controlled by interactions between SCN activity and the underlying regulation of circadian clock genes. To estimate the amplitude and phase characteristics of process C, analysis of certain body rhythms can be useful (e.g. core body temperature changes) (Czeisler et al., 1989;Dijk and Czeisler, 1995).

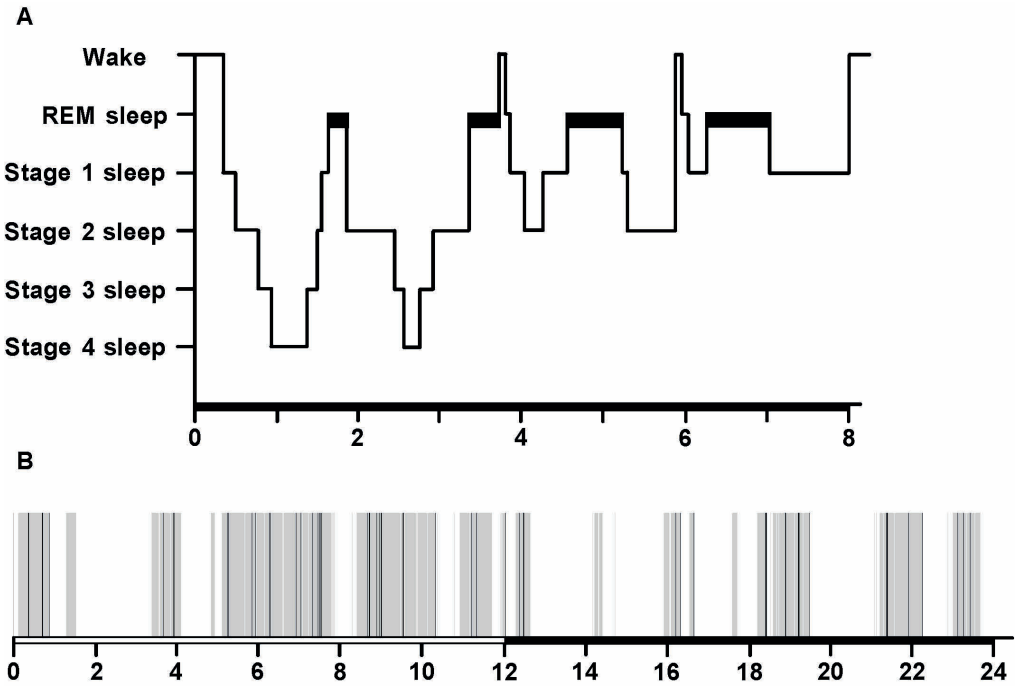

Figure 3: Representation of a human and a mouse hypnogram (human hypnogram adapted from Strollo, 1998; mouse hypnogram own data, unpublished).

Depicted are representative hypnograms of a monophasic, i.e. human (A), and a polyphasic, i.e. mouse (B), sleeper. In humans typically one main sleeping phase occurs with four to six recurrent cycles of shallow to deep NREMS followed by REMS (y-axis indicates vigilance states). In mice, sleep cycles are shorter (white areas: wake; grey bars: NREMS; black bars: REMS), more frequent, and distributed throughout the day, although the larger proportion of sleep appears in the light (inactive) period. The x-axes indicate time in hours, the open and filled horizontal bars on the x-axis the light and dark period, respectively. Note that the human hypnogram shows only the sleeping phase, while the mouse hypnogram displays both the light (inactive) and dark (active) period.

2.2.2 Neuronal sleep-wake regulation in mammals

2.2.2.1 Wake regulation

Von Economo pioneered in proposing an ascending arousal pathway that maintains wakefulness, when excessive sleep was observed in patients suffering from encephalitis lethargica (later termed “Von Economo’s Disease”) who had lesions around the junction of the midbrain and the posterior hypothalamus (Economo, 1930). Later Moruzzi and Magoun as well as others in recent years contributed to the identification of the arousal system which originates in the rostral pons and ascends through the midbrain reticular formation to the diencephalon where it branches into two major pathways (Moruzzi and Magoun, 1949;Starzl et al., 1951) (Figure 4, page 11). The first branch projects to the thalamus and activates thalamic relay neurons which are essentially involved in information transmission to the cerebral cortex. The major input of this pathway comes from the cholinergic pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei (Edley and Graybiel, 1983;Hallanger and Wainer, 1988;Rye et al., 1987), and topographically projects to the thalamus including the intralaminar nuclei, the thalamic relay nuclei, and the reticular nucleus of the thalamus (Berendse and Groenewegen, 1990;Herkenham, 1980). It is demonstrated that neurons of the PPT/LDT show highest firing rates during the states when cortical activation occurs such as wake and REMS (Strecker et al., 2000). Consequently an involvement of cholinergic signals in gating thalamocortical transmission is important for wakefulness (McCormick, 1989;Steriade et al., 1993). The second branch originates from the upper brainstem and caudal hypothalamus, including the noradrenergic locus coeruleus (LC) (Jones and Cuello, 1989;Jones and Yang, 1985), the serotonergic dorsal (DR) and median raphe nuclei (Vertes et al., 1999;Vertes and Kocsis, 1994), the dopaminergic ventral periaqueductal grey (vPAG) (Beckstead et al., 1979) as well as the histaminergic tuberomammillary (TMN) neurons (Brown et al., 2001;Haas and Panula, 2003;Panula et al., 1989). It bypasses the thalamus and projects to the lateral hypothalamic area (LH), the basal forebrain (BF), and throughout the cerebral cortex (Jones, 2003;Saper, 1985;Saper et al., 2001). Further contribution to the wake-regulatory systems derives from orexinergic neurons of the LH (Peyron et al., 1998) and cholinergic or GABAergic neurons of the BF (Gritti et al., 1997;Rye et al., 1984) that additionally project to the cerebral cortex. Most monoaminergic neurons are maximally active during wake, less active during NREMS and inactive during REMS (Aston-Jones and Bloom, 1981;Fornal et al., 1985;Steininger et al., 1999), whereas orexinergic neurons are most active during wakefulness (Alam et al., 2002;Koyama et al., 2002) and most cholinergic neurons of the BF during both wake and REMS (Alam et al., 1999;Detari et al., 1984;Szymusiak and McGinty, 1986).

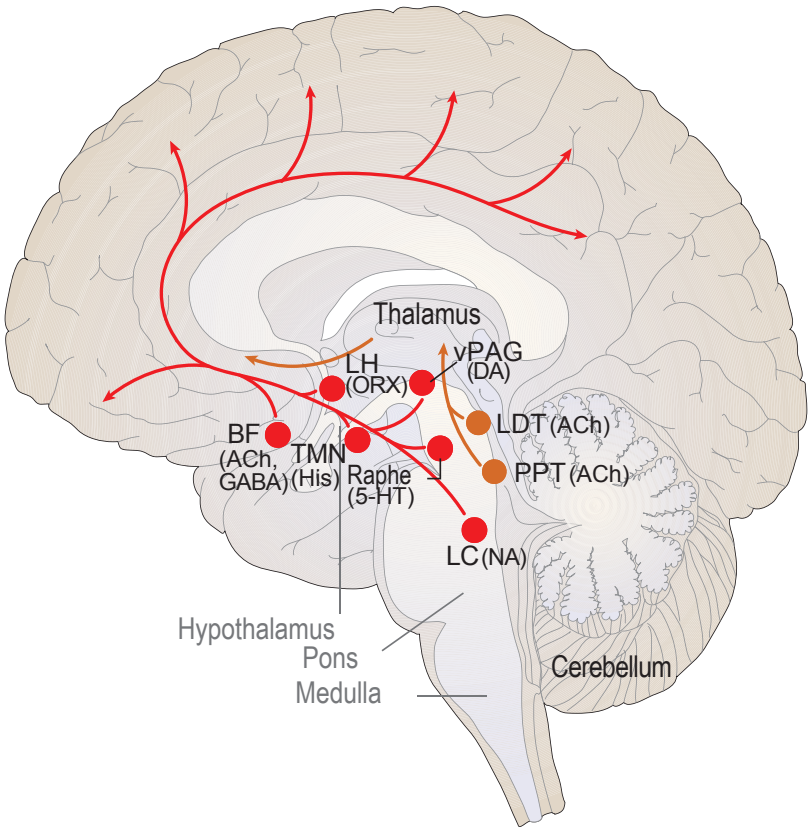

Figure 4: The ascending arousal system of the brain (adapted from Saper et al., 2005).

The two branches of the ascending arousal system originate in the rostral pons (exemplified in a human brain). The first branch (indicated in orange) arises from the cholinergic pedunculopontine (PPT) and laterodorsal (LDT) tegmental nuclei and projects to the thalamus. Relay neurons of the thalamus are essentially involved in thalamocortical information transmission. The second branch (indicated in red) originates from the noradrenergic locus coeruleus (LC), the serotonergic dorsal (DR) and median raphe nuclei, the dopaminergic ventral periaqueductal grey (vPAG) as well as histaminergic tuberomammillary (TMN) neurons. It bypasses the thalamus and projects to the lateral hypothalamic area (LH), the basal forebrain (BF), and throughout the cerebral cortex. Further contribution to the wake-regulatory systems derives from orexinergic neurons of the LH and cholinergic or GABAergic neurons of the BF. Abbreviations: 5-HT: serotonin; ACh: acetylcholine; DA: dopamine; GABA: gamma-aminobutyric acid; His: histamine; NA: noradrenaline; ORX: orexin.

2.2.2.2 Non-rapid eye movement sleep regulation

Basic prerequisite for the initiation of sleep is a reduction of neuronal activity in the arousal system concomitant with a cessation of sensory input transmission through thalamic neurons to the cortex. Unlike the complex regulation of REMS, NREMS is initiated rather simply by activation of inhibitory GABAergic neurons in the preoptic area (PO; midline, medial, lateral, and ventrolateral areas) of the hypothalamus (Datta and MacLean, 2007) (Figure 5, page 13). However, the localisation of sleep-inducing neurons is not limited to the PO but extends to the lower brainstem reticular formation, the solitary tract nucleus, the rostral hypothalamus, and the BF (Jones, 2005). The firing rate of cortically projecting GABAergic neurons in the BF positively correlates with EEG delta activity (Lee et al., 2004). GABAergic neurons in the BF and the ventrolateral PO (VLPO) are furthermore most active in association with cortical slow wave synchronisation and/or cortical deactivation (i.e. sleep) (Manns et al., 2000;Szymusiak et al., 1998). Most of the wake-promoting brain areas, including the TMN (histaminergic), LH (orexinergic), LC (noradrenergic), DR (serotonergic), and PPT/LDT (cholinergic), receive inhibitory input from the PO (Gritti et al., 1994;Jones and Cuello, 1989;Saper et al., 2001;Sherin et al., 1998;Steininger et al., 2001). Whereas the dense cluster of the VLPO innervates the wake-associated TMN, the extended part projects to the REMS-related LC and DR (Ko et al., 2003;Lu et al., 2002). The VLPO in turn receives afferent input from all of the major monoaminergic systems (Chou et al., 2002), and its neuronal activities are inhibited by noradrenaline and serotonin (Gallopin et al., 2000) as well as histaminergic neurons via GABA (Vincent et al., 1982). Therefore GABAergic VLPO neurons get disinhibited when monoaminergic neurons cease firing during drowsiness, thus increase their activity and promote NREMS. Additionally, many brainstem areas presumably contain sleep-active GABAergic interneurons and axons which might locally inhibit wake-promoting neurons (Jones, 1995;Maloney et al., 1999;Maloney et al., 2000). GABAergic thalamic reticularis neurons promote the generation of sleep spindles (12-14 Hz) by inhibiting (and thereby pacing) thalamic relay neurons (Steriade et al., 1994). This GABA-derived hyperpolarisation of thalamic relay neurons finally stimulates the development of delta oscillations.

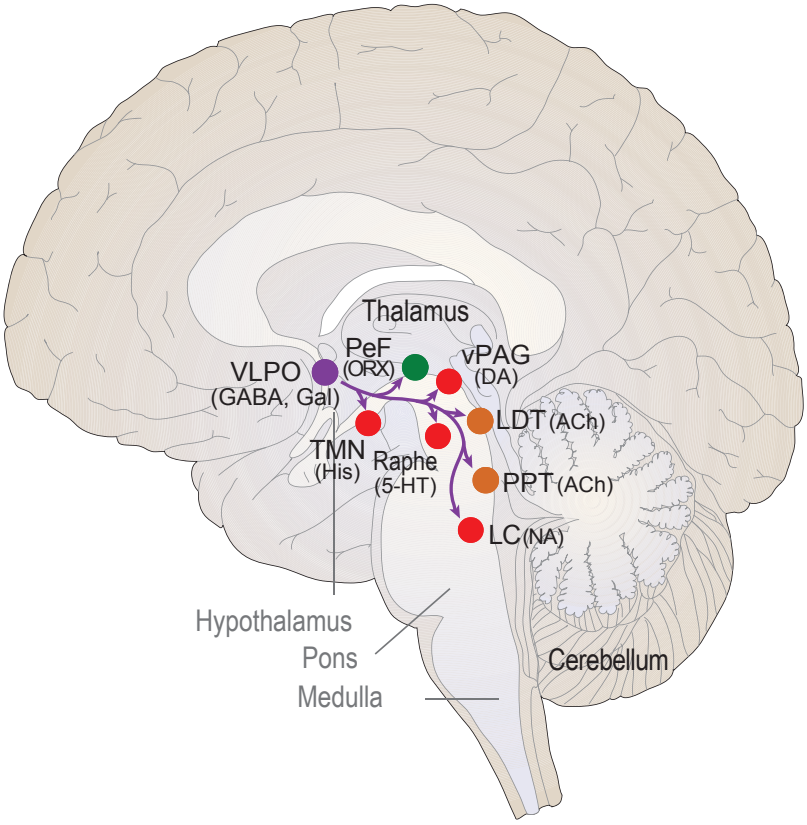

Figure 5: Sleep promoting pathways, Part 1: NREMS (adapted from Saper et al., 2005).

The ventrolateral preoptic area (VLPO) sends inhibitory inputs (indicated in purple) to most of the wake-promoting centres, including the monoaminergic cell groups (indicated in red) of the histaminergic tuberomammillary nucleus (TMN), the noradrenergic locus coeruleus (LC), the serotonergic dorsal raphe (DR), and the dopaminergic ventral periaqueductal grey (vPAG). Further inhibitory projections terminate in the orexinergic (indicated in green) perifornical (PeF) neurons of the lateral hypothalamus (LH) as well as the cholinergic (indicated in orange) pedunculopontine (PPT), and laterodorsal (LDT) tegmental nuclei. Abbreviations: 5-HT: serotonin; ACh: acetylcholine; DA: dopamine; GABA: gamma-aminobutyric acid; Gal: galanin; His: histamine; NA: noradrenaline; ORX: orexin.

2.2.2.3 Rapid eye movement sleep regulation

One of the most influential experiments, enabling allocation of important centres for REMS generation to the lower brainstem, was a transection study by Jouvet and colleagues conducted in cats (Jouvet, 1962). However, the exact identification of the REMS regulatory brain areas and their respective neurotransmitters is still under investigation. A complex interplay of aminergic (LC and DR) and cholinergic neurons (LDT, PPT, and medial pontine reticular formation (mPRF)) responsible for the generation of REMS and the manifestation of REMS signs (e.g. muscle atonia, rapid eye movements (REM), ponto-geniculo-occipital (PGO) waves) was postulated in the reciprocal interaction model (McCarley and Hobson,

1975). The core of most REMS regulation models (e.g. reciprocal interaction model, cellular-molecular-network model) is a group of cholinergic REMS-on neurons of the PPT/LDT (Datta and MacLean, 2007;McCarley and Hobson, 1975). These cholinergic neurons depolarise neurons of effector systems crucial for REMS sign generation (e.g. muscle atonia, REM, cortical activation). Glutamatergic neurons in the reticular formation constitute one of these effector systems, affecting cholinergic PPT/LDT neurons to increase their activity and thus contribute to the maintenance of REMS (Leichnetz et al., 1989;Mitani et al., 1988;Semba, 1993). Further, putatively excitatory efferents from the reticular formation project to noradrenergic LC and serotonergic raphe nucleus (RN) REMS-off neurons (Aston-Jones and Bloom, 1981;Foote et al., 1983;McCarley and Hobson, 1975;Sakai et al., 1983). Norepinephrine and serotonin in turn might inhibit the activity of the PPT/LDT neurons either through direct projections or excitation of GABAergic interneurons (Berridge and Waterhouse, 2003;Jones and Yang, 1985;Vertes et al., 1999;Vertes and Kocsis, 1994). There is also evidence of autoinhibition of LC and RN REM-off neurons by recurrent collaterals (McCarley and Hobson, 1975). Both neuronal groups receive GABAergic inputs or contain GABA locally (Ennis and Aston-Jones, 1989;Gervasoni et al., 1998). Instead of excitatory inputs from REMS-on onto REMS-off cells, an intrinsic pacemaker function of neurons in the LC might be responsible for REM-off cell activation (Datta and MacLean, 2007).

More recent evidence encouraged Lu and colleagues to propose an alternative REMS regulation model (i.e. the flip-flop switch) (Lu et al., 2006) (Figure 6, page 15). Accordingly, brainstem cholinergic and aminergic cells are REMS modulators, but not generators. In fact, they postulate the existence of three REMS-on cell groups with distinct projections and neurotransmitters. The first group of REMS-on cells is localised in the sublaterodorsal tegmental nucleus (SLD) (Boissard et al., 2002;Onoe and Sakai, 1995;Sakai et al., 2001) and sends glutamatergic projections to the spinal cord and GABAergic projections to REMS-off neurons of the ventral periaqueductal grey (vPAG) and the lateral pontine tegmentum (LPT) (Lu et al., 2006). The second and third group of REMS-on cells are located in the precoeruleus (PC) and parabrachial nucleus (PB), respectively, with glutamatergic projections to the BF and medial septum (Lu et al., 2006). The postulated switch consists, on the one hand, of GABAergic REMS-off neurons within the vPAG/LPT that send inhibitory signals to all three REMS-on groups and, on the other hand, GABAergic REMS-on SLD neurons that in turn inactivate the REMS-off neurons. Therefore the major difference from the original reciprocal interaction model is the involvement of GABAergic REMS-on and REMS-off populations instead of an interaction between aminergic and cholinergic cells (Fuller et al., 2007;Lu et al., 2006).

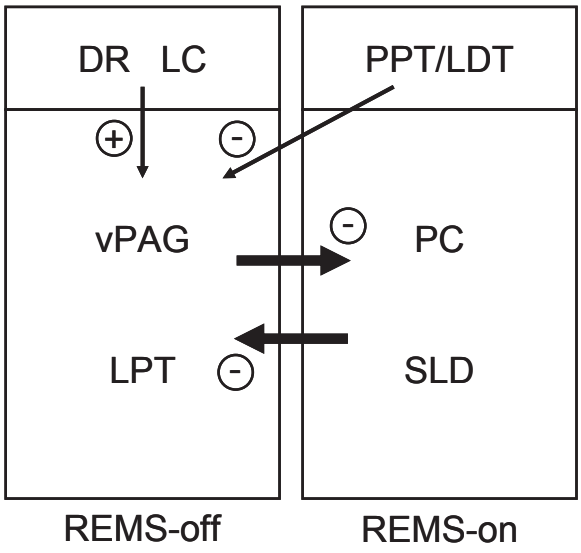

Figure 6: Sleep promoting pathways, Part 2: Schematic representation of the flip-flop switch model for REMS regulation (adapted from Lu et al., 2006).

According to the model, REMS-off neurons (indicated on the left) can be found in the ventral periaqueductal grey (vPAG) and the lateral pontine tegmentum (LPT). REMS-on neurons (indicated on the right) are located in the sublaterodorsal tegmental nucleus (SLD) as well as the precoeruleus (PC) and parabrachial (PB) nucleus. The GABAergic REMS-off neurons presumably display a reciprocal inhibitory interaction (indicated by thick arrows) with GABAergic REMS-on neurons of the SLD and additionally inhibit REMS generation in the PC. Serotonergic dorsal raphe (DR) and noradrenergic locus coeruleus (LC) neurons, on the one hand, and cholinergic REMS-on neurons in the pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei, on the other hand, play a modulatory role by activating or inhibiting (indicated by thin arrows) REMS-off cells, respectively.

2.2.3 Humoral sleep-wake regulation in mammals

Beside a contribution of various neuronal systems and their neurotransmitters, it cannot be disregarded that there are numerous kinds of neuromodulators which influence neural activities involved in sleep-wake regulation and thereby affect sleep-wake changes. Certain hormones, neuropeptides, cytokines, and nucleosides belong to these neuromodulators. For instance, hormones like galanin (Murck et al., 1999;Murck et al., 2004), ghrelin (Weikel et al., 2003), neuropeptide Y (Antonijevic et al., 2000a;Ehlers et al., 1997), and growth hormone-releasing hormone (Obal, Jr. et al., 1996;Steiger et al., 1992) were shown to promote sleep, whereas the corticotropin-releasing hormone (CRH) (Ehlers et al., 1986;Holsboer et al., 1988), vasopressin (Arnauld et al., 1989), and somatostatin (Beranek et al., 1999;Ziegenbein et al., 2004) seem to impair sleep. Two reciprocal hormonal systems, the hypothalamo-pituitary-somatotrophic (HPS) and the hypothalamo-pituitary-adrenocortical (HPA) system, presumably interact in this regulation (Weitzman, 1976). Despite certain correlations (e.g.

sleep-related variations in plasma concentration), the physiological significance of those hormones in sleep-wake regulation remains elusive. Further research is inevitably needed to clarify whether sleep-related changes in hormonal dynamics are merely secondary phenomena (e.g. synchronised to sleep/wake by circadian regulators without causal relationship), or if they have a regulatory function. There is strong evidence from clinical and preclinical studies supporting a crucial involvement of CRH and/or the HPA axis in sleep-wake regulatory processes (Buckley and Schatzberg, 2005). The investigation of the putative participation of central CRH in sleep-wake regulation is the objective of this thesis.

Box 2: Why do we sleep?

This question is the greatest enigma in sleep research and to date no convincing answer is available. Many studies insinuate a role of sleep in ecological adaptation, energy conservation, recovery, and learning and synaptic plasticity. The currently available theories can be subsumed in three main groups:

1. **We sleep to conserve energy** (wake is only profitable at certain times of the day [e.g. prey availability], otherwise sleep is preferable to reduce energy consumption; e.g. Zepelin et al., 2005)
2. **We sleep to facilitate learning and memory** (wake is associated with energetically unsustainable learning, while sleep is linked to synaptic depression and downscaling; e.g. Tononi and Cirelli, 2006)
3. **We sleep to restore key cellular components** (e.g. macromolecules are consumed during wake and replenished during sleep; e.g. Mackiewicz et al., 2007)

2.3 The hypothalamo-pituitary-adrenocortical axis

Living organisms are continuously exposed to changing environments and challenging situations. To maintain a physiological homeostasis (a complex dynamic equilibrium; Cannon, 1929), organisms respond with a variety of physiological answers to external or internal changes perceived as threats. Selye conceptualised the general adaptation syndrome (Selye, 1936) to diverse noxious stimuli and introduced the term “stress” as a situation challenging homeostasis and evoking respective physiological reactions. Later on allostasis, a permanent adaptive organismic effort to maintain homeostasis in the presence of stressors, was introduced to extend the concept of homeostasis (Sterling and Eyer, 1988). Whereas homeostasis defines biological set points to maintain physiological stability, allostasis involves modulations, including those of set points, to adapt to the environment.

The behavioural and physiological responses to stressors are mediated by to major, highly conserved and complementary systems, i.e. the autonomic nervous system (immediate and short response; fight-or-flight) and the HPA axis (long-lasting, amplifying response). Activation of these systems leads to increased arousal and awareness, increased blood pressure, heart rate, and cardiovascular tone as well as energy mobilisation. In contrast, immune responses, feeding behaviour, digestion, and reproductive functions are decreased (Ulrich-Lai and Herman, 2009).

Many brain regions communicate upon distinct stress-related stimuli with the paraventricular nucleus (PVN) of the hypothalamus, the superior centre of the HPA axis, and are involved in activation or inhibition of the HPA axis. Major excitatory input to the PVN arises from the brainstem nucleus of the solitary tract (Cunningham, Jr. and Sawchenko, 1988;Plotsky, 1987) as well as the dorsal and medial RN (Jorgensen et al., 1998;Sawchenko et al., 1983). Further excitatory input derives from the amygdala (medial and basolateral nuclei) (Canteras et al., 1995;Cullinan et al., 1996;Feldman et al., 1990). However, most parts of the limbic system like the hippocampus (key regulator of negative feedback) (Feldman and Conforti, 1980;Furay et al., 2008;van Haarst et al., 1997), the medial prefrontal cortex (Cerqueira et al., 2008;Sullivan and Gratton, 1999), the lateral septum (Risold and Swanson, 1996), and the posterior division of the bed nucleus of the stria terminalis (BNST) send direct or indirect inhibitory signals to the PVN, although the anterior division of the BNST sends excitatory signals to the PVN (Crane et al., 2003;Dunn, 1987;Sawchenko and Swanson, 1983). Further inhibitory input originates in the peri-PVN (Boudaba et al., 1996;Cullinan et al., 2008;Herman et al., 2002), the medial preoptic area, and the dorsomedial nucleus of the hypothalamus (Cullinan et al., 1996;Roland and Sawchenko, 1993). In general, glutamatergic and noradrenergic stimulation activates, whereas GABAergic input inactivates hypophysiotropic

neurons in the medial parvocellular subdivision of the PVN (Cole and Sawchenko, 2002;Liposits et al., 1986;Miklos and Kovacs, 2002;van den Pol et al., 1990).

These hypophysiotrophic neurons of the PVN synthesise CRH and arginine vasopressin (AVP) (Frank and Landgraf, 2008;Landgraf, 2006). CRH is regarded as the major activator of the stress system, orchestrating different responses to stressors by activating the HPA axis and the autonomic nervous system. The focus of this thesis lies on the action of CRH and its role regarding sleep/wake regulation. The neurons in the PVN project to the external layer of the median eminence where CRH is released into hypophyseal portal blood vessels (Figure 7, page 19). Within the anterior pituitary CRH binds to CRH receptor type 1 (CRH-R1) on the surface of corticotrophs to stimulate the expression of the hormonal precursor molecule proopiomelanocortin (POMC). Subsequently adrenocorticotropic hormone (ACTH) is secreted from the anterior pituitary into the blood circulation (Engelmann et al., 2004). Additionally AVP acts synergistically with CRH by activating V1b receptors and thereby potentiates the ACTH release (Lamberts et al., 1984;Rivier and Vale, 1983). ACTH in turn triggers glucocorticoid release from the zona fasciculata of the adrenal glands into the blood. Whereas cortisol is the main HPA axis effector in humans, it is corticosterone (CORT) in rodents (i.e. mice and rats) (Dawson, Jr. et al., 1984;Payne and Hales, 2004). Glucocorticoids act on two distinct receptors of the steroid nuclear receptor family (Evans, 1988), the glucocorticoid receptor (GR) and with a ten-fold higher affinity the mineralocorticoid receptor (MR) (de Kloet et al., 1998;de Kloet et al., 2008;Reul and de Kloet, 1985). Glucocorticoids negatively feedback to terminate HPA axis activity, suppressing both *Pomc* transcription as well as ACTH release within the anterior pituitary (APit) and CRH and AVP synthesis in the hypothalamus (Keller-Wood and Dallman, 1984;Papadimitriou and Priftis, 2009;Tasker et al., 2006).

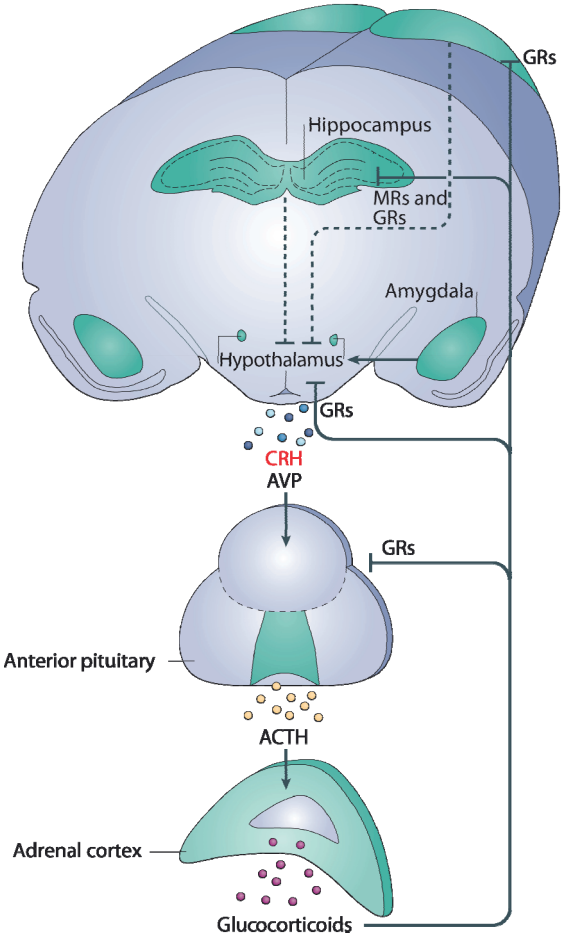

Figure 7: Schematic representation of the HPA axis (adapted from Lupien et al., 2009).

Hypophysiotrophic neurons of the paraventricular nucleus (PVN) of the hypothalamus synthesise CRH (indicated in red) and AVP. These neurons project to the external layer of the median eminence where CRH (and AVP) is released into hypophyseal portal blood vessels upon stress. In the anterior pituitary, CRH stimulates the expression of *Pomc* and the release of ACTH into the blood circulation. ACTH in turn triggers glucocorticoid release from the adrenal cortex into the blood. Glucocorticoids negatively feedback by acting on GR and MR receptors to terminate HPA axis activity suppressing *Pomc* transcription and ACTH release within the anterior pituitary, as well as CRH, and AVP synthesis in the hypothalamus. Abbreviations: ACTH: adrenocorticotropic hormone; AVP: arginine vasopressin; CRH: corticotropin-releasing hormone; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; PVN: paraventricular nucleus of the hypothalamus.

In addition to stress-adaptation activation, the HPA axis receives a circadian regulation under control of the SCN (Cascio et al., 1987;Kalsbeek et al., 1996;Moore and Eichler, 1972). In diurnal species (e.g. humans) plasma glucocorticoid levels peak (acrophase) in the early morning before the onset of the activity period, and are lowest (nadir) in the evening before the resting/sleeping period (Keller-Wood and Dallman, 1984;Ungar and Halberg, 1962;van Cauter, 1990;Weitzman et al., 1971). Nocturnal animals (e.g. mice and rats) respectively show an opposite pattern (Dallman et al., 1978). Glucocorticoid release occurs in a pulsatile

fashion (approximately one pulse per hour) (Droste et al., 2008), and the varying magnitude of pulses (to a lesser extend a changing frequency) entails increasing or decreasing plasma glucocorticoid levels towards the end of inactivity (e.g. sleep) and activity periods, respectively (Veldhuis et al., 1989;Veldhuis et al., 1990;Walker et al., 2010;Windle et al., 1998).

2.4 The corticotropin-releasing hormone (CRH)

2.4.1 The CRH peptide family

Phylogenetically CRH has existed functionally prior to the evolution of tetrapods and teleosts (Chang and Hsu, 2004). This suggests its fundamental involvement in homeostatic maintenance of an organism (Lovejoy and Balment, 1999;Valdez and Koob, 2004). In 1955 *in vitro* experiments primarily pointed out a function of CRH as a releasing factor of pituitary hormones (Guillemin and Rosenberg, 1955). As lately as 1981, CRH was first isolated from ovine brain, characterised as a 41 amino acid peptide, and synthesised by Vale and colleagues (Vale et al., 1981). CRH has been identified in various other mammalian species as well as fish and frogs (Lovejoy and Balment, 1999). Further CRH-like peptides sharing more or less sequence homology with CRH have been found, such as urocortin I, urocortin II, urocortin III, sauvagine, and urotensin I (Dautzenberg and Hauger, 2002;Donaldson et al., 1996;Lederis et al., 1982;Montecucchi and Henschen, 1981;Vaughan et al., 1995). These peptides seem to originate from one ancestral peptide precursor (Lovejoy and Balment, 1999). Apart from distinct distribution patterns and functions of the different CRH-like peptides, the main focus of this thesis is on CRH. Another peptide, CRH-binding protein, circulating in the blood and expressed on the cell surface of brain tissue in some species (Potter et al., 1991), is implicated in modulation of HPA axis activation and of CRH effects by limiting availability of free CRH (Grammatopoulos and Chrousos, 2002).

2.4.2 The distribution of CRH

Immunolabeling, radioimmunoassay, and mRNA expression studies could demonstrate a widespread distribution of CRH-containing neurons within the brain of humans (Charlton et al., 1987), rats (Fischman and Moldow, 1982;Sakanaka et al., 1987;Sawchenko and Swanson, 1985;Sawchenko and Swanson, 1990), and mice (Nakane et al., 1986) (Figure 8, page 21). Two main sources of CRH can be distinguished in the brain; one within the HPA axis and the other in non-HPA axis sites. Within the brain and particularly along the HPA

axis, the PVN of the hypothalamus represents the major source of CRH in mammals (Sakanaka et al., 1987;Sawchenko and Swanson, 1990;Swanson and Simmons, 1989). Amongst others, extra-HPA axis sources of CRH in the brain consist of the hippocampal formation, the lateral septum, the central nucleus of the amygdala (CeA), the BNST, the nucleus accumbens, and several hypothalamic nuclei (e.g. supraoptic, medial preoptic, periventricular preoptic nucleus). Sources of CRH are also found in the brainstem, notably in the LDT, the periaqueductal grey (PAG), the LC, and the caudal nucleus of the solitary tract (Cummings et al., 1983;Merchenthaler, 1984;Sakanaka et al., 1987;Sawchenko and Swanson, 1990).

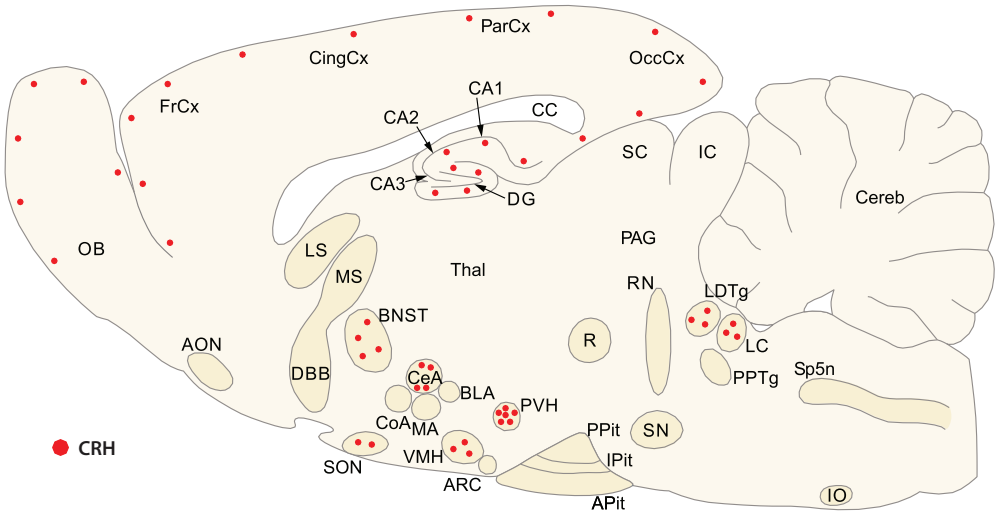

Figure 8: Distribution of CRH mRNA in the rodent brain (courtesy of Deussing JM, Max Planck Institute of Psychiatry, Munich, Germany; unpublished data, adapted).

This saggital section of a rodent brain highlights brain areas where CRH mRNA expression (red dots) can be detected. The PVN constitutes the major source of CRH in mammals. Furthermore, CRH mRNA is consistently expressed in several cortical areas, the olfactory bulb, the supraoptic nucleus, the bed nucleus of the stria terminalis, the hippocampus, and the central nucleus of the amygdala. CRH mRNA can also be detected in brain areas that are involved in sleep-wake regulation such as the laterodorsal tegmentum and the locus coeruleus. The most relevant abbreviations for the present study are indicated as follows: LDTg: laterodorsal tegmentum; LC: locus coeruleus; PVH: paraventricular nucleus of the hypothalamus.

Besides the PVN projecting to the external layer of the median eminence (see HPA axis), pathways of CRH-containing neurons include axons from the CeA to the parvocellular region of the PVN. Additionally, CRH fibres from the PVN, the CeA, and the BNST descend to the brainstem, e.g. the LC (Valentino et al., 1993;Valentino and van Bockstaele, 2008) and the dorsal and medial RN (Swanson and Simmons, 1989). Furthermore the CeA, PVN, and

BNST are interconnected by CRHergic neurons. Other CRHergic pathways comprise ascending projections from the hypothalamus to the lateral septum, and projections originating in the brainstem to the hypothalamus or the spinal cord. Interneurons and neurons expressing CRH are localised to layers II and III of the cortex and to the neocortex (particularly the prefrontal cortex), respectively (Holsboer, 1999;Sakanaka et al., 1986;Sakanaka et al., 1987).

Within the central nervous system (CNS), CRH is synthesised at specific synapses and, as demonstrated by immunohistochemical studies (Cain et al., 1991), stored at nerve terminals. Under appropriate conditions CRH may be released or co-released along with classical neurotransmitters. In the hippocampus, CRH has been identified within GABAergic neurons (Yan et al., 1998), and in the LC within both glutamatergic and GABAergic neurons (Valentino et al., 2001). Synaptic release of CRH appears to be comparable (release properties and kinetics) to that of biogenic amines (Whim, 2006). However, the effects of CRH at specific synapses and the respective functions of these synapses have to be elucidated.

2.4.3 Stress-related effects of CRH

As mentioned before (see HPA axis), CRH was originally implicated within the HPA axis as the major stress hormone. Activation of the HPA axis by CRH results in glucocorticoid release from the adrenal cortex and subsequent physiological effects (e.g. energy mobilisation) as well as potentiation of various sympathetically mediated effects (e.g. peripheral vasoconstriction) (Ulrich-Lai and Herman, 2009). Chronic HPA axis activation caused by centrally overexpressed CRH has been implicated in the development of Cushing's syndrome-like symptoms in mice (Stenzel-Poore et al., 1992). Moreover a CRH-mediated hyperactivity of the HPA system is now related to the manifestation of affective diseases, such as major depression, in humans (for review please refer to: Arborelius et al., 1999;Holsboer, 1999;Nemeroff, 1988;Reul and Holsboer, 2002). The facts that increased numbers of CRH-secreting neurons in the PVN (Raadsheer et al., 1994) and elevated concentrations of CRH in the cerebrospinal fluid (CSF) (Nemeroff et al., 1984) are reported in patients suffering from acute, severe depression are in line with this assumption. However, numerous reports highlighted non-HPA axis but CNS-specific neuromodulatory roles of CRH (for review please refer to: Bale and Vale, 2004;Dunn and Berridge, 1990;Guillemin, 2005;Holsboer, 1999). Intracerebroventricular (i.c.v.) and site-specific local injection studies demonstrated a CRH-mediated induction of anxiety-like behavioural and autonomic effects even in hypophysectomised animals (for review please refer to: Dunn and

Berridge, 1990). Autonomic effects of i.c.v. CRH in rats comprehend increases of plasma glucose levels (Brown et al., 1982), mean arterial pressure, and heart rate (Fisher et al., 1982;Fisher et al., 1983). Furthermore central application of CRH inhibits gastric acid secretion (Tache et al., 1983), decreases gastric emptying, and inhibits small bowel while increasing large bowel transit in rats (Lenz et al., 1988). Anxiogenic and fear-related behavioural responses after CRH injections in rats and mice include increased grooming and freezing behaviour and decreased ingestion in novel environments (Britton et al., 1982;Morley and Levine, 1982;Sutton et al., 1982). Additionally, in rats CRH increases stress-induced freezing (Sherman and Kalin, 1988), potentiates acoustic startle (Liang et al., 1992b;Swerdlow et al., 1989), suppresses social interaction (Dunn and File, 1987), and decreases exploratory behaviour (Butler et al., 1990). Genetic approaches (e.g. transgenic mice centrally overexpressing CRH) confirmed the role of CRH in eliciting anxiety-like behaviours (e.g. increased grooming, time spent rearing, and digging in the home cage) (van Gaalen et al., 2002). Taken together, the importance of CRH in mediating stress-related behaviours either by activating the HPA axis or central pathways seems to be established and is further corroborated in studies which apply antisense oligodeoxynucleotides against mRNA of CRH or its receptors, thereby demonstrating increases in anxiolytic-like behaviours (Heinrichs et al., 1997;Liebsch et al., 1999;Skutella et al., 1994).

2.4.4 Sleep-wake regulatory effects of CRH

Accumulating evidence from human studies suggests an involvement of CRH in spontaneous and stress-induced sleep-wake regulation. Single intravenous (i.v.) injections of CRH to healthy young men during the sleep onset of nocturnal sleep decreased SWS while increasing shallow sleep (Tsuchiyama et al., 1995). Similar results were obtained after repetitive i.v. injections, furthermore REMS decreased (Holsboer et al., 1988). This effect was ascribed to inhibition of central CRH synthesis and release by negative feedback, since cortisol application was shown to increase SWS and decrease REMS in humans (Bohlhalter et al., 1997;Born et al., 1991;Friess et al., 1994;Friess et al., 2004). Inhibition of cortisol synthesis (e.g. by metyrapone) led to elevated levels of ACTH and subsequently to decreases in SWS, however REMS remained unchanged (Jahn et al., 1996). Since ACTH and cortisol application suppressed REMS (Steiger and Holsboer, 1997), REMS reduction after CRH administration was linked to increased cortisol levels after acute HPA axis activation. Decreased SWS on the other hand was linked to a central action of CRH.
Most sleep-endocrine studies in depressed patients reported elevated levels of plasma ACTH and cortisol throughout the day in comparison to healthy controls (Antonijevic et al., 2000b;Benca et al., 1992). One of the cardinal symptoms of human depression, associated

with sleep-endocrine changes (Steiger, 2002), is impaired sleep (e.g. early awakening, disturbed sleep continuity, decreased SWS, and REMS disinhibition) (Benca et al., 1992;Reynolds and Kupfer, 1987). Thus a relationship between impaired sleep and HPA axis hyperactivity was suggested. Furthermore SD has been proven effective in relieving depressive symptoms (including sleep disturbances) although the underlying mechanism of action is currently unclear (Borbély and Wirz-Justice, 1982;Wirz-Justice and van den Hoofdakker, 1999). After clinical remission sleep-EEG changes persisted (Kupfer et al., 1993) possibly representing a biological scar due to metabolic changes during acute depression (Steiger, 2003). Interestingly ageing and depression exhibit synergistic effects on sleep-endocrine activity (Antonijevic et al., 2000b;Steiger, 2003), thus sleep-endocrine disturbances are most pronounced in elderly depressed patients.

Further support for the participation of CRH in sleep-wake regulation is denoted by a number of animal studies. In mice, rats, and rabbits, i.c.v. injection of CRH elicits increases in wakefulness and decreases in NREMS (Ehlers et al., 1983;Ehlers et al., 1986;Opp et al., 1989;Sanford et al., 2008). Another experiment demonstrated that i.c.v. application of an antisense oligodeoxynucleotide probe directed against CRH mRNA before the activity phase of the animals reduced spontaneous waking and increased NREMS in Sprague-Dawley (Sp-D) rats during this period (Chang and Opp, 2004). In Lewis rats, in which CRH synthesis and its secretion from the hypothalamus are decreased, reduced amounts of waking and increased NREMS in comparison to Fischer 344 and Sp-D rats, in which the level of the neuropeptide in the brain is normal, could be observed (Opp, 1998). Transgenic mice centrally overexpressing CRH displayed increased wakefulness and REMS and slightly decreased NREMS under baseline conditions in comparison to controls (Kimura et al., 2010). Furthermore, sleep deprived rats injected i.c.v. with CRH displayed enhanced REMS and reduced NREMS (Marrosu et al., 1990). As outlined above, there is ample evidence for the participation of CRH in spontaneous and stressor-induced sleep-wake regulation from both the clinical and preclinical sides.

2.5 The corticotropin-releasing hormone receptor system

2.5.1 The CRH receptor family

Two types of CRH receptors, the CRH receptor type 1 and 2 (CRH-R1 and CRH-R2), have been identified in various vertebrate species (Chang et al., 1993;Chen et al., 1993;Dautzenberg et al., 1997;Kishimoto et al., 1995;Liaw et al., 1996;Lovenberg et al., 1995b;Myers et al., 1998;Palchaudhuri et al., 1998;Palchaudhuri et al., 1999;Vita et al., 1993;Yu et al., 1996) to transmit signals of CRH and its related peptides across the cell membrane. These receptors are encoded by distinct genes however share 70% homology at the amino acid level (Dautzenberg et al., 2001a;Dautzenberg and Hauger, 2002;Grammatopoulos and Chrousos, 2002;Hillhouse et al., 2002). Both receptor types contain seven hydrophobic α-helical transmembrane domains and belong to the class II G-protein-coupled receptor (GPCR) superfamily, which includes receptors e.g. for GHRH (Grammatopoulos and Chrousos, 2002). The highest sequence conservation between CRH-R1 and CRH-R2 can be found in the putative transmembrane domains (TM1-7; ~85% identity) and intracellular loops (i1-i4; ~90% identity), especially the i3 loop, the putative G-protein binding site, being identical in all CRH receptors identified so far (Arai et al., 2001;Dautzenberg et al., 1997;Dautzenberg et al., 2001a). The lowest sequence conservation is found in the extracellular domains (e1-e4; ~60% identity), particularly the N-terminal e1 domain (~45% identity) (Dautzenberg et al., 1997). Whereas in the case of CRH-R1 e1 probably determines ligand binding (Perrin et al., 1998), e2 and the junction of e3 and TM5 seem to be essential for CRH-R2 (Dautzenberg et al., 2000;Liaw et al., 1997a, b). Mammalian CRH-R1 exhibits a high affinity for CRH and urocortin I (Vaughan et al., 1995), CRH-R2 on the other hand shows a preference for urocortin I, II, and III (Dautzenberg et al., 2001a, b; Lewis et al., 2001;Reyes et al., 2001).

Beside the functional 415-amino acid protein CRH-R1α, several splice variants of CRH-R1 mRNA have been isolated (i.e. CRH-R1β, -R1c, -R1d, -R1e, -R1f, -R1g, and -R1h; Figure 9 A, page 26). Most are restricted to humans and all are deficient in ligand binding and/or signalling (Dautzenberg et al., 2001a;Grammatopoulos et al., 1999;Pisarchik and Slominski, 2001;Ross et al., 1994). Three functional splice variants of CRH-R2 (i.e. CRH-R2α, -R2β, -R2γ; Figure 9 B, page 26) are known which, although differing in their N-terminal sequences, display similar pharmacological profiles (Dautzenberg et al., 2001a;Kostich et al., 1998;Liaw et al., 1996;Valdenaire et al., 1997).

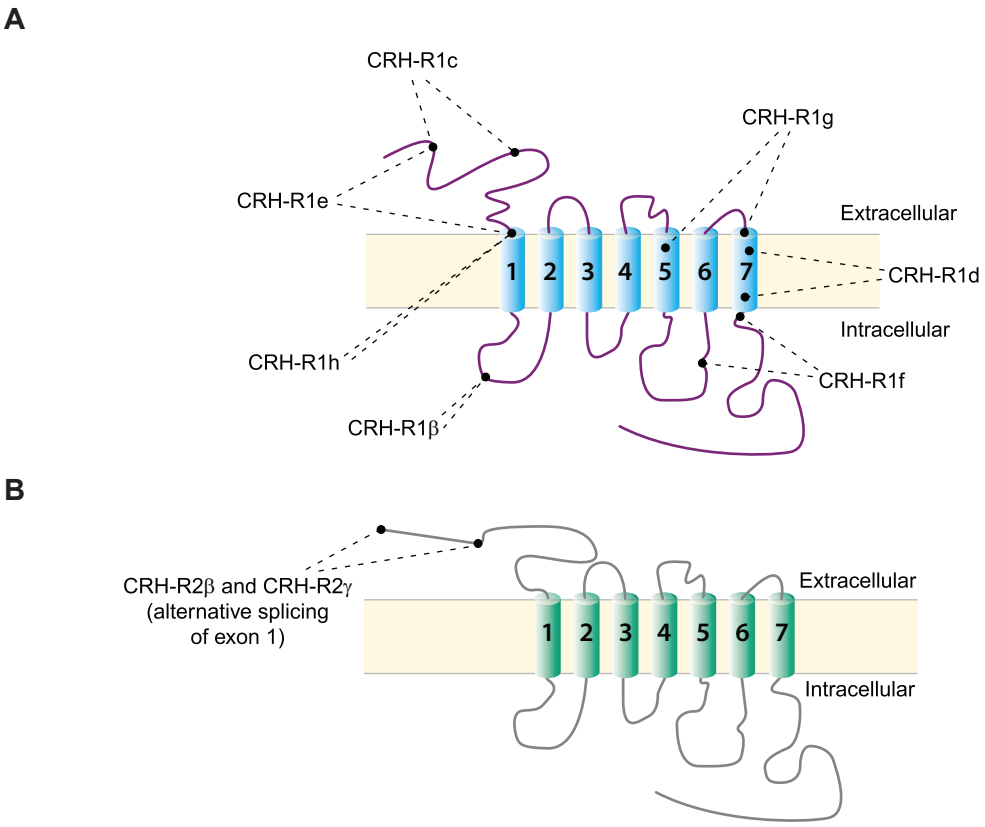

Figure 9: The CRH receptors (taken from Grammatopoulos and Chrousos, 2002).

The two types of CRH receptors, R1 (A) and R2 (B) are encoded by separate genes. There are several isoforms of CRH-R1α (R1β-R1h; A), which are generated by alternative splicing. These isoforms are deficient in ligand binding or signaling properties caused either by amino acid insertions or deletions (indicated by black dots and dashed lines). Similarly CRH-R2α has two isoform (R2β and R2γ) which differ in their N-termini (indicated by black dots and dashed lines) but are otherwise identical. The numbers (1-7) indicate the transmembrane domains of the CRH receptors.

Ligands upon binding to CRH-R1 or CRH-R2 in most tissues initiate an activation of adenylyl cyclase (AC), entailing increases of intracellular cyclic adenosine monophosphate (cAMP) (Grammatopoulos et al., 1994;Heldwein et al., 1996;Olianas et al., 1995). Increased cAMP subsequently activates the protein kinase A (PKA) and its associated transcription factor cAMP response element binding protein (CREB) (Dautzenberg and Hauger, 2002;Grammatopoulos and Chrousos, 2002), as well as other cAMP pathways. Although CRH receptors are primarily coupled to the G_s-adenylyl cyclase signalling cascade, growing evidence suggests a tissue-specific coupling to different G-proteins (e.g. G_i and G_q) and an activation of alternative signalling pathways (Grammatopoulos et al., 2000;Grammatopoulos et al., 2001;Karteris et al., 2000;Ulisse et al., 1990). Thus, activation of CRH receptors might cell-specifically trigger different intracellular pathways mediating various tissue-dependent physiological responses. This, amongst others, includes activation of phospholipase C (PLC)

and protein kinase C (PKC) as well as activation of the orphan nuclear receptor Nur77, and the nuclear factor-κB (NF-κB) (Arzt and Holsboer, 2006;Hillhouse et al., 2002). The transcription factors CREB, Nur77, and NF-κB appear to be implicated in the control of the expression of the gene *Pomc* in corticotrophs after activation by CRH (Karalis et al., 2004;Philips et al., 1997). Additionally to an expression on post-synaptic membranes, literature suggests a pre-synaptic localisation of CRH receptors pointing to a possible role as synaptic auto-receptors (Gallagher et al., 2008;Lawrence et al., 2002;Rominger et al., 1998;Swinny et al., 2003).

2.5.2 The distribution of the CRH receptors

Immunohistochemistry, in situ hybridisation, and PCR studies have demonstrated a differential distribution of CRH-R1 and CRH-R2 receptors in the periphery and across the brain of mice, rats, monkeys and humans. While peripheral CRH-R2 has been detected in the heart, skeletal muscle, lung, epidydimis, kidney, and intestines of rats (Kishimoto et al., 1995;Lovenberg et al., 1995a, b;Perrin et al., 1995;Stenzel et al., 1995), CRH-R1 has been demonstrated in skin, testis, ovary, and adrenal gland (Kageyama et al., 1999;Nappi and Rivest, 1995;Palchaudhuri et al., 1998;Slominski et al., 1995;Slominski et al., 2004;Vita et al., 1993). In rodents CRH-R2β is the major splice variant found in the periphery, however, in humans it is CRH-R2α (Dautzenberg et al., 2001a). Furthermore CRH-R2β has been detected in non-neuronal structures of the brain, such as the choroid plexus and arterioles (Chalmers et al., 1995;Lovenberg et al., 1995a).
In the mammalian brain CRH-R2α is the dominant splice variant which can be found with highest densities in the PVN, the ventromedial hypothalamus, the lateral septum, the amygdala, the hippocampal formation, and the brainstem PAG and dorsal and median RN (Chalmers et al., 1995;de Souza, 1987;Palchaudhuri et al., 1999;Potter et al., 1994;Sanchez et al., 1999;van Pett et al., 2000) (Figure 10, page 28). The CRH-R2γ, which has been reported only in humans so far, is expressed in the lateral septum, the hippocampus, the amygdala, midbrain areas, and the frontal cortex (Kostich et al., 1998). Highest densities of centrally expressed CRH-R1 are detected in several cortical areas, the cerebellum, the olfactory bulb, the amygdala, the hippocampus, and the BNST (Chalmers et al., 1995;de Souza, 1987;Sanchez et al., 1999;van Pett et al., 2000) (Figure 10, page 28). Furthermore CRH-R1 is also detected in several other brain areas including neuronal systems implicated in arousal and sleep-wake regulation such as the hypothalamus, the BF, and the brainstem (e.g. brainstem reticular formation, LDT, PPT) (Arzt and Holsboer, 2006;de Souza, 1987;Jones, 2003). In the pituitary, CRH-R1 expression is detected in the anterior lobe, whereas, more recently, CRH-R2 expression was found in the posterior lobe (Müller et al.,

2001;Sanchez et al., 1999;van Pett et al., 2000). Some controversy exists as to the expression of CRH-R1 within the PVN and the LC. Although some studies suggest an expression of CRH-R1 in the LC of mice (Sauvage and Steckler, 2001), only in non-human primates expression of this receptor has been conclusively detected within both nuclei (Sanchez et al., 1999). Induced CRH-R1 expression in the PVN is reported in response to stress exposure in rats but not mice (Imaki et al., 2001;Imaki et al., 2003;Konishi et al., 2003).

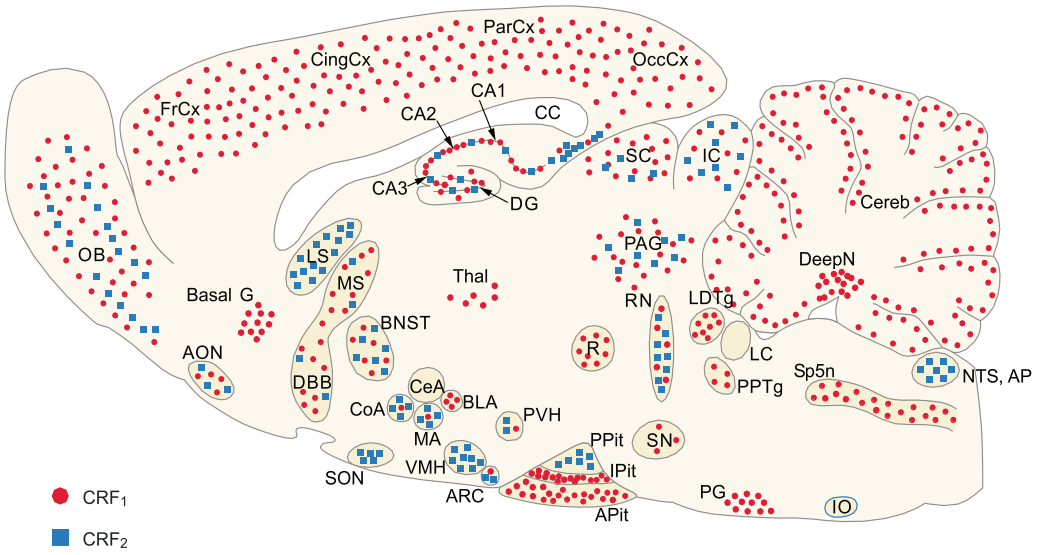

Figure 10: The CRH receptor distribution in the brain (taken from Reul and Holsboer, 2002).

This saggital view of a rodent brain illustrates the distribution of CRH-R1 (red dots) and CRH-R2 (blue squares). CRH-R1 is expressed in several cortical areas, the cerebellum, the olfactory bulb, the amygdala, the hippocampus, and the BNST. It is also found in several brain areas involved in sleep-wake regulation such as the hypothalamus, the BF, the brainstem reticular formation, the LDT, and the PPT. CRH-R2 is located in the PVN, the ventromedial hypothalamus, the lateral septum, the amygdala, the hippocampal formation, the PAG, and the dorsal and median RN as well as the posterior pituitary. Relevant abbreviations for this study are indicated as follows: APit: anterior pituitary; BF: basal forebrain; BLA: basolateral amygdala; BNST: bed nucleus stria terminalis; CoA: cortical nucleus of the amygdala; LDTg: laterodorsal tegmentum; MA: medial amygdala; PAG: periaqueductal grey; PPTg: pedunculopontine tegmentum; PVH: paraventricular nucleus of the hypothalamus; RN: raphe nucleus; VMH: ventromedial hypothalamus.

2.5.3 The stress-related function of the CRH receptors

Studies using CRH-R1 antisense oligodeoxynucleotides or antagonists (e.g. the antalarmin analogue CP-154,526) generally reported attenuation of anxiety-related behaviour as rats spent more time on and make more entries into the open arms of an elevated plus maze (Liebsch et al., 1999;Lundkvist et al., 1996). In addition, reduced fear conditioning (Hikichi et al., 2000) and decreased ultrasonic vocalisations by pups separated from their dams have been reported (Kehne et al., 2000). Clinical studies applying specific, small compound CRH-R1 antagonists (i.e. NBI30775/RS121919 and NBI-34041) to depressed patients reported decreased anxiety and depressive symptoms (Nickel et al., 2003;Zobel et al., 2000) as well as a reduced response to psychosocial stress after treatment (Ising et al., 2007). Studies in knockout mouse models support the critical involvement of CRH-R1 in HPA axis activation and mediation of anxiety-related behaviour. Compared with controls, mice deficient for CRH-R1 displayed a blunted response to restraint stress, while maintaining normal baseline levels of HPA axis activity (Smith et al., 1998;Timpl et al., 1998). A compensatory increase of AVP mRNA and protein in the PVN, assumingly maintaining basal stress hormone levels, was reported in one study (Müller et al., 2000), however, not confirmed by others (Bale et al., 2002;Smith et al., 1998). Furthermore CRH-R1 deficient mice showed increased anxiolytic-like behaviour as they spent more time on and make more entries into the open arms of an elevated plus maze (Smith et al., 1998). Additionally the latency to enter the lit compartment was decreased in the light-dark box test, and time spent in the light was increased (Timpl et al., 1998). Another mouse model, with a conditional restriction of the CRH-R1 deficiency to limbic brain areas, could highlight the importance of limbic CRH-R1 in modulating anxiety-related behaviour independently of HPA axis function (Müller et al., 2003). Taken together, the role of CRH-R1 in stress and anxiety seems to be established; on the other hand the function of CRH-R2 remains still obscure.

Three independent laboratories studied three differently generated CRH-R2 knockout (CRH-R2 KO) mouse lines regarding behavioural and endocrine activity. Only two groups reported increases of anxiety-related behaviours (e.g. less entries and less time spent on open arms of an elevated plus maze) (Bale et al., 2000;Kishimoto et al., 2000). Whereas only one of these two observed a stressor-induced augmentation of ACTH and CORT release (Bale et al., 2000), the third group showed opposite endocrine changes with an absence of anxiety-related behaviours (Coste et al., 2000). In contrast an impaired cardiovascular function and altered feeding behaviour after food deprivation as well as decreased grooming in a novel open field were all detected in these three groups (Bale et al., 2000;Coste et al., 2000;Kishimoto et al., 2000). Their findings suggest a role of CRH-R2 at least in stress adaptation. Other studies in CRH-R2 KO mice showed increased immobility time in the forced swim test indicating depression-like behaviour (Bale and Vale, 2003;Coste et al.,

2006). Increased levels of CRH mRNA in the CeA (Bale et al., 2000), an important nucleus for stress signal transduction (Liang et al., 1992a;Müller et al., 2003), of CRH-R2 KO mice might explain the stress sensitivity and increases in anxiety- and depression-like behaviours observed in these animals. Additionally, lesioning studies in the septum, a nucleus expressing high amounts of CRH-R2 and modulating activity of the amygdala (Lee and Davis, 1997;Melia and Davis, 1991), reported decreased levels of ACTH following restraint stress (Beaulieu et al., 1986;Marcilhac and Siaud, 1996). Studies in mice deficient for both CRH receptors revealed sexually dichotomous responses, as females displayed anxiolytic-like behaviours in contrast to males showing anxiogenic-like behaviours (Bale et al., 2002). Taken together, CRH-R1 is crucially involved in the induction of stress responses, while CRH-R2 seems to be involved in modification and adaptation of stress responses.

2.5.4 The functional role of the CRH receptors in sleep-wake regulation

Literature is limited regarding the contribution and involvement of the different CRH receptors in sleep-wake regulation. In rats blocking the function of pituitary CRH receptors by i.v. injection of different CRH receptor antagonists (i.e. astressin and α-helical CRH (α-hCRH)) shortly before the onset of the dark period reduced spontaneous waking during the active phase of the animals (Chang and Opp, 1999). In the same conditions, i.c.v. administration of the antagonists likewise reduced spontaneous waking in rats (Chang and Opp, 1998). In contrast antagonist application shortly before the sleeping-period of rats (i.e. light period) was proven ineffective (Chang and Opp, 1998) since at that time endogenous CRH levels are low (Yokoe et al., 1988). Another study in rats could show that an i.v. application of urocortin induced increases of wake and decreases of NREMS, which were attenuated by intraperitonealy pretreating animals with low doses of the CRH receptor antagonist astressin (Uchida et al., 2007). Whereas α-hCRH is a more potent antagonist of CRH-R2 than CRH-R1 (Dieterich et al., 1997;Turnbull and Rivier, 1997), astressin binds to both CRH receptors with similar affinity (Rivier et al., 1996). Therefore α-hCRH more effectively blocks responses mediated by CRH receptors in the brain than in the pituitary (Fisher et al., 1991;Rivier et al., 1996). The reduction of wakefulness observed after i.c.v. administration of α-hCRH is more likely attributable to blockade of central subcortical CRH-R2 (Chang and Opp, 1998). Astressin on the other hand is effective in blocking CRH receptors both in the brain and pituitary (Rivier et al., 1996). In rats selectively bred for either high or low anxiety-related behaviour (HAB or LAB) (Liebsch et al., 1998), a subcutaneous administration of a CRH-R1-specific antagonist, i.e. R121919, attenuated stress-induced (i.e. citrate buffer application) changes in sleep-wake patterns of HAB animals stronger than in LAB animals (Lancel et al., 2002). As HAB animals possess a hyper-reactive HPA axis (Landgraf et al., 1999) compared

to LAB animals, the difference in the effectiveness of the antagonist was related to the pathophysiological condition in the CRH system of the rats under baseline conditions (Lancel et al., 2002). In depressed patients with a hyperactive CRH system, an application of R121919 was shown to ameliorate sleep disturbances as SWS increased while the number of awakenings and REM density decreased (Held et al., 2004).
Given the localisation of the two CRH receptors (CRH-R1 and CRH-R2) within the mammalian CNS and according to the research summarised above, an involvement of CRH in spontaneous and stress-related sleep-wake regulation seems likely. However, literature is biased to an importance of CRH-R1, thus the functional role of CRH-R2 still remains to be elucidated. Furthermore it currently remains unclear whether an activation of pituitary or central CRH-R1 is the driving force behind the CRH-mediated sleep-wake regulation.

2.6 Aim of the thesis

As outlined above the assumption of a contribution of CRH to the regulation of sleep and wake seems reasonable. However, the limited availability of water-soluble, high specificity antagonists for either CRH receptor precluded an unambiguous distinction of each receptor's intrinsic involvement in this regulation. For the same reasons, earlier experiments were yet unsatisfactorily distinguishing the sleep-wake effects of CRH as a neuromodulator within the CNS from its role as an HPA axis activator. Taking a more sophisticated gene technological approach, this study has the advantage of using conventional CRH-R2 knockout mice as well as previously unavailable conditional CNS specific CRH-R1 knockouts. Against the background of a differentially functional CRH receptor system in the presence of a fully functional HPA axis, this study is entitled to address above mentioned questions. The aim of the project is, first to clarify whether CRH effects on sleep/wake are CNS mediated or not, and second which of the CRH receptors is essentially involved in this regulatory process.

III Materials and Methods

3.1 Experimental animals

In this study, adult (9-12 weeks old) male mice of three different mouse lines were used: conditional CNS-specific CRH-R1 knockout (CRH-R1 CKO), conventional CRH-R2 knockout (CRH-R2 KO), and their respective control littermate (CRH-R1 and -R2 CL) mice as well as commercially available C57BL/6J mice (Harlan Winkelmann GmbH, Borchen, Germany), the major background strain of our knockout models. Both genetically modified mouse lines were provided by the research group “Molecular Neurogenetics” of the Max Planck Institute of Psychiatry in Munich, Germany. Although generation of the mice was not part of this study, it will briefly be described as follows.

CNS-restricted deletion of CRH-R1 was achieved by breeding mice with a homozygous floxed *Crhr1* gene, where exons 9-13 (encoding transmembrane domains four to seven, and a G-protein coupling domain) are flanked by *loxP* sites (Müller et al., 2003), to *nestin-cre* mice (Tronche et al., 1999). In this transgenic mouse line *Cre* expression is controlled by the nestin (*Nes*) promoter and neural enhancer, which drives *Cre* expression in neuronal and glial precursors as early as embryonic day 10.5. Resulting heterozygous *Crhr1*$^{+/loxP}$ *nestin-cre* and *Crhr1*$^{+/loxP}$ F_1 animals were intercrossed to obtain the F_2 generation animals of the desired genotypes: *Crhr1*$^{loxP/loxP}$ *nestin-cre* (conditional knockout; CKO) and *Crhr1*$^{loxP/loxP}$ (control littermate; CL). Subsequently, the conditional CNS-specific CRH-R1 knockout mouse line was maintained by breeding *Crhr1*$^{loxP/loxP}$ *nestin-cre* and *Crhr1*$^{loxP/loxP}$ animals. In *Crhr1*$^{loxP/loxP}$ *nestin-cre* animals CRH-R1 expression is disrupted throughout the brain but not in the pituitary. The CRH-R1 CKO and CL mice were kept on a C57BL/6J x 129S2/SvPas background and have been bred to transgenic *nestin-cre* mice that were generated using (C57BL/6J x SJ)F_2 oocytes (Schmidt et al., 2006).

The CRH-R2 knockout mouse line (courtesy of Stenzel-Poore MP, Oregon Health & Science University, Portland, USA) was originally generated using 129X1/SvJ-derived RW-4 embryonic stem cells (Coste et al., 2000). Chimaeras were bred to C57BL/6J mice to generate offspring harbouring the mutant allele. The sequence coding for transmembrane domains three and four has been substituted by a neomycin resistance cassette. At the Max Planck Institute of Psychiatry mice were backcrossed to C57BL/6J for four generations and then maintained by brother-sister mating. Homozygous *Crhr2*$^{-/-}$ knockout animals (CRH-R2 KO) and *Crhr2*$^{+/+}$ wildtype control littermates (CRH-R2 CL) were obtained from heterozygous breedings (*Crhr2*$^{+/-}$ $\times$ *Crhr2*$^{+/-}$).

3.2 Animal housing

During sleep recordings, animals were single-housed in custom-made Lucite® cages (transparent Lucite® walls, 35cm height; replaceable, grey Lucite® floor, 26cm x 26cm) placed in an noise reduced recording chamber under constant environmental conditions (a 12h/12h light/dark cycle, lights on at 09:00 a.m., lights off at 09:00 p.m., ~100lx during the light period, 22°C ± 1°C, 50% ± 10% humidity). The open top side of the cages allowed free access to the animals and optimal mobility for the swivel system used for EEG and EMG recordings (see EEG/EMG recordings). A feeding rack and a water bottle were attached to the front panel of the cage allowing animals to access food and water *ad libitum*. When the animals were subjected to automated sleep deprivation (ASD), they were kept in the same cages except for a round recess (15cm diameter) in the rear panel of each cage, which was closable by insertion of a lucent divider (see Automated sleep deprivation).
All animal studies were conducted according to the guidance of the European Community Council Directive. Experimental protocols were approved by the local commission for the Care and Use of Laboratory Animals of the State Government of Bavaria, Germany.

3.3 Surgery

During surgery, animals were anaesthetised using inhalation narcosis (custom-made vaporising device) with an isoflurane/oxygen mixture (Isofluran®, DeltaSelect GmbH, Dreieich, Germany). To maintain a constant position of the head, mice were fixed with a stereotaxic apparatus (Type LabStandard™, Stoelting Co., Wood Dale, USA). Before and after surgery, animals received subcutaneously atropine sulfate (0.05mg/kg, Atropin®, Braun Melsungen AG, Melsungen, Germany) to stabilise circulation, and meloxicam (0.5mg/kg, Metacam®, Braun Melsungen AG, Melsungen, Germany) as a postoperative analgesic, respectively. The scalp was incised longitudinally, the skull exposed and holes for the implantation of the EEG electrodes (one in the frontal and one in the parietal field of each hemisphere; Figure 11, page 34) and the i.c.v. guide cannula (only for injection experiments) were drilled in the cranial bone with a dental drill (KaVo-5 Type EWL4970, Kaltenbach und Voigt Elektronisches Werk GmbH, Leutkirch, Germany). The i.c.v. guide cannula was inserted stereotaxically into the left lateral ventricle. The position of the cannula was defined according to the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997) and adjusted to the different mouse lines (distance from bregma to hole in µm: C57BL/6J: x = 1.0, y = 0.4, z = -1.3; CRH-R1 CKO/CL: x = 0.8, y = 0.4, z = -1.3; CRH-R2 KO/CL: x = 0.8, y = 0.4, z = -1.5). After positioning, the i.c.v. cannula was fixed to the skull with dental acrylic

resin (Paladur, Heraeus Kulzer, Hanau, Germany). Four electrodes made of gold wires with ball-shaped ends were placed epidurally on the cortex for EEG recordings, and two more of these electrodes were inserted in the neck muscle to record EMG signals. All electrodes were soldered to an 8-pin connector (BCP socket connector, <0.5g; Compona GmbH, Switzerland) which was flanked by two jeweller's screws and affixed to the skull with Paladur. To increase footing, the screws, the EEG electrodes, the sides of the connector and the i.c.v. cannula (if present) were covered with Paladur. At the end of the surgery, the incision was sutured, and mice were allowed to recover from surgery for two weeks in their experimental cages before initiation of baseline recordings. When experiments were completed, animals were euthanised with an overdose of sodium pentobarbital. Brains were removed and processed for Nissl staining (cresyl violet acetate, Sigma-Aldrich, St. Louis, USA) to examine the positions of the i.c.v. cannulae (tissue lesions). Only animals with correctly placed cannulae were accounted for further analysis.

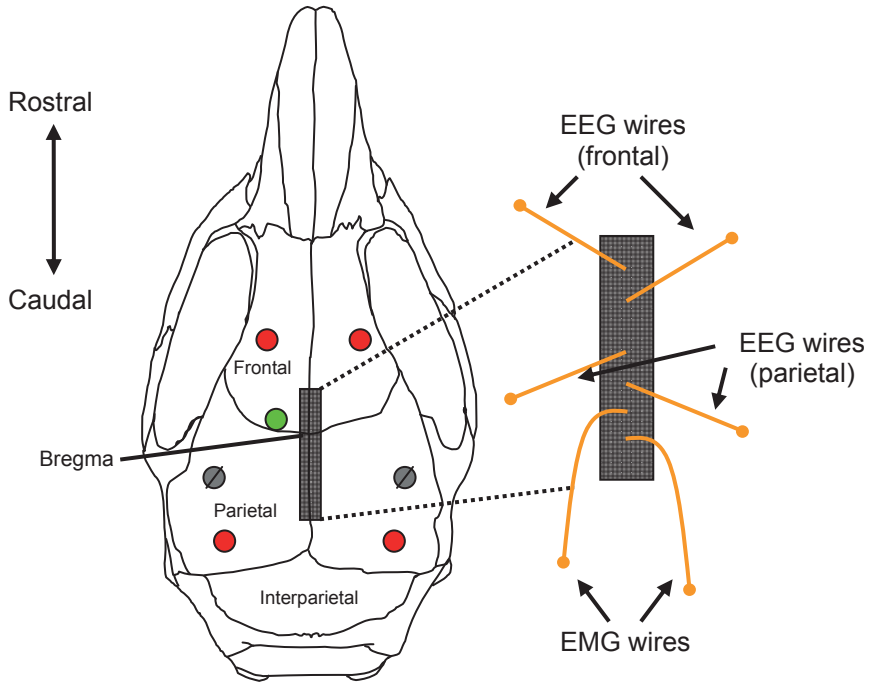

Figure 11: Schematic drawing of a dorsal view of a mouse skull and the 8-pin connector (skull adapted from www.informatics.jax.org).

Indicated on the left hand side are the locations of the holes for the i.c.v. guide cannula (●), the EEG electrodes (●), and the jeweller's screws (●). On the right hand side a schematic representation of the 8-pin connector is given. Golden lines with ball-shaped ends represent the two frontal and the two parietal EEG electrodes, as well as the two EMG electrodes which were placed into the neck muscle of the animal.

3.4 EEG/EMG recordings

After the recovery period an 8-pole recording cable was plugged into the connector and attached to an electric swivel system (Type SW-921.18, Precisor Messtechnik, Munich, Germany) which allowed EEG and EMG recordings from the freely behaving animal. All signals were preamplified (1000-fold, custom made) and sent to a main amplifier (10-fold, custom made) before transformation by an analogue/digital card (64Hz sampling rate; National Instruments, Austin, USA) and storage on a computer (Figure 12, page 36). The EEG signals were analogue bandpass-filtered (0.5-29Hz, filter frequency roll off 48dB/octave), and root mean square was applied to the non-filtered EMG signals before its digital conversion (sample rate: 64Hz). Continuous EEG/EMG recordings were made for 23 hours per experimental day to allow animal care and maintenance of the recording setup during the remaining hour, if necessary. Data obtained in this manner were analysed by a LabView-based acquisition programme (EGEraVigilanz, SEA, Cologne, Germany), which enabled semiautomatic classification of vigilance states applying a FFT algorithm adapted from a report by Louis et al (Louis et al., 2004). The frequency bands were defined as follows: δ (0.5-5Hz), θ (6-9Hz), α (10-15Hz), η (16-22.75Hz), and β (23-31.75Hz). For the computer-based semi-automatic analysis the delta and theta band were further specified (δ = δ x α / η x β and θ = θ x θ / δ x α). According to changes in the EEG and EMG signals and manually set thresholds, sleep stages were defined in three steps as follows: (1) EMG-amplitude above threshold → WAKE. (2) δ-values above threshold and (3) θ-values below threshold → NREMS. All other cases were defined as REMS. Vigilance states were defined as wake, NREMS or REMS, in 4-second epochs each. Semi-automatically scored data were confirmed and corrected by visual rescoring according to the following criteria. Epochs were scored as NREMS in the case of a high amplitude, low frequency (delta range) EEG, and as REMS if theta activity was dominant concomitant with muscle atonia (no EMG signal). All other cases were scored as wake.

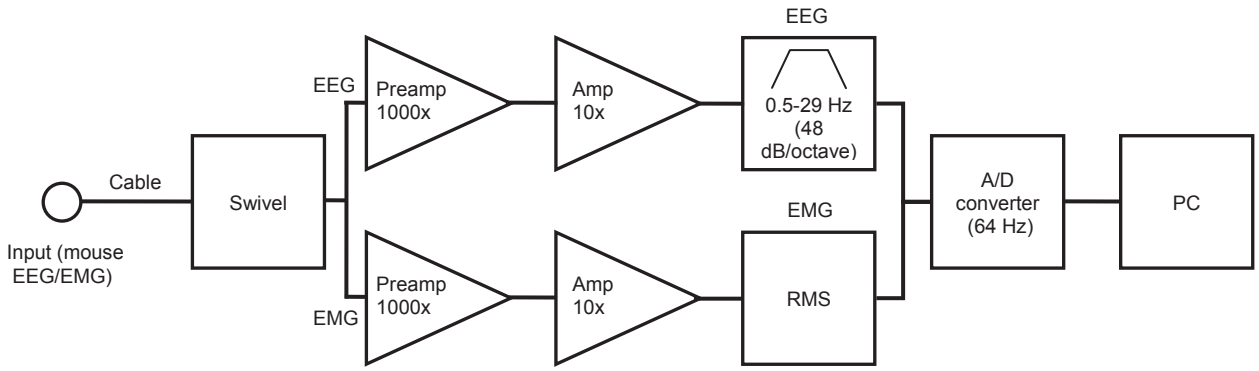

Figure 12: Block diagram of the EEG/EMG recording setup.

The mouse EEG and EMG signals were sent to a preamplifier (1000-fold) and to a main amplifier (10-fold). Whereas the EEG was bandpass-filtered (0.5-29Hz, filter frequency roll off 48dB/octave), root mean square was applied to the unfiltered EMG signal. Both signals were converted analogue to digital (sample rate: 64Hz) and stored on a computer for later offline-analysis. Abbreviations: Preamp: preamplifier; Amp: main amplifier; RMS: root mean square; PC: personal computer.

3.5 Experimental procedures for testing CRH and ASV-30

CRH was administered i.c.v. to 14 C57BL/6J, 12 CRH-R1 CKO, 9 CRH-R1 CL, 7 CRH-R2 KO, and 8 CRH-R2 CL mice, and the dose-dependent changes in vigilance states were analysed. For central application of CRH, a bolus injection was achieved i.c.v. by using a Hamilton syringe (Type 700 Hamilton Microliter®, Banaduz, Switzerland) with an injection cannula (inner diameter: 0.11mm). Each experimental animal either received 3µl of a control vehicle (sterile water, Braun Melsungen AG, Melsungen, Germany) or three different doses of human/rat CRH (0.3, 1.0, 3.0µg; h/rCRF, Bachem AG, Bubendorf, Switzerland) dissolved into an equal volume of vehicle. Based on previous findings that i.c.v. administered CRH at 1.0µg/mouse elevated levels of plasma CORT (Dunn and Berridge, 1990), one lower and one higher dose than that were chosen with the intention to determine differences in dose responses. Upon injection the animals were gently restrained, and the injection cannula was inserted into the guide cannula. Each i.c.v. application was finished within 30 seconds after insertion of the injection cannula, and animals were released into their respective recording cage. All injections were completed within the last 30 minutes of the dark period under red light conditions. To include manipulatory effects of injections, EEG and EMG recordings started one hour before the onset of the light period. The order of CRH application at several doses was randomised in the same animal including two baseline days between treatment days to prevent accumulation of drug effects (Figure 13, page 37).

Another set of experiments was designed to permit a more specific dissection of the presumably different involvement of the CRH receptors in sleep-wake regulation. Centrally pretreating CRH-R1 CKO and CL animals with a specific CRH-R2 antagonist should allow a temporary blockade of either both central CRH receptors (CRH-R1 CKO) or CRH-R2 (CRH-R1 CL). Subsequent central application of CRH (with or without CRH-R2 antagonist) should provide additional evidence by putative variations in sleep-wake responses to differentiate a participation of the CRH receptors in the sleep-wake regulatory process. In this set of experiments, the selective CRH-R2 antagonist antisauvagine-30 (ASV-30, Bachem AG, Bubendorf, Switzerland) was i.c.v. injected at 10nmol without or together with 1.0µg of CRH in 13 CRH-R1 CKO and 8 CRH-R1 CL. With the following exceptions, the same experimental conditions and injection protocols were applied, as mentioned above. Animals received two injections of either vehicle control alone, or a combination of vehicle control and CRH, ASV-30 and vehicle control, or ASV-30 and CRH. Injections were set in 15 minute intervals whereupon ASV-30, if scheduled, was administered first.

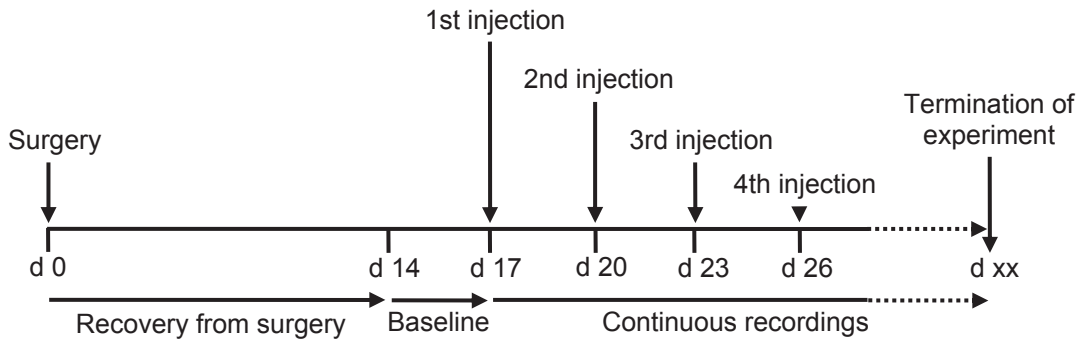

Figure 13: Schematic representation of the schedule for the injection experiments.

In all experiments, animals were allowed to recover from surgery (d 0) for two weeks (d 14) before initiation of baseline recordings. On day 17 (d 17) animals received the first i.c.v. injection (randomised treatment). To avoid accumulation of drug effects two baseline days were inserted. The second i.c.v. injection followed on day 20 (d 20). Depending on the number of different treatments, the injection schedule was maintained until termination of the experiment (indicated by dotted line and d xx). The arrows and the arrowhead indicate main events of a set of experiments.

3.6 Automated sleep deprivation

To challenge sleep homeostasis and investigate the involvement of the different CRH receptors in homeostatic sleep-wake regulation, a total of 8 C57BL/6J, 7 CRH-R1 CKO, 7 CRH-R1 CL, 8 CRH-R2 KO, and 8 CRH-R2 CL mice were subjected to ASD. According to differences in the sleep-wake profile following ASD, a different contribution of CRH receptors to homeostatic sleep-wake control might be evaluated. Running wheels (15cm diameter) were attached to the recess in the rear panel of each home cage. All animals had access to the locked running wheels as part of their individual home cages three days before initiation of the first baseline recording and throughout the whole experiment. After one day of baseline recordings, animals were automatically sleep deprived for six hours from the beginning of the light period (inactive phase; Zeitgeber Time (ZT) 0). On the consecutive day after ASD, EEG recordings were also monitored to examine sleep patterns of the recovery day. After ten days animals underwent a second ASD. Upon termination of this ASD (ZT 6), blood samples were immediately collected from the tail vein of the animals to investigate plasma CORT levels. Basal levels of plasma CORT were determined from time-matched blood samples taken five days after the first ASD (Figure 14).

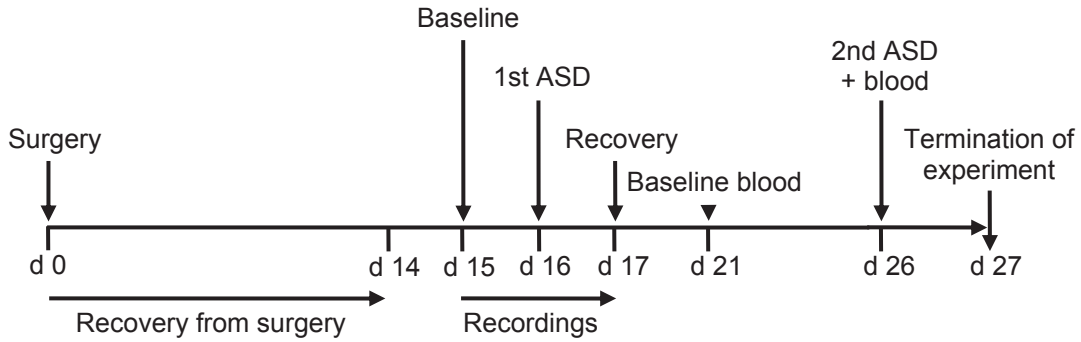

Figure 14: Schematic representation of the schedule for the ASD experiment.

Animals were allowed to recover from surgery (d 0) for two weeks (d 14) before initiation of baseline recordings on day 15 (d 15). The first ASD was performed on day 16 (d 16) and recordings were continued on day 17 (d 17) to examine sleep patterns on the recovery day. Five days after the first ASD, baseline blood samples were collected (d 21). On day 26 (d 26), ten days after the first, the second ASD was accomplished and blood samples were taken. The experiment ended on day 27 (d 27). The arrows and the arrowhead indicate main events of the experiment.

During ASD, animals were kept inside the running wheels by introducing a lucent divider (supplied with a water bottle and a feeding rack) between the exit of the running wheel and the rest of the home cage. The technical details of the ASD setup have been described

elsewhere (Fenzl et al., 2007). Basically the progression to sleep was prevented by initiating the rotation of the running wheels according to the magnitude of muscle tone (EMG). The EMG signal of each mouse was picked up online after preamplification (see EEG/EMG recordings) and sent to the control unit of each individual running wheel (Figure 15). A low amplitude EMG signal, equal to inactivity of the respective animal and a possible transition to sleep, was detected whenever the amplitude of the EMG signal fell short of a threshold set manually at the control unit. Consequently a geared motor (series 950D, type 454-0855, gear ratio 810:1, RS-Components GmbH, Mörfelden-Walldorf, Germany) started driving the running wheel thereby enforcing movement of the animal. To avoid activation of the running wheel during short periods of quiet wakefulness (awake but inactive animal) a manually settable delay line (delay time: 0-10 seconds) was connected upstream to the motor. Furthermore the rotational direction of the running wheels was manually adjustable to prevent habituation of the mice to the same rotation direction. At the end of the ASD the lucent divider was removed, and the animals were released into their home cages. Unlike gentle handling, a widespread sleep deprivation routine, ASD not only provides a more standardised method but seemingly a very efficient one, as more than 98% of NREMS and REMS were blocked in all experiments. This suppression level is hardly acquired with gentle handling.

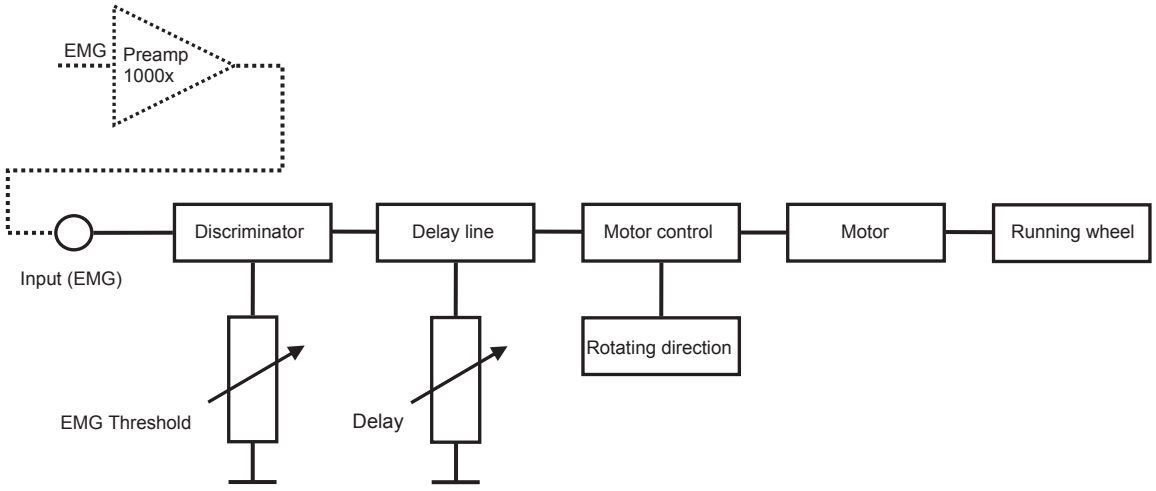

Figure 15: Block diagram of the running wheel control (adapted from Fenzl et al., 2007).

The preamplified EMG signal (denoted by the dashed symbol and lines) of each mouse was sent to the control unit of the respective running wheel. A discriminator compared the incoming EMG signal with the manually set EMG threshold. In case of a low amplitude EMG (indicating transition to sleep) the motor control unit triggered the motor drive of the running wheel, which consequently started to rotate. To avoid reactions to every short bout of inactivity (e.g. quiet wake) a delay line (delay time: 0-10s) was connected upstream to the motor. Additionally the rotating direction (integrated in the motor control) could be adjusted manually to avoid habituation of the animals to the same rotation direction. Abbreviations: Preamp: preamplifier.

3.7 Slow wave activity

As a reflection of sleep depth/intensity or pressure, SWA (encompassing a slightly narrower frequency range as the EEG delta band; 0.5-4Hz) served as a quantifiable indicator of accumulated homeostatic sleep pressure. To asses the different reactivity of the various mouse lines to homeostatic challenges (i.e. ASD), SWA was extracted with the help of a LabView-based (custom made; SWA programme) analysis programme. According to the semi-automatically generated and manually corrected scoring files, the SWA programme was able to identify all NREMS epochs, and the EEG power density therein, of the FFT transformed recording data. Epochs containing recording artefacts were excluded, and SWA (EEG power density in the 0.5-4Hz frequency band) was calculated for each remaining epoch identified as NREMS. To compare SWA among multiple strains, the data values had to be normalised. From the total amount of NREMS epochs, SWA was calculated for light and dark conditions separately in one hour means averaged from all animals of one particular mouse line. All data were referred to the mathematical mean of SWA baseline activity (corresponding to 100%, relative SWA) and deviations (positive or negative) represent the individual one hour means (±SEM) for baseline, ASD, and recovery.

3.8 Sleep architecture

Following the i.c.v. CRH injection, changes in sleep architecture parameters were analysed with a LabView-based (custom made; Classification) classification programme to assess possible treatment-induced differences in sleep composition of the various mouse lines. The mean frequency of entries (i.e. mean number) in a vigilance state and the respective mean duration spent in this state were calculated for each treatment and mouse line during the light (12h) or the dark period (10h). The data for the mean number of the vigilance states will not be shown, since they can be deducted from the mean length and the total amount of the respective state within a certain time period. The semi-automatically generated and manually corrected scoring files were processed by the Classification programme. Afterwards the total number of respective vigilance episodes (wake, NREMS, and REMS) per every 12h or 10h of recording was summed. Furthermore the length of each individual vigilance episode and subsequently the total time spent in a certain vigilance state within these 12h or 10h were determined. By dividing the total time spent in a given vigilance state with the number of respective vigilance episodes (both within 12h or 10h each), the mean duration (in epochs) spent in one bout of the vigilance state was determined. As an output the Classification programme provided the average number of four second epochs spent in a wake, NREMS or

REMS episode within the respective 12h or 10h means. These epoch-number means were then converted to means in minutes (±SEM).

3.9 Analysis of plasma corticosterone levels

To quantify HPA axis reactivity in our CRH-R1 CKO animals (n = 19) and their CL (n = 19) in response to CRH administration, blood samples were collected from the tail vein of the animals. Baseline control blood (15µl) was collected one week before and an equal volume of sample blood after vehicle or 1.0µg of CRH treatment was taken two hours after injection (ZT 2). To assess HPA axis activation by ASD, blood samples (C57BL/6J, CRH-R2 KO, and CL: n = 8; CRH-R1 CKO and CL: n = 7) were immediately collected upon termination (ZT 6) of the second ASD from the tail vein of the animals to investigate plasma CORT levels. Basal levels of plasma CORT were determined from time-matched blood samples taken five days after the first ASD.

All blood samples were analysed (repeated determination) for plasma CORT levels by radioimmunoassay (Corticosterone-^{125}I RIA Kit, MP Biomedicals, Orangeburg, USA). The sensitivity of the CORT RIA kit was 25ng/ml. After collection, blood samples were centrifuged immediately (3500 rpm, 15 minutes, 4°C) and separated plasma supernatant was collected and stored at -80°C until later analysis. The test protocol, as provided by the vendor, in short is as follows. Test plasma samples were diluted 1:150. In a first step 0.2ml of radioactively labelled CORT (Corticosterone-^{125}I) were added to the samples followed by 0.2ml of a rabbit anti-corticosterone antiserum. Samples were briefly vortexed and incubated for two hours at room temperature. Subsequently 0.5ml of a goat-anti-rabbit precipitation solution were added and samples were vortexed again. After centrifugation (2500 rpm, 15 minutes, 4°C) the supernatant was aspirated and radioactivity in the precipitate measured in a gamma-counter (COBRA^{TM} II Auto-Gamma®, Canberra-Packard GmbH, Dreieich, Germany). The content of CORT in the plasma samples was determined by comparison of the measured radioactivity in the given samples with the radioactivity count in standard controls of known CORT amount (standard solutions with 25, 50, 100, 250, 500, or 1000ng/ml of CORT). Plasma CORT samples were measured in duplicates and obtained values were averaged. The intra-assay coefficient of variation was <12% for CORT.

3.10 Statistical analysis

Time spent in wake, NREMS, and REMS was calculated in consecutive two hour intervals, and effects of different treatments and time intervals on these vigilance states were statistically evaluated for significance in each time period (light or dark) and each mouse group by two-factorial analyses of variances (ANOVA) with a repeated measures design. Thereby the two influential factors were dose and time, both within-subjects factors with 5 levels (baseline, vehicle, 0.3µg, 1.0µg, and 3.0µg CRH) for the i.c.v. CRH injection studies or 4 levels (baseline, vehicle + vehicle, ASV-30 + vehicle, ASV-30 + CRH) for the antagonist study and 6 (light period; 2h intervals) or 5 levels (dark period; 2h intervals) in both studies, respectively. SWA was calculated in consecutive one hour intervals and effects of different treatments and time intervals on SWA were statistically evaluated for significance in each time period (light or dark) and each mouse group by two-factorial ANOVA with a repeated measures design as well. The two influential factors were treatment and time, both within-subjects factors with 3 levels (baseline, ASD, and recovery) and 12 (light period; 1h intervals) or 10 levels (dark period; 1h intervals), respectively. Furthermore, the mean duration of the wake, NREMS, and REMS episodes within the light and dark period were calculated, and the effects of treatment, time period and mouse groups on given means were statistically analysed by three-factorial ANOVA. As mentioned previously besides dose and time, group was an additional influential factor with 5 levels: C57BL/6J, CRH-R1 CKO, CRH-R1 CL, CRH-R2 KO, and CRH-R2 CL. Data for plasma CORT levels were compared between given mouse groups and different treatments by two-factorial analysis of variance (ANOVA) with a repeated measures design. In the event of significant main or interaction effects in ANOVA, post-hoc tests for simple effects (contrasts tests or Student-Neumann-Keuls tests) were applied in order to locate significant differences among the levels of the within- or between-subjects factors, respectively. As nominal level of significance $\alpha = 0.05$ was accepted. All post-hoc tests (tests with contrasts and/or Student-Neumann-Keuls tests) were performed at a reduced level of significance (Bonferroni adjusted), in order to keep the type 1 error less or equal to 0.05. Values presented in tables and graphs indicate mean values ±SEM.

IV Results

4.1 Sleep and wake responses to CRH injections

4.1.1 C57BL/6J

In C57BL/6J mice, significant interaction effects of treatment and time on wake were found during the light period ($F_{treatment\ x\ time}$ (20/220) = 18.74, P < 0.001) but in the dark period only significant treatment effects could be detected ($F_{treatment}$ (4/44) = 33.78, P < 0.001). Compared to the baseline recording, injection itself even with vehicle elicited a significant increase in wakefulness during the first two hours (contrasts tests, P = 0.039), which afterwards returned to and remained at baseline levels (Figure 16 A, page 45). CRH application, however, caused significantly higher rises of wake by postinjection hour two in comparison to vehicle and thus baseline (contrasts tests, P < 0.05). Arousal levels remained significantly elevated in a dose-dependent manner during most of the entire light period. The highest dose of CRH caused significantly increased wakefulness for up to ten hours after injection (contrasts tests: ZT 2-8: P < 0.001; ZT 10: P ≤ 0.014), whereas the wake-promoting effects of CRH disappeared roughly six and eight hours after application of 0.3µg or 1.0µg (contrasts tests, P < 0.001), respectively. At the beginning of the dark period, a dose-dependent reduction in time spent awake was observed in all animals injected with CRH in comparison to vehicle treatment. This reduction in wake persisted to be significant at the two higher doses (ZT16-22; contrasts tests: 1.0µg: P ≤ 0.012; 3.0µg: P < 0.001) during most of the dark period.

Regarding NREMS, interaction effects of treatment and time were significant during the light period ($F_{treatment\ x\ time}$ (20/220) = 18.521, P < 0.001), whereas during the dark period only significant treatment effects arose ($F_{treatment}$ (4/44) = 65.694, P < 0.001). Vehicle injections showed as compared with baseline levels, only a slight and non-significant reduction of NREMS during the first two hours (Figure 16 B, page 45). Application of CRH, however, induced clear, dose-dependent decreases of this vigilance state in comparison to control and therefore baseline levels. The higher the dose of applied CRH, the larger were the changes and effect durations observed in NREMS. While 0.3µg of CRH significantly reduced time spent in NREMS for up to four hours, 1.0µg and 3.0µg of CRH affected significant reductions for six and ten hours, respectively (contrasts tests, P < 0.001). After transition to the dark period, all CRH-treated animals showed increases in NREMS compared with vehicle treated animals, although the dose dependency was not as clear as seen for wake. Whereas NREMS after treatment with 0.3µg of CRH was only slightly elevated and reached significantly higher levels only at one timepoint (ZT 16; contrasts tests, P ≤ 0.022), injections of 1.0µg and 3.0µg of CRH similarly led to more distinct rises in NREMS levels. Both doses

entailed significant increases of NREMS at several timepoints of the dark period (ZT 16, 18, and 22; contrasts tests, $P \leq 0.035$).

In the case of REMS, significant interaction effects were found in the light period ($F_{treatment \times time}(20/220) = 9.675$, $P < 0.001$), whereas significant treatment effects could be observed during the dark period ($F_{treatment}(4/44) = 24.356$, $P < 0.001$). Vehicle injections significantly suppressed REMS appearance for up to two hours compared with baseline (contrasts tests, $P = 0.008$; Figure 16 C, page 45). However, no further differences in REMS amounts could be detected in comparison to baseline levels. As for other vigilance states, effects induced by CRH treatment were dose-dependent. The lowest dose of CRH (0.3µg) significantly reduced REMS levels for four post-injection hours compared to vehicle treatment and baseline (contrasts tests, $P \leq 0.025$). The two other doses of CRH, 1.0µg and 3.0µg, almost completely blocked REMS for the first four or six hours respectively, and significant reduction of REMS lasted for eight hours after application (contrasts tests, $P \leq 0.017$). On the other hand, CRH promoted REMS during the dark period. At 0.3µg of CRH, REMS levels were slightly increased, reaching significant levels at two timepoints (ZT 16 and 22; contrasts tests, $P \leq 0.025$). The higher doses exhibited distinct effects on REMS enhancement. Elevation of REMS remained significant almost throughout the entire dark period after treatment with 1.0µg (contrasts tests, $P \leq 0.013$) and 3.0µg of CRH (contrasts tests, $P < 0.001$).

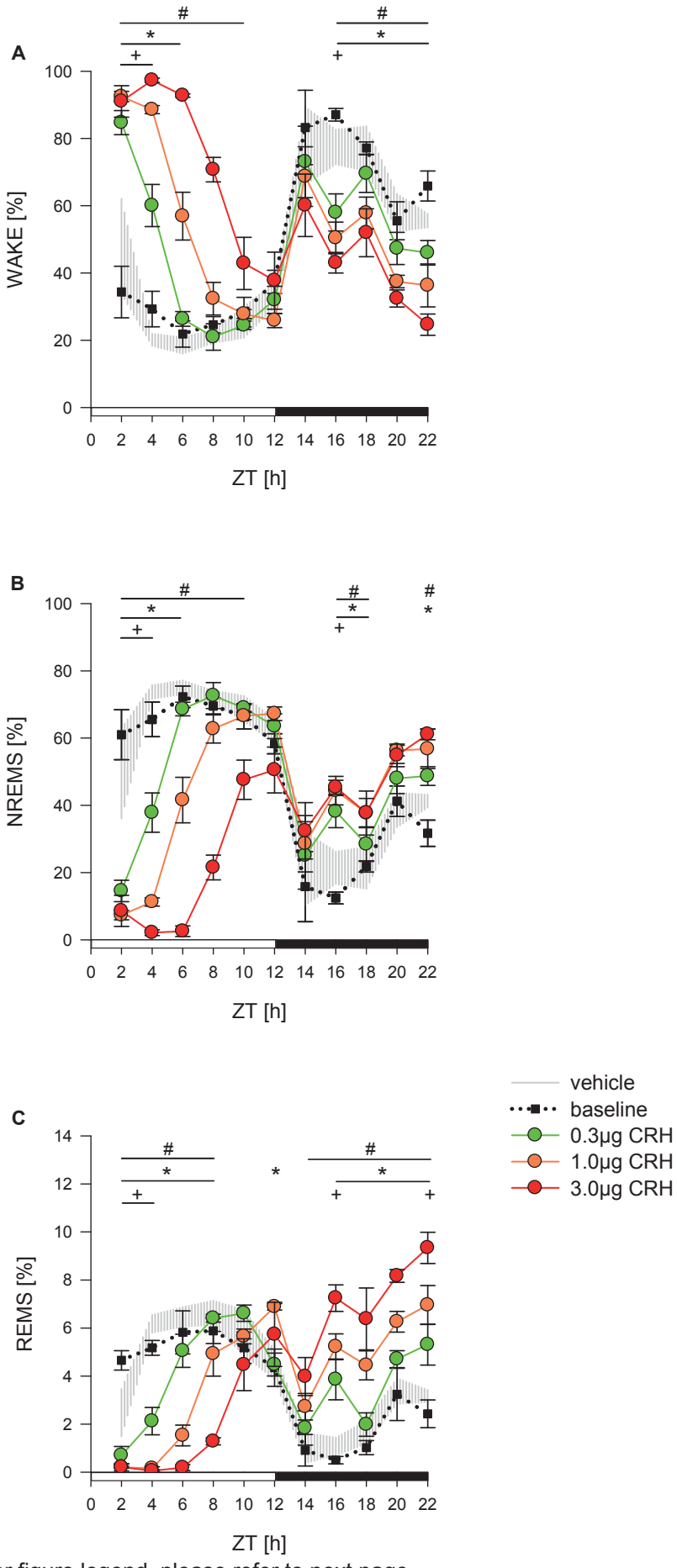

Figure 16 (C57BL/6J): For figure legend, please refer to next page.

Figure 16: Effects of i.c.v. CRH injections on vigilance states in C57BL/6J (n = 14) mice.

Corticotropin-releasing hormone (CRH) dose-dependently increased wake levels during the light period for up to ten hours after application and decreased those levels for almost the entire course of the dark period. Contrariwise NREMS was dose dependently decreased during the light period and showed increases during the successive dark period. REMS was similarly reduced immediately after CRH application and almost totally blocked for up to four and six hours after injection of 1.0µg and 3.0µg CRH, respectively. Shortly after transition to the dark period, a dose-dependent increase in REMS was observed. Depicted are the percentage of the given vigilance states (indicated on the y-axis) of two hour means ±SEM for baseline (■) and each CRH treatment (●: 0.3µg, ●: 1.0µg, ●: 3.0µg). The hatched area indicates mean values ±SEM after vehicle treatment. The symbols "+", "*" and "#" denote statistically significant differences by comparison of vehicle and baseline versus treatment with 0.3µg, 1.0µg or 3.0µg of CRH, respectively (contrasts tests in ANOVA, $P < 0.05$). Solid lines under those symbols connect consecutive timepoints showing statistical differences. The open and filled bar on the x-axis indicates the light and dark period, respectively.

4.1.2 CRH-R1 CKO and CL

In CRH-R1 CKO and CL mice, significant interaction effects of treatment and time on wake were detected only during the light period (CRH-R1 CKO: $F_{treatment\ x\ time}$ (20/220) = 4.552 , $P < 0.001$; CRH-R1 CL: $F_{treatment\ x\ time}$ (20/160) = 13.353, $P < 0.001$). As similarly seen in C57BL/6J mice, vehicle treatment led to a slight but significant, short lasting increase of wake levels in both CRH-R1 CKO and CL animals during the first two hours after injection in comparison to baseline (contrasts tests, $P \leq 0.008$). No further effects of vehicle treatment on wakefulness could be observed for the rest of the recording session if compared to baseline (Figure 17 A and D, page 48). However, CRH application in CL mice elicited a significant, dose-dependent increase in wakefulness in comparison to baseline and vehicle conditions, as similarly seen in C57BL/6J mice. These effects were significant for up to eight hours after injection of 3.0µg of CRH and up to six and two hours after injection of 1.0µg or 0.3µg of CRH respectively (contrasts tests, $P \leq 0.002$; Figure 17 A). In contrast, such a wake-inducing effect of CRH did not appear in CRH-R1 CKO animals. Although all CRH treatments led to slight elevations in wake levels at the beginning of the experiment (Figure 17 D), no significant differences from vehicle and baseline levels could be detected with the exception of wake levels at ZT 2 after application of 0.3µg CRH, ZT 4 after application of 1.0µg CRH, and ZT 6 after application of 3.0µg CRH (contrasts tests, $P \leq 0.047$). During the dark period, contrary to the effects in C57BL/6J animals, no reduction in wakefulness could be induced after any dose, neither in CL nor in CRH-R1 CKO animals.

Concerning NREMS, treatment and time effects showed a significant interaction during the light period in CRH-R1 CL animals only ($F_{treatment\ x\ time}$ (20/160) = 11.794, $P < 0.001$). Vehicle

treatment induced a slight but significant decrease within the first two hours after injection (contrasts tests, $P \leq 0.035$) in comparison to baseline levels in both CL littermates and CRH-R1 CKO animals (Figure 17 B and E, page 48). No further vehicle effect could be observed for the rest of the recording session in either of the two animal lines. As similarly seen in C57BL/6J, CRH in CL elicited profound and significant dose-dependent reductions in NREMS (Figure 17 B). Whereas reduced levels returned to baseline by two hours after injecting 0.3µg of CRH (contrasts tests, $P < 0.001$), 1.0µg and 3.0µg of CRH respectively prolonged this time effect to six (contrasts tests, $P \leq 0.005$) and eight hours (contrasts tests, $P < 0.001$). In CRH-R1 CKO animals, all CRH treatments failed to induce significant decreases in NREMS in comparison to vehicle control and baseline (Figure 17 E). During the dark period, as seen in wake responses, no treatment effects on NREMS levels could be detected in both genotypes.

Similar to wake, significant interaction effects between treatment and time factors on REMS were shown during the light period of both CRH-R1 CKO and CL mice (CRH-R1 CKO: $F_{treatment\ x\ time}(20/220) = 10.255$, $P < 0.001$; CRH-R1 CL: $F_{treatment\ x\ time}(20/160) = 7.562$, $P < 0.001$). Vehicle injection itself decreased REMS significantly only at ZT 2 in both genotypes (contrasts tests, $P = 0.002$) but induced no further changes (Figure 17 C and F, page 48). The clear responses of REMS to CRH injections were blurred in both CRH-R1 CL and CKO mice compared with those in C57BL/6J. Although REMS levels decreased in a dose-dependent fashion in comparison to baseline and were almost totally blunted after 3.0µg of CRH (ZT 4-6; Figure 17 C) in CRH-R1 CL, these decreases reached significant levels in comparison to vehicle levels at the highest dose and ZT 6 only (contrasts tests, $P \leq 0.002$). Reduced REMS started returning to baseline values after two, four and six hours respectively according to the applied dose of CRH, and remained comparable to vehicle and baseline levels except for the case after treatment with 3.0µg of CRH. After this highest dose of CRH, REMS levels significantly increased from the end of the light period (ZT 12) to the beginning of the dark period (ZT 14-16) in comparison to the control conditions (contrasts tests, $P \leq 0.018$; Figure 17 C). In CRH R1 CKO animals, the dose-dependent effects of CRH on REMS in comparison to baseline were also evident (Figure 17 F). In a dose-dependent fashion, REMS levels in comparison to vehicle and baseline were significantly reduced at ZT 4 and returned to vehicle and baseline levels by ZT 6 (1.0µg CRH) and ZT 8 (3.0µg CRH) respectively (contrasts tests, $P \leq 0.011$). Contrary to their CL, CRH-R1 CKO animals displayed no further significant CRH effects on REMS levels during the dark period, although REMS was slightly elevated after 1.0µg and 3.0µg of CRH (ZT 16-22) if compared to baseline.

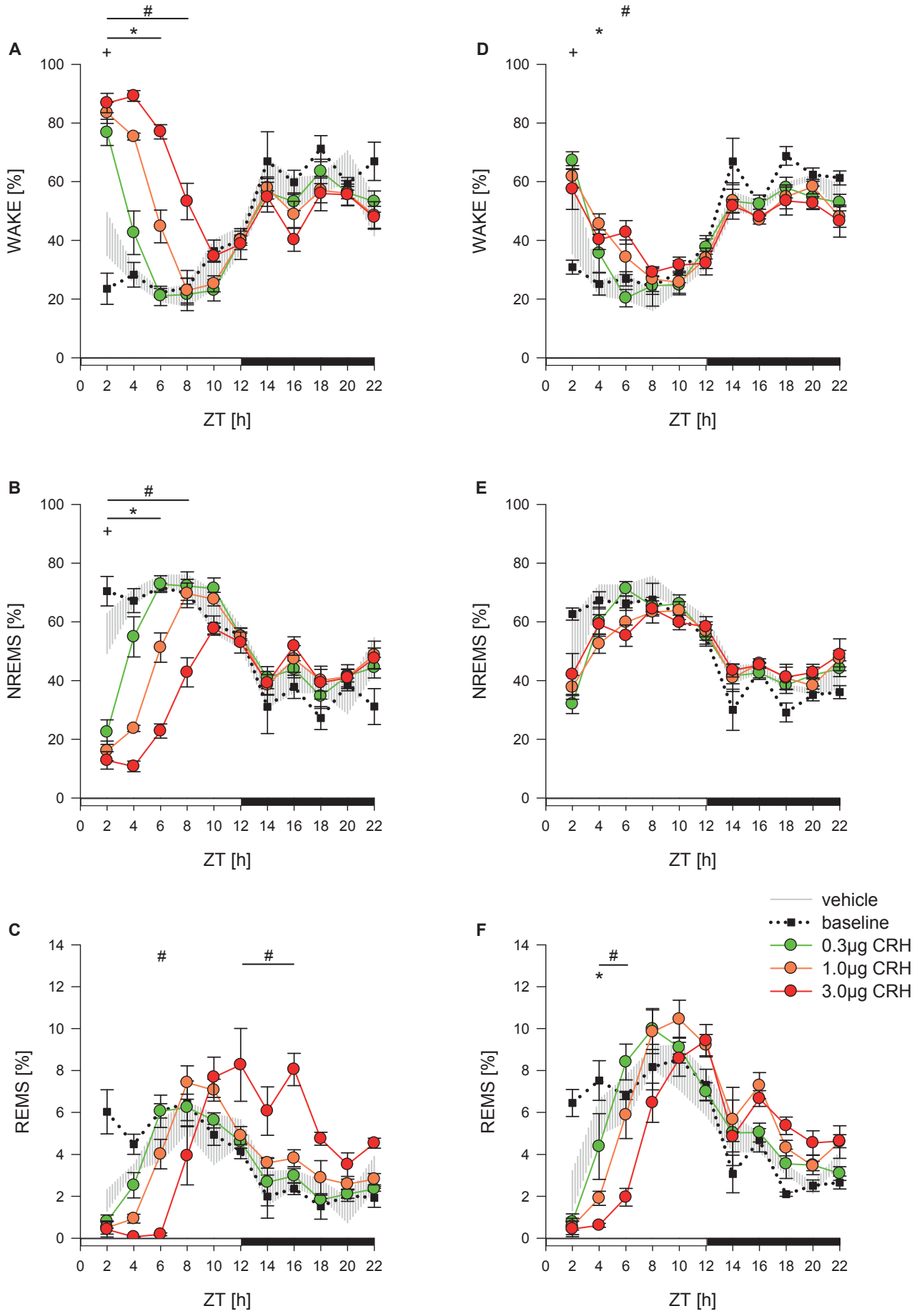

Figure 17 (CRH-R1 CL: A-C; CRH-R1 CKO: D-F): For figure legend, please refer to next page.

Figure 17: Effects of i.c.v. CRH injections on vigilance states in CRH-R1 CL (n = 9; A-C) and CRH-R1 CKO (n = 12; D-F) mice.

Corticotropin-releasing hormone (CRH) dose-dependently increased wake levels in CRH-R1 CL mice up to eight hours during the light period and decreased NREMS amounts at the same time. REMS reductions tended to occur dose-dependently, although only the highest dose of CRH induced significant reductions in comparison to vehicle and baseline treatment six hours after injection. No effects on wake and NREMS occurred during the dark period, whereas REMS was significantly increased after 3.0µg CRH during the first half of the dark period. In the conditional knockout animals, in contrast, wake-promoting and NREMS-reducing effects of CRH were almost totally blunted. Dose-dependent REMS reductions could still be induced by CRH injections, however REMS was only affected during the light period.

For graph denotations please refer to figure legend of figure 16, page 46.

4.1.3 CRH-R2 KO and CL

In CRH-R2 KO and CL mice, significant interaction effects of treatment and time on wake were detected during the light period (CRH-R2 KO: $F_{treatment\ x\ time}$ (20/120) = 13.890, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (20/140) = 7.849, P < 0.001). During the dark period significant interaction effects of treatment and time could be shown for CRH-R2 KO animals ($F_{treatment\ x\ time}$ (16/96) = 2.649, P < 0.003), whereas only significant treatment effects could be observed in CRH-R2 CL ($F_{treatment}$ (4/28) = 4.726, P < 0.006). Similar to the other control mouse lines, in CRH-R2 CL vehicle treatment induced a significant increase in wake levels during the first two post-injection hours if compared to baseline (contrasts tests, P ≤ 0.007; Figure 18 A, page 52). With the exception of ZT22, where significant decreases of wakefulness occurred (P = 0.016), no further differences to baseline levels were evident. In CRH-R2 KO animals, in contrast, vehicle treatment did not induce any significant changes in wake levels compared to baseline, neither during the light nor the dark period (Figure 18 D, page 52). After CRH treatment in CRH-R2 CL, significant and dose-dependent increases in wakefulness appeared, lasting for four, six, or up to ten hours after injection of 0.3µg, 1.0µg or 3.0µg of CRH, respectively (contrasts tests, P ≤ 0.001). Similar effects could be observed after CRH treatment in CRH-R2 KO animals. Whereas 0.3µg of CRH led to significant increased levels of wakefulness for four hours, effects of 1.0µg and 3.0µg of CRH lasted for up to six hours (contrasts tests, P < 0.001). Although wake levels were elevated after 3.0µg of CRH at ZT 8, this increase was only significant if compared to baseline (contrasts tests, P ≤ 0.023), but not in comparison to vehicle treatment. During the dark period, CRH treatment in CRH-R2 CL either with the two higher (ZT 16 and 20) or all CRH doses (ZT 22), entailed slight decreases in wakefulness, reaching significance levels only in comparison to baseline (contrasts tests, P ≤ 0.027) but not vehicle treatment. In CRH-R2 KO, however, 3.0µg of CRH significantly reduced wake levels (ZT 18-20) in comparison to both vehicle treatment

and baseline (contrasts tests, P ≤ 0.025), whereas at ZT 22 all CRH treatments significantly reduced wakefulness in comparison to baseline only (contrasts tests, P ≤ 0.002).

Regarding NREMS, significant interaction effects of treatment and time were detected during the light period (CRH-R2 KO: $F_{treatment\ x\ time}$ (20/120) = 13.509, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (20/140) = 7.633, P < 0.001). As for wakefulness, during the dark period significant interaction effects of treatment and time could be shown for CRH-R2 KO animals ($F_{treatment\ x\ time}$ (16/96) = 2.728, P = 0.002), whereas only significant treatment effects occurred in CRH-R2 CL ($F_{treatment}$ (4/28) = 3.990, P = 0.013). After vehicle treatment in CRH-R2 CL, significant reductions of NREMS levels at ZT 2 and increases at ZT 22 could be observed in comparison to baseline (contrasts tests, P ≤ 0.032; Figure 18 B, page 52). No further changes after vehicle treatment were evident. In CRH-R2 KO, a comparison of baseline and vehicle treatment revealed no effects during the whole recording period (Figure 18 E, page 52). Injecting CRH-R2 CL mice with 0.3µg, 1.0µg, and 3.0µg of CRH dose-dependently and significantly reduced NREMS for four, six, and ten hours after injection, respectively (contrasts tests, P < 0.001). During the dark period, only if referred to baseline, significant increases in NREMS in CRH-R2 CL could be identified at the beginning for 3.0µg of CRH (ZT 16; contrasts tests, P = 0.041) and towards the end for either both higher doses (ZT 20; contrasts tests, P ≤ 0.031) or all CRH doses (ZT 22; contrasts tests, P ≤ 0.032). During the light period, treatment with CRH in CRH-R2 KO resulted in significant and dose-dependent decreases of NREMS for four (0.3µg) or six (1.0µg and 3.0µg) hours after injection (contrasts tests, P < 0.001) compared to vehicle and thus baseline. During the second half of the dark period (ZT 18-20), only treatment with 3.0µg of CRH entailed significant increases in NREMS levels in comparison to both vehicle and baseline conditions (contrasts tests, P < 0.03). However, the increase in NREMS at ZT 22 observed in CRH-R2 KO mice after all CRH treatments was significant only if compared to baseline levels (contrasts tests, P ≤ 0.003).

Significant interaction effects of treatment and time on REMS were detected during the light period in both genotypes (CRH-R2 KO: $F_{treatment\ x\ time}$ (20/120) = 3.945, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (20/140) = 4.836, P < 0.001), however, during the dark period only in CRH-R2 CL ($F_{treatment\ x\ time}$ (16/112) = 1.761, P = 0.048). Vehicle treatment induced significant decreases in REMS within the first two post-injection hours only in CRH-R2 CL (contrasts tests, P = 0.003; Figure 18 C, page 52), reductions in CRH-R2 KO on the other hand only developed into a trend (Figure 18 F, page 52). No further effects of vehicle treatment could be detected in either genotype for the rest of the recording. Whereas at ZT 2 suppression effects of all CRH treatments in CRH-R2 CL mice only differed from baseline levels, an increasing dose of CRH furthermore resulted in significant, dose-dependent REMS

decreases for four (0.3µg; contrasts tests, $P = 0.022$), six (1.0µg; contrasts tests, $P < 0.001$), and eight (3.0µg; contrasts tests, $P < 0.001$) hours in comparison to vehicle treatment. From the beginning of the dark period, CRH significantly increased REMS for four (1.0µg, ZT 14-16; contrasts tests, $P \leq 0.026$) and six (3.0µg, ZT 14-18; contrasts tests, $P \leq 0.029$) hours. Increases observed at the end of the dark period (1.0µg, ZT 20; 3.0µg, ZT 22) only differed from baseline level (contrasts tests, $P \leq 0.008$). In CRH-R2 KO animals in contrast, all treatments showed a trend towards decreasing REMS levels during the light period. The distinct dose-response effect after CRH treatments, as seen for REMS reduction in C57BL/6J and CRH-R2 CL mice, was blurred in CRH-R2 KO as at ZT 4 treatment effects only significantly differed to baseline (contrasts tests, $P \leq 0.006$). Furthermore at ZT 6 only the highest dose of CRH (3.0µg) induced significant REMS reductions compared to vehicle control and baseline (contrasts tests, $P \leq 0.011$). Although REMS was slightly elevated after all CRH treatments throughout the dark period in CRH-R2 KO mice, no significant differences could be demonstrated.

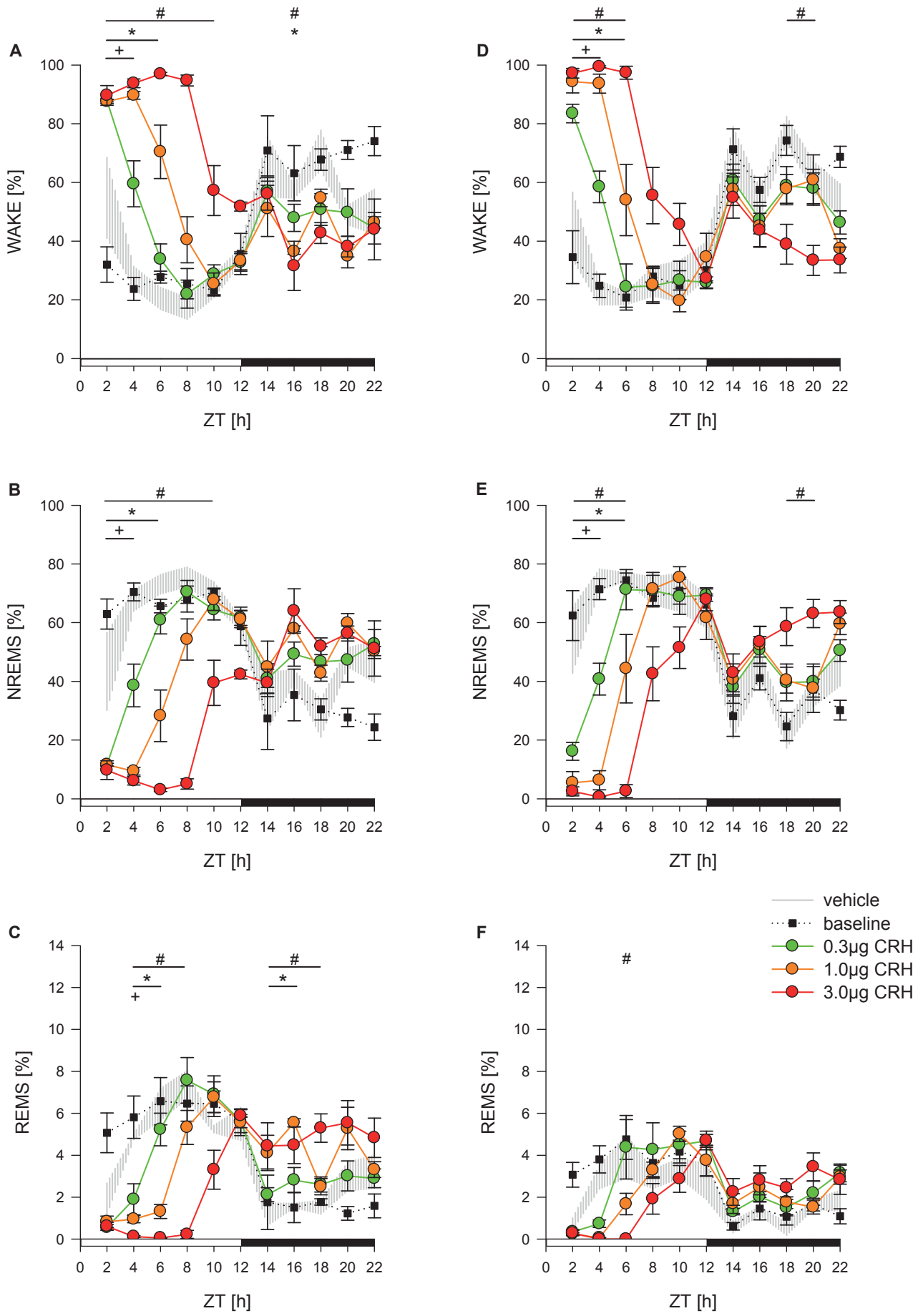

Figure 18 (CRH-R2 CL: A-C; CRH-R2 KO: D-F): For figure legend, please refer to next page.

Figure 18: Effects of i.c.v. CRH injections on vigilance states in CRH-R2 CL (n = 8; A-C) and CRH-R2 KO (n = 7; D-F) mice.

Corticotropin-releasing hormone (CRH) dose-dependently increased wake levels in CRH-R2 CL mice during the light period and contrariwise decreased NREMS amounts. REMS was dose-dependently decreased up to eight hours after injection of CRH. Although wake was significantly decreased after the two higher doses and in one time point only, a trend towards decreased levels of wake after CRH treatment was obvious during the dark period. Similarly NREMS showed a trend towards increased levels during the dark. REMS on the other hand significantly increased in the middle of the dark period after 1.0µg and 3.0µg of CRH. In CRH-R2 KO, wake and NREMS was significantly increased and decreased, respectively, for up to six hours after CRH injections. REMS showed a dose-dependent decrease in comparison to baseline, but only after 3.0µg of CRH a significant decrease after six hours could be detected compared to baseline and vehicle. Towards the end of the dark period, a significant decrease of wake and similarly an increase in NREMS emerged after 3.0µg of CRH. In contrast, despite slight increases, no effects on REMS could be detected in CRH-R2 KO during the dark period.
For graph denotations please refer to figure legend of figure 16, page 46.

4.2 Plasma CORT level changes after CRH injection in CRH-R1 CKO and CL

Under baseline conditions at ZT 2 (Figure 19, page 54) both genotypes showed similar levels of plasma CORT (CRH-R1 CKO: 16.2 ± 3.0ng/ml, n = 19; CRH-R1 CL: 22.7 ± 5.2ng/ml, n = 19). Two hours after vehicle treatment (ZT 2), an increase in plasma CORT levels was detected in both mouse lines (CRH-R1 CKO: 43.7 ± 11.8ng/ml, n = 8; CRH-R1 CL: 70.5 ± 11.8ng/ml, n = 9). However, differences in these levels were insignificant, neither within nor between the two genotypes. On the other hand, treatment with 1.0µg of CRH distinctly increased plasma CORT in CRH-R1 CL (423.2 ± 49.5ng/ml, n = 8) after two hours in comparison to their vehicle and baseline values ($F_{treatment}$ = 44.57, P < 0.001; F_{group} = 4.768, P = 0.034; contrasts tests, P < 0.001). A similar effect could be seen in CRH-R1 CKO mice, where CRH treatment significantly increased plasma CORT levels (323.2 ± 65.8ng/ml, n = 8) two hours after injection as well (contrasts tests, P < 0.001). Although increased levels of plasma CORT were lower in CRH-R1 CKO mice than in CL mice (Student-Neumann-Keuls test, P = 0.007), i.c.v. injected CRH was able to stimulate the HPA axis even with a deficiency of central CRH-R1.

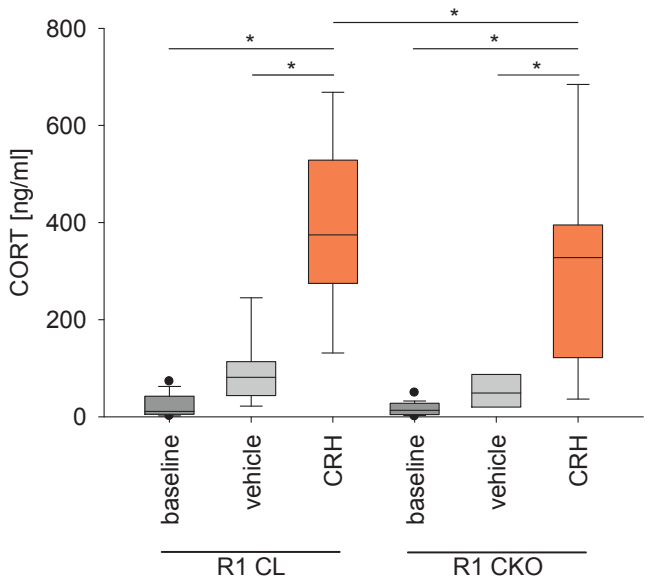

Figure 19: Effects of i.c.v. CRH injections on CORT levels in CL and CRH-R1 CKO mice.

Compared to baseline (CRH-R1 CKO: 16.2 ± 3.0ng/ml, n = 19; CRH-R1 CL: 22.7 ± 5.2ng/ml, n = 19) and vehicle (CRH-R1 CKO: 43.7 ± 11.8ng/ml, n = 8; CRH-R1 CL: 70.5 ± 11.8ng/ml, n = 9) conditions, a significant increase in plasma CORT was induced by 1.0µg of CRH by two hours after application in both CL (423.2 ± 49.5ng/ml, n = 8) and CRH-R1 CKO (323.2 ± 65.8ng/ml, n = 8) mice, although CL animals displayed significantly higher CORT levels after neuropeptide treatment compared to CKO animals. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10% and 90% percentiles (whiskers). Asterisks indicate significant differences in obtained CORT values in respect of different treatment conditions (contrasts tests in ANOVA, $P < 0.05$) and their according genotypes (Student-Neumann-Keuls tests, $P < 0.05$).

4.3 Sleep and wake responses in CRH-R1 CKO and CL to CRH injections with ASV-30 treatment

Statistical analysis revealed significant interaction effects of treatment and time on wake in CRH-R1 CKO and CL littermates during the light period (CRH-R1 CKO: $F_{treatment\ x\ time}$ (20/160) = 4.152, $P < 0.001$; CRH-R1 CL: $F_{treatment\ x\ time}$ (20/140) = 26.314, $P < 0.001$). In the dark period, CRH-R1 CKO mice expressed significant treatment effects (CRH-R1 CKO: $F_{treatment}$ (4/32) = 15.649, $P < 0.001$), whereas CRH-R1 CL displayed significant interaction effects of treatment and time (CRH-R1 CL: $F_{treatment\ x\ time}$ (16/112) = 1.867, $P < 0.031$). Injections of vehicle or ASV-30 alone significantly increased wake levels in both genotypes at ZT 2 (contrasts tests, $P < 0.001$; Figure 20 A and D, page 57). Afterwards wake returned to and remained at baseline levels with the exception of ZT 22 in CRH-R1 CKO where vehicle and ASV-30 treatment significantly decreased wake (contrasts tests, $P \leq 0.006$; Figure 20 D). No differences between vehicle and ASV-30 effects on wakefulness could be observed neither

in the two genotypes nor the light or dark period. After injecting 1.0µg of CRH alone or in combination with ASV-30, wakefulness in CRH-R1 CL significantly increased compared to vehicle and baseline levels for four (CRH; contrasts tests, $P < 0.001$) and six (ASV-30 + CRH; contrasts tests, $P < 0.001$) hours, respectively (Figure 20 A). In contrast to the single injection experiments, a significant reduction of wake levels could be observed during the dark period. Whereas wakefulness was diminished almost through the whole dark period in comparison to baseline levels, significant reductions compared to both vehicle and baseline levels were detectable only at ZT 16 and 22 (CRH; contrasts tests, $P \leq 0.019$) and ZT 16 and 20-22 (ASV-30 + CRH; contrasts tests, $P \leq 0.014$). As similarly seen in the earlier experiments, CRH effects on wakefulness in CRH-R1 CKO animals were blunted (Figure 20 D). As vehicle, both treatments (CRH and ASV-30 + CRH) slightly but significantly increased wake levels at ZT 2 (contrasts tests, $P < 0.001$) compared to baseline, however after ASV-30 + CRH the increases remained significant at ZT 4 (contrasts tests, $P \leq 0.002$) if compared to baseline and vehicle. Unlike the CRH effects of the earlier experiments in CRH-R1 CKO, both treatments (CRH and ASV-30 + CRH) induced significant decreases of wakefulness during the dark period at ZT 22 (contrasts tests, $P \leq 0.025$).

In both genotypes, significant interaction effects of treatment and time on NREMS were detected during the light period (CRH-R1 CKO: $F_{treatment \times time}$ (20/160) = 3.520, $P < 0.001$; CRH-R1 CL: $F_{treatment \times time}$ (20/140) = 25.279, $P < 0.001$). Similar to the dark period of wake, in CRH-R1 CKO mice only significant treatment effects (CRH-R1 CKO: $F_{treatment}$ (4/32) = 14.331, $P < 0.001$), but in CRH-R1 CL significant interaction effects of treatment and time were found (CRH-R1 CL: $F_{treatment \times time}$ (16/112) = 1.740, $P < 0.049$). As for wake, vehicle and ASV-30 treatment significantly decreased NREMS of both genotypes during the first two hours after injection (contrasts tests, $P \leq 0.003$; Figure 20 B and E, page 57), however only in CRH-R1 CKO vehicle and ASV-30 treatment additionally increased NREMS at ZT 22 (contrasts tests, $P = 0.011$). No differences between vehicle and ASV-30 effects on NREMS could be shown neither in the two genotypes nor the light or dark period. In CRH-R1 CL NREMS was significantly decreased for four or six hours after CRH or ASV-30 + CRH treatment (contrasts tests, $P < 0.001$), respectively, and afterwards returned to vehicle and baseline levels (Figure 20 B). During the whole dark period, NREMS was slightly elevated in CRH-R1 CL in comparison to baseline levels, however, significant increases compared to vehicle and baseline occurred after CRH treatment at ZT 16 and 22 (contrasts tests, $P \leq 0.021$) and after ASV-30 + CRH at ZT 16 and 20-22 (contrasts tests, $P \leq 0.03$). Following the initial significant decrease in NREMS of CRH-R1 CKO at ZT 2 after treatment with CRH or ASV-30 + CRH compared to baseline, only ASV-30 in combination with CRH led to persisting, significant decreases in NREMS for another two hours (ZT 4; contrasts tests, $P \leq 0.019$) compared to

baseline and vehicle (Figure 20 E). During the dark period, with the exception of the significant increases at ZT 22 in comparison to control conditions (contrasts tests, $P \leq 0.041$), NREMS only slightly increased after CRH and ASV-30 + CRH in CRH-R1 CKO in comparison to baseline levels.

Concerning REMS, significant interaction effects of treatment and time were detected during the light period in both genotypes (CRH-R1 CKO: $F_{treatment \times time}$ (20/160) = 4.088, $P < 0.001$; CRH-R1 CL: $F_{treatment \times time}$ (20/140) = 7.192, $P < 0.001$). During the dark period only in CRH-R1 CL significant interaction effects of treatment and time arose (CRH-R1 CL: $F_{treatment \times time}$ (16/112) = 2.998, $P < 0.001$). In both genotypes, REMS was significantly reduced after vehicle and ASV-30 treatment (ZT 2; contrasts tests, $P < 0.001$; Figure 20 C and F, page 57) but remained at comparable levels to baseline throughout the rest of the recording period. As for the other vigilance states, no differences in either genotype concerning effects of vehicle and ASV-30 treatment could be observed. In CRH-R1 CL treatment with CRH or ASV-30 + CRH significantly decreased REMS during the first four (contrasts tests, $P \leq 0.023$) or six (contrasts tests, $P \leq 0.013$) hours (Figure 20 C). During the dark period CRH treatment entailed significant increases of REMS in CRH-R1 CL if compared to vehicle and baseline at ZT 18 and 22 (contrasts tests, $P \leq 0.034$). After ASV-30 + CRH REMS was significantly increased at ZT 16 and 20-22 (contrasts tests, $P \leq 0.032$). In CRH-R1 CKO animals, CRH and ASV-30 + CRH induced significant reductions in REMS for six hours, only if compared to baseline (contrasts tests, $P \leq 0.03$; Figure 20 F). No significant difference in reduction levels compared to vehicle control could be detected. During the dark period REMS levels remained comparable to vehicle and baseline levels after any treatment.

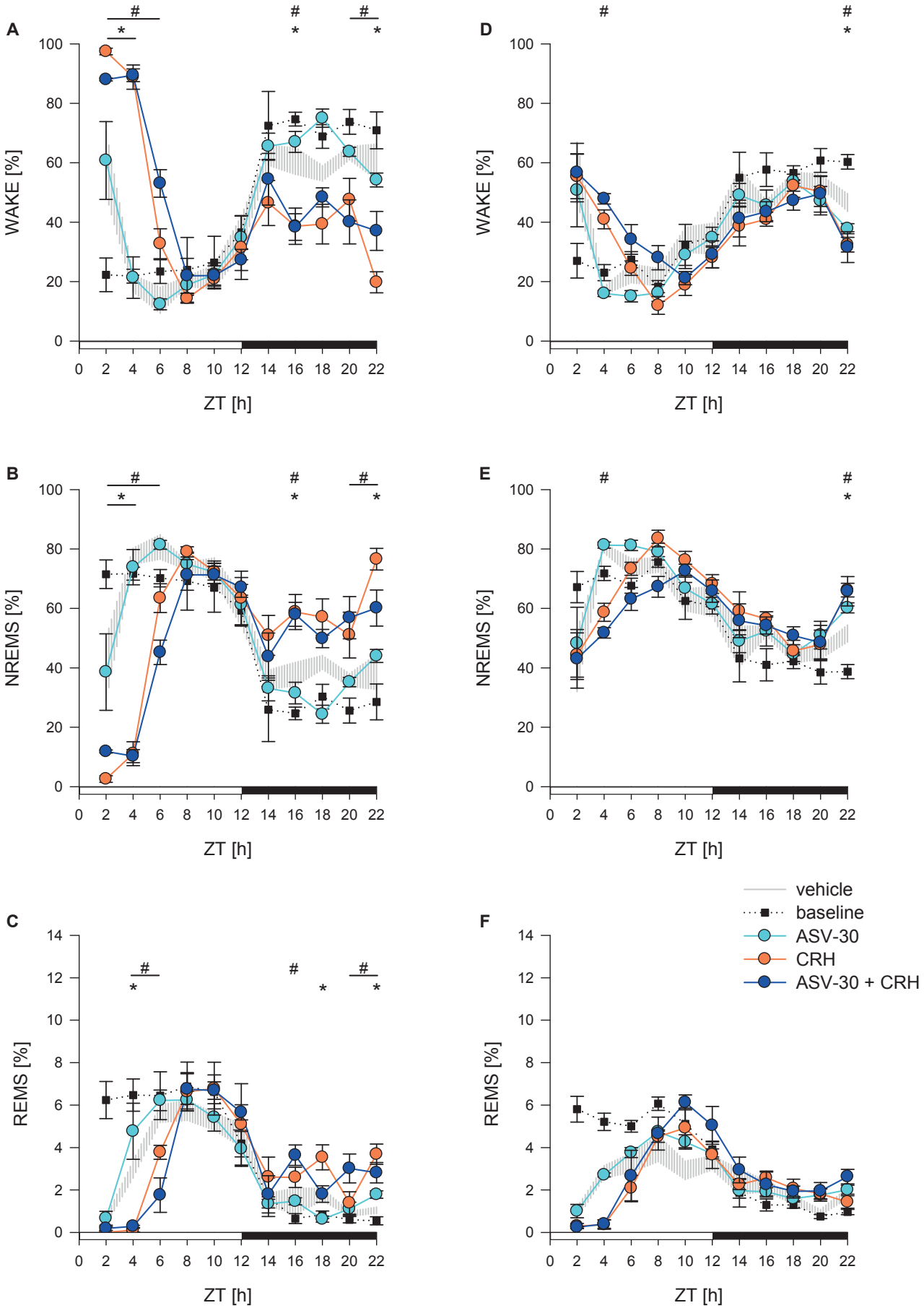

Figure 20 (CRH-R1 CL: A-C; CRH-R1 CKO: D-F): For figure legend, please refer to next page.

Figure 20: Effects of i.c.v. CRH and ASV-30 injections on vigilance states in CRH-R1 CL (n = 8; A-C) and CRH-R1 CKO (n = 13; D-F) mice.

No differences in effects of i.c.v. injected ASV-30 compared to vehicle injections could be determined in either genotype. In CRH-R1 CL CRH injections even after pretreatment with ASV-30, significantly increased wake and decreased NREMS levels for up to six hours during the light period. Similarly REMS was significantly suppressed for up to six hours. During the dark period, CRH treatment significantly diminished wake and contrariwise elevated NREMS and REMS. In contrast, CRH effects in CRH-R1 CKO on wake and NREMS were severely blunted. Although no significant differences compared to vehicle and ASV-30 could be detected, a decrease of REMS after CRH injections compared to baseline was obvious. Depicted are the percentage of the given vigilance states (indicated on the y-axis) of two hour means ±SEM for baseline (■) and each treatment (●: ASV-30, ●: CRH, ●: ASV-30 + CRH). The hatched area indicates mean values ±SEM after vehicle treatment. The symbols "#" and "*" denote statistically significant differences by comparison of vehicle and baseline versus treatment with ASV-30 + CRH and CRH, respectively (contrasts tests in ANOVA, $P < 0.05$). Solid lines under those symbols connect consecutive timepoints showing statistical differences. The open and filled bar on the x-axis indicates the light and dark period, respectively.

4.4 CRH-induced changes in sleep architecture

4.4.1 C57BL/6J

The effects of CRH on sleep were further analysed to investigate whether CRH affected more episode frequency or duration in sleep architecture. C57BL/6J mice displayed sleep-wake distributions typical of nocturnal animals. ANOVA with treatment, phase and group as influential factors showed that during the resting phase (light period) the mean duration of wake episodes was significantly shorter than during the active phase (contrasts tests, $P < 0.001$; Table 1, page 59). However, the higher the dose of CRH injected, the longer a wake bout became during the inactive phase. After the lowest dose of CRH (0.3µg), wake bouts during the light and dark period were equal in length. With 1.0µg of CRH, mean wake episode length during the light period already significantly exceeded bout lengths during the dark period (contrasts tests, $P < 0.001$). At the highest dose of CRH (3.0µg), the difference between mean wake episode length during the light versus the dark period was even more distinct (contrasts tests, $P < 0.001$). After this dosage, the diurnal rhythmicity appeared to be inverted, opposing total wake levels seen after vehicle treatment, and the mean wake episode length during the light period was greater than that after the treatment of either vehicle or lower doses of CRH (contrasts tests: 0.3µg, $P \leq 0.001$; 1.0µg, $P = 0.052$). Within the dark period, the magnitude of decrease in wake bout length depended on the applied dose of CRH and developed into a trend.

Similarly seen on wake, vehicle treatment had no effect on the length of NREMS or REMS episodes if compared to baseline. For both NREMS (Table 1) and REMS (Table 1), episode length was significantly greater in the resting than in the active period of the animals (contrasts tests, $P < 0.001$). However, application of CRH caused a dose-dependent decrease in NREMS and REMS episodes during the light period. At the lowest dose of CRH the mean NREMS or REMS bout during the resting period remained significantly longer than that in the active period of the animals (contrasts tests, $P \leq 0.001$). However, this difference disappeared after the two higher doses of CRH. Within the light period the decrease in length of NREMS and REMS bouts was dependent on the applied dose of CRH (contrasts tests, $P \leq 0.025$). During the course of the dark period, CRH seemed to affect neither NREMS nor REMS bout duration.

	C57BL/6J		
	WAKE	**NREMS**	**REMS**
vehicle	1.52 ±0.13 ‡	2.53 ±0.09 ‡	0.61 ±0.06 ‡
	12.53 ±3.93 ‡	1.57 ±0.10 ‡	0.28 ±0.02 ‡
0.3µg CRH	3.39 ±1.21	2.04 ±0.10 ‡	0.52 ±0.05 ‡
	4.58 ±0.79	1.54 ±0.06 ‡	0.28 ±0.02 ‡
1.0µg CRH	9.87 ±2.49 ‡	1.82 ±0.09	0.38 ±0.03
	4.15 ±0.51 ‡	1.61 ±0.08	0.35 ±0.03
3.0µg CRH	17.42 ±4.90 ‡	1.46 ±0.20	0.26 ±0.04
	3.19 ±0.54 ‡	1.49 ±0.09	0.31 ±0.02
P value	< 0.001	≤ 0.025	≤ 0.006

Table 1: Effects of i.c.v. CRH injections on mean durations of all vigilance episodes in C57BL/6J (n = 14).

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of all vigilance states and for all treatments in C57BL/6J.
During the light period, a dose-dependent increase in mean wake bout length and a decrease in NREMS and REMS were evident. Mean wake episode length decreased during the dark period, whereas NREMS and REMS episode length was unaffected by CRH treatments. Significant effects of treatment are denoted by P-values (least significance level given) in the bottom line of the table. The symbol "‡" indicates significant differences between the light and dark period (contrasts tests in ANOVA, $P < 0.05$).

4.4.2 CRH-R1 CKO and CL

Under baseline conditions episode length of all vigilance states was comparable between CRH-R1 CL and CRH-R1 CKO. Mice of both genotypes consistently displayed longer wake bouts and shorter NREMS and REMS bouts during their active phase than during their resting phase under baseline and vehicle conditions, which reached significant levels except for wake in both animal lines (contrasts tests, $P \leq 0.016$; Table 2, page 61). In CRH-R1 CL animals, vehicle treatment did not affect wake and NREMS bout length, but slightly reduced REMS bout length during the inactive phase of the animals (contrasts tests, $P < 0.001$; data not shown) if compared to baseline levels, whereas no differences in each bout length between baseline and vehicle treatment were detected in CRH-R1 CKO animals. Following CRH treatment, wake bout length in CRH-R1 CL mice was increased dose-dependently during the light period, albeit only the highest dose entailed significant changes compared to all other treatments (contrasts tests, $P \leq 0.006$; Table 2, page 61). After 3.0µg of CRH wake episodes were also significantly longer in CRH-R1 CL as compared to CRH-R1 CKO (Student-Neumann-Keuls test, $P < 0.001$). Already at the lowest dose of CRH, wake bout lengths were equalised over the day. Towards the dark period, CRH treatment did not show any significant impact on wake episode lengths any longer. However, in CRH-R1 CKO animals no differences in wake episode length were to be found during the light period after any dose of CRH, although mean wake episode length during the light and dark period converged (Table 2, page 61). Regarding NREMS (Table 2) and REMS (Table 2), CRH injections also influenced the episode duration in CRH-R1 CL animals. With increasing amounts of injected CRH, NREMS and REMS episodes became shorter during the light period. After 1.0µg and 3.0µg of CRH, NREMS and REMS durations were significantly decreased (contrasts tests, $P \leq 0.004$), resulting in disappearance of circadian differences in NREMS and REMS episode length between the light and dark period. During the dark period CRH treatments did not induce significant changes in episode length in CRH-R1 CL. In CRH-R1 CKO animals, CRH treatment had no influence on NREMS episode length at all (Table 2). Meanwhile, REMS episodes were exiguously shortened during the light period with increasing doses of CRH, but only after the highest dose this reduction reached a significant level (contrasts tests, $P = 0.035$; Table 2). Effects of i.c.v. injected CRH on sleep architecture appeared to a lesser extent in CRH-R1 CKO than in CL mice.

In the antagonist study, application of ASV-30 had no influence on wake, NREMS and REMS episode length, neither in CRH-R1 CL nor in CRH-R1 CKO animals. Injection of 1.0µg of CRH alone or together with ASV-30 elicited the same responses regarding episode lengths in CRH-R1 CL and CRH-R1 CKO as described above (data not shown).

	WAKE		NREMS		REMS	
	R1 CL	**R1 CKO**	**R1 CL**	**R1 CKO**	**R1 CL**	**R1 CKO**
vehicle	1.20 ±0.09	1.18 ±0.09	1.89 ±0.09 ‡	1.57 ±0.10 ‡	0.31 ±0.03 ‡	0.31 ±0.02 ‡
	2.08 ±0.20	2.12 ±0.33	1.18 ±0.20 ‡	0.93 ±0.08 ‡	0.20 ±0.02 ‡	0.21 ±0.08 ‡
0.3µg CRH	2.34 ±0.66	1.58 ±0.16	1.79 ±0.18 ‡	1.40 ±0.12 ‡	0.29 ±0.04 ‡	0.31 ±0.03 ‡
	2.29 ±0.43	1.76 ±0.19	1.24 ±0.11 ‡	0.93 ±0.07 ‡	0.20 ±0.02 ‡	0.24 ±0.02 ‡
1.0µg CRH	3.92 ±0.98	1.48 ±0.17	1.47 ±0.17	1.41 ±0.16 ‡	0.25 ±0.02	0.30 ±0.04
	1.99 ±0.23	1.58 ±0.11	1.22 ±0.10	0.88 ±0.08 ‡	0.22 ±0.02	0.24 ±0.02
3.0µg CRH	7.44 + ±2.66 ‡	1.72 # ±0.34	1.13 ±0.13	1.55 ±0.19 ‡	0.22 ±0.03	0.26 ±0.03
	1.95 ±0.23 ‡	1.78 ±0.28	1.20 ±0.09	0.95 ±0.09 ‡	0.25 ±0.02	0.24 ±0.02
P value	≤ 0.006		< 0.001		< 0.004	≤ 0.035

Table 2: Effects of i.c.v. CRH injections on mean durations of all vigilance episodes in CRH-R1 CL (n = 9) and CRH-R1 CKO (n = 12).

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of all vigilance states and for all treatments in CRH-R1 CL and CRH-R1 CKO. Whereas CRH treatments induced dose-dependent increases of mean wake bout length and decreases in NREMS and REMS during the light period in CRH-R1 CL, only REMS bout length was affected in CRH-R1 CKO. During the dark period in both genotypes no CRH effects could be determined. Significant effects of treatment are denoted by P-values (least significance level given) in the bottom line of the table. The symbol "‡" indicates significant differences between the light and dark period (contrasts tests in ANOVA, $P < 0.05$). The symbols "#" and "+" denominate significant differences between CRH-R1 CL and CRH-R1 CKO (Student-Neumann-Keuls tests, $P < 0.05$).

4.4.3 CRH-R2 KO and CL

During the light period CRH-R2 CL displayed slightly shorter NREMS episode length if compared to CRH-R2 KO (Student-Neumann-Keuls test, $P = 0.032$) under baseline conditions. No further differences in mean vigilance episode length could be detected between CRH-R2 CL and CRH-R2 KO. Animals of both genotypes consistently displayed longer wake (Table 3, page 63) and shorter NREMS (Table 3) bouts during their active phase than during their resting phase under baseline conditions, though only the difference in NREMS was significant (contrasts tests, $P \leq 0.03$). In contrast, REMS (Table 3) bouts developed an equal mean length between the light and dark period. In both lines, CRH-R2 KO and CL, no effects of vehicle treatment on sleep architecture in comparison to baseline levels could be detected. After treatment with CRH, both genotypes responded with an increase in mean wake bout length during the light period (Table 3). CRH-R2 CL showed slightly higher increases if compared to CRH-R2 KO, and after 0.3µg of CRH mean wake bout length already exceeded that of the dark period. Injection of 1.0µg or 3.0µg of CRH significantly increased wake bout lengths in comparison to the dark period (contrasts tests, $P \leq 0.009$). Although wake bouts during the light period exceeded those of the dark period after 1.0µg of CRH, significant differences could be detected only after injection of 3.0µg CRH (contrasts tests, $P < 0.001$). In the light period a dose-dependent decrease of mean NREMS bout length could be observed in CRH-R2 CL (Table 3, page 63). After 1.0µg and more distinctly after 3.0µg of CRH, mean NREMS bout length was not only significantly shorter compared to baseline and vehicle conditions during the light (contrasts tests, $P < 0.002$), but also significantly shorter than during the dark period (contrasts tests, $P < 0.011$). Similarly CRH-R2 KO showed a trend towards decreased mean NREMS bout lengths, however only after 3.0µg of CRH bout means significantly decreased in comparison to baseline and vehicle (contrasts tests, $P \leq 0.003$; Table 3, page 63). No differences in the length between the light and the dark period could be detected in CRH-R2 KO after CRH treatment. Mean REMS bout duration was unaffected by CRH treatments both during the light period and across genotypes (Table 3).

Although mean wake bout length was dose-dependently decreasing during the dark period in both genotypes, except for the increase after 3.0µg of CRH in CRH-R2 CL, no significant treatment effects could be identified. Similarly NREMS and REMS bout lengths remained at comparable levels during the dark period in both genotypes, and no significant effects of CRH could be determined.

	WAKE		NREMS		REMS	
	R2 CL	**R2 KO**	**R2 CL**	**R2 KO**	**R2 CL**	**R2 KO**
vehicle	1.54 ±0.15	1.55 ±0.13	1.88 ◊ ±0.02 ‡	2.49 ■ ±0.03 ‡	0.33 ±0.01	0.26 ±0.02
	3.39 ±0.49	5.17 ±1.27	1.34 ±0.02 ‡	1.72 ±0.03 ‡	0.31 ±0.01	0.31 ±0.01
0.3µg CRH	5.24 ±2.49	2.67 ±0.46	1.58 ±0.02	1.56 ±0.04	0.34 ±0.01	0.31 ±0.01
	2.67 ±0.40	3.02 ±0.57	1.56 ±0.03	1.67 ±0.06	0.31 ±0.01	0.29 ±0.01
1.0µg CRH	9.16 ±1.95 ‡	7.41 ±2.14	1.11 ◊ ±0.05 ‡	1.87 ■ ±0.04	0.33 ±0.01	0.24 ±0.01
	2.13 ±0.37 ‡	3.56 ±0.80	1.63 ±0.03 ‡	1.65 ±0.06	0.37 ±0.01	0.23 ±0.01
3.0µg CRH	28.52 ±3.21 ‡	24.51 ±4.07 ‡	0.92 ±0.09 ‡	1.36 ±0.04	0.28 ±0.01	0.30 ±0.01
	3.77 ±1.21 ‡	2.56 ±0.63 ‡	1.63 ±0.03 ‡	1.95 ±0.03	0.35 ±0.01	0.27 ±0.01
P value	≤ 0.009	< 0.001	≤ 0.002	≤ 0.003		

Table 3: Effects of i.c.v. CRH injections on mean durations of all vigilance episodes in CRH-R2 CL (n = 8), and CRH-R2 KO (n = 7).

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of all vigilance states and for all treatments in CRH-R2 CL and CRH-R2 KO. Treatment with CRH induced dose-dependent increases of mean wake bout length and decreases in NREMS in both genotypes during the light period, whereas mean REMS episode length was unaffected. During the dark period in both genotypes no effects of CRH could be determined. Significant effects of treatment are denoted by P-values (least significance level given) in the bottom line of the table. The symbol "‡" indicates significant differences between the light and dark period (contrasts tests in ANOVA, $P < 0.05$). The symbols "■" and "◊" denominate significant differences between CRH-R2 CL and CRH-R2 KO (Student-Neumann-Keuls tests, $P < 0.05$).

4.4.4 Comparison of sleep architecture between the mouse lines

In CRH-R1 CKO and CL, some differences in sleep architecture were observed in comparison with C57BL/6J mice under baseline and vehicle conditions. During the light period, NREMS (Table 5, page 66) and REMS (Table 6, page 67) bouts were significantly longer in C57BL/6J mice than in CRH-R1 CKO or CL animals (Student-Neumann-Keuls test, $P < 0.001$). During the dark period, however, wake (Table 4, page 65) and NREMS episodes displayed significantly greater durations in C57BL/6J as compared to those of CRH-R1 CKO or CL animals (Student-Neumann-Keuls test, $P \leq 0.005$).
Likewise CRH-R2 KO and CL demonstrated differences in sleep architecture under baseline and vehicle control conditions as compared to C57BL/6J, CRH-R1 CKO, and CRH-R1 CL. Mean wake bout length of CRH-R2 KO and CL mice was in a similar range with all other mouse lines during the light period (Table 4, page 65). On the other hand mean NREMS bouts of CRH-R2 KO were comparable to C57BL/6J and thus significantly longer in contrast to the similar bout means of CRH-R1 CKO, CRH-R1 CL, and CRH-R2 CL (Student-Neumann-Keuls test, $P < 0.001$; Table 5, page 66). Mean REMS bout lengths during the light period in both genotypes were indistinguishable from those of CRH-R1 CKO and CL and thus significantly shorter than those of C57BL/6J animals (Student-Neumann-Keuls test, $P < 0.001$; Table 6, page 67). Although the mean wake bout length of CRH-R2 KO and CL during their activity phase (dark period) was significantly shorter than that of C57BL/6J (Student-Neumann-Keuls test, $P \leq 0.012$), both were slightly but insignificantly longer in comparison to those of CRH-R1 CKO and CRH-R1 CL. Whereas CRH-R2 KO displayed the longest mean NREMS bout length during the dark period, that of CRH-R2 CL ranged between those of C57BL/6J and CRH-R1 CKO/CL. Similarly to C57BL/6J, in CRH-R2 KO NREMS episode length was significantly longer as compared to CRH-R1 CKO and CRH-R1 CL mice. Mean REMS bout length was comparable between all mouse lines during the dark period.
All animal lines, except for CRH-R1 CKO, responded to CRH treatments with a dose-dependent increase in mean wake episode length during the light period (Table 4, page 65). After 3.0µg of CRH mean wake bout length in CRH-R1 CL was significantly longer than in CRH-R1 CKO, however increases in C57BL/6J, CRH-R2 KO, and especially in CRH-R2 CL were even larger (Student-Neumann-Keuls test, $P \leq 0.031$). Differences in NREMS (Table 5, page 66) and REMS (Table 6, page 67) observed during the light period under baseline and vehicle conditions across all animal lines disappeared with an increasing dose of CRH. During the dark period, CRH treatment entailed decreases in mean wake bout length in C57BL/6J and to a lesser extend in CRH-R2 KO and CL, and after 1.0µg of CRH no differences in mean wake episode length could be detected between any mouse lines (Table 4, page 65). Whereas CRH-R1 CKO displayed the shortest mean NREMS episodes during the dark period after all treatments, differences between the other mouse lines vanished

(Table 5, page 66). No differences between mouse lines in mean REMS bout length could be detected during the dark period (Table 6, page 67).

	WAKE				
	C57BL/6J	**CRH-R1 CL**	**CRH-R1 CKO**	**CRH-R2 CL**	**CRH-R2 KO**
vehicle	1.52 ±0.13 ‡	1.20 ±0.09	1.18 ±0.09	1.54 ±0.15	1.55 ±0.13
	12.53 #+■◊ ±3.93 ‡	2.08 * ±0.20	2.12 * ±0.33	3.39 * ±0.49	5.17 * ±1.27
0.3µg CRH	3.39 ±1.21	2.34 ±0.66	1.58 ±0.16	5.24 ±2.49	2.67 ±0.46
	4.58 #+ ±0.79	2.29 * ±0.43	1.76 * ±0.19	2.67 ±0.40	3.02 ±0.57
1.0µg CRH	9.87 #+ ±2.49 ‡	3.92 * ±0.98	1.48 *■◊ ±0.17	9.16 + ±1.95 ‡	7.41 + ±2.14
	4.15 ±0.51 ‡	1.99 ±0.23	1.58 ±0.11	2.13 ±0.37 ‡	3.56 ±0.80
3.0µg CRH	17.42 #+■ ±4.90 ‡	7.44 *+■◊ ±2.66 ‡	1.72 *#■◊ ±0.34	28.52 #+* ±3.21 ‡	24.51 #+ ±4.07 ‡
	3.19 ±0.54 ‡	1.95 ±0.23 ‡	1.78 ±0.28	3.77 ±1.21 ‡	2.56 ±0.63 ‡
P value	< 0.001	≤ 0.006		≤ 0.009	< 0.001

Table 4: Comparison of effects of i.c.v. CRH injections on mean duration of wake episodes between C57BL/6J (n = 14), CRH-R1 CL (n = 9), CRH-R1 CKO (n = 12), CRH-R2 CL (n = 8), and CRH-R2 KO (n = 7) mice.

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of wake for all treatments and all mouse lines.
During the light period, a dose-dependent increase in mean wake bout length was evident in all mice treated with CRH, except for CRH-R1 CKO. Significant effects of treatment are denoted by P-values (least significance level given) in the bottom line of the table. The symbol "‡" as appendix to the mean episode duration of a group points to significant differences between the light and dark period for that group (contrasts tests in ANOVA, $P < 0.05$), whereas symbols "∗", "#", "+", "■", and "◊" denominate significant differences between the actual group and C57BL/6J (∗), or CRH-R1 CL (#), or CRH-R1 CKO (+), or CRH-R2 CL (■), or CRH-R2 KO (◊), respectively (Student-Neumann-Keuls tests, $P < 0.05$).

	NREMS				
	C57BL/6J	**CRH-R1 CL**	**CRH-R1 CKO**	**CRH-R2 CL**	**CRH-R2 KO**
vehicle	2.53 $^{\#+\blacksquare}$ ±0.09 ‡	1.89 $^{*\lozenge}$ ±0.09 ‡	1.57 $^{*\lozenge}$ ±0.10 ‡	1.88 $^{*\lozenge}$ ±0.02 ‡	2.49 $^{\#+\blacksquare}$ ±0.03 ‡
	1.57 $^{\#+}$ ±0.10 ‡	1.18 $^{*\lozenge}$ ±0.20 ‡	0.93 $^{*\lozenge}$ ±0.08 ‡	1.34 ±0.02 ‡	1.72 $^{\#+}$ ±0.03 ‡
0.3µg CRH	2.04 $^{+}$ ±0.10 ‡	1.79 ±0.18 ‡	1.40 * ±0.12 ‡	1.58 ±0.02	1.56 ±0.04
	1.54 $^{+}$ ±0.06 ‡	1.24 ±0.11 ‡	0.93 $^{*\blacksquare\lozenge}$ ±0.07 ‡	1.56 $^{+}$ ±0.03	1.67 $^{+}$ ±0.06
1.0µg CRH	1.82 $^{\blacksquare}$ ±0.09	1.47 ±0.17	1.41 ±0.16 ‡	1.11 $^{*\lozenge}$ ±0.05 ‡	1.87 $^{\blacksquare}$ ±0.04
	1.61 $^{+}$ ±0.08	1.22 ±0.10	0.88 $^{*\blacksquare\lozenge}$ ±0.08 ‡	1.63 $^{+}$ ±0.03 ‡	1.65 $^{+}$ ±0.06
3.0µg CRH	1.46 ±0.20	1.13 ±0.13	1.55 ±0.19 ‡	0.92 ±0.09 ‡	1.36 ±0.04
	1.49 $^{+}$ ±0.09	1.20 $^{\lozenge}$ ±0.09	0.95 $^{*\blacksquare\lozenge}$ ±0.09 ‡	1.63 $^{+}$ ±0.03 ‡	1.95 $^{\#+}$ ±0.03
P value	≤ 0.025	< 0.001		≤ 0.002	≤ 0.003

Table 5: Comparison of effects of i.c.v. CRH injections on mean duration of NREMS episodes between C57BL/6J (n = 14), CRH-R1 CL (n = 9), CRH-R1 CKO (n = 12), CRH-R2 CL (n = 8), and CRH-R2 KO (n = 7) mice.

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of NREMS for all treatments and all mouse lines. Significant and dose-dependent reductions in mean NREMS episode length during the light period were detected in all mice treated with CRH, except for CRH-R1 CKO.

For table denotations please refer to table 4, page 65.

	REMS				
	C57BL/6J	**CRH-R1 CL**	**CRH-R1 CKO**	**CRH-R2 CL**	**CRH-R2 KO**
vehicle	0.61 # + ■ ◊ ±0.06 ‡	0.31 * ±0.03 ‡	0.31 * ±0.02 ‡	0.33 * ±0.01	0.26 * ±0.02
	0.28 ±0.02 ‡	0.20 ±0.02 ‡	0.21 ±0.08 ‡	0.31 ±0.01	0.31 ±0.01
0.3µg CRH	0.52 # + ■ ◊ ±0.05 ‡	0.29 * ±0.04 ‡	0.31 * ±0.03 ‡	0.34 * ±0.01	0.31 * ±0.01
	0.28 ±0.02 ‡	0.20 ±0.02 ‡	0.24 ±0.02 ‡	0.31 ±0.01	0.29 ±0.01
1.0µg CRH	0.38 # ◊ ±0.03	0.25 * ±0.02	0.30 ±0.04	0.33 ±0.01	0.24 * ±0.01
	0.35 ±0.03	0.22 ±0.02	0.24 ±0.02	0.37 ±0.01	0.23 ±0.01
3.0µg CRH	0.26 ±0.04	0.22 ±0.03	0.26 ±0.03	0.28 ±0.01	0.30 ±0.01
	0.31 ±0.02	0.25 ±0.02	0.24 ±0.02	0.35 ±0.01	0.27 ±0.01
P value	≤ 0.006	< 0.004	≤ 0.035		

Table 6: Comparison of effects of i.c.v. CRH injections on mean duration of REMS episodes between C57BL/6J (n = 14), CRH-R1 CL (n = 9), CRH-R1 CKO (n = 12), CRH-R2 CL (n = 8), and CRH-R2 KO (n = 7) mice.

The table shows 12h/10h means (light or dark period, indicated by light and dark boxes, respectively) of average bout lengths in minutes (±SEM) of REMS for all treatments and all mouse lines.

Treatment with CRH dose-dependently and significantly decreased mean REMS episode length in C57BL/6J, CRH-R1 CL, and CRH-R1 CKO mice but produced no effects regarding mean REMS duration in CRH-R2 CL and CRH-R2 KO.

For table denotations please refer to table 4, page 65.

4.5 Automated sleep deprivation-induced changes in sleep-wake profiles

4.5.1 C57BL/6J

In the ASD experiment with C57BL/6J, significant interaction effects of treatment and time on wake were detected during the light period of ($F_{treatment\ x\ time}$ (10/70) = 29.3, P < 0.001). However, during the dark period only significant treatment effects were found ($F_{treatment}$ (2/14) = 14.118, P < 0.001). As expected during ASD animals showed significant increases in wakefulness (ZT 2-6; contrasts tests, P < 0.001; Figure 21 A, page 70), which returned to baseline levels after eight hours. Within the subsequent dark period, wake was significantly reduced at several timepoints (ZT 14-16 and 22) as compared to baseline (contrasts tests, P ≤ 0.014). During the light period of the following recovery day, wake levels were still significantly decreased compared to baseline levels for the first four hours (ZT 2-4; contrasts tests, P ≤ 0.013), however returned to and remained at baseline levels for the rest of the recording day.

Regarding NREMS of C57BL/6J, significant interaction effects of treatment and time were detected during the light period ($F_{treatment\ x\ time}$ (10/70) = 29.544, P < 0.001), whereas only significant treatment effects were observed during the dark period ($F_{treatment}$ (2/14) = 12.762, P < 0.001). Levels of NREMS were significantly suppressed during the ASD procedure (ZT 2-6; contrasts tests, P < 0.001; Figure 21 B, page 70) and returned to baseline after termination of sleep deprivation. At the beginning and the end of the dark period, NREMS significantly increased compared to baseline (ZT 14-16 and 22; contrasts tests, P ≤ 0.023). The next day (recovery day) NREMS continued to be significantly elevated compared to baseline during the first four hours of the light period (ZT 2-4; contrasts tests, P ≤ 0.015), but subsequently reverted to and stayed at baseline levels.

As for the other vigilance states of C57BL/6J, statistical analysis on REMS revealed significant interaction effects of treatment and time during the light period ($F_{treatment\ x\ time}$ (10/70) = 13.434, P < 0.001) and significant treatment effects during the dark period ($F_{treatment}$ (2/14) = 17.291, P < 0.001). In the light period REMS was totally suppressed for the duration of the ASD (contrasts tests, P < 0.001; Figure 21 C, page 70) and afterwards returned to levels indistinguishable from baseline. In the dark period REMS was constantly elevated, however only at ZT 16-18 and 22 significant increases could be remarked (contrasts tests, P ≤ 0.039). On the following recovery day, the usual basal distribution pattern of REMS within the light period was flattened. In the first four hours REMS was slightly, but at ZT 4 significantly (contrasts tests, P = 0.033) elevated compared to baseline levels. However, at ZT 6-8 significant reduced amounts of REMS were observed (contrasts tests, P ≤ 0.02).

From ZT 10 on to the end of the dark period, REMS levels were again similar to baseline levels.

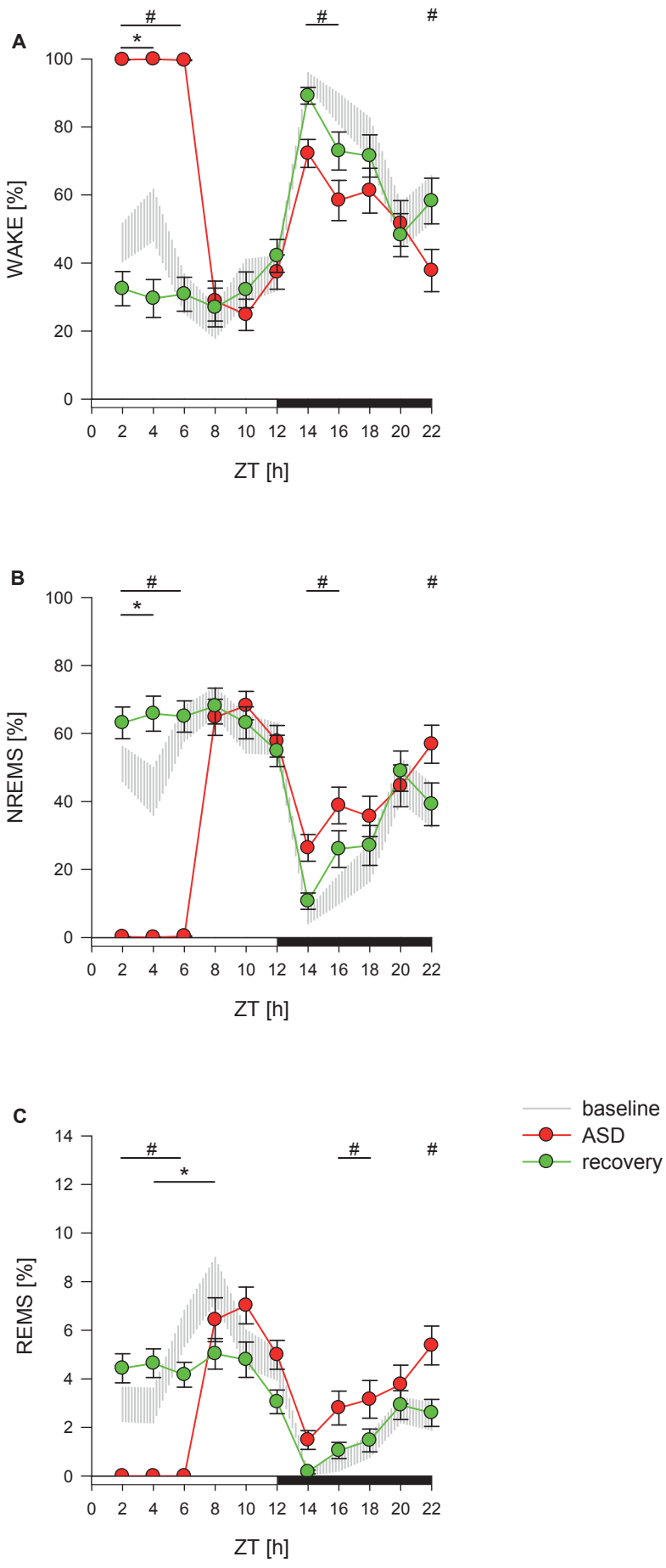

Figure 21 (C57BL/6J): For figure legend, please refer to next page.

Figure 21: Effects of ASD on vigilance states in C57BL/6J (n = 8) mice.

During ASD wake was significantly increased while an almost total or a complete blockade of NREMS and REMS was achieved, respectively. During the subsequent dark period following sleep deprivation, wakefulness was significantly decreased and meanwhile NREMS and REMS increased. On the recovery day wake levels were significantly reduced below while NREMS levels were increased above baseline at the beginning of the light period. In the middle of the light period recovery REMS displayed a significantly different pattern in comparison to baseline as its course was flattened. Depicted are the percentage of the given vigilance states (indicated on the y-axis) of two hour means ±SEM for each treatment (●: ASD, ●: recovery). The hatched area indicates mean values ±SEM during baseline. The symbols "#" and "*" denote statistically significant differences by comparison of baseline versus ASD or recovery, respectively (contrasts tests in ANOVA, $P < 0.05$). Solid lines under those symbols connect consecutive timepoints showing statistical differences. The open and filled bar on the x-axis indicates the light and dark period, respectively.

4.5.2 CRH-R1 CKO and CL

In CRH-R1 CKO and CL animals, significant interaction effects of treatment and time on wake could be obtained both during the light (CRH-R1 CKO: $F_{treatment\ x\ time}$ (10/60) = 79.409, $P < 0.001$; CRH-R1 CL: $F_{treatment\ x\ time}$ (10/60) = 61.014, $P < 0.001$) and the dark period (CRH-R1 CKO: $F_{treatment\ x\ time}$ (8/48) = 3.111, $P < 0.007$; CRH-R1 CL: $F_{treatment\ x\ time}$ (8/48) = 3.212, $P < 0.005$). ASD was effective in CRH-R1 CL as wakefulness was significantly increased during the first six hours of sleep deprivation compared to baseline (contrasts tests, $P < 0.001$; Figure 22 A, page 74). Over large parts of the ensuing dark period, wake was diminished in CRH-R1 CL reaching significance levels at ZT 14 and 20-22 (contrasts tests, $P \leq 0.009$) if compared to baseline. During recovery day CRH-R1 CL animals displayed an unsteady wake pattern as wake levels first rose significantly (ZT 6; contrasts tests, $P < 0.001$) above, but subsequently significantly dropped below (ZT 8; contrasts tests, $P = 0.002$) baseline levels. Similarly towards the end of the dark period, wake levels were first significantly elevated (ZT 18; contrasts tests, $P < 0.019$) but then significantly diminished (ZT 20; contrasts tests, $P < 0.004$) in CRH-R1 CL. As seen in CRH-R1 CL, sleep deprivation efficiently prevented CRH-R1 CKO animals from falling asleep, as wake levels were significantly increased during the first six hours of the light period (contrasts tests, $P < 0.001$; Figure 22 D, page 74). At the transition to and over large parts of the dark period, wake levels were significantly reduced (contrasts tests, $P \leq 0.024$). In the first six hours of the recovery day, the amount of wake fluctuated around baseline levels, as wake was significantly decreased at ZT 2 and 6 (contrasts tests, $P < 0.001$), but significantly increased at ZT 4 (contrasts tests, $P = 0.002$). No further differences could be detected for the rest of the recovery day in CRH-R1 CKO.

In both genotypes significant interaction effects of treatment and time on NREMS were detected during the light (CRH-R1 CKO: $F_{treatment\ x\ time}$ (10/60) = 80.874, P < 0.001; CRH-R1 CL: $F_{treatment\ x\ time}$ (10/60) = 61.639, P < 0.001) as well as the dark period (CRH-R1 CKO: $F_{treatment\ x\ time}$ (8/48) = 3.122, P < 0.006; CRH-R1 CL: $F_{treatment\ x\ time}$ (8/48) = 3.155, P < 0.006). Contrary to wake, NREMS was suppressed during ASD in CRH-R1 CL (ZT 2-6; contrasts tests, P < 0.001; Figure 22 B, page 74). After being kept awake, animals showed a rebound of NREMS during the dark period with significant increases at ZT 14 and 20-22 (contrasts tests, P ≤ 0.007). As for wake, CRH-R1 CL animals developed a disturbed NREMS pattern during the recovery day. Compared to baseline, in the light period at ZT 6 NREMS was significantly decreased and at ZT 8 significantly increased. Similarly NREMS was significantly increased at ZT 12 (transition to dark) and ZT 20 (contrasts tests, P ≤ 0.05), whereas significant decreases occurred at ZT 18 (contrasts tests, P = 0.01). Sleep deprivation in CRH-R1 CKO was likewise efficient, as NREMS was significantly decreased (ZT 2-6; contrasts tests, P < 0.001; Figure 22 E, page 74). Additionally NREMS rebound appeared during the dark period with significant increased NREMS levels (ZT 12-16 and 22; contrasts tests, P ≤ 0.026). On the recovery day CRH-R1 CKO showed similar fluctuations of NREMS as seen in CRH-R1 CL. Whereas NREMS was significantly increased at ZT 2 and 6 (contrasts tests, P ≤ 0.005), a significant decrease was found at ZT 4 (contrasts tests, P < 0.001). However, unlike in CRH-R1 CL mice no further differences could be observed by comparison of recovery and baseline day.

Concerning REMS, significant interaction effects of treatment and time were detected during the light period in both genotypes (CRH-R1 CKO: $F_{treatment\ x\ time}$ (10/60) = 6.49, P < 0.001; CRH-R1 CL: $F_{treatment\ x\ time}$ (10/60) = 10.192, P < 0.001), however only in CRH-R1 CL also during the dark period (CRH-R1 CL: $F_{treatment\ x\ time}$ (8/48) = 2.997, P < 0.008). In CRH-R1 CL REMS was almost totally blocked in the first six hours of the light period by ASD (contrasts tests, P ≤ 0.005; Figure 22 C, page 74) as compared to baseline. After returning to baseline levels for the rest of the light period, REMS rebound significantly occurred over large parts of the dark period (ZT 14-16 and 20-22; contrasts tests, P ≤ 0.048). On the other hand, during the first few hours of the recovery day, not only the REMS distribution was flattened but also the REMS amount decreased (ZT2 and 6; contrasts tests, P ≤ 0.048) as compared to baseline. At the end of the light and the beginning of the dark period, REMS levels on recovery and baseline days were comparable. However, REMS significantly increased again towards the end of the dark period in CRH-R1 CL (ZT 20-22; contrasts tests, P ≤ 0.023). In CRH-R1 CKO appearance of REMS was also almost totally blocked by ASD in comparison to baseline (contrasts tests, P < 0.001; Figure 22 F, page 74). In contrast to CRH-R1 CL, no further effects of ASD on REMS could be observed. On the following recovery day, REMS

was slightly reduced during the light period, however, significant decreases could only be detected at the beginning (ZT 2 and 4; contrasts tests, $P \leq 0.016$). Throughout the rest of the recovery day, REMS levels in CRH-R1 CKO mice were indistinguishable from baseline levels.

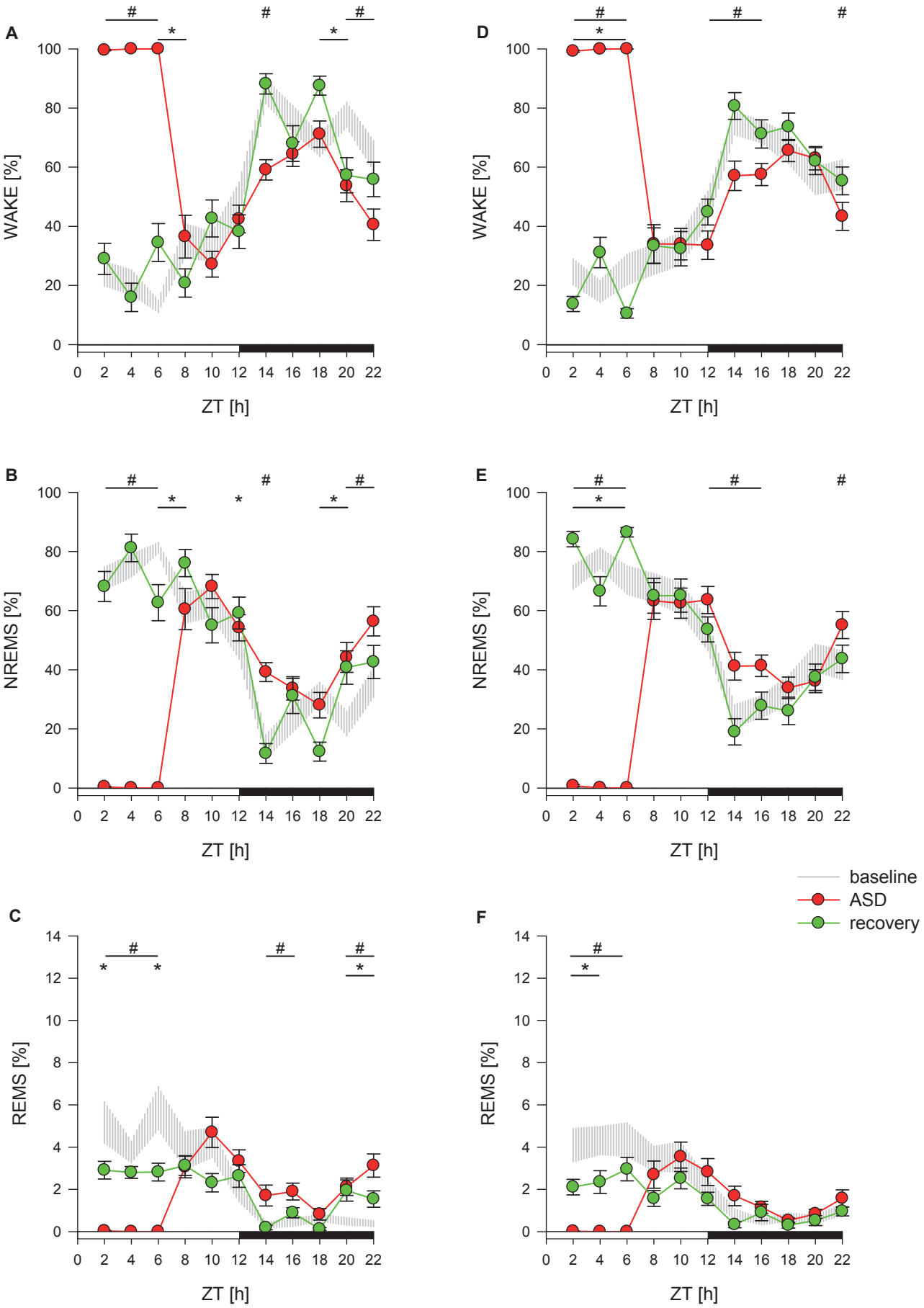

Figure 22 (CRH-R1 CL: A-C; CRH-R1 CKO: D-F): For figure legend, please refer to next page.

Figure 22: Effects of ASD on vigilance states in CRH-R1 CL (n = 7; A-C) and CRH-R1 CKO (n = 7; D-F) mice.

ASD efficiently increased wake, decreased NREMS and almost totally blocked REMS in both genotypes. During the following dark period wake was significantly reduced below baseline levels. On the other hand NREMS was significantly elevated over levels under baseline conditions. Whereas REMS was significantly increased during the dark period in CRH-R1 CL, no differences in REMS amounts could be detected in CRH-R1 CKO animals. In both genotypes wake and NREMS fluctuated near baseline values in the light period of the recovery day, and significant differences could be detected. At the same time REMS was significantly reduced in both CRH-R1 CL and CRH-R1 CKO during the first half of the light period of the recovery day. Similarly seen in the light period, CRH-R1 CL displayed fluctuations of wake and NREMS levels during the second half of the dark period; in CRH-R1 CKO such fluctuations could not be identified. Furthermore, if compared to baseline only in CRH-R1 CL REMS levels were significantly elevated towards the end of the dark period.
For graph denotations please refer to figure legend of figure 21, page 71.

4.5.3 CRH-R2 KO and CL

In CRH-R2 KO as well as CRH-R2 CL, significant interaction effects of treatment and time on wake could be determined during the light period (CRH-R2 KO: $F_{treatment\ x\ time}$ (10/70) = 85.249, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (10/70) = 64.188, P < 0.001). Whereas during the dark period in CRH-R2 KO significant interaction effects of treatment and time (CRH-R2 KO: $F_{treatment\ x\ time}$ (8/56) = 3.283, P < 0.004), in CRH-R2 CL only significant treatment effects could be shown (CRH-R2 CL: $F_{treatment}$ (2/14) = 4.341, P < 0.034). As similarly seen in the other mouse lines, ASD efficiently increased wake of CRH-R2 CL during deprivation in comparison to baseline (ZT 2-6; contrasts tests, P < 0.001; Figure 23 A, page 77). Following sleep deprivation, wakefulness showed a trend towards decreased levels during the first half of the dark period, reaching significant levels only at ZT 12-14 (contrasts tests, P ≤ 0.035). Although at ZT 6 of the recovery day wakefulness was significantly diminished, no further differences could be detected (contrasts tests, P = 0.035). Likewise CRH-R2 KO mice showed significant increases of wakefulness during the six hours of ASD (contrasts tests, P < 0.001; Figure 23 D, page 77), but in large parts of the dark period slightly, within the first part significantly, reduced amounts of wake (ZT 14-16; contrasts tests, P ≤ 0.002). During recovery day in CRH-R2 KO, the only differences from baseline were significant increases of wake at ZT 8 (contrasts tests, P = 0.01) and decreases at ZT 18 (contrasts tests, P = 0.024).

Regarding NREMS, significant interaction effects of treatment and time were detected during the light period for CRH-R2 KO and CRH-R2 CL (CRH-R2 KO: $F_{treatment\ x\ time}$ (10/70) = 89.227, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (10/70) = 63.674, P < 0.001). However, during the dark

period significant interaction effects could only be shown for CRH-R2 KO (CRH-R2 KO: $F_{treatment\ x\ time}$ (8/56) = 3.062, P < 0.006). As CRH-R2 CL were efficiently kept awake during ASD, NREMS was almost totally blocked for six hours (ZT 2-6; contrasts tests, P < 0.001; Figure 23 B, page 77). Following ASD, NREMS returned to baseline levels. At the end of the light period NREMS was significantly increased (ZT 12; contrasts tests, P = 0.043) over baseline levels. Although NREMS remained elevated for almost the whole dark period, no further differences from baseline could be detected. During the subsequent recovery day, NREMS levels remained comparable to baseline and only significantly increased at ZT 6 (contrasts tests, P = 0.031). In CRH-R2 KO animals, NREMS was similarly suppressed during ASD (ZT 2-6; contrasts tests, P < 0.001; Figure 23 E, page 77). In contrast to CRH-R2 CL, however, NREMS rebound significantly occurred over large parts of the dark period (ZT 14-18; contrasts tests, P ≤ 0.037). During recovery CRH-R2 KO mice displayed a NREMS distribution pattern similar to baseline, except for ZT 8 and ZT 18 with significantly decreased (contrasts tests, P = 0.004) and increased (contrasts tests, P = 0.023) NREMS, respectively.

REMS of both genotypes was significantly affected by interaction effects of treatment and time during the light (CRH-R2 KO: $F_{treatment\ x\ time}$ (10/70) = 12.565, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (10/70) = 17.261, P < 0.001) as well as the dark period (CRH-R2 KO: $F_{treatment\ x\ time}$ (8/56) = 3.164, P < 0.005; CRH-R2 CL: $F_{treatment\ x\ time}$ (8/56) = 2.629, P < 0.016). Concomitant with the suppression of NREMS in CRH-R2 CL, a total blockade of REMS was achieved by ASD (ZT 2-6; contrasts tests, P < 0.001; Figure 23 C, page 77) in comparison to baseline. After termination of sleep deprivation, REMS returned to baseline levels but with the transition to the dark period a significant rebound of REMS occurred (ZT 12-18 and 22; contrasts tests, P ≤ 0.038). From the beginning of the recovery day, REMS of CRH-R2 CL had reverted and remained comparable to baseline levels for the rest of the recording, except for a significant increase at ZT 20 (contrasts tests, P = 0.024). In CRH-R2 KO mice ASD also effectively blocked REMS (ZT 2-6; contrasts tests, P < 0.001; Figure 23 F, page 77) and resulted in a significant rebound compared to baseline from the light/dark transition on to the end of the dark period (ZT 12-16 and 20; contrasts tests, P ≤ 0.02). However, no statistical differences were evident throughout the recovery day as REMS displayed a distribution pattern comparable to baseline.

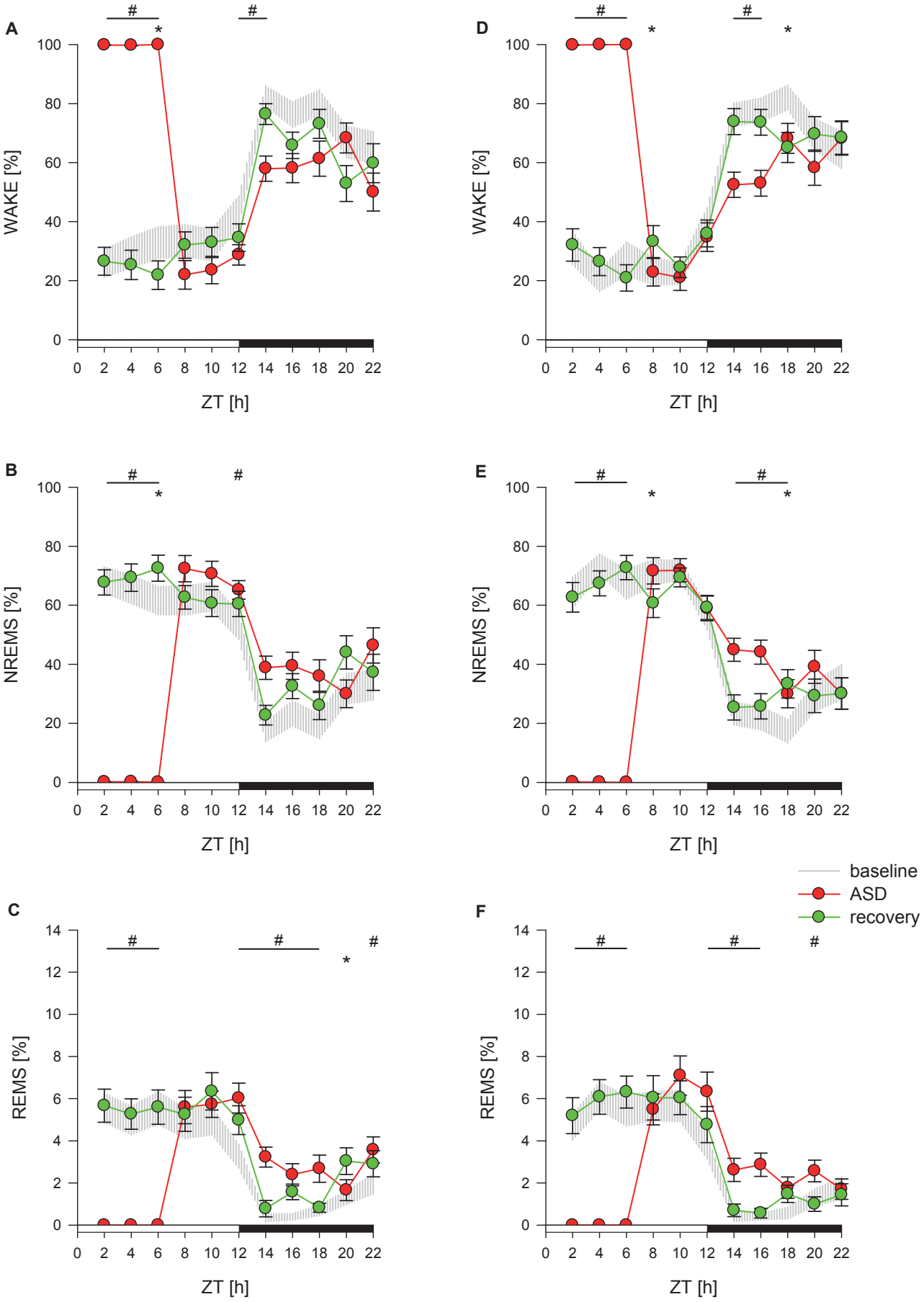

Figure 23 (CRH-R2 CL: A-C; CRH-R2 KO: D-F): For figure legend, please refer to next page.

Figure 23: Effects of ASD on vigilance states in CRH-R2 CL (n = 8; A-C) and CRH-R2 KO (n = 8; D-F) mice.

In both genotypes ASD significantly increased wake, decreased NREMS and blocked REMS. In the subsequent dark period, wake was significantly reduced in both CRH-R2 CL and CRH-R2 KO. Although NREMS was slightly increased in CRH-R2 CL in response to ASD, significant differences from baseline were more pronounced during the dark period of CRH-R2 KO. In contrast significant increases in REMS over baseline levels after ASD were comparable within the dark period in both genotypes. During most parts of the recovery day, wake and NREMS levels of CRH-R2 CL and CRH-R2 KO mice were comparable to baseline, and only few significant differences emerged both during the light and the dark period. The distribution of REMS during the recovery day in both genotypes was similar to that of baseline, although CRH-R2 CL displayed significantly increased REMS levels towards the end of the dark period.
For graph denotations please refer to figure legend of figure 21, page 71.

4.6 Accumulation of slow wave activity in response to automated sleep deprivation

After termination of the sleep deprivation experiment SWA, as a measure of sleep depth or intensity, was calculated in one hour means for all mouse lines on baseline, ASD, and recovery day. Under baseline conditions all mice displayed a typical distribution of SWA. During the first few hours of the light period, SWA was elevated in comparison to the daily mean SWA (reference value as 100%) but decreased below this level around the middle of the light period. SWA remained decreased till the middle of the dark period and afterwards started to increase over the mean SWA. Although SWA started to decline towards the end of the dark period, levels remained above or around the mean SWA. All mice tested, similarly reacted to ASD in terms of SWA, as significant increases in the power density between 0.5 and 4.0Hz were observed. However, the SWA response profile slightly differed between the various genotypes.

4.6.1 C57BL/6J

Statistical analysis revealed significant interaction effects of treatment and time on SWA in C57BL/6J during both the light ($F_{treatment\ x\ time}$ (22/154) = 1.537, P < 0.049) and the dark period ($F_{treatment\ x\ time}$ (18/126) = 1.283, P < 0.048). After ASD a distinct and significant increase in SWA, compared to baseline, could be observed for two hours (ZT 7-8; contrasts tests, P < 0.001; Figure 24, page 79), after which SWA values remained slightly but insignificantly elevated until ZT 15. With the exception of a significant increase at ZT 18 (contrasts tests, P = 0.004), SWA remained at baseline levels for the rest of the dark period. On the following

recovery day, the course of the SWA profile was similar to baseline, and a significant increase over baseline levels arose only at ZT 15 (contrasts tests, P = 0.032).

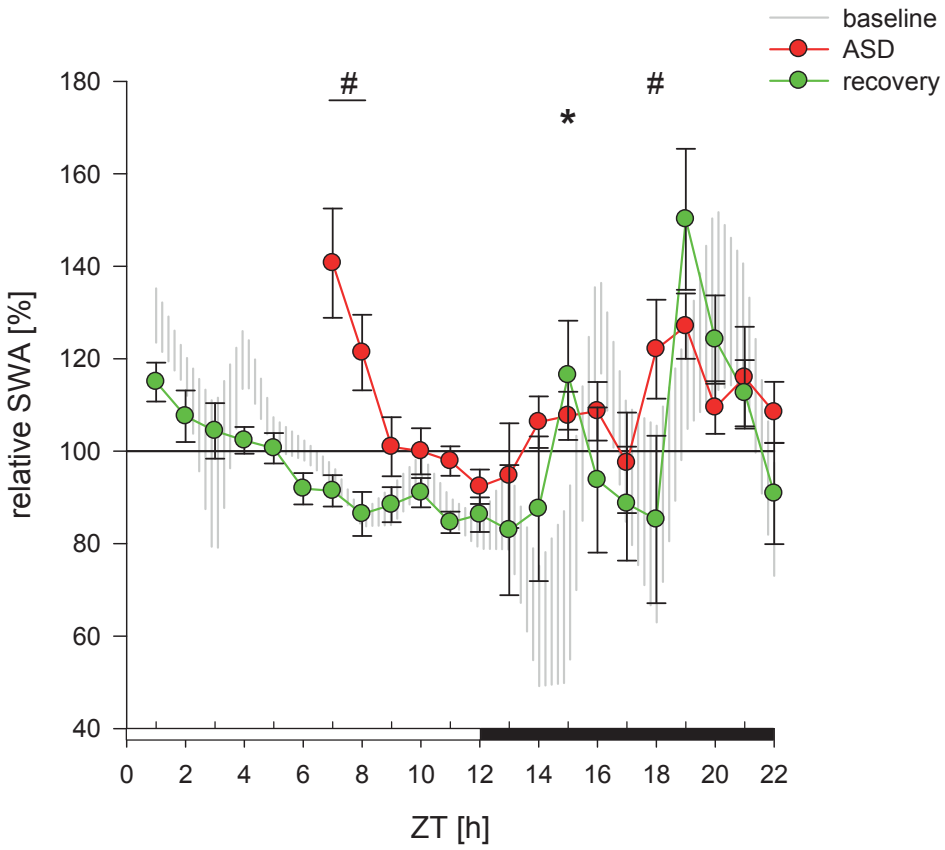

Figure 24: Slow wave activity after ASD in C57BL/6J (n = 8) mice.

Baseline SWA of C57BL/6J developed a typical distribution with increased values in the first half of the light period, decreased levels up to the middle of the dark period, and again increased levels during the rest of the dark period. After termination of ASD, animals responded with distinct increases in SWA for two hours. Although slightly increased, SWA developed a distribution comparable to baseline and only significantly rose above baseline at ZT 18. SWA during recovery day remained comparable to baseline, except for a significant increase at ZT 15. Depicted is the percentage of the given relative SWA (indicated on the y-axis) of one hour means ±SEM for each treatment (●: ASD, ●: recovery). The hatched area indicates mean values ±SEM during baseline. The black horizontal line indicates the mean baseline SWA as 100% reference. The symbols "#" and "*" denote statistically significant differences by comparison of baseline versus ASD or recovery, respectively (contrasts tests in ANOVA, P < 0.05). Solid lines under those symbols connect consecutive timepoints showing statistical differences. The open and filled bars on the x-axis indicate the light and dark period, respectively.

4.6.2 CRH-R1 CKO and CL

In CRH-R1 CKO and CRH-R1 CL, significant interaction effects of treatment and time on SWA could be detected during the light (CRH-R1 CKO: $F_{treatment\ x\ time}$ (22/132) = 31.607, P < 0.001; CRH-R1 CL: $F_{treatment\ x\ time}$ (22/132) = 12.409, P < 0.001), but only for CRH-R1 CL also during the dark period (CRH-R1 CL: $F_{treatment\ x\ time}$ (18/108) = 10.189, P < 0.003). Similarly seen in C57BL/6J animals, CRH-R1 CL displayed significantly increased SWA values for two hours after ASD (ZT 7-8; contrasts tests, P ≤ 0.004; Figure 25 A, page 81). As for C57BL/6J, SWA steeply declined within these two hours. However, afterwards SWA displayed a flattened course in comparison to baseline and thus at first was slightly elevated (ZT 9-13) but then slightly decreased (ZT 16-19). On the following recovery day, SWA of CRH-R1 CL remained at levels comparable to baseline, and the only significant difference was found with its sudden drop below baseline at ZT 17 (contrasts tests, P < 0.001). In contrast to all other mouse lines tested, CRH-R1 CKO demonstrated significant increases in SWA levels for five successive hours after termination of ASD (ZT 7-10: contrasts tests, P < 0.001; ZT 11: contrasts tests, P = 0.018; Figure 25 B, page 81). Furthermore within these hours SWA declined slower than those of the other mouse lines. However, as seen in the other mouse lines SWA stayed slightly elevated afterwards (ZT 12-15). No further differences to baseline could be detected. During the subsequent recovery day, SWA proceeded almost identical to baseline.

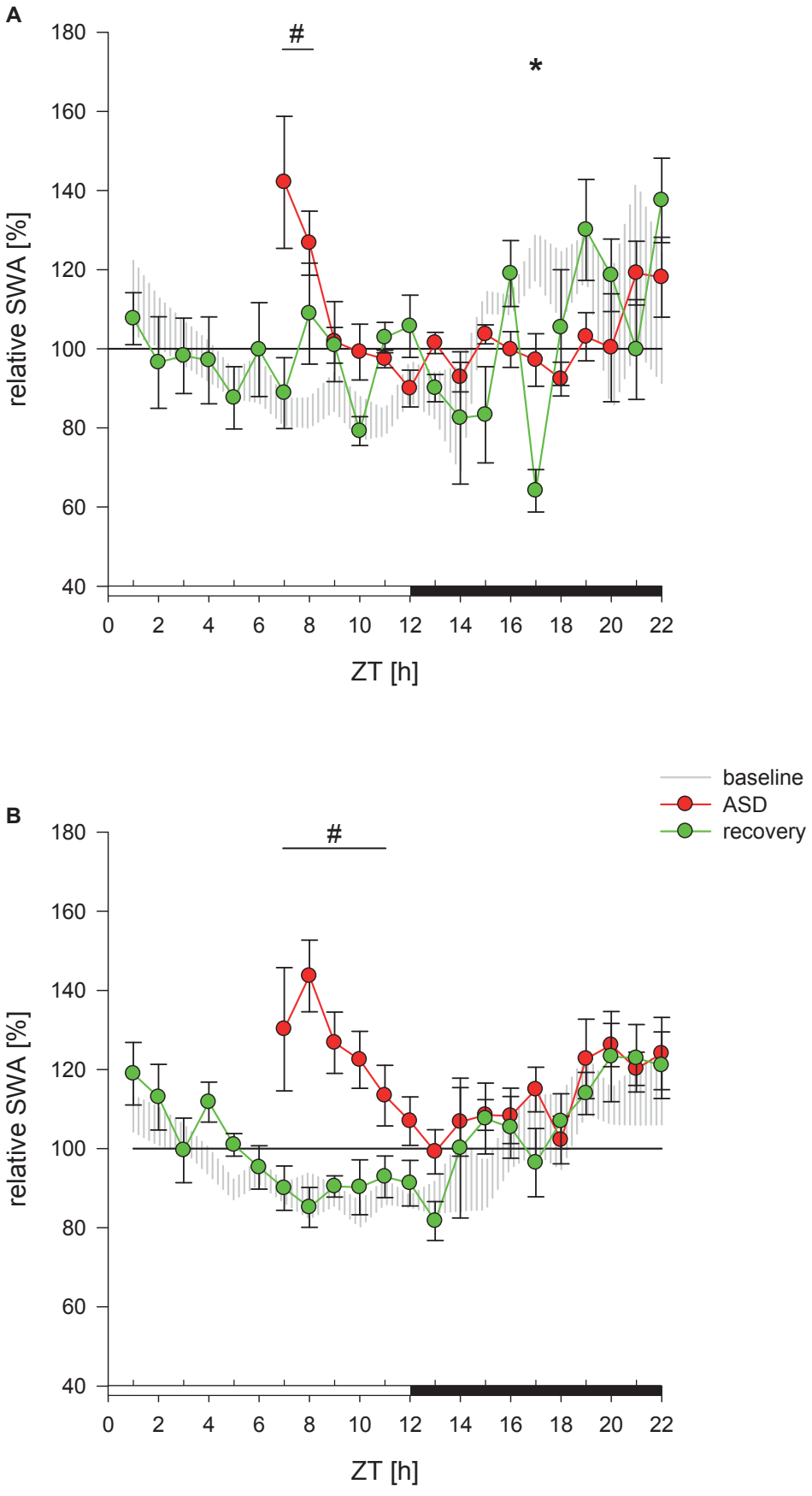

Figure 25 (CRH-R1 CL: A; CRH-R1 CKO: B): For figure legend, please refer to next page.

Figure 25: Slow wave activity after ASD in CRH-R1 CL (n = 7; A) and CRH-R1 CKO (n = 7; B) mice.

Both genotypes displayed a typical distribution of SWA during baseline. Initially SWA was increased and subsequently declined during the light period. After the middle of the dark period, increases in SWA could be detected again. Both CRH-R1 CL and CRH-R1 CKO responded to ASD with a distinct increase in SWA after termination of sleep deprivation, however the time course of the response differed. CRH-R1 CL showed increased SWA values for two hours with a steep decline towards baseline levels, whereas SWA of CRH-R1 CKO remained significantly elevated for five hours. Furthermore CRH-R1 CKO exhibited a slower decline in SWA. Although SWA was slightly decreased in the middle of the following dark period in CRH-R1 CL, no significant differences between baseline SWA and after ASD could be observed in either genotype. Except for a significant decrease of SWA in CRH-R1 CL at ZT 17, SWA on the recovery day remained comparable to baseline levels in CRH-R1 CL and CRH-R1 CKO.
For graph denotations please refer to figure 24, page 79.

4.6.3 CRH-R2 KO and CL

In CRH-R2 KO and CRH-R2 CL mice, SWA was significantly affected by interaction effects of treatment and time during the light period (CRH-R2 KO: $F_{treatment\ x\ time}$ (22/154) = 74.278, P < 0.001; CRH-R2 CL: $F_{treatment\ x\ time}$ (22/154) = 67.179, P < 0.001). Whereas during the dark period no effects could be determined in CRH-R2 CL, significant treatment effects were identified in CRH-R2 KO (CRH-R2 KO: $F_{treatment}$ (2/14) = 4.656, P < 0.028). After ASD, SWA of CRH-R2 CL showed significant increases for up to four hours (ZT 7-10; contrasts tests, P ≤ 0.02; Figure 26 A, page 83). Within that time frame SWA steeply declined as similarly seen in C57BL/6J and CRH-R1 CL. SWA remained at baseline levels for the rest of the light and the beginning of the dark period. From the middle of the dark period towards its end, SWA was slightly decreased compared to baseline. During the recovery day, SWA of CRH-R2 CL developed a similar pattern as under baseline conditions. Although SWA was slightly decreased around the middle of the dark period, no significant differences could be found. Likewise SWA significantly increased for three hours after ASD in CRH-R2 KO (ZT 7-9; contrasts tests, P ≤ 0.006; Figure 26 B, page 83), however quickly returned to baseline levels afterwards. Although SWA was slightly decreased in some parts of the dark period, except for significant decreases at ZT 17 and 21 (contrasts tests, P ≤ 0.012), no further differences to baseline were detectable. During recovery, SWA of CRH-R2 KO remained at levels similar to baseline, though within the second half of the dark period SWA was slightly diminished, with significant decreases at ZT 17 and 21 only (contrasts tests, P ≤ 0.019).

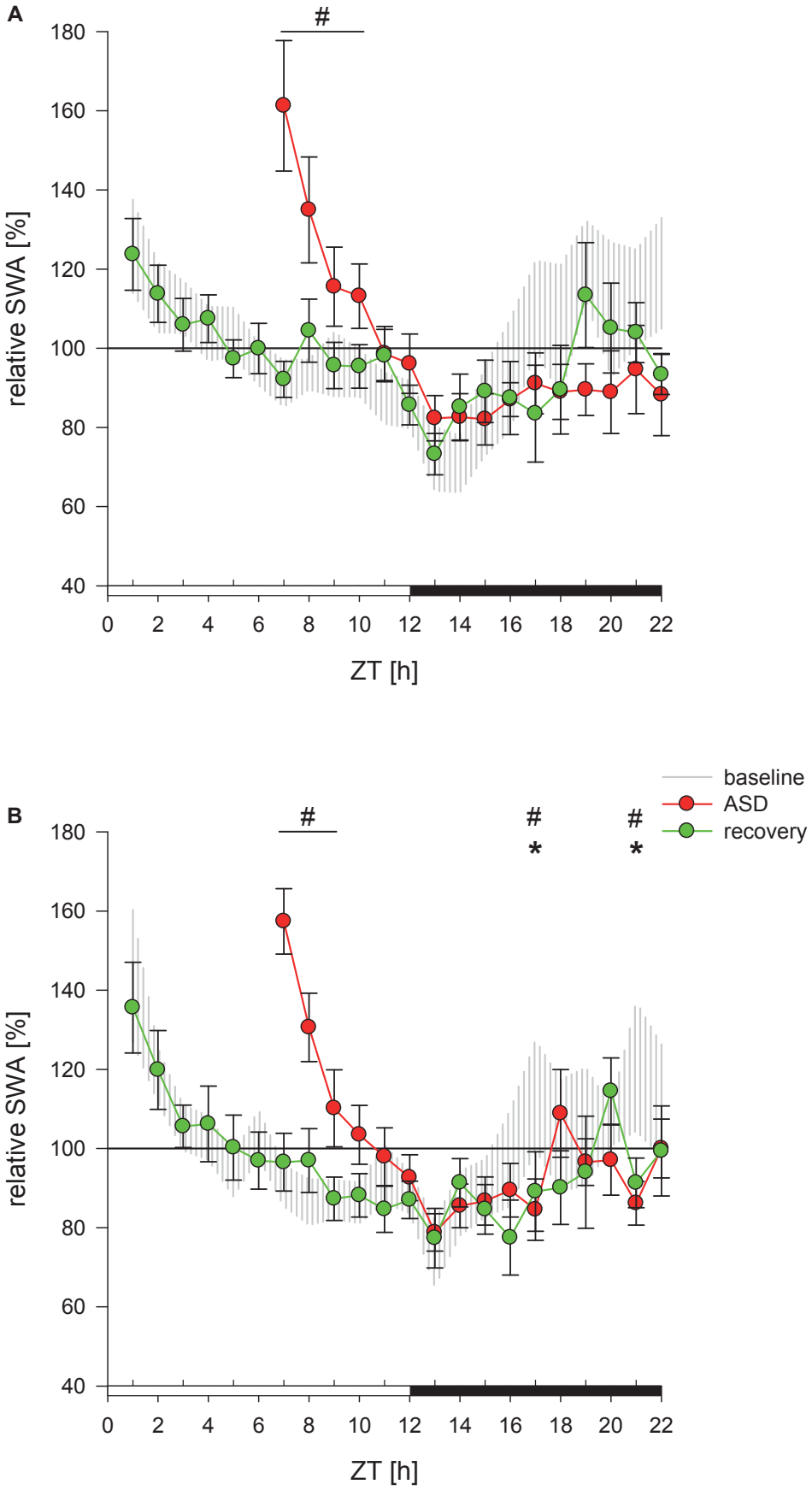

Figure 26 (CRH-R2 CL: A; CRH-R2 KO: B): For figure legend, please refer to next page.

Figure 26: Slow wave activity after ASD in CRH-R2 CL (n = 8; A) and CRH-R2 KO (n = 8; B) mice.

In both, CRH-R2 CL and CRH-R2 KO mice, a typical SWA distribution developed under baseline conditions. Whereas SWA levels were elevated at the beginning of the light period and subsequently declined, an increase in SWA could be observed within the second half of the dark period. After ASD both genotypes displayed a significantly elevated SWA. Although increased SWA could be detected in CRH-R2 CL for four hours and in CHR-R2 KO only for three, both animal lines similarly showed a steep decline of SWA towards baseline levels. The only significant difference from baseline during the dark period was a reduced SWA at ZT 17 and 21 in CRH-R2 KO mice. These differences were additionally persistent during the recovery day. SWA levels during the recovery day otherwise remained comparable to baseline levels in both genotypes.
For graph denotations please refer to figure 24, page 79.

4.7 Plasma CORT level changes in response to automated sleep deprivation

Statistical analysis revealed significant differences in plasma levels of CORT under baseline conditions as well as after treatment within and between the various mouse groups ($F_{treatment}$ = 96.232, P < 0.001; F_{group} = 7.663, P < 0.001). CORT concentrations in the time-matched baseline blood samples from all mouse lines were within a comparable range. However, C57BL/6J (n = 8) mice displayed the highest mean value and individual variation of plasma CORT levels (77.43 ± 18.33ng/ml) under baseline conditions, which was significantly higher than that of CRH-R2 CL (5.35 ± 0.34ng/ml, n = 8; Student-Neumann-Keuls test, P = 0.011; Figure 30, page 87). Whereas CRH-R2 CL exhibited the lowest CORT levels under baseline conditions, CORT levels of CRH-R2 KO mice (23.1 ± 3.42ng/ml, n = 8; Figure 30, page 87) ranged within those of CRH-R1 CL (36.46 ± 8.65ng/ml, n = 7) and CRH-R1 CKO (30.1 ± 10.82ng/ml, n = 7). All animals similarly responded to ASD with an increase of plasma CORT values in comparison to their baseline. Highest plasma CORT values (182.99 ± 22.57ng/ml, n = 8), significantly increased over baseline levels (contrasts tests, P < 0.001), were measured in C57BL/6J (Figure 27, page 85). Plasma CORT levels in CRH-R1 CL (100.07 ± 13.27ng/ml, n = 7) and CRH-R1 CKO (154.63 ± 32.54ng/ml, n = 7) mice were also significantly increased after ASD in comparison to baseline (CRH-R1 CKO: contrasts tests, P < 0.001; CRH-R1 CL: contrasts tests, P = 0.008; Figure 28, page 86). In contrast to CRH-R1 CKO animals, in which CORT levels increased in the same range as in C57BL/6J, CRH-R1 CL showed increased CORT levels after ASD closer to CRH-R2 KO and CRH-R2 CL (Figure 30, page 87). In both genotypes, CRH-R2 CL (95.24 ± 11.04ng/ml, n = 8) and CRH-R2 KO (93.45 ± 16.61ng/ml, n = 8), significantly increased CORT levels, if compared to baseline, could be demonstrated after ASD (CRH-R2 KO and CRH-R2 CL; contrasts tests, P < 0.001; Figure 29, page 86). Possessing similar mean values after ASD, both C57BL/6J and to a

weaker extent CRH-R1 KO exhibited significantly increased mean CORT levels by comparison to CRH-R1 CL, CRH-R2 KO, and CRH-R2 CL mice (C57BL/6J: Student-Neumann-Keuls test, P ≤ 0.002; CRH-R1 CKO: Student-Neumann-Keuls test, P ≤ 0.049; Figure 30, page 87).

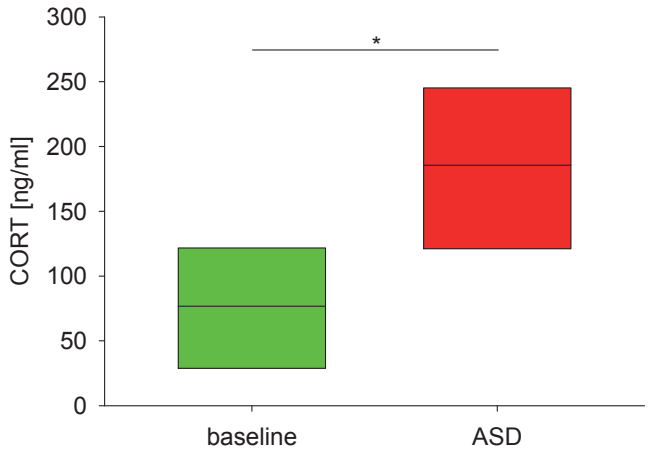

Figure 27: Plasma CORT levels after ASD in C57BL/6J (n = 8) mice.

Compared to the baseline control (77.43 ± 18.33ng/ml), plasma CORT levels after ASD (182.99 ± 22.57ng/ml) were significantly increased pointing to a treatment induced activation of the HPA axis. Data are given as box plots showing medians (lines in the boxes) as well as 25% and 75% percentiles (boxes). The asterisk indicates the significant difference in obtained CORT values in respect of different treatment conditions (contrasts tests in ANOVA, P < 0.05).

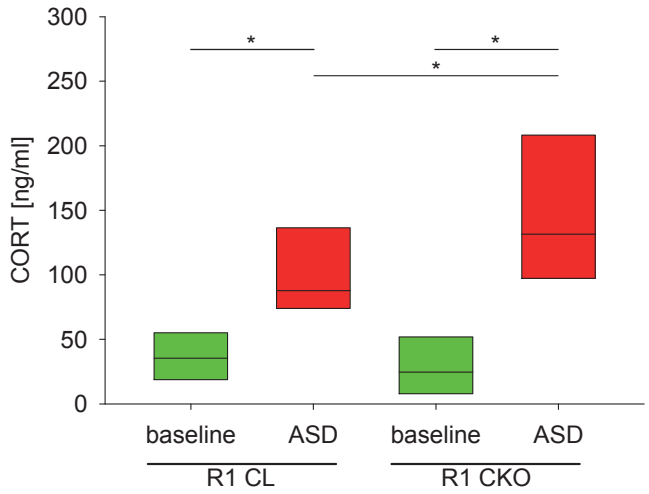

Figure 28: Plasma CORT levels after ASD in CRH-R1 CL (n = 7) and CRH-R1 CKO (n = 7) mice.

Indicating a treatment-derived HPA axis activation, plasma CORT levels of both genotypes were significantly increased after ASD (CRH-R1 CL: 100.07 ± 13.27ng/ml; CRH-R1 CKO: 154.63 ± 32.54ng/ml) as compared to baseline controls (CRH-R1 CL: 36.46 ± 8.65ng/ml; CRH-R1 CKO: 30.1 ± 10.82ng/ml). Furthermore CRH-R1 CKO showed significantly higher plasma CORT levels after ASD than their wildtype controls. Data are given as box plots showing medians (lines in the boxes) as well as 25% and 75% percentiles (boxes). Asterisks indicate significant differences in obtained CORT values in respect of different treatment conditions (contrasts tests in ANOVA, $P < 0.05$) and their according genotypes (Student-Neumann-Keuls tests, $P < 0.05$).

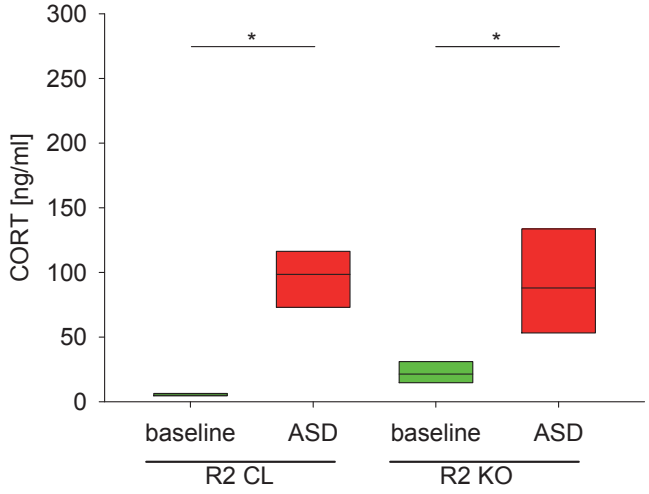

Figure 29: Plasma CORT levels after ASD in CRH-R2 CL (n = 8) and CRH-R2 KO (n = 8) mice.

In comparison to baseline controls (CRH-R2 CL: 5.35 ± 0.34ng/ml; CRH-R2 KO: 23.1 ± 3.42ng/ml), plasma CORT levels after ASD were significantly increased in both genotypes (CRH-R2 CL: 95.24 ± 11.04ng/ml; CRH-R2 KO: 93.45 ± 16.61ng/ml), suggesting a treatment-induced HPA axis activation. Data are given as box plots showing medians (lines in the boxes) as well as 25% and 75% percentiles (boxes). Asterisks indicate significant differences in obtained CORT values in respect of different treatment conditions (contrasts tests in ANOVA, $P < 0.05$) and their according genotypes (Student-Neumann-Keuls tests, $P < 0.05$).

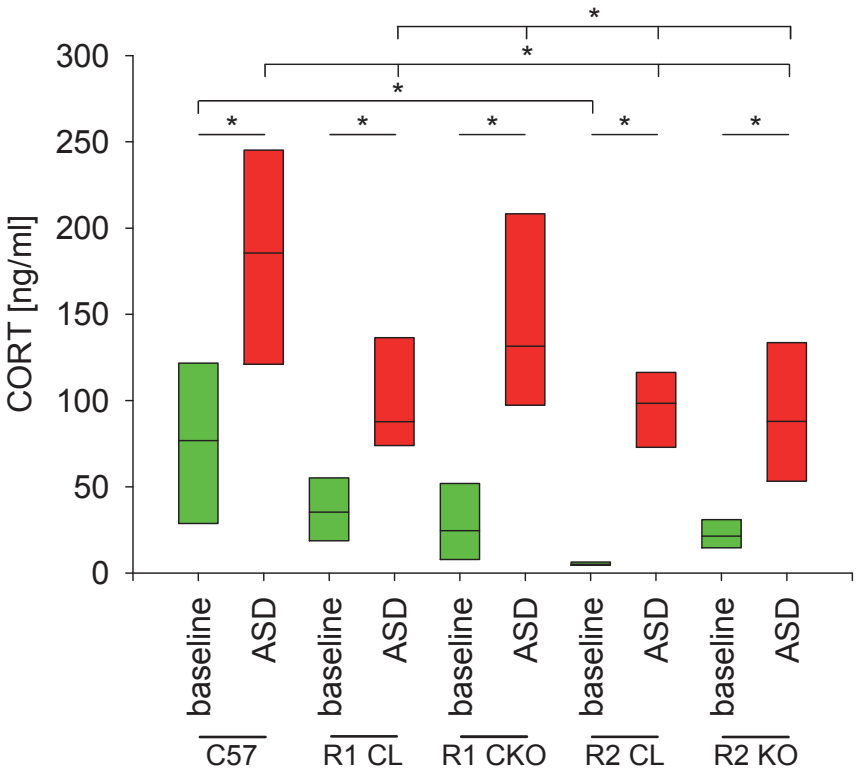

Figure 30: Comparison of plasma CORT levels under baseline conditions and after ASD between C57BL/6J (n = 8), CRH-R1 CL (n = 7), CRH-R1 CKO (n = 7), CRH-R2 CL (n = 8), and CRH-R2 KO (n = 8) mice.

Whereas a significant difference between C57BL/6J (77.43 ± 18.33ng/ml), displaying the highest, and CRH-R2 CL (5.35 ± 0.34ng/ml), displaying the lowest CORT levels, could be detected under baseline conditions, mean CORT levels of CRH-R2 KO (23.1 ± 3.42ng/ml), CRH-R1 CL (36.46 ± 8.65ng/ml), and CRH-R1 CKO (30.1 ± 10.82ng/ml) were within a comparable range. After ASD all animals responded with significant increases in CORT levels. However, after sleep deprivation CORT levels in C57BL/6J (182.99 ± 22.57ng/ml) and CRH-R1 CKO (154.63 ± 32.54ng/ml) were significantly higher than those in CRH-R1 CL (100.07 ± 13.27ng/ml), CRH-R2 CL (95.24 ± 11.04ng/ml), and CRH-R2 KO (93.45 ± 16.61ng/ml). Data are given as box plots showing medians (lines in the boxes) as well as 25% and 75% percentiles (boxes). Asterisks indicate significant differences in obtained CORT values in respect of different treatment conditions (contrasts tests in ANOVA, $P < 0.05$) and their according genotypes (Student-Neumann-Keuls tests, $P < 0.05$).

V Discussion

5.1 Central CRH-R1 mediates effects of CRH on sleep-wake regulation

The results demonstrated that i.c.v. injected CRH immediately promotes wakefulness dose-dependently and in turn suppresses sleep, namely both NREMS and REMS, in C57BL/6J mice. The effects of CRH on the sleep-wake patterns were also evident when CRH was injected into CRH-R1 CL and CRH-R2 KO/CL mice. In all mentioned mouse strains, wake-promoting effects of CRH were characterised by prolonging the episode duration but not increasing the episode occurrence. However, responses to CRH in CRH-R1 CKO mice were quite different from the other mouse lines. Acute effects of i.c.v. injected CRH that were supposed to increase waking and decrease NREMS almost disappeared, suggesting that wake-promoting and NREMS-suppressing effects of CRH are mediated by central CRH-R1. Since CRH effects on REMS still partly remained in CRH-R1 CKO mice, CRH-R1 might not be the sole mediator of REMS suppression. Despite acute effects of CRH seen in the light period, only C57BL/6J mice distinctly responded to CRH for a longer period, resulting in increases in NREMS/REMS and suppression of wakefulness during the dark period. Injections of CRH at the beginning of the light period most likely would not directly affect sleep-wake changes observed during the subsequent dark period. Since those changes were also shown in a dose-dependent fashion, the loss of sleep produced in the light period might have caused the rebound of sleep during the dark. Such rebound responses were obscure in CRH-R1 CL mice. In the experiment where different doses of CRH were administered, no distinct sleep rebound was apparent during the dark period. In the follow-up study, application of CRH alone or together with the CRH-R2 antagonist entailed a significant and distinct sleep rebound in CRH-R1 CL mice. In CRH-R2 KO and CL, a trend of dose-dependent rebound sleep was shown during the dark period. Therefore all mouse lines, except for CRH-R1 CKO, reacted with a more or less pronounced rebound after CRH-induced loss of sleep. The mechanism accounting for differences in CRH effects between C57BL/6J, CRH-R1 CL and CRH-R2 CL remains unclear.

5.1.1 Genetic background might influence baseline sleep profiles in control strains

Slight variations seen in the basal levels of sleep and wakefulness of the control lines (i.e. C57BL/6J, CRH-R1 CL and CRH-R2 CL) might have been caused by differences in genetic backgrounds. The influence of genetic background on the homeostatic regulation of sleep and the characteristics of the EEG (e.g. power densities) has been the topic of many studies

extensively discussed for the last decade (Franken et al., 1999;Franken et al., 1998;Franken et al., 2001). Different inbred mouse strains are reported to display marked variations in sleep-wake distribution, mean duration of vigilance episodes, SWA and EEG power density under baseline, as well as under certain experimental conditions (Franken et al., 1998;Valatx et al., 1972;Valatx and Bugat, 1974). In the present study, C57BL/6J mice showed greater baseline mean durations for wake during the light and REMS episodes during the dark period in comparison to those in CRH-R1 CKO/CL and CRH-R2 KO/CL mice. Mean episode length of NREMS in C57BL/6J and CRH-R2 KO was similar throughout the day and longer as compared to CRH-R1 CKO/CL and CRH-R2 CL animals. Nevertheless, under baseline conditions, sleep in the knockout mouse models, especially CRH-R1 CKO and CL, was more fragmented than in C57BL/6J. During the dark period C57BL/6J mice displayed higher wake and lower NREMS levels than the knockout models, whereas REMS levels were comparable between all breeding lines, although slightly elevated in CRH-R1 CKO and slightly reduced in CRH-R2 KO mice. Differences in time spent in wake and NREMS during the light versus the dark period under baseline conditions were most prominent in C57BL/6J mice and least in CRH-R1 CKO. However, the baseline sleep profile of the CRH-R1 CKO/CL and CRH-R2 KO/CL mouse lines resembles more the one of 129/SvJ mice described in literature than that of C57BL/6J mice (Huber et al., 2000).

Despite the close inheritance to homozygous C57BL/6J mice, mixed genetic lineage with 129/SvJ mice could contribute to variations in sleep architecture of CRH-R1 CKO/CL and CRH-R2 KO/CL mice from that of C57BL/6J mice, and possibly influence the appearance of CRH effects in general.

5.1.2 CRH receptor deficiency might affect baseline sleep profiles

Dissimilarities in spontaneous sleep architecture and in response to exogenous CRH between CRH-R1 CKO and CL as well as CRH-R2 KO and CL animals should be attributed to the lack of either central CRH-R1 or systemic CRH-R2 in the knockout mice. In contrast to earlier studies on spontaneous wake control by CRH (Chang and Opp, 2001;Opp, 1995), no specific changes in the amount of wakefulness or its architecture were evident in the present models of central CRH-R1 or systemic CRH-R2 deficiency. In the case of sleep, differences were found during baseline recordings and under vehicle conditions regarding the mean duration of NREMS and the total amount of REMS. Compared with their CL, CRH-R1 CKO mice demonstrated shorter NREMS episodes, but slightly increased total REMS due to more frequent episode entry. Regarding REMS, results obtained from the later performed antagonist study differed, as CRH-R1 CKO and CL animals showed comparable amounts of

REMS under baseline conditions. In contrast, CRH-R2 KO compared to their CL displayed significantly longer NREMS episodes but slightly reduced total REMS due to less frequent episode entries. This might point to a contrarian involvement of the CRH receptors in REMS regulation. According to the present data this assumption is speculative and further studies regarding specific effects of CRH and its receptors in brain regions involved in the regulation of REMS are needed.

Under baseline conditions, no differences in wakefulness could be detected, but REMS slightly varied within the knockout mouse lines. According to the present data, no critical role of either CRH-R1 or CRH-R2 in the regulation of baseline sleep phenotypes can be proposed. Slight changes in the sleep architecture could also be indirectly acquired as a result in order to compensate a lack of CRH signalling via CRH-R1 or CRH-R2 during ontogenesis.

5.1.3 Central application of CRH and ASV-30

In this study, C57BL/6J mice were used to determine the dose-dependent impact of CRH on sleep-wake regulation, and should serve as a comparison whether a similar dose-response relationship would appear in the case of central CRH-R1 or systemic CRH-R2 deficiency. The CRH doses for this study were carefully selected to elicit clear behavioural and neuroendocrine effects (Dunn and Berridge, 1990). One previous study in rats extensively examined whether i.c.v. injected CRH and an unspecific CRH receptor antagonist could reach CRH receptor-expressing neurons within various parts of the brain (Bittencourt and Sawchenko, 2000). A clear dose-dependent induction or attenuation of neuronal activity could be demonstrated within several brain areas after CRH or antagonist administration, respectively. From this study it might be deduced that by i.c.v. application CRH or CRH receptor antagonists similarly access the brains of mice. Although h/rCRH has a higher affinity for CRH-R1 than for CRH-R2 (Dautzenberg et al., 2001a;Vaughan et al., 1995), it remains a natural ligand for CRH-R2 at sufficiently high local concentrations (Keck et al., 2005), especially in the present model of central CRH-R1 deficiency. CRH executes its regulatory mechanism through a central neurotransmitter-like pathway (Brown et al., 1982;Fisher et al., 1982;Koob, 1999) either alone, or in combination with its activating effect upon the HPA system (Rivier et al., 2003;Vale et al., 1981). To clarify which pathway(s) would participate in sleep-wake modulation, relatively high CRH doses were tested to mimic a situation how stress-provoked excess secretion of CRH affects sleep in central CRH-R1 or systemic CRH-R2 knockout mice. A recent study compared effects of i.c.v. injected CRH on sleep-wake behaviour in several mouse strains, including C57BL/6J, endowed with a

differential responsiveness to stress (Sanford et al., 2008). Although the dose range used in that study was presumably too low to stimulate HPA axis activity, the authors demonstrated that CRH caused only at the highest dose (0.4µg CRH) significant increases in wake and decreases in NREMS and REMS. As indicated above, in the present study a low (non stress simulating) dose of CRH (0.3µg) and two higher doses (1.0 and 3.0µg) that were expected to reliably evoke HPA axis activity were chosen. One might assume that the latter dose used was too high, as it may increase HPA axis activity up to a non-physiological level. Still it was necessary to examine how CRH-R1 CKO and CRH-R2 KO animals would respond to the highest dose in comparison to their CL, as stimulation of HPA axis hormone release might differ.

Administration of both CRH and CRH receptor antagonists by i.c.v. injection is a reliable tool to test drug-induced changes in sleep-wake responses, as both compounds gain access to brain areas relevant for sleep-wake regulation.

5.1.4 Influences of exogenous CRH on non-rapid eye movement sleep and wake regulation

Unlike in CRH-R2 KO mice, in CRH-R1 CKO animals no distinct effects of CRH on wake and NREMS could be observed, although plasma CORT levels were increased. Thus, one might propose that CRH effects on NREMS suppression were mediated centrally through CRH-R1 rather than CRH-R2. The effects on NREMS were furthermore not influenced by CRH-mediated HPA axis activation and subsequent peripheral release of CORT, which might have acted back on sleep regulation by activating central corticosteroid receptors (Friess et al., 1994;Friess et al., 2004). These findings further suggest that the wake-promoting action of CRH is at least to a certain extent dissociated from its action on the HPA system. Activation of the HPA axis, as evidenced by increased plasma CORT levels after CRH application, did not increase arousal in CRH-R1 CKO mice. The main purpose of this study was to assess whether CRH directly modulates sleep-wake behaviour in mice through central CRH receptor type 1 or 2, or if its impact on sleep is rather mediated as a secondary side effect of HPA axis components and arousal. CRH has long been implicated to be a mediator of arousal or spontaneous wakefulness, as mentioned before. Furthermore, it could be shown in rats that i.c.v. injections of low amounts of CRH dose-dependently affect EEG activity (e.g. EEG spectral power) by increasing brain excitability and alertness (Ehlers et al., 1983). It is known that many parts of the brain contribute to sleep-wake regulation such as the BF, the hypothalamus, the thalamus and the brainstem (for review please refer to: Datta and MacLean, 2007; Jones, 2003; the introduction). Secreting various excitatory and inhibitory

neurotransmitters, the arousal systems are implicated in regulating different behavioural and emotional states. Whereas the brainstem reticular formation (glutamatergic neurons) seems to be essential for the maintenance of arousal and cortical activation in general (Lindsley et al., 1950), other brainstem arousal systems like the LC (noradrenergic neurons) have been shown to discharge maximally during aroused conditions such as stress by local release of CRH (Aston-Jones et al., 1999;Valentino et al., 1991;Valentino and van Bockstaele, 2008). The RN (serotonergic neurons) are linked to arousal processes associated with positive emotions and rhythmic movements such as grooming (Datta and MacLean, 2007;Jacobs and Fornal, 1991), and the ponto-mesencephalic areas, such as the LDT and the PPT nuclei (cholinergic neurons) are active during behaviourally quite wake periods (Jones, 2003). In all of the above mentioned areas, CRH receptor-like immunoreactivity has been detected (predominantly CRH-R1, except for the CRH-R2 dominated RN) to a more or less strong extent (Sauvage and Steckler, 2001;van Pett et al., 2000), and many of the wake-promoting or arousal-stimulating systems contain neurons expressing CRH themselves, notably the LH, the LDT, and the LC (Koob, 1999;Smagin et al., 2001). Thus it seems likely that CRH is in a position to participate in sleep-wake control not only during stress, but also within its diurnal rhythm under non-stressful conditions, although the current results comparing baseline sleep of knockout mice and control littermates cannot directly support this possibility. Particular brain regions expressing CRH-R1 may be more crucial for affecting arousal than other regions. Such spatial specificities regarding CRH effects and CRH-R1 or CRH-R2 function should be further considered in future studies.

Central deficiency of CRH-R1 attenuates wake-promoting and NREMS-suppressing effects of exogenously applied CRH. Even an elicited activity of the HPA system was not accompanied by pronounced increases in wakefulness or decreases in NREMS. A functional HPA axis does not seem to be essential for a CRH-mediated wake and NREMS regulation.

5.1.5 Influences of exogenous CRH on rapid eye movement sleep regulation

In contrast to wake and NREMS, a dose-dependent suppression of REMS was observed in C57BL/6J, CRH-R1 CL, and CRH-R1 CKO animals, pointing to a potential role of CRH-R2 and its involvement in suppression of REMS in CRH-R1 CKO animals. The expression of CRH-R2 was not altered in the brain of conditional CRH-R1 knockout animals compared to control littermates, similar to conventional CRH-R1 knockout animals (Timpl et al., 1998). Regarding a mechanism of REMS regulation, Lu et al. proposed a putative flip-flop switch model consisting of REM-off and REM-on neurons (see introduction Figure 6, page 15), which enables the brain to perform sharp switches from NREMS to REMS and REMS to

wake (Lu et al., 2006). These neurons presumably inhibit each other's activity reciprocally via GABAergic projections, which could be influenced by several other brain areas or nuclei. The orexin (hypocretin) system, the serotonergic DR, and the noradrenergic LC probably excite REM-off cells, whereas inhibitory input to REM-off cells derives from the GABAergic extended part of the ventrolateral preoptic nucleus, the GABAergic sublaterodorsal nucleus, and the cholinergic PPT and LDT (Lu et al., 2006). In most of these areas, either CRH-R1 (PPT, LDT, LC, and orexinergic neurons) or CRH-R2 (DR and orexinergic neurons) is expressed (Sauvage and Steckler, 2001;van Pett et al., 2000;Winsky-Sommerer et al., 2004). Therefore an influence of CRH on REMS regulation could be mediated centrally by both receptor types. Apparently in CRH-R2 KO animals the presence of CRH-R1 would suffice to suppress REMS as a consequence of NREMS inhibition and wake promotion. Given the fact that REMS in CRH-R2 KO was similarly suppressed as in the other mouse strains regarding time course and magnitude, an involvement of central CRH-R2, even as a modulator, seems unlikely. Further corroborating this notion are the results from the present CRH-R2 antagonist study in CRH-R1 CKO and CL mice. Interestingly ASV-30 application produced no significant effects on sleep and wake in both animals, as neither sleep or wake amounts nor architecture were changed at all. ASV-30 has been demonstrated to exhibit a very high affinity and specificity for mouse CRH-R2 (Brauns et al., 2001;Higelin et al., 2000;Rühmann et al., 1998). Additionally it has been repeatedly proven effective in blocking CRH-R2 if injected i.c.v. in rats and mice applying a comparable dose and administration composition as in the present study (de Groote et al., 2005;Maruyama et al., 2007;Risbrough et al., 2003;Risbrough et al., 2004). However, the CRH-induced REMS reduction persisted in CRH-R1 CKO mice even following pretreatment with the CRH-R2 antagonist. It is thus reasonable to propose that central CRH-R2 is at least not directly involved in REMS regulation or that of NREMS or wake. Apart from that, REMS regulation could be affected by another pathway rather than central CRH receptors. Since i.c.v. injected CRH increased plasma CORT concentrations even in animals with a central CRH-R1 deficiency, the activation of the HPA axis through peripheral CRH-R1 might have also contributed to REMS alteration in this experimental condition. Elevated plasma CORT possibly affects CNS functions including sleep via GR and/or MR along the neuroendocrine negative feedback loop. In several human studies it could be demonstrated that injections of cortisol or its analogues (e.g. dexamethasone) profoundly suppress REMS, especially via central GR feedback (Born et al., 1987;Born et al., 1989;Born et al., 1991;Fehm et al., 1986;Gillin et al., 1972).

As evidenced in the present study, centrally administered CRH can enhance HPA activity in CRH-R1 CKO mice. Thus, it is likely that elevated peripheral hormones were responsible for the perpetual (or "sustained") REMS suppression. Therefore, the role of central CRH-R1

regarding REMS regulation still remains partially obscure. As reported previously CRH-R1 receptors in the periphery, including the pituitary, are still intact in CRH-R1 CKO animals (Schmidt et al., 2006), thus centrally injected CRH most probably triggered this HPA axis response. So far, it has been shown that i.c.v. injected CRH is actively transported out of the brain across the blood-brain barrier and capable of reaching peripheral sites of action (Martins et al., 1997). Recently it was demonstrated that conditional CRH-overexpressing mice with an intact HPA system characteristically show elevated REMS, which can be normalised with CRH-R1 antagonism suggesting that regional-specific overexpression of CRH accounts for REMS enhancement (Kimura et al., 2010). In contrast, other studies have shown REMS suppression by CRH, but more recently bi-directional REMS responses (i.e. increases and decreases) to CRH that are mediated through CRH-R1 located in the central nucleus of the amygdala have been reported (Pawlyk et al., 2006;Wellman et al., 2009;Yang et al., 2009). Indeed, REMS is a very fragile state, therefore depending on the experimental design the CRH system could contribute to either case triggering increases or reductions in REMS.

Brain regions possibly involved in spontaneous REMS regulation express CRH receptors; however, a temporary blockade of both central CRH receptors did not attenuate the CRH-induced REMS suppression. Activation of the HPA axis might have resulted in REMS suppression.

In summary, the present study using conditional CNS-specific CRH-R1 and conventional CRH-R2 knockout mice demonstrates that CRH is able to centrally modulate sleep-wake regulation via CRH-R1. Although a possible role of central CRH-R2 in sleep-wake control cannot be totally neglected, the results suggest that central CRH-R1 plays a dominant role regarding the effects on arousal. Enhanced HPA axis activity seemingly is not a prerequisite for CRH exerting NREMS suppression, whereas REMS suppression might be influenced by increased stress hormone release in the periphery.

5.2 Central CRH-R1 participates in modulating sleep homeostasis

Partial or total SD, by a manual (e.g. gentle handling (Franken et al., 1993)) or automated (e.g. flower pot technique (Mendelson et al., 1974)) method, is widely accepted as a reliable tool to study homeostatic sleep regulation (Lancel et al., 1991;Takahashi et al., 1981;Tobler and Borbély, 1990). As reported in literature, ASD is a standardised alternative to existing sleep deprivation methods and highly efficient in terms of NREMS and REMS inhibition (Fenzl et al., 2007). In all animals of the present study, an almost total blockade of sleep for six hours could be achieved, which subsequently resulted in the rebound of sleep during the following dark period.

5.2.1 Central CRH-R1 deficiency attenuates REMS rebound after automated sleep deprivation

In contrast to rats, six hours of SD are sufficient enough to obtain a profound sleep rebound in mice, since four hours of SD are similarly effective, while ten hours of SD not further augment the rebound response (Huber et al., 2000;Tobler and Borbély, 1990). Although minor variations between the mouse groups regarding the magnitude of rebound and the distribution of sleep on the recovery day were detected, dissimilarities in genetic background responsible for variations in their baseline sleep profiles (as discussed above) might account for these phenomena (Franken et al., 1999;Huber et al., 2000) besides differences in CRH receptor system functionality. Yet all animals displayed a rebound in NREMS which is well documented in literature (Feinberg and Campbell, 1993;Franken et al., 1991;Lancel et al., 1991;Morrow and Opp, 2005). The most striking finding in the present study was the absence of REMS rebound in CRH-R1 CKO animals which, in contrast, was clearly displayed by all other mice. Acute stressors, such as immobilisation stress, applied over a short period of time (less than two hours) have been shown to evoke a distinct REMS rebound, even without SD (Bonnet et al., 2000;Marinesco et al., 1999;Meerlo et al., 2001;Rampin et al., 1991). A prominent role of the LC in REMS regulation was proposed, since REMS rebound after acute stress and after SD could be attenuated by pharmacologically lesioning the noradrenergic components of the LC (Gonzalez et al., 1995;Gonzalez et al., 1996). More recently, CRH was suggested as a mediator of stress-induced REMS rebound, as i.c.v. application of a CRH receptor antagonist suppressed both the stress- and SD-induced REMS rebound (Gonzalez et al., 1995;Gonzalez et al., 1996;Kimura et al., 2010). In all animals of this study, plasma CORT levels were significantly elevated after ASD. Although some reports claim that sleep deprivation constitutes no stressor for the animals (Palchykova et al., 2006;Tobler et al., 1983), it is questionable

whether a prolonged loss of sleep can be achieved without stressing an animal. One of the studies reported marked rises in plasma CORT levels induced by a short period of forced locomotion (Tobler et al., 1983), which might in part be explanatory for the increases in plasma CORT levels observed in the present ASD study. At least to a certain degree, stress seems to amplify SD induced effects (Tobler and Borbély, 1990). It is however noteworthy that most of the six hours of ASD (>70%) were spent awake voluntarily, with the animals grooming or moving within the running wheels, and not due to forced locomotion by rotation of the wheels (Fenzl et al., 2007 and personal observations). Furthermore, the distance the animals had to move involuntarily during the present ASD study was markedly shorter as compared to the distance voluntarily covered by rodents in freely accessible running wheels (Hanagasioglu and Borbély, 1982;Vyazovskiy et al., 2006) and comparable to the enforced distance during the disk-over-water SD method (Bergmann et al., 1989;Rechtschaffen et al., 1983), which is generally considered as a light locomotor load (Rechtschaffen et al., 1999). Still it is likely that the increased levels of plasma CORT, indicative of an activated HPA axis, were, at least partly, contributing to the consequences of sleep loss by ASD. Several studies confirm an increased CRH synthesis or CRH activity within the brain after different sleep deprivation paradigms (Fadda and Fratta, 1997;Gonzalez et al., 1996;Gonzalez and Valatx, 1998;Koban et al., 2006) which is, although not directly evidenced, in line with the present results.

Since CRH-R1 CKO animals displayed no REMS rebound, and given the fact that according to literature stress- and SD-induced increases in REMS could be suppressed by CRH receptor antagonism, an involvement of central CRH and its pathways in a regulatory mechanism in response to SD or sleep loss is suggested. Unlike CRH-R2, central CRH-R1 seems to be essentially involved in homeostatic sleep regulatory processes underlying a recovery mechanism from sleep loss.

5.2.2 Sleep intensity is influenced by central CRH-R1

The significant increase in power densities across low frequency EEG bands following sleep deprivation (Borbély et al., 1981) was considered as an indicator of homeostatic sleep intensity, and subsequently SWA (EEG power density between 0.5-4 Hz) during NREMS was proposed as an indicator of homeostatic sleep pressure (Borbély, 1982a;Borbély et al., 1984). A large number of studies support this notion as similar increases in SWA following partial sleep restriction or total SD have been reported in rats (Endo et al., 1997;Franken et al., 1991;Tobler and Borbély, 1990), cats (Tobler and Scherschlicht, 1990), rabbits (Opp et al., 1997;Tobler et al., 1990), and humans (Finelli et al., 2000). The significance of SWA has

further been extended to and been validated for different mouse lines (Huber et al., 2000). The significant increase in SWA after ASD reported in all mice of the present study presumably reflects enhanced sleep intensity. However, the time course and magnitude of SWA changes slightly differed between the various genotypes. Except for CRH-R1 CKO, all mice similarly displayed a profoundly increased SWA after termination of ASD followed by an exponential decline to baseline levels, which is typically observed in mice (Franken et al., 1999;Huber et al., 2000). CRH-R1 CKO mice initially showed increased SWA levels comparable to the other mouse lines but contrariwise a distinctly slower (more parabolic) decline towards baseline. It is tempting to reason that the more lengthy increase in SWA after ASD, which was shown in CRH-R1 CKO mice, might reflect a deeper and more efficient sleep in these animals as compared to the other mice. This implies that without central CRH-R1 mice sleep better after a deficit of sleep. Further corroborating this assumption are studies in human patients suffering from affective disorders. In general, hyperactivity of the CRH system is well recognised as one of the main causes for the development of depressive disorders, frequently concomitant with severe sleep impairments such as decreased SWS and sleep intensity (Holsboer, 1999;Holsboer and Ising, 2008;Nemeroff et al., 1984;Raadsheer et al., 1994;Steiger, 2002;Steiger, 2007). The specific CRH-R1 antagonist R121919, which is able to cross the blood brain barrier (Keck et al., 2001), has been shown to ameliorate these sleep symptoms in humans (Held et al., 2004), as SWS and thus sleep intensity was increased while REM density decreased.

Although SWA was significantly increased in CRH-R2 KO a longer period than in C57BL/6J and CRH-R1 CL, the difference between SWA during baseline and after ASD was considerably smaller than that seen in CRH-R1 CKO mice. In consequence, a more likely involvement of central CRH-R1 in the homeostatic control of sleep is suggested by our results, whereas the role of CRH-R2 seems to be, if at all, of a minor nature.

To summarise, the general effects of ASD on sleep in the mouse lines of the present study are in line with current literature. Sleep loss over a period of six hours elicits rebound sleep and a significant increase in SWA as typically observed in mice. However, in contrast to CRH-R2 deficiency the lack of central CRH-R1 entails some differences in the response to ASD. The absence of REMS rebound and the characteristically altered SWA course imply an involvement of central CRH-R1 in sleep regulatory processes underlying a mechanism of recovery sleep.

VI Summary and Conclusions

The different mouse lines demonstrated dissimilarities in their baseline sleep profiles, which in part might be reflected by genetic background influences. More importantly, with the exception of CRH-R1 CKO animals, all mice reacted to i.c.v. CRH injections with dose-dependent increases in wake and decreases in NREMS. In contrast, mice from all breeding lines responded with more or less pronounced dose-dependent REMS suppression. It can be concluded that central CRH-R1 is essentially involved in CRH-mediated effects on wake and NREMS, but not REMS. According to the results, CRH-R2, a presumed modulator of HPA axis activity, plays a minor, if at all, role in sleep regulation. Furthermore, HPA axis activation, as evidenced in the study of CRH injections in CRH-R1 CKO, presumably is no prerequisite for an impact of CRH on wake and NREMS regulation. However, peripheral stress hormone release might affect REMS suppression.

In response to ASD, all mice showed significant increases in plasma levels of CORT, SWA, and additionally NREMS rebound and, except for CRH-R1 CKO mice, also REMS rebound. Elevated CORT levels indicate that ASD is a stressor. Since no REMS rebound occurred in CRH-R1 CKO animals, the central CRH-R1 system might be a mediator in this process. Furthermore sleep intensity was markedly enhanced for a longer period in the mouse model of central CRH-R1 deficiency as compared to all other mice. CRH-R1 assumingly participates in controlling sleep quality and the stress-related rebound of REMS.

In conclusion, the results only insinuate a contribution of CRH to spontaneous sleep-wake regulation. Most importantly this study considerably reveals for the first time a crucial role of central CRH and CRH-R1 in stressor-induced changes in the sleep-wake behaviour of mice.

VII Outlook

The experiments and results presented in this thesis certainly have to be regarded as a first step towards understanding the differential role of the CRH receptors in the regulatory mechanism by which CRH influences sleep-wake behaviour. Further experiments in mice will contribute to obtaining a more detailed picture of the regulatory processes involved in CRH-induced arousal or sleep suppression.

The variation in the baseline sleep profiles indicates a genetic influence on sleep-wake regulation caused by differences in their genetic background. Microarray approaches might identify dissimilarities in the expression of genes which probably are associated with the homeostatic regulation of sleep. This could provide an informative basis for the variation detected in sleep architecture (i.e. mean duration of vigilance states).

To avoid the ontogenetic acquisition of compensatory mechanisms due to a lack of CRH signalling through CRH-R2, conditional CRH-R2 knockout mice would be needed. A repetition of the experiments with conditional transgenic mice potentially produces results evidencing a more differentiated role of CRH-R2 in sleep, and especially in REMS regulation.

The contribution of CORT to REMS suppression should be further investigated in the CRH-R1 CKO and CL mice. The inhibition of CORT release after CRH treatment by either adrenalectomy or preferentially a pharmacological blockade of CORT synthesis (e.g. metyrapone administration) might resolve the issue of CORT-mediated REMS suppression in CRH-R1 CKO mice.

Microdialysis experiments or multiunit recordings with chronically implanted electrodes (e.g. targeting the noradrenergic LC or serotonergic DR) together with brain-site-specific local microinjections of CRH (e.g. amygdala) could give more insight into putative modulatory effects of CRH on the activity patterns of neuronal systems involved in sleep-wake regulation. Together with immunohistochemical approaches, the interaction of brain-site-specific CRH and other neurotransmitter systems might be traced. Inclusion of the different CRH receptor knockout models into this experiment might additionally highlight a disrupted communication between CRH and neurotransmitter systems that normally would interact in mediating arousal or suppression of NREMS and REMS.

Other models of transgenic mice could further discriminate various interactions of CRH and its receptors with different neurotransmitter systems. Mice specifically overexpressing or

underexpressing CRH in certain neuronal subpopulations (e.g. glutamatergic neurons in the LC) might further substantiate an interaction of CRH and the specific neurotransmitter system in mediating influences on different vigilance states. Furthermore, experiments that investigated the basic sleep profile of mice either overexpressing CRH in the whole CNS or only the forebrain (including limbic structures) should be complemented by observation of sleep in mice specifically overexpressing CRH within the hindbrain. In general, approaches including conditional CRH overexpressing mice are inevitable to investigate effects of a chronically activated CRH system on vigilance states.

In my opinion, research in mouse models holds the potential to answer several questions concerning sleep-wake regulatory influences of CRH.
In the long run, experiments in these animals will conduce to a better understanding of how and where exactly CRH mediates its effects upon the different vigilance states. The possibility of simultaneously applying various approaches (e.g. genetic and pharmacologic) to tackle specific problems is one of the great advantages of experiments in mice.

VIII References

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IX List of Abbreviations

5-HT	Serotonin
α-hCRH	Alpha-helical CRH
AC	Adenylyl cyclase
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variances
Amp	Main amplifier
APit	Anterior pituitary
ASD	Automated sleep deprivation
ASV-30	Antisauvagine-30
AVP	Arginine vasopressin
BF	Basal forebrain
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of the amygdala
CNS	Central nervous system
CoA	Cortical nucleus of the amygdala
CREB	Cyclic adenosine monophosphate response element binding protein
CRH or CRF	Corticotropin-releasing hormone or factor
CRH-R1	Corticotropin-releasing hormone receptor type 1
CRH-R1 CKO	Conditional CNS-specific corticotropin-releasing hormone receptor type 1 knockout
CRH-R1 CL	Corticotropin-releasing hormone receptor type 1 wildtype control littermate
CRH-R2	Corticotropin-releasing hormone receptor type 2
CRH-R2 CL	Corticotropin-releasing hormone receptor type 2 wildtype control littermate
CRH-R2 KO	Conventional corticotropin-releasing hormone receptor type 2 knockout
CSF	Cerebrospinal fluid

DA	Dopamine
DR	Dorsal raphe nucleus
e	Extracellular domain
EEG	Electroencephalogram
EMG	Electromyogram
EOG	Electrooculogram
FFT	Fast Fourier Transformation
GABA	Gamma-aminobutyric acid
Gal	Galanin
GHRH	Growth hormone-releasing hormone
GPCR	G-protein-coupled receptor
GR	Glucocorticoid receptor
HAB	High anxiety-related behaviour
His	Histamine
HPA	Hypothalamo-pituitary-adrenocortical
HPS	Hypothalamo-pituitary-somatotrophic
h/rCRH or h/rCRF	Human/rat corticotropin-releasing hormone or factor
i	Intracellular loop
i.c.v.	Intracerebroventricular
i.d.	Inner diameter
i.v.	Intravenous
LAB	Low anxiety-related behaviour
LC	Locus coeruleus
LDT and LDTg	Laterodorsal tegmentum (or tegmental nuclei)
LH	Lateral hypothalamus
LPT	Lateral pontine tegmentum
MA	Medial amygdala
mPRF	Medial pontine reticular formation

MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NF-κB	Nuclear factor-κB
NREMS	Non-rapid eye movement sleep
ORX	Orexin
PAG	Periaqueductal grey
PB	Parabrachial nucleus
PC	Precoeruleus
PCR	Polymerase chain reaction
PeF	Perifornical
PGO	Ponto-geniculo-occipital
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PO	Preoptic area
POMC	Proopiomelanocortin (protein)
PPT and PPTg	Pedunculopontine tegmentum (or tegmental nuclei)
Preamp	Preamplifier
PVN and PVH	Paraventricular nucleus of the hypothalamus
REM	Rapid eye movement
REMS	Rapid eye movement sleep
RMS	Root mean square
RN	Raphe nucleus

SCN	Suprachiasmatic nucleus (of the hypothalamus)
SD	Sleep deprivation
SLD	Sublaterodorsal tegmental nucleus
Sp-D	Sprague-Dawley
SWA	Slow wave activity
SWS	Slow wave sleep
TM	Transmembrane domain
TMN	Tuberomammillary nucleus
VLPO	Ventrolateral preoptic area
VMH	Ventromedial hypothalamus
vPAG	Ventral periaqueductal grey
ZT	Zeitgeber time

X List of Figures, Tables, and Boxes

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XII Curriculum Vitae

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ABSTRACTS AND POSTERS

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