The role of corticotropin-releasing hormone in REM sleep regulation:

A possible mechanism through the cholinergic system

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Maria Letizia Curzi

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1. Gutachter: Prof. Dr. Rainer Landgraf
2. Gutachter: Prof. Dr. Christian Leibold

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“Your real duty is to save your dreams”

-Amedeo Modigliani-

(1884-1920)

Ai miei genitori

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1 Abstract

The neuropeptide corticotropin-releasing hormone (CRH) coordinates neuroendocrine and behavioral responses to stress. Its prolonged hypersecretion produces several signs and symptoms of depression, and is associated with a severe impairment of sleep, in particular reduced sleep intensity, disinhibition of rapid eye movement sleep (REMS), and early morning awakenings. It was recently demonstrated that REMS is upregulated in a conditional mouse model that overexpresses CRH in the forebrain including limbic structures. The results suggest that overexpression of CRH in the forebrain including limbic structures contributes to enhanced REMS, which may apply similarly to the case of depressed patients. However, how limbic CRH affects REMS is still not clear. In general, during REMS, dynamic changes in neurotransmitter activity occur. For example, monoaminergic systems are low, while cholinergic activity becomes high. REMS upregulation seen in depressed patients might be the product of complex interactions between CRH and a neurotransmitter system known to play a role in REMS regulation.

In this thesis the role of CRH in the regulation of REMS was further explored. Polysomnographic recordings combined with microinjections, quantitative microdialysis and immunohistochemistry were used to examine whether overexpressed CRH in the forebrain contributes to REMS enhancement by altering the cholinergic system known to play a role in REMS generation. Since CRH overexpression is also present in the limbic system, the present study mainly focused on the amygdala. This limbic structure is strongly implicated in emotional responses closely related to REMS, although only few studies so far have described its interaction with REMS.

The results show that injection of a muscarinic antagonist into the central nucleus of the amygdala (CeA) decreases upregulated REMS of homozygous forebrain-specific CRH (CRH-COE Cam) overexpressing mice. Furthermore, homozygous CRH-COE Cam mice possess higher extracellular levels of acetylcholine (ACh) in the CeA than their control littermates, whereas spontaneous locomotor activity is comparable in both genotypes. This suggests that higher ACh is not due to an increase in locomotor activity but is reflected by REMS enhancement. These results indicate that CRH

overexpression appears capable of stimulating the cholinergic activity in the amygdala which in turn may lead to upregulated REMS. As seen in depressed patients, this animal model may possess hyper-cholinergic sensitivity that may contribute to REMS disinhibition.

Immunohistochemical studies were carried out to confirm this hypothesis: Activation of CRH receptors by microinjection of CRH into the CeA induced an increase of c-Fos expression in cholinergic structures in the brainstem in normal C57BL/6J mice, suggesting that amygdaloid CRH is able to influence the neuronal activity in REMS regulating structures such as the laterodorsal tegmental nucleus (LDT) and the sublaterodorsal tegmental nucleus (SLD). Further, cholinergic neurons in the LDT become more active in homozygous CRH-COE Cam mice than controls in response to sleep deprivation, when REMS rebound occurs, indicating that in this animal model CRH intensifies the mesopontine cholinergic system, which may at least in part result in upregulated REMS.

This thesis emphasizes that REMS upregulation seen in depressed patients might be the product of complex interactions between CRH and the cholinergic system. Further investigations will need to complete a detailed picture of the underlying mechanism by which CRH influences REMS.

2 Introduction

2.1 What is sleep and why do we need it?

Sleep is a complex behavior characterized by significantly reduced sensory responsiveness, the adoption of a specific posture, and the occupation of a sheltered site. In the physiological sense, sleep is a state characterized by rapid reversibility to the wakeful condition, characteristic changes in the electroencephalogram (EEG), and a compensatory sleep rebound following deprivation of the state. The physiological definition is valid for birds and mammals, but in other animals whose central nervous system (CNS) is not well developed, the behavioral definition is more often used (Tobler, 1995, Zeppelin et al., 2005, Siegel, 2008). An important property of sleep is its spontaneous occurrence with endogenous periodicity that is independent of other corporal needs and environmental signs, including variations in ambient temperature. This distinguishes sleep from hibernation and torpor, both associated with variations in temperature and accessibility of food and water (Zeppelin et al., 2005).

More than 100 species have been studied in order to describe this particular behavior. Among all the studied species, humans, cats, rats, and, more recently, many mouse strains have been the most frequent subjects of sleep research (Zeppelin et al., 2005). Despite intense investigations, it is very surprising that almost 85 years after the EEG discovery by Hans Berger (Berger, 1929) and 75 years after the first sleep research application by Frédéric Bremer (Bremer, 1935), the key function of sleep still remains unclear.

Sleep is frequently viewed as an extremely vulnerable state that endangers the propagation of the species. The fact that sleeping situations are potentially dangerous has led to the assumption that sleep has been conserved in evolution because of its fundamental vital function (Siegel, 2009). As a matter of fact, animals cannot survive without sleep (Rechtschaffen, 1998). Indeed, Rechtschaffen and colleagues demonstrated in a series of experiments that sleep deprivation (SD) produces a serious syndrome including death when rats were sleep deprived more than 11 days (Everson et al., 1989, Rechtschaffen et al., 1989). The significance of

this syndrome caused by interfering with the function of sleep is not entirely clear, but the physiological changes caused by chronic SD suggested that sleep may be necessary for effective thermoregulation (Rechtschaffen et al., 1989). Besides the homeothermal aspect of sleep (Parmeggiani, 2003, Krauchi and Deboer, 2010) other functions have been suggested: energy conservation (Walker and Berger, 1980, Berger and Phillips, 1995), memory consolidation (Stickgold, 2005, Diekelmann and Born, 2010), neuronal plasticity (Tononi and Cirelli, 2006), tissue turnover and immune restoration (Krueger and Obal, 2003).

Taken together, this great variety of theories that attempt to explain the function of sleep indicates that sleep is vitally requisite for us.

2.2 Regulation of sleep and wakefulness

2.2.1 Classification of vigilance states

Vigilance states in rodents are defined similarly as in humans. Thus, based on EEG and electromyogram (EMG) recordings, three distinct vigilance states can be identified in mammals and birds: wakefulness, non-rapid eye movement sleep (NREMS) or slow wave sleep, and paradoxical or rapid eye movement sleep (REMS). In humans, NREMS can be further divided into light (S1-S2) and deep (S3-S4) stages. During the night, NREMS and REMS stages appear cyclically.

Human sleep begins with S1, continues through S2, S3 and S4, and is concluded with REMS. This cycle is repeated every 90 to 110 minutes, four to five times a night (Rechtschaffen, 1968, Carskadon, 2011).

In rodents, the length of a sleep-cycle is only ~10 to 12 minutes. They spend ~50 to 65% of their time asleep per day, while their sleeping phase primarily occurs the light period of the day (80% of the day and 20% of the night are spent asleep). The sleeping phase is not consolidated like in humans, and the periods of NREMS and REMS are interrupted by activity bouts, a phenomenon recognized as polyphasic sleep (Tobler, 1995) (Figure 1).

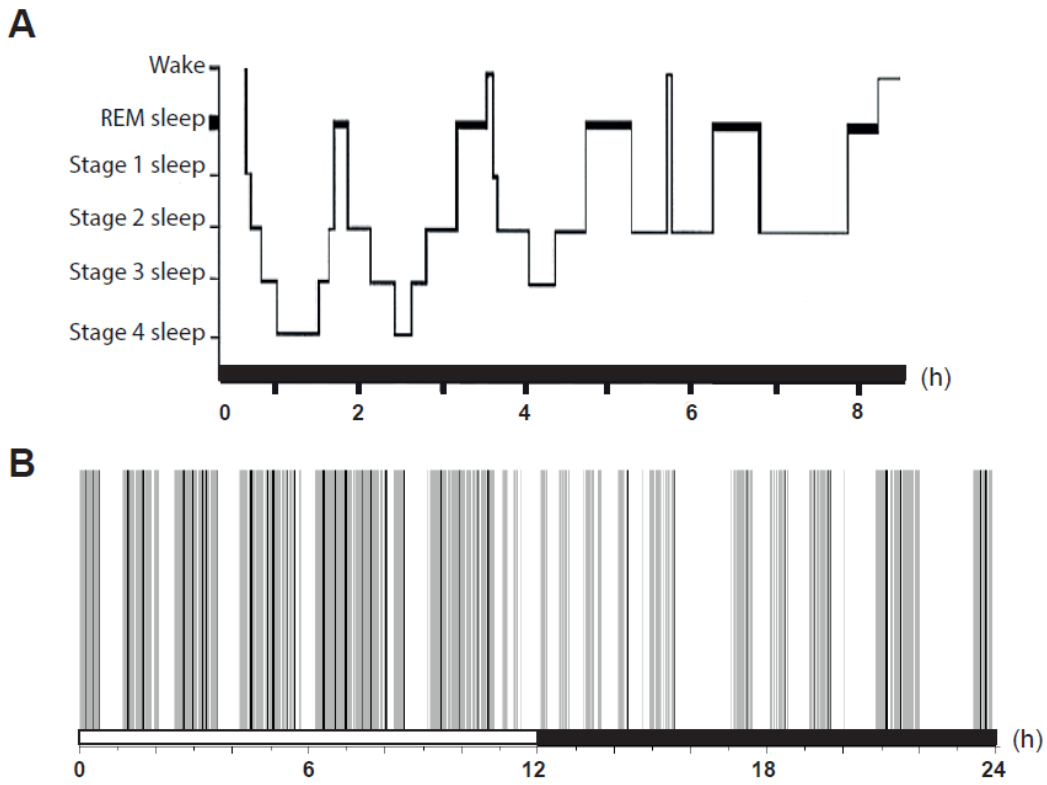

Figure 1: Representative hypnograms from a human (A) and a mouse (B).
Humans have a monophasic sleep pattern. Typically one main sleeping phase occurs with four to six regular cycles of shallow to deep NREMS followed by REMS (y axis indicates vigilance states). Rodents on the other hand display polyphasic sleep. Their sleep cycles are shorter (white areas: wake; grey bars: NREMS; black bars: REMS), occur more frequently, and are distributed throughout 24h, even though the greater amount of sleep takes place in the light (inactive) period. The x axes indicate time in hours (h), the white and the black horizontal bars represent the light and dark period, respectively. Note that the human hypnogram shows only the sleeping dark phase, while the mouse hypnogram exhibits both the inactive light and the active dark period. Human hypnogram adapted from Kamel, 2006; mouse hypnogram, own data, unpublished.

Wakefulness is determined by low-amplitude, fast activity in the EEG and the presence of muscle tone in the EMG. Active exploratory behaviors and attentive wakefulness are dominated by high theta activity (above 7 Hz), nevertheless, the beta (15-30 Hz) and gamma (30-60 Hz) ranges are also present in the waking EEG (Steriade, 2006). In transition to the drowsy state, when the sleep pressure is increasing, the slower EEG frequencies become more prevalent: delta (0.5-4 Hz) and low theta (4-7 Hz) waves occur.

NREMS is defined by high voltage, low frequency synchronized cortical activity in the EEG, and decreased muscle tonus. Normally, three main EEG components are associated with NREMS: slow oscillations (0.5-1 Hz), delta waves (1-5 Hz), and sleep spindles (12-15 Hz). The amount of slow oscillations and delta waves is referred to as slow wave activity (SWA), and also indicates sleep intensity. In human sleep, spindles are present in stage 2 of NREMS (Dijk, 2009), and in rodents they occur shortly before the transition from NREMS to REMS (Vyazovskiy et al., 2004).

REMS was first described nearly 60 years ago (Aserinsky and Kleitman, 1953, Dement, 1958, Jouvet and Michel, 1959). This sleep state is defined by the appearance of fast, theta activity dominant (6-9 Hz), desynchronized, low voltage rhythm in the cortical EEG, rapid eye movements, limb twitching, and complete loss of muscle tone. Since the REMS EEG resembles that of the waking state, REMS has been alternatively named paradoxical sleep. To distinguish these two sleep states, EMG recordings are required (Jouvet and Michel, 1959) (Figure 2).

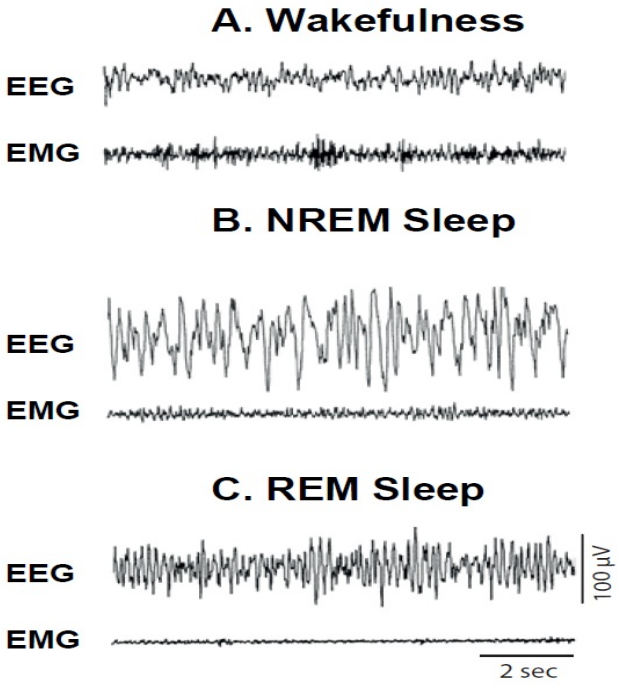

Figure 2: Vigilance state-specific polygraphic recording of rodent sleep

EEG and EMG traces recorded from a mouse during states of wakefulness **(A)**, NREMS **(B)**, and REMS **(C)** Each representative sample consists of a 10 seconds (sec) trance. Adapted from Datta, 2007.

2.2.2 The two process model of sleep regulation

Sleep regulation depends on three different processes: The (1) homeostatic process; (2) circadian, and (3) ultradian process occurring during sleep timing. The timing and structure of sleep are established by the interaction of the homeostatic and the circadian process, as described in the two-process model of sleep regulation (Borbély, 1982, Borbély and Achermann, 1999) (Figure 3).

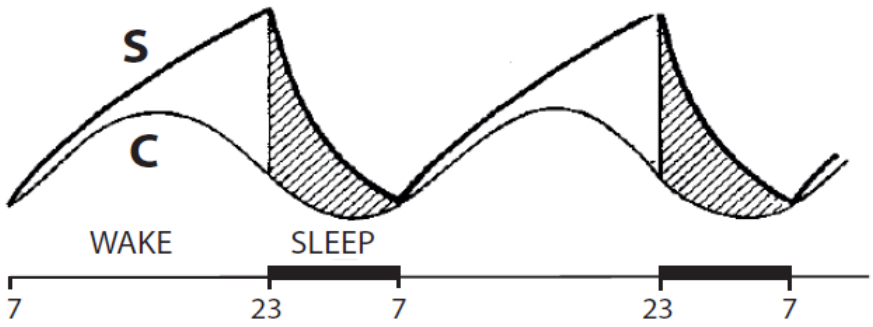

Figure 3: The two process model

Sleep is regulated by an interaction of homeostatic sleep pressure (Process S; upper curve) and the circadian rhythm (Process C; lower curve), leading to alternating periods of wakefulness (white areas) and sleep (black bars). During wake, Process S increases in an exponential way. Sleep is initiated when Process S reaches plateau and Process C declines whereas when sleep is initiated Process S decreases exponentially. Adapted from Borbély and Achermann, 2000.

In this model the homeostatic process (Process S) increases during waking when the sleep demand becomes higher and decreases during sleep. The circadian process (Process C) does not depend directly on previous sleep-wake amount, but affects the timing of sleep according to the intrinsic circadian rhythm of about 24 hours (Takahashi et al., 2008). In humans the circadian rhythm is slightly longer, whereas that of rodents is slightly shorter than 24 hours. Brain lesion studies have shown that the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, is an important brain area that regulates the circadian rhythm (Moore and Eichler, 1972, Edgar et al., 1993).

Process C and Process S work together to control the timing and intensity of sleep. During the active period the circadian signal induces wakefulness, resulting in the

elevation of homeostatic sleep pressure. When the homeostatic sleep propensity reaches a critical level and simultaneously the circadian signal reaches its nadir, sleep will be induced.

2.2.3 Neuronal mechanisms of sleep and wakefulness

At the beginning of the twentieth century, von Economo provided the most important insight on neuronal structures involved in the control of sleep and wake. While investigating the pandemic flu of 1917-1920, he observed patients suffering from excessive sleepiness or extreme insomnia following encephalitis lethargica (Von Economo, 1926). Examination of their brains allowed him to conclude that the anterior hypothalamus contains sleep-inducing centers whereas the posterior hypothalamus contains the wake promoting areas. However, current knowledge of brain circuitry and neurotransmitters that shape the sleep-wake cycle has mostly been generated by neurochemical studies in cats and more recently in rats and mice, the latter being more accessible for electrophysiological/genetic approaches. Fortunately, results in these animals can often be applied to humans since the basic neuronal system implicated in sleep-wake regulation seems to be well conserved throughout evolution (Brown et al., 2008).

Wake regulation

Moruzzi and Magoun were the first to describe the ascending reticular activating system, a brainstem netlike core of neurons that is capable of inducing low-voltage fast EEG activity in the cortex typical for wakefulness (Moruzzi and Magoun, 1949). Studies in the 1970s and 1980s revealed that the wake inducing neurons were not part of the undifferentiated reticular formation but consisted of monoaminergic and cholinergic neurons of specific cell groups (Jones, 2003). The ascending arousal system has two main pathways that project to the cerebral cortex (Starzl et al., 1951, Jones, 2003). The first pathway is relayed in the thalamus and the major input comes from the cholinergic pedunculopontine and laterodorsal tegmental nuclei (PPT and LDT, respectively) (Satoh and Fibiger, 1986, Hallanger et al., 1987). The firing rate of PPT/LDT neurons is high during wake and REMS and the lowest during NREMS, indicating their contribution to cortical activation during wake and REMS (el Mansari et al., 1989, Steriade, 1993). The second pathway extends through the hypothalamus,

bypasses the thalamus and is conveyed to the cerebral cortex by the basal forebrain (Saper, 1985, Saper et al., 2001, Jones, 2003). It includes the serotonergic dorsal and median raphe nuclei (DR/MRN), the noradrenergic locus coeruleus (LC), dopaminergic neurons from the ventral periaqueductal gray (vPAG), and the histaminergic neurons from the tuberomammillary nucleus (TMN) (Dahlstrom and Fuxe, 1964, Panula et al., 1989, Kocsis et al., 2006, Lu et al., 2006a). In general, monoaminergic neurons fire most actively during wake, fire less active during NREMS, and stop firing during REMS (Aston-Jones and Bloom, 1981, Fornal et al., 1985, Steininger et al., 1999). Other important subparts in the wake regulatory systems are a group of orexinergic neurons found in the lateral hypothalamus (LH) (de Lecea et al., 1998, Peyron et al., 1998), which fires only during wake (Lee et al., 2005), and a cluster of cholinergic and GABAergic neurons in the basal forebrain (Gritti et al., 1997).

REM sleep regulation

The circuitry responsible for the generation of REMS is very different and intricate from that generating wake or NREMS. Furthermore after more than 50 years following the discovery of this unique state, the exact identification of REMS regulatory brain structures and their respective neurotransmitters is still under debate. One of the most influential studies which could enable the allocation of important REMS promoting centers to the lower brainstem, was a transection study conducted on cats in 1962 (Jouvet, 1962). Afterwards, pharmacological experiments suggested that the cholinergic and the monoaminergic systems interact in the control of REMS generation (Karczmar et al., 1970).

The early studies by Jouvet and others guided the development of McCarley and Hobson's "reciprocal interaction" model in 1975 (McCarley and Hobson, 1975) (Figure 4), which has since been the most widely accepted explanation for REMS regulation (Pace-Schott and Hobson, 2002). Their model described an interplay among the monoaminergic (LC and DR) and cholinergic LDT and pedunculopontine PPT, and medial pontine reticular formation) neurons at the synaptic level responsible for the rhythmic cycling of NREMS and REMS (McCarley and Hobson, 1975, Pace-Schott and Hobson, 2002) The essence of this model is represented by a group of cholinergic REM-on neurons in the LDT and PPT of the brainstem. Activated cholinergic neurons are inhibited by REM-off monoaminergic neurons located in the

serotonergic DR and noradrenergic LC during other vigilance states, either through direct projections or excitation of inhibitory GABAergic interneurons (Jones and Yang, 1985, Vertes and Kocsis, 1994, Berridge and Waterhouse, 2003). As REM-off neurons reduce their firing during NREMS, REM-on neurons are disinhibited and REMS is generated (Brown, 2008). The REMS state is stabilized reciprocally through excitatory interactions between cholinergic neurons in the LDT/PPT and glutamatergic effector neurons in the reticular formation that are responsible for generating REMS-specific features such as muscle atonia, rapid eye movements and cortical activation (Mitani et al., 1988, Semba, 1993, Brown, 2008). Further, there is also evidence that REM-on neurons from LDT/PPT might send excitatory projections to LC, and DR neurons so that monoaminergic REM-off neurons gradually become more active when the REM state extends (McCarley and Hobson, 1975, Aston-Jones and Bloom, 1981, Sakai et al., 1983, Berridge and Waterhouse, 2003). A more sophisticated version incorporates an intrinsic pacemaker function of neurons in the LC which might be responsible for monoaminergic REM-off cell activation. Furthermore, GABAergic neurons are hypothesized to control both the monoaminergic REM-off and the glutamatergic REM-on neurons (Datta and Maclean, 2007). In turn, GABAergic neurons may be under the control of LDT/PPT neurons (McCarley and Massaquoi, 1986, McCarley, 2004).

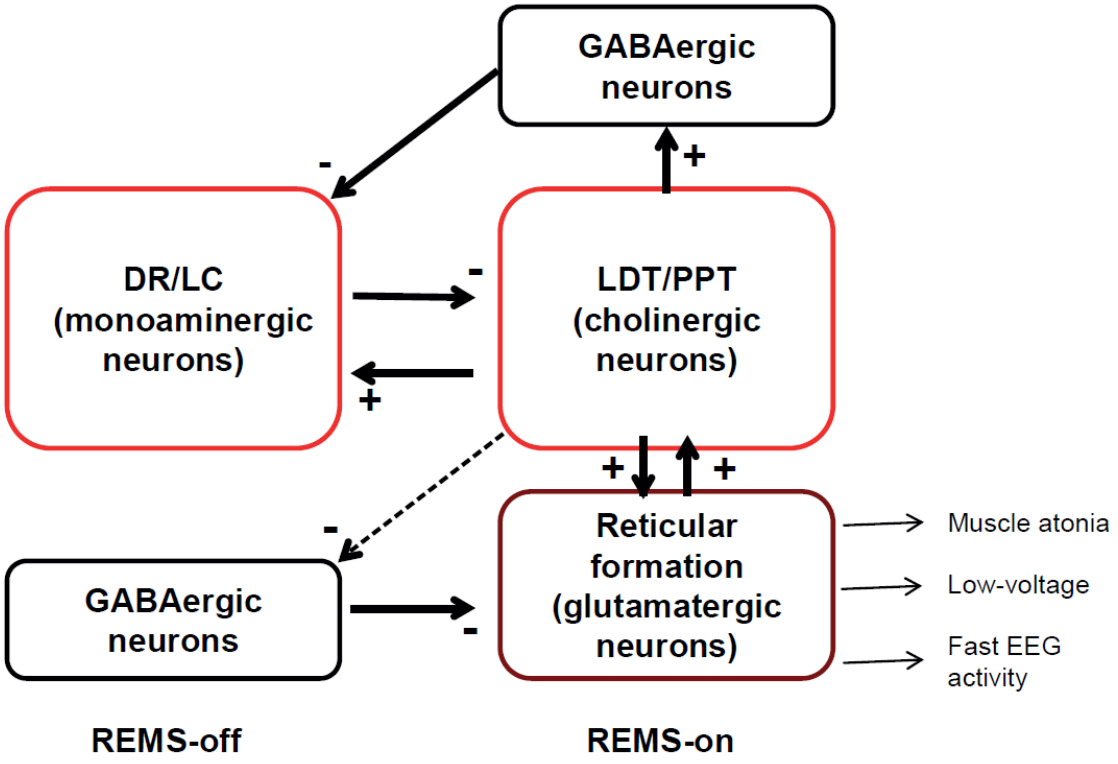

Figure 4: Schematic representation of the reciprocal interaction model of REMS regulation
REM-on neurons in the laterodorsal (*LDT*) and pedunculopontine (*PPT*) nuclei are inhibited by REM-off aminergic neurons in the serotonergic dorsal raphe (*DR*) and the noradrenergic locus coeruleus (*LC*) during NREMS and wake. REMS is stabilized by reciprocally excitatory interactions between the cholinergic and glutamatergic effector neurons in the reticular formation. REMS is ended by renewed activity in aminergic neurons, produced by excitatory projections from the cholinergic neurons. GABAergic neurons control both the monoaminergic REM-off and the glutamatergic REM-on neurons. Furthermore, GABAergic neurons may be in turn under the control of LDT/PPT neurons (dotted arrows). Adapted from Brown, 2008.

In principal, neuropharmacological and electrophysiological studies have strongly supported the reciprocal interaction model. Nevertheless, more recent incongruities between this cholinergic-monoaminergic model and new experimental data encouraged Lu and colleagues to perform a series of experiments that delineate an alternative brainstem regulation model for REMS (i.e. the flip flop switch) (Lu et al., 2006b, Fuller et al., 2007) (Figure 5). Their work has revealed an important role for non-cholinergic and non-monoaminergic REM-on and REM-off GABAergic cell populations in areas within the brainstem, whereas the cholinergic and monoaminergic cell groups are described as REMS modulator and not generators. Specifically, three REM-on groups with specific projections and neurotransmitters

have been postulated (Lu et al., 2006b). The first REM-on group is located in the sublaterodorsal tegmental nucleus (SLD) (Sakai et al., 2001, Boissard et al., 2002) and sends glutamatergic projections to the spinal cord and GABAergic projections to REM-off neurons in the vPAG and the lateral pontine tegmentum (LPT) (Lu et al., 2006b). The second and third REM-on groups are contained in the precoeruleus (PC) and parabrachial nucleus (PB), respectively, with glutamatergic projections to the basal forebrain and medial septum (Lu et al., 2006b). In this alternative REM switching circuitry model, GABAergic REM-on neurons in the SLD inhibit GABAergic REM-off neurons in the vPAG/LPT and LPT, whereas GABAergic REM-off neurons in turn send inhibitory signals to all three REM-on groups (Lu et al., 2006b, Fuller et al., 2007).

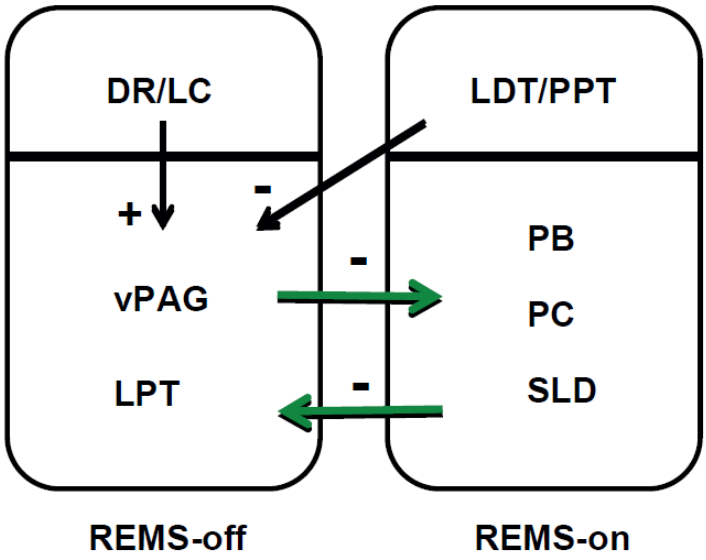

Figure 5: Schematic representation of the flip-flop switch model for REMS regulation
REM-off neurons are located in the ventral periaqueductal grey (*vPAG*) and the lateral pontine tegmentum (*LPT*). REM-on neurons can be found in the sublaterodorsal tegmental nucleus (*SLD*) as well as the precoeruleus (*PC*) and parabrachial (*PB*) nucleus. According to the model, GABAergic REM-off neurons send inhibitory signals to all three REM-on groups. On the other hand GABAergic REMS-on SLD neurons in turn inactivate the REM-off neurons. Cholinergic neurons in the laterodorsal tegmental (*LDT*) and in the pedunculopontine (*PPT*) nuclei together with the serotonergic dorsal raphe (*DR*) and the noradrenergic locus coeruleus (*LC*), play a modulatory role by inhibiting or activating REM-off cells, respectively. Adapted from Lu et al., 2006.

A further significant current research extends the study of REMS regulation mechanisms rostrally from the brainstem to the forebrain structures such as the

amygdala in the limbic system. Recent findings demonstrated that the amygdala has reciprocal connections with brainstem regions involved in REMS initiation (Pace-Schott, 2002). Studies in cats showed a promotion of REMS in response to a cholinergic or electric stimulation of the central nucleus of the amygdala (CeA) (Smith and Miskiman, 1975, Calvo et al., 1996) and a spontaneous increase in the discharge rate of CeA neurons during REMS (Frysinger et al., 1988). Furthermore, inactivating the CeA with muscimol or tetrodotoxin (TTX) in rats was able to produce a significant decrease in REMS (Martin and Ghez, 1999, Sanford et al., 2002). An association between amygdala activation and REMS was also proposed by fMRI studies demonstrating that the amygdala is activated during this state in humans (Maquet et al., 1996). These findings prove that the amygdala might play a role in REMS regulation via modifying brainstem activity, thus in turn influencing REMS (Pace-Schott, 2002).

Interestingly, another aspect of REMS is a commonality that might share a neurobiological mechanism with depressive phenomena, hypothesized by McCarley and supported by clinical data (McCarley, 1982). First, the brainstem aminergic system is able to suppress both REMS and depressive symptoms whereas the cholinergic system promotes both REMS and depression (Janowsky et al., 1980, Risch et al., 1980, Silberman et al., 1980). Furthermore as in REMS regulation, the control of depressive phenomena involves a balance between the monoaminergic and cholinergic systems, rather than absolute activity levels. As proposed by McCarley and Hobson in the reciprocal interaction model, REMS occurs when cholinergic activity becomes dominant with the gradual inhibition of the monoaminergic nuclei (LC and RN) (McCarley and Hobson, 1975). Therefore, weakened monoaminergic inhibition in depression results in a faster discharge from inhibition of the REMS-promoting cholinergic neurons, initiating a cycle of REMS (decreased REMS latency) with stronger REM activity, i.e., increased REM density (McCarley, 1982).

NREM sleep regulation

Unlike the intricate regulation of REMS, NREMS is initiated in a different but relatively simple pathway by the activation of two groups of inhibitory GABAergic neurons located in the ventrolateral preoptic area (VLPO) and the median preoptic area (MnPO) of the preoptic anterior hypothalamus (POAH) (Sherin et al., 1996, Suntsova

et al., 2002, Gong et al., 2004, Sakai, 2011). Both the sleep-inducing VLPO and MnPO send inhibitory GABAergic projections to the monoaminergic wake promoting brain areas including the orexinergic LH. Thus, by inhibiting the wake regulatory systems, the VLPO and MnPO can promote NREMS. Furthermore, it has been demonstrated that the presence of GABAergic interneurons and axons in the brainstem areas might inhibit wake-promoting neurons (Maloney et al., 1999, 2000).

2.2.4 Humoral sleep-wake regulation

The complexity of sleep and wake regulation is further increased by the actions of neuromodulators that compose humoral mechanisms. Modulators such as specific inflammatory factors, hormones, neuropeptides, and nucleosides are able to influence neuronal activities involved in sleep-wake regulation and thus affect sleep-wake changes. The hypothesis that sleep is in part regulated by humoral factors was first proposed by Aristotle (Krueger et al., 1998) whereas the modern experimental pursuit began with Ishimori (Ishimori, 1909) and Piéron (Piéron, 1913). Both demonstrated the presence of a sleep promoting substance, named “hypnotoxin” in the cerebrospinal fluid (CSF) of sleep-deprived dogs. Afterwards several research groups pursued similar approaches to identify those substances (Pappenheimer et al., 1975, Inoué, 1989). Nowadays it is known that many neuromodulators can affect sleep, although persuasive evidence for the involvement in physiological sleep regulation is limited to only small number of these modulators. The list of sleep-promoting substances includes cytokines, e.g. interleukin-1 (Krueger et al., 1984) and tumor necrosis factor (Fang et al., 1997), prostaglandin D_2 (Hayaishi, 1988), adenosine (Porkka-Heiskanen, 1997), and hormones like prolactin (Roky et al., 1995), vasoactive intestinal peptide (Bourgin et al., 1997), galanin (Murck et al., 2004), ghrelin (Weikel et al., 2003), neuropeptide Y (Antonijevic et al., 2000) and growth hormone-releasing hormone (Steiger et al., 1992). Contrarily, other hormones such as corticotropin-releasing hormone (CRH) (Holsboer et al., 1988), vasopressin (Arnauld et al., 1989), and somatostatin (Ziegenbein et al., 2004) seem to impair sleep. Future studies are needed to clarify how these substances interact with various neural systems and their neurotransmitters, where they act to affect sleep, and what cell types are involved.

2.3 The cholinergic system in the central nervous system

2.3.1 Acetylcholine

Acetylcholine (ACh) is an essential neurotransmitter which plays a crucial role in synaptic transmission in both the peripheral and central nervous system (CNS) (Webster, 2001, Halbach, 2002). ACh was discovered as the first neutransmitter. In 1914 Dale could show that esters of choline produced physiological effects (Dale, 1914). Later in 1921 Loewi demonstrated that stimulation of the vagus liberated the release of a chemical substance (Loewi, 1921). Five years later the chemical substance was confirmed to be choline ester and accordingly identified as ACh (Loewi and Navratil, 1926).

The process of synthesis, storage, and release of ACh requires different specific enzymes (Figure 6): ACh is synthesized in a reaction catalyzed by the enzyme choline acetyltransferase (ChAT) in the cytosol of nerve terminals, using mitochondrial acetyl-coenzyme A supplied by glucose metabolism and choline derived from phosphatidylcholine and dietary sources (Tucek, 1966, Halbach, 2002). Following synthesis, ACh is taken up and subsequently stored in synaptic vesicles via the vesicular ACh transporter (VAChT) (Weihe et al., 1996, Arvidsson et al., 1997, Amenta and Tayebati, 2008). If an axon potential reaches the cholinergic axon terminal, the synaptic vesicles attach to the presynaptic membrane and release ACh into the synaptic cleft via exocytosis. From the synaptic cleft, ACh diffuses to the postsynaptic site and interacts with respective receptors (nicotinic or muscarinic).

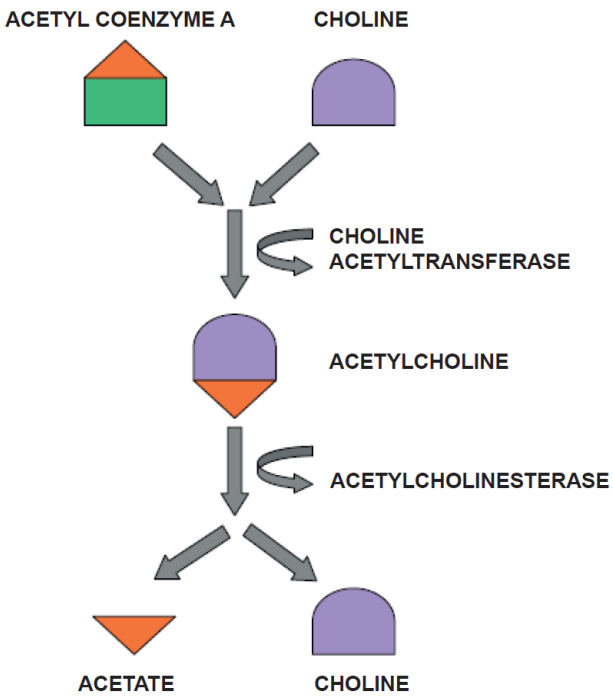

Figure 6: Bionsynthesis and degradation of acetylcholine.
Acetylcholine is synthesized by the enzyme choline acetyltransferase from the compounds choline and acetyl-coenzyme A. The enzyme acetylcholinesterase converts acetylcholine into the inactive metabolites choline and acetate. Adapted from Nirogi et al., 2009.

Upon release, ACh is hydrolyzed by acetylcholinesterase (AChE) into choline and acetate. Liberated choline is transported back to the presynaptic terminal by a sodium-dependent, high affinity active transport system, and reutilized in ACh synthesis (Suszkiw and Pilar, 1976, Ducis and Whittaker, 1985). The remaining choline can be catabolised or incorporated into phospholipids, which can serve again as a source of choline (Amenta and Tayebati, 2008, Nirogi et al., 2010).

2.3.2 Cholinergic receptors

Cholinergic receptors, also known as ACh receptors (AChRs), consist of two groups: the muscarinic ACh receptors (mAChRs) and nicotinic ACh receptors (nAChRs). They can be classified according to the binding activity by natural alkaloids, i.e., nicotine and muscarine, to mimic the effects of ACh as a neurotransmitter. This classification introduced originally in 1914 by Dale is still valid (Dale, 1914), even

though several subtypes of nicotinic and muscarinic receptors have been described in the meantime.

The muscarinic receptors are monomers consisting of 440-540 amino acids folded into seven transmembrane-spanning domains, the N-terminus on the extracellular side and the C-terminus on the intracellular side (Halbach, 2002). The muscarinic receptors are coupled to G proteins which modulate a large group of effector responses including adenylate cyclase attenuation, guanylate cyclase stimulation, Ca^{2+} channel activity, K^{+} channel activity and phosphatidyl inositol turnover (Kerlavage et al., 1987). By the use of selective radioactively labeled agonist and antagonist substances, five subtypes of muscarinic receptors have been identified, named M_1-M_5 (Peralta et al., 1988). Muscarinic receptor subtypes M_1, M_3 and M_5 are coupled to the G_q proteins, which activate several ion channels and phospholipases (A2, C and D), ultimately leading to the activation of different second messenger systems. Muscarinic receptor subtypes M_2 and M_4 are coupled to G_i proteins. Activation of these subtypes reduces the levels of cyclic adenosine monophosphate (cAMP) through the inhibition of adenylate cyclase (Felder, 1995, Halbach, 2002). Although the muscarinic receptor subtypes are distributed throughout the entire brain, their proportions vary in different areas. For example, RNA *in situ* hybridization studies revealed that messenger RNA (mRNA) of M_1 is formed in the cerebral cortex, limbic area and in the striatum. By contrast, mRNA of M_2 is more abundant in the basal forebrain, midbrain, medulla, pons region and cerebellum. mRNA of M_3 is, similarly to M_1, abundant in the cortex and hippocampus but not in the striatum, while M_4 expression is highest in the striatum but low in the cortex and hippocampus. Only small amounts of the M_5 subtype have been discovered, and its distribution in the CNS is not fully understood (Levey et al., 1991, Hersch et al., 1994, Wess, 1996, Webster, 2001, Halbach, 2002). Muscarinic receptors are activated by muscarine and are blocked by atropine and scopolamine. Further, amongst pharmacological agonists are carbachol, pilocarpine, arecholine and oxoremorine, while pirenzepine and telenzepine, exert antagonistic effects (Halbach, 2002, Tripathi, 2004).

The nicotinic receptors are part of the ligand-gated ion channel superfamily and, in contrast to the muscarinic receptors, no second messengers are involved in the signal transduction. The receptor is composed of four distinct protein subunits (α, β, δ and γ) which form the ion channel (Halbach, 2002). In the CNS the nicotinic receptor

subunits can be composed of a combination of different heterodimers: α (2–7) and β (2–4). Homomeric assembled receptors are also found: α7, α8 and α9 (Karlin, 2002, Picciotto et al., 2012). To form a functional receptor, numerous combinations of subunits are possible, but so far the α4β2 heteromer and the α7 homomer showed the highest affinity for ACh (Zoli et al., 1995, Webster, 2001, Tripathi, 2004, Ferreira et al., 2008). In general, nicotinergic signaling is not nearly as prominent in the CNS as muscarinergic signaling. For example, some areas such as the limbic system seem to utilize only muscarinergic receptors. Nevertheless, they are present in the cerebral cortex, the hippocampus, the hypothalamus, the thalamus, the superior colliculus, and in some cholinergic nuclei of the brain stem and forebrain (Halbach, 2002). Nicotinic receptors can be activated by nicotine and inhibited by curare. Additional pharmacological agonists are carbachol, butyrylcholine and tetramethylammonium, whereas hexamethonium, dihydri- β-erythroidine, mecamylamine and bungarotoxin are antagonists (Halbach, 2002, Tripathi, 2004).

2.3.3 Cholinergic projections

The two major cholinergic groups of projecting neurons, found in the basal forebrain and in the brainstem, have been identified by the use of immunohistochemical staining for ChAT. This specific enzyme is located in neurons that synthetize ACh for synaptic transmission, and therefore considered “cholinergic” (Mesulam et al., 1983, Woolf, 1991, Butcher, 1995). The first report describing cholinergic neurons and their projections was published by Lewis and Shute in 1967 (Lewis and Shute, 1967, Lewis et al., 1967, Shute and Lewis, 1967). Sixteen years later, Mesulam and coworkers established a nomenclature to distinguish different groups of cholinergic projecting neurons which is still widely used today (Mesulam et al., 1983).

Based upon Mesulam’s nomenclature, the cholinergic system is divided into six major groups of projecting neurons (Ch1-Ch6; Figure 7).

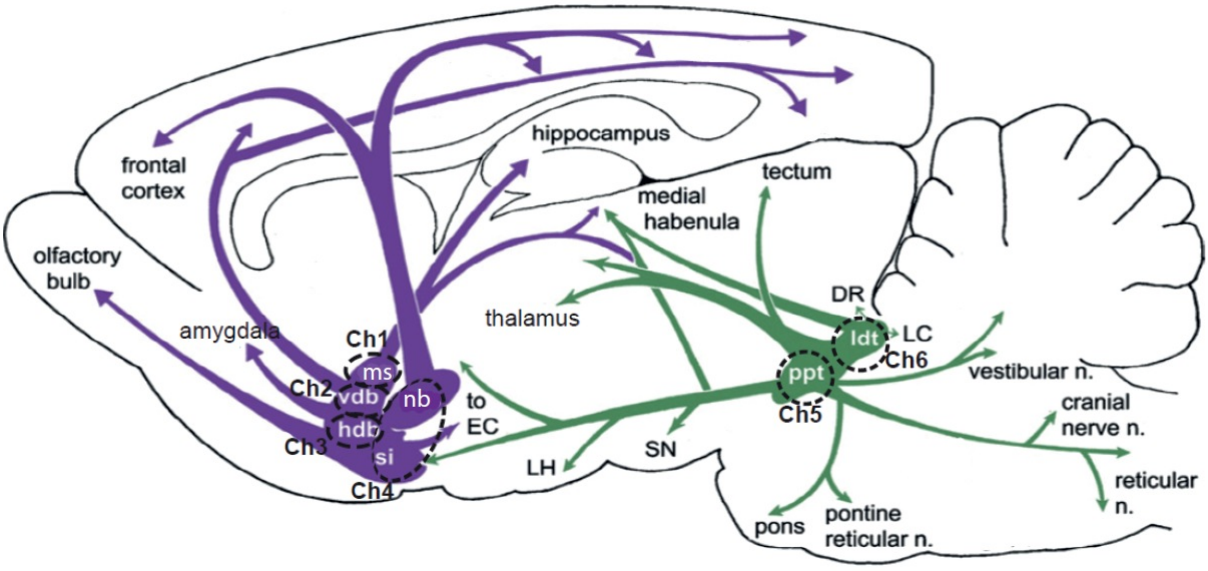

Figure 7: Schematic representation of cholinergic neurons and their projections.
Abbreviations: *Ch*, cholinergic group of neurons; *DR*, dorsal raphe; *EC*, entorhinal cortex; *hdb*, horizontal diagonal band nucleus; *LC*; locus coeruleus; *ldt*, laterodorsal tegmental nucleus; *LH*, lateral hypothalamus; *ms*, medial septal nucleus; *nb*, nucleus basalis; ppt, pedunculopontine nucleus; *si*, substantia innominata; *SN*, substantia nigra; *vdb*, vertical diagonal band nucleus. Adapted from Woolf, 2011.

The Ch1-Ch4 groups of cholinergic cells are located in the basal forebrain and innervate the entire cerebral cortex. The medial septum (MS) and the vertical limb of the diagonal band of Broca (VDB) (Ch1 and Ch2, respectively) are located in the most rostral part of the basal forebrain and send their projections mainly to the hippocampus, including CA1-CA4 and the dentate gyrus (Mesulam et al., 1983, Woolf and Butcher, 2011). Group Ch3 includes cholinergic neurons located in the horizontal limb of the diagonal band (HDB) and provides the major source of cholinergic projections to the olfactory bulb (Zaborszky et al., 1986). The last cholinergic group in the basal forebrain is referred to as Ch4, and it comprises neurons located in the magnocellular preoptic area, the nucleus basalis (NB) and the substantia innominata (SI). These neurons project to the amygdala and to the cerebral cortex (Mesulam et al., 1983, Woolf et al., 1984, Woolf, 1991).

The Ch5-Ch6 groups of cholinergic projecting neurons are located in the brainstem. Members of groups Ch5 and Ch6 are situated in the PPT and in the LDT,

respectively. These nuclei have ascending projections to the hypothalamus, thalamus, basal forebrain and medial prefrontal cortex as well as descending projections to the pons, the nucleus vestibularis, the LC and the DR (Satoh and Fibiger, 1986, Steckler et al., 1994).

2.3.4 Involvement of cholinergic centers in sleep-wake regulation

In the context of control of sleep and waking the cholinergic system is well recognized to play a primary role in generating the brain-activated states of wake and REMS (Jones, 2005, Brown, 2008, Lydic, 2008, Watson et al., 2010). Specifically cholinergic projections from neurons located in the brainstem (LDT/PPT) and the basal forebrain are known to promote the cortically activated states of wake and REMS (Lydic, 2008).

Cholinergic LDT/PPT neurons send their major projection to the thalamus, which in turn stimulate the cerebral cortex (Mesulam et al., 1983, Steriade et al., 1990, McCormick, 1992, Jones, 1995). ACh release in these areas has been shown to be maximal during wakefulness and REMS (Jasper and Tessier, 1971, Williams et al., 1994, Leonard and Lydic, 1997). Similarly, single unit recording studies indicate that the activity of cholinergic neurons in the LDT/PPT is at their highest rates during wake and REMS (el Mansari et al., 1989, Steriade et al., 1990). Moreover c-Fos expression, which reflects neural activity, occurs in cholinergic LDT/PPT neurons following REMS rebound after SD (Maloney et al., 1999). Another important LDT/PPT projection acts on the brainstem reticular formation (Greene et al., 1989, Jones, 2005) through muscarinic receptors. Specifically, the M2 and M3 subtypes came out to be the most important ones in the reticular formation (Buckley et al., 1988, Baghdoyan, 1997). Furthermore, pharmacological and genetic studies revealed that these are the major subtypes responsible for REMS regulation (Datta et al., 1993, Sakai and Onoe, 1997, Baghdoyan and Lydic, 1999, Marks and Birabil, 2000, Goutagny et al., 2005). Several studies have performed injections of cholinergic agonists to the reticular formation, demonstrating that cholinergic input into the reticular formation generates REMS (Mitler and Dement, 1974, Sitaram et al., 1976, Hobson et al., 1983, Baghdoyan et al., 1984, Vanni-Mercier et al., 1989, Yamamoto et al., 1990). When LDT/PPT is stimulated electrically, ACh release

increases in the reticular formation (Lydic and Baghdoyan, 1993) and REMS is enhanced (Thakkar et al., 1996). Moreover the release of ACh in the reticular formation is higher during REMS than the other behavioral states (Kodama et al., 1990, Leonard and Lydic, 1997). Based on these studies it is evident that cholinergic projections from the LDT/PPT to the reticular formation induce REMS (Watson et al., 2010).

The basal forebrain cholinergic neurons project throughout the entire cerebral cortex and to the hippocampus (Mesulam et al., 1983). ACh release from the hippocampus has been shown to be maximal during both wake and REMS (Marrosu et al., 1995). Furthermore, ACh seems to act in the cortex mostly through muscarinic receptors (McCormick, 1992, Jones, 2004). A microdialysis study showed that ACh release in the basal forebrain is high during REMS, low during quiet wake, and lowest during NREMS (Vazquez and Baghdoyan, 2001). Similarly, cortical ACh release is enhanced during REMS and wake as compared to NREMS (Marrosu et al., 1995, Materi et al., 2000). Taken together, these studies support that cholinergic projections from the basal forebrain can induce cortical activation during wake and REMS (Watson et al., 2010).

2.4 The hypothalamic-pituitary-adrenocortical (HPA) axis

One of the most important requirements for a living organism is its capacity to maintain a dynamic equilibrium, or homeostasis. The concept of homeostasis was first introduced by Cannon in 1929. He emphasized the importance of all physiological processes in order to maintain such equilibrium operated by the organism (Cannon, 1929). In the classical idea of stress, this equilibrium is constantly challenged by specific physical and psychological adverse stimuli, termed “stressors” (Selye, 1936, Chrousos and Gold, 1992, de Kloet et al., 2005). Thus, stress can be defined as an actual disruption or an anticipated threatened homeostasis (Charmandari et al., 2005, Chrousos, 2009, Ulrich-Lai and Herman, 2009). The responses to stress intend to adjust physiological integrity by involving two major, highly conserved systems: the autonomic nervous system (ANS) and the HPA axis. The ANS activation represents the classical “fight or flight” response and provides an

immediate and short-term response, whereas the HPA axis ensures a long-lasting and amplified response. Activation of these two systems provide complementary actions in the body, including energy mobilization and increased blood pressure, heart rate and cardiovascular tone (Ulrich-Lai and Herman, 2009).

The HPA axis is an elaborate ensemble of interactions between the hypothalamus, the pituitary and the adrenal glands (Figure 8). Hypophysiotrophic neurons in the medial parvocellular subdivisions of the paraventricular nucleus (PVN) of the hypothalamus synthesize corticotropin-releasing hormone (CRH) and arginin vasopressin (AVP) (Landgraf, 2006). CRH is then transported axonally to the median eminence and released into the hypophyseal portal blood (Antoni, 1986, Arborelius et al., 1999). When CRH reaches the anterior pituitary, it binds to CRH receptor type 1 on the corticotrophs and stimulates the expression of the precursor polypeptide pro-opiomelanocortin (POMC) and subsequently the release of the POMC-derived peptide, adrenocorticotropin (ACTH), into the blood circulation (Arborelius et al., 1999, Engelmann et al., 2004). Moreover, AVP is a strong synergistic factor with CRH in potentiating ACTH release; however, AVP possesses little ACTH-releasing activity alone. Thus, CRH is normally considered as the major ACTH stimulator (Chang and Opp, 2001, Herman et al., 2002, Tsigos and Chrousos, 2002). ACTH then triggers the synthesis and the secretion of glucocorticoids: corticosterone in rodents and cortisol in primates (de Kloet et al., 1998) from the adrenal cortex, which operate as the last effectors of the HPA axis (Arborelius et al., 1999).

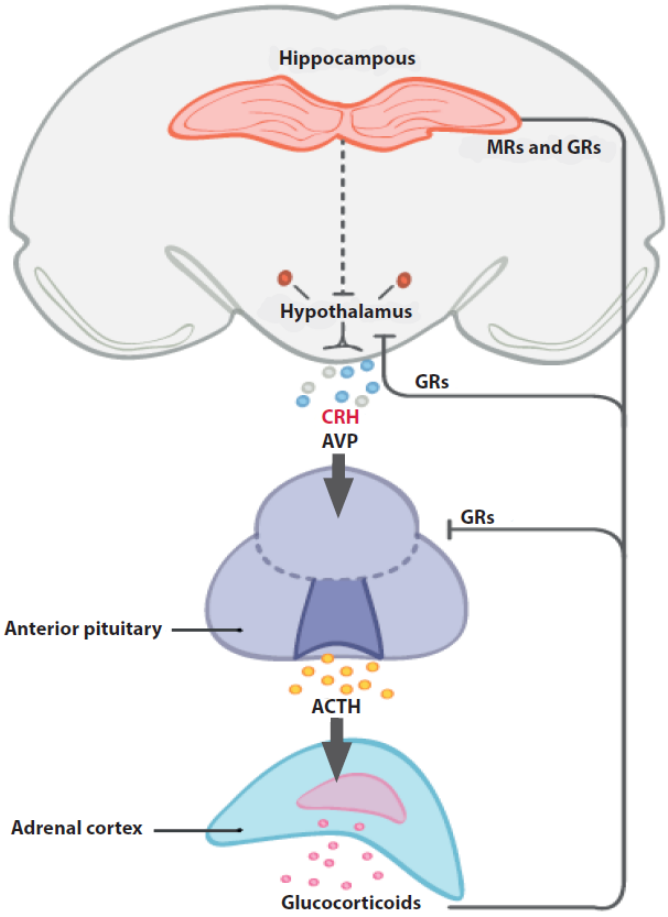

Figure 8: HPA axis.

Activation of the HPA axis leads to CRH and AVP production in the paraventricular nucleus of the hypothalamus. These hormones are released into the blood circulation, leading to secretion of ACTH from the anterior pituitary. ACTH stimulates the synthesis and release of glucocorticoids from the adrenal cortex into the blood. Regulatory control over the HPA axis is mediated via a negative feedback by glucocorticoids acting on GR and MR receptors at the level of the pituitary as well as from the anterior hypothalamus and the hippocampus. *ACTH*: adrenocorticotropin; *AVP*: arginine vasopressin; *CRH*: corticotropin-releasing hormone; *GR*: glucocorticoid receptor; *MR*: mineralocorticoid receptor. Adapted from Schloesser, 2012.

In nonstressful situations, CRH is released in a circadian, pulsatile fashion from the parvocellular cells of the PVN (Engler et al., 1989, Tsigos and Chrousos, 2002, Buckley and Schatzberg, 2005). In diurnal species, the amplitude of the CRH pulses increases early in the morning and becomes low towards the evening before the resting period starts (Horrocks et al., 1990). On the contrary, rats (nocturnal animals) show an opposite pattern: CRH levels rise throughout the nocturnal active period, drop in the morning and decrease during the daytime resting period (Watts et al., 2004). During stressful events, the amplitude of the CRH pulsation in the hypophyseal portal blood markedly increases, leading to an increase of ACTH and glucocorticoid secretory episodes (Tsigos and Chrousos, 2002).

The effects of glococorticoids are mediated via two types of receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reul and de Kloet, 1985, de Kloet et al., 1998, de Kloet et al., 2008). The distribution of MR receptors is mainly restricted to the limbic structures while GR are found throughout the brain (de Kloet, 1991, Arborelius et al., 1999). Glucocorticoids operate in a negative feedback to turn off neuroendocrine responses at two levels in the hypothalamus and the pituitary gland. This suppresses the synthesis and release of CRH and AVP, as well as the POMC-derived peptides in the pituitary (Gulyas et al., 1995, Chang and Opp, 2001, Papadimitriou and Priftis, 2009).

The HPA system is controlled by a various number of stress-sensitive brain regions located in the brainstem and forebrain limbic areas, which are able to send inhibitory or excitatory projections to neurons of the PVN. (Herman et al., 2003, Ulrich-Lai and Herman, 2009). The PVN receives a substantial stress-excitatory input from the nucleus of the solitary tract (Swanson and Kuypers, 1980, Cunningham and Sawchenko, 1988) as well as the DR, the TMN (Ulrich-Lai and Herman, 2009), and the anteroventral division of the bed nucleus of stria terminalis (Gray et al., 1993, Choi, 2007). Additional excitatory drive originates from the medial and basolateral amygdala (Canteras et al., 1995, Cullinan et al., 1996, Dayas et al., 2001). Activation of the PVN is inhibited by many hypothalamic circuits such as the medial preoptic area, the dorsomedial hypothalamus and local neurons in the peri-PVN (Herman et al., 2003, Cullinan et al., 2008). Further inhibitory input originates in forebrain limbic areas such as the hippocampus (Jacobson and Sapolsky, 1991, Herman et al., 2003), the medial prefrontal cortex (Diorio et al., 1993, Figueiredo et al., 2003, Gerrits, 2003),

the lateral septum (Risold and Swanson, 1996) and the posterior regions of the bed nucleus of stria terminalis (Cullinan et al., 1993). In general, many neurotransmitters are implicated in the regulation of CRH release. Glutamate, ACh, serotonin, noradrenalin and histamine stimulate the activation of the HPA axis, whereas GABA inhibits it (Decavel and Van Den Pol, 1990, Cole and Sawchenko, 2002, Majzoub, 2006).

2.5 The corticotropin releasing hormone (CRH)

2.5.1 General aspects and distribution

CRH is regarded as the major activator of the HPA axis and is also known as corticotropin-releasing factor (CRF) or corticoliberin. Besides controlling the HPA axis during baseline and under stress, CRH also acts as neurotransmitter in the brain, where it modulates for example anxiety-related behavior, the sleep-wake cycle, learning and memory as well as locomotor activity. It was first described by Guillemin and Rosenberg in 1955. They proved the presence of a hypothalamic factor, which was able to stimulate the secretion of ACTH from anterior pituitary cells in vitro (Guillemin, 1955). The chemical identification of CRH remained indefinable until 1981, when Vale and colleagues succeeded to isolate and characterize a 41 amino acid hypothalamic ovine CRH (Vale et al., 1981). The sequence of CRH has been identified in many other species including humans, rats, pigs, goats and cows (Dunn and Berridge, 1990). In all species, the primary protein structure of CRH is very much conserved in humans, rats and mice, differing from ovine CRH only by seven amino acids (De Souza, 2002, Halbach, 2002). In addition to original CRH, two nonmammalian CRH-related analogues have been identified in teleost fishes and frogs, named urotensin and sauvagine, respectively (Montecucchi and Henschen, 1981, Lederis et al., 1982). Furthermore, there exist three mammalian CRH peptide analogues called Urocortin I, II and III, which have been demonstrated to share a high sequence homology with CRH (Vaughan et al., 1995, Donaldson et al., 1996).

Through immunohistochemical analysis, radioimmunoassay and mRNA expression studies, CRH was found to be widely distributed within the CNS (Swanson et al., 1983, Sakanaka et al., 1987, Sawchenko, 1990) (Figure 9).

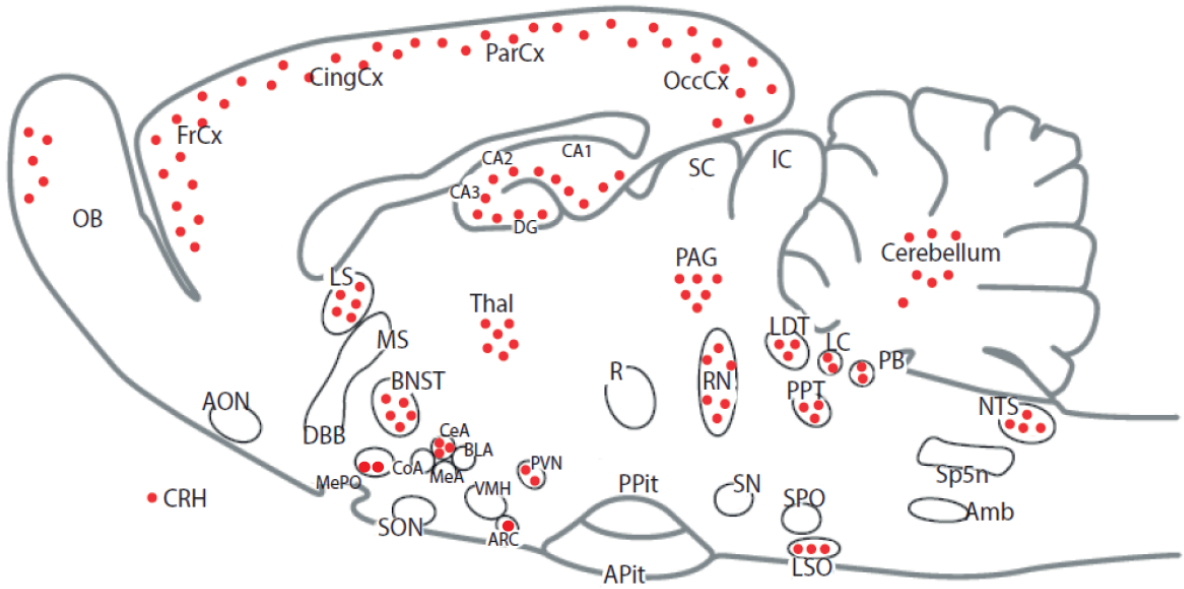

Figure 9: Distribution of CRH-expressing cells in the central nervous system.
Saggital section of a rodent brain, and structures where CRH (red dots) can be detected. The PVN represents the major source of CRH. Moreover, CRH is expressed in numerous cortical areas, the olfactory bulb, the supraoptic nucleus, the bed nucleus of the stria terminalis, the hippocamus, and the central nucleus of the amygdala. CRH can also be identified in brain areas that are involved in sleep-wake regulation such as the laterodorsal tegmental nucleus, the locus coeruleus and the parabrachial nucleus. The most important abbreviations for the present study are indicated as follows: *LDT*: laterodorsal tegmentum nucleus; *LC*: locus coeruleus; *PB*: parabrachial nucleus; *CeA*: central nucleus of the amygdala. Adapted from Warnock 2006.

It is clearly demonstrated that the major source of CRH is the PVN of the hypothalamus; within the hypothalamic area, CRH is also expressed in the medial preoptic area, dorsomedial nucleus, arcuate nucleus, posterior hypothalamus, and the mammillary nuclei (Sawchenko et al., 1983, Sakanaka et al., 1987, Sawchenko, 1990, De Souza, 2002). Besides the hypothalamus, CRH-containing neurons are present in the central nucleus of the amygdala (CeA), the hippocampal formation, the thalamic nuclei, the lateral septum, the bed nucleus of stria terminalis (BNST), the nucleus accumbens, the olfactory bulb and the cerebellum. CRH expressing neurons

also locate in the brainstem, particularly in the RN, the LC, the LDT, the PPT, the substantia nigra, the periaqueductal grey and the nucleus of the solitary tract. Furthermore, scattered CRH-containing interneurons and neurons are found in the second and third layers of the cortex and in the neocortex (specifically the prefrontal, insular and cingulated areas), respectively (Merchenthaler, 1984, Sakanaka et al., 1987, Sawchenko et al., 1990, Sawchenko, 1990, Holsboer, 1999, De Souza, 2002).

Two main CRH pathways can be distinguished in the brain; the one within the HPA axis (hypothalamic pathways) and the other in non-HPA axis areas (extra-hypothalamic pathways) (Holmes et al., 2003). Hypothalamic CRH pathways originate in the PVN and project to the anterior pituitary (as described in the section of the HPA axis). The extra hypothalamic pathways comprise axons from the CeA to the parvocellular regions of the PVN. Furthermore, the non-HPA axis circuits include descending fibers from the PVN, the BNST and the CeA to the brainstem areas, such as the LC (Van Bockstaele et al., 1998, Valentino and Van Bockstaele, 2008), the RN (Price et al., 1998) and the parabrachial nucleus (PBN) (Sawchenko, 1990). Additionally, CRH axons also interconnect the CeA with the BNST and the PVN. Other extra hypothalamic pathways consist of ascending fibers from the brainstem to a variety of anterior brain areas such as the lateral septum, the medial prefrontal cortex, the thalamus and the hypothalamus (Merchenthaler, 1984, Sakanaka et al., 1987, Holsboer, 1999, De Souza, 2002). To date, not all of the CRH projections have been clearly examined, for example the brainstem possesses many adjacent CRH cell groups but it is uncertain if all of these receive inputs from the same projection originating from the CeA. As mentioned already, CRH has been hypothesized to act as both a neurohormone and a neurotransmitter within the CNS (Pavcovich and Valentino, 1997). In fact, a prerequisite for being considered a neurotransmitter is the localization within presynaptic terminals, as demonstrated for CRH by immunohistochemical studies (Cain et al., 1991) and a wide distribution of CRH expressing neurons and binding sites in the brain (Swanson et al., 1983, Sakanaka et al., 1987, De Souza, 1995, De Souza, 2002). Specific neuronal groups have been reported to coexpress CRH with classical neurotransmitters and to innervate various brain areas. For instance, CRH has been identified within cholinergic neurons in the LDT (Crawley et al., 1985) and PPT (Austin et al., 1995), and in glutamatergic and GABAergic neurons within the LC (Valentino et al., 2001). Furthermore, GABAergic neurons of the hippocampus have been reported to coexpress CRH (Yan et al.,

1998). Additionally CRH is known to colocalize and corelease with other neuropeptides such as angiotensin II, AVP, cholecystokin and neurotensin (Sawchenko, 1990, Cain et al., 1991).

2.5.2 The CRH receptors

A decade after the characterization of CRH, expression cloning technique identified and characterized the first CRH receptor from a human Cushing's corticotropic adenoma (Chen et al., 1993). To date, two different CRH receptor subtypes have been described in humans and other mammals: the CRH receptor type 1 (CRHR1) and the CRH receptor type 2 (CRHR2) (Chang et al., 1993, Vita et al., 1993, Chalmers et al., 1995, Kishimoto et al., 1995, Lovenberg et al., 1995b, Liaw et al., 1996). Different genes encode the two CRH receptor families, nevertheless they share 70% sequence homology with each other (Lovenberg et al., 1995b, Dautzenberg and Hauger, 2002, Grammatopoulos and Chrousos, 2002). Both subtypes contain seven transmembrane domains and appertain to the superfamily G protein coupled receptor, which includes other neuropeptides receptors such as the growth-hormone-releasing hormone (GHRH) receptor (De Souza, 2002, Grammatopoulos and Chrousos, 2002). The CRHR1 exists in different isoforms (i.e. CRHR1α, -R1β, -R1c, -R1d, R1e, -R1f, -R1g and -R1h) however, only the 415-amino acid protein CRHR1α seems to be functional (Grammatopoulos et al., 1999, Dautzenberg et al., 2001, Pisarchik and Slominski, 2001). The CRHR2 is currently known to exist in three different isoforms (CRHR2α, CRHR2β, and CRHR2γ). These subtypes are showing differences at their N-terminus; however their pharmacological characteristics are similar (Ross et al., 1994, Lovenberg et al., 1995b, Kostich et al., 1998, Dautzenberg et al., 2001, De Souza, 2002). Recently, a possible third CRHR was characterized in catfish; however, this subtype still hasn't been found in other species yet (Arai et al., 2001, Majzoub, 2006).

Many studies have analyzed the distribution of CRHRs within the brain in different species of animals and have demonstrated a heterogeneous expression of the two subtypes. *In situ* hybridization, immunohistochemistry and RNAse protection assays showed an almost exclusive expression of CRHR1 in cortical areas, the amygdala (BLA and CeA), the cerebellum, the basal forebrain, the superior colliculus, the red

nucleus, the trigeminal nuclei, in the anterior lobe of the pituitary and the LC in rodents (Chalmers et al., 1995, Lacroix and Rivest, 1996, Ambrosio et al., 1997, Sanchez et al., 1999, Chen et al., 2000). Additionally, CRHR1 is also expressed in the brainstem cholinergic nuclei (LDT and PPT) (De Souza, 1987, Holsboer, 1999), which seem to be implicated in REMS modulation. On the other hand, CRHR2α, the major isoform located at neuronal membranes, is more strongly distributed in the PVN, the lateral septum, the ventromedial hypothalamus, the cortical and medial nuclei of the amygdala, and the RN. It has been further reported a mixed CRHR1α and CRHR2α population within the hippocampus, the BNST, the periaqueductal grey (PAG) and the olfactory bulb (Chalmers et al., 1995, Lacroix and Rivest, 1996, Ambrosio et al., 1997, Sanchez et al., 1999, Van Pett et al., 2000). The CRHR2β, this splice variant is expressed predominantly in non-neuronal structures, i.e. the choroid plexus and arterioles, while in the periphery is detectable in the heart and skeletal muscle (Chalmers et al., 1995, Lovenberg et al., 1995a) The isoform CRHR2γ is expressed in the lateral septum, the hippocampus, the frontal cortex, the amygdala and in midbrain areas; however this form has been reported only in humans so far (Kostich et al., 1998).

Using a double-immunocytochemical staining, it was shown that definite groups of neurons co-express CRHR1 with ACh. Specifically, all cholinergic basal forebrain nuclei except the NB were found to express CRHR1 (Sauvage and Steckler, 2001). A strong colocalization was also found in the brainstem such as the LDT and PPT. These results showed that the cholinergic system provides direct anatomical substrates for CRH action through the CRHR1 (Sauvage and Steckler, 2001, Warnock et al., 2006). Furthermore detection of CRHR1 immunoreactivity was found in dopaminargic and noradrenergic neurons within the LC, the ventral tegmental area and the substantia nigra (Sauvage and Steckler, 2001).

2.5.3 Sleep-wake regulatory effects of CRH

Accumulating evidences from human and animal studies support that CRH is involved in spontaneous and stressor-induced sleep-wake regulation. For example, in healthy humans, single intravenous (i.v.) injections and repetitive i.v. injections of CRH produced a decrease in SWS (Tsuchiyama et al., 1995) and REMS (Holsboer

et al., 1988). Contrary to this, cortisol application was shown to increase SWS and decrease REMS in humans. Hence, CRH and acute cortisol administration exert quite opposite sleep effects. It appears likely that the observed results were due to a negative feedback inhibition of central CRH (Born et al., 1991, Friess et al., 1994, Bohlhalter et al., 1997, Friess et al., 2004). Furthermore, since ACTH and cortisol application suppressed REMS (Steiger and Holsboer, 1997), REMS reduction observed after CRH application seems to be a result from increased cortisol levels after HPA axis activation. On the other hand, decreased SWS or increased wakefulness were due to a central action of CRH. Additional examples showing the participation of CRH in sleep-wake regulation are supported by animal studies. Intracerebroventricular (i.c.v) injection of CRH to rats, mice and rabbits resulted in enhanced wakefulness and decreased NREMS (Ehlers et al., 1986, Opp et al., 1989, Sanford et al., 2008, Romanowski et al., 2010). These results from both clinical and preclinical sides suggest that the activation of the HPA axis through CRH contributes to wake responses. However, it remained unclear whether central administration of CRH acted on sleep directly or indirectly through all the stress hormones. Transgenic mice centrally overexpressing CRH allowed unravelling the role of CRH in sleep-wake regulation. These animals displayed increased wakefulness and REMS and vaguely decreased NREMS under baseline conditions compared to controls (Kimura et al., 2010). Literature is limited regarding the implication of the different CRH receptors in sleep-wake regulation. However, several studies in rats and mice were focussed on CRHR1 (Chang and Opp, 1998, Lancel et al., 2002, Kimura et al., 2010, Romanowski et al., 2010), while the functional role of CRHR2 still needs to be elucidated (Jakubcakova et al., 2011).

2.5.4 Stress-related effects of CRH

As already mentioned before, CRH is the major stress hormone. In fact, the most important role of CRH is to coordinate neuroendocrine responses to stress by activating the HPA axis, resulting in glococorticoids release from the adrenal cortex and subsequent physiological effects (Ulrich-Lai and Herman, 2009). It has been reviewed extensively that central CRH is also capable of mediating specific autonomic and behavioral responses to stress independently from HPA axis activation. For example, central administration of CRH in laboratory animals induced

alterations in the ANS such as increased heart rate, blood pressure and blood sugar. Additionally, i.c.v. administered CRH provoked behavioral effects, e.g., suppression of exploratory behavior in a new environment, increased conflict behavior, generation of grooming behavior, and decreased reproductive behavior and food intake (Dunn and Berridge, 1990, Owens and Nemeroff, 1991, Koob et al., 1993, Arborelius et al., 1999, Holsboer, 1999, Bale and Vale, 2004, Guillemin, 2005). Currently, it is hypothesized that central CRH-mediated HPA hyperactivity is associated with major depression (Nemeroff, 1998, Arborelius et al., 1999, Holsboer, 1999, Reul and Holsboer, 2002). A compelling number of clinical reports in depressed patients documented elevated CSF CRH concentration, elevated CRH concentration in the PVN, decreased CRH receptor binding sites in the prefrontal cortex of suicide victims, supporting the hypothesis and suggesting that high levels of CRH could derive from both hypothalamic and extrahypothalamic neurons (Nemeroff et al., 1984, Nemeroff et al., 1988, Raadsheer et al., 1994). Similar changes seen in patients (i.e. long-standing CRH hyperactivity) have also been found in animals that have been subjected to early-life stress (Plotsky and Meaney, 1993, Coplan et al., 1996, Ladd et al., 1996). Since centrally administered CRH has been shown to stimulate anxiogenic reactions, which can be blocked by either a CRH antisense oligodeoxynucleotide or a receptor antagonist, it seems likely that central CRH is also involved in anxiety disorders (Dunn and Berridge, 1990, Skutella et al., 1994, van Gaalen et al., 2002). Along this line, conventional transgenic mice that overexpress CRH exhibit increased anxiety-like behavior (Stenzel-Poore et al., 1994, van Gaalen et al., 2002). Overall, these findings evidence the role of central CRH in stress-related disorders such as depression and anxiety. Strongly supported by mouse genetic studies, CRHR1 was identified as the mediator of defensive and anxiogenic behavior (Smith et al., 1998, Timpl et al., 1998), whereas the role of CRHR2α would mediate anxiolytic effects (Bale et al., 2000, Kishimoto et al., 2000).

2.6 Animal models of stress-related disorders

2.6.1 Stress and REM sleep

Stress-related disorders such as depression and anxiety can be initiated by an overactivity of the HPA axis (de Kloet et al., 2005). These disorders are characterized by an enhanced stress hormone secretion (Arborelius et al., 1999, Holsboer, 1999) as well as alterations in the sleep-wake cycle (Steiger and Kimura, 2010). Polysomnographic sleep recordings have revealed that, besides disturbances of sleep continuity, stress-related disorders are associated with upregulated REMS and a reduction of SWS (Benca et al., 1992, Thase et al., 1997).

In order to clarify the fundamental biological mechanisms that lead to depression, a great number of animal models for depression have been developed over the last 50 years (Willner, 1991, McArthur and Borsini, 2006). These animals are interesting models for defining the interrelationships among depression and sleep disturbances. Of particular interest are selectively bred helplessness mice (El Yacoubi et al., 2003). They show sleep patterns that are similar to those observed in depressed patients, notably a lighter and fragmented sleep, with an increased pressure of REMS; compared to nonhelpless mice they further displayed higher basal corticosterone (El Yacoubi et al., 2003). Further interesting animal models which have been studied by Dugovic and colleagues are the Wistar-Kyoto (Gomez et al., 1996, Dugovic et al., 2000, Solberg et al., 2001) and the prenatally stressed rats (Dugovic et al., 1999). Similarly to the helpless mouse line and to other animal models for depression, they both display increased spontaneous REMS including hyper responsiveness of the HPA axis to stress (Dugovic et al., 1999, Dugovic et al., 2000).

Different studies focused on the influence of stress exposure on sleep changes and found similar variations in the sleep-wake cycle as reported in above mentioned animal models of depression (Rampin et al., 1991, Cespuglio et al., 1995, Meerlo et al., 2001). Rampin and colleagues were the first to describe that a stressful stimulus, such as immobilization stress (IS) is able to alter sleep in rats (Rampin et al., 1991). In fact one hour of IS, performed at the beginning of the dark period, was enough to induce an increase in REMS (Rampin et al., 1991). These results were confirmed by Gonzalez and colleagues who could also prove an involvement of CRH (Gonzalez

and Valatx, 1997). Indeed, they showed that the contribution of endogenous CRH in REMS enhancement depends on the environmental conditions (Gonzalez and Valatx, 1997).

2.6.2 The conditional CRH overexpressing mouse model

Taken into account that stress-related disorders such as depression are accompanied with elevated CRH levels in humans CSF (Nemeroff et al., 1984), the investigation would be worthwhile in animal models that show high CRH activity. Several lines of CRH-overexpressing mice have been created so far (Stenzel-Poore et al., 1994, Kolber et al., 2010). Nevertheless, in all cases the unrestricted CRH overexpression resulted in increased corticosterone levels (Groenink et al., 2002) accompanied by symptoms of Cushing-like syndrome (Stenzel-Poore et al., 1992), therefore limiting their usefulness for studies of sleep physiology. This problem was circumvented by designing conditional mutants overexpressing CRH under the CNS-specific Nestin and forebrain-specific Camk2α promotors (CRH-COE-Nes and CRH-COE-Cam, respectively) (Lu et al., 2008). Both lines do not show explicit behavioural or endocrine abnormalities, under resting conditions, but react with increased active stress-coping behavior and corticosterone release under stress conditions (Lu et al., 2008, Kimura et al., 2010). Furthermore, upregulated REMS has been demonstrated in both lines, suggesting that overexpressed CRH in the forebrain contributes to enhanced REMS.

In the present study, CRH-COE-Cam mice were used in order to unravel the role of forebrain CRH on REMS regulation. Briefly, homologous recombination in embryonic stem cells was used to knock into the ubiquitously active ROSA26 (R26) locus a single copy of the murine CRH cDNA headed by a loxP-flanked (floxed) transcriptional stop sequence (Lu et al., 2008). To achieve forebrain-specific overexpression of CRH, homozygous mice carrying the altered R26 allele (R26 $^{flopCrh/flopCrh}$), which is Cre-recombinase sensitive, were bred with the Camk2α-Cre transgenic line (Lu et al., 2008). In this conditional mouse line, Cre expression is controlled by the Camk2α promotor (Cam) (Minichiello et al., 1999), which drives Cre mediated CRH overexpression to principal neurons of the anterior forebrain including limbic structures from around postnatal day 15 (Minichiello et al., 1999). Resulting

heterozygous R26 $^{+/flopCrh}$ and R26 $^{+/flopCrh}$ Cam-cre F1 were intercrossed to obtain the F2 generation of the desired genotypes: R26 $^{flopCrh/flopCrh}$ (CRH-COE^{con}-Cam; controls) and R26 $^{flopCrh/flopCrh}$ Camk2a-cre (CRH-COE^{hom}-Cam; homozygous) (Lu et al., 2008).

3 Aim of the study

Although it was previously suggested that CRH overexpression in the forebrain including limbic structures contributes to enhanced REMS, the mechanism of how CRH drives REMS increase and contributes to stress-related sleep disorders is not yet fully uncovered. Therefore, this thesis aimed to explore the role of CRH in the regulation of REMS by examining the involvement of an altered neurotransmitter system.

This work addressed the following questions:

Study I Can characteristically upregulated REMS in CRH-COE Cam mice be decreased with a muscarinic antagonist, and is this action mediated through the amygdala?

Study II Do CRH-COE Cam mice have a higher cholinergic activity in the amygdala, and does CRH modulate ACh release via stimulation of the CRHR1?

Study III Is the amygdala able to activate pontine REMS regulating structures after CRH stimulation?

Study IV Does overexpressed CRH in the forebrain affect cholinergic neuronal activity in pontine REMS regulating structures in response to SD?

4 Materials and Methods

4.1 Animals

In the present study, adult (8-12 weeks old) male homozygous forebrain-specific CRH-overexpressing (CRH-COE Cam) and control littermates as well as C57BL/6J mice (Harlan Winkelmann GmbH, Borchen, Germany) were used. CRH-COE-Cam mice were provided by the research group “Molecular Neurogenetics” of the Max Planck Institute of Psychiatry, Munich, Germany. All animals were maintained under standard laboratory conditions (temperature 24°C ± 1°C; humidity 50% ± 10%) on a 12h/12h light dark cycle (lights on at 09:00 h, lights off at 21:00 h). Standard rodent pellets and water were provided *ad libitum*. All animal experiments conducted in this thesis were approved by the local commission for the Care and Use of Laboratory Animals of the State Government of Upper Bavaria. Accordingly to different settings for each experiment, animals were housed as follows.

Study I: Sleep recording and atropine microinjection

CRH-COE-Cam mice (controls n=14; homozygous n=14) were single housed in Plexiglas cages (length x width x height = 25 x 25 x 35 cm) and placed in a sound-attenuated recording chamber.

Study II: Microdialysis for ACh measurement

CRH-COE-Cam mice (controls n=12; homozygous n=12) were placed in the microdialysis experimental room which had similar environmental conditions as the recording chamber and single housed in Plexiglas cages (length x width x height = 25 x 25 x 35 cm). The cages were divided into two compartments using a Plexiglas separation wall. Mice were housed in the large section of the cage (length x width = 25 x 15 cm).

Study III: CRH microinjection and ChAT/c-Fos Immunohistochemistry

C57BL/6J mice (n=15) were single housed in Plexiglas cages (length x width x height = 25 x 25 x 35 cm) and placed in a sound-attenuated chamber. The open top side of the cages allowed free access to the animals for the microinjection procedure.

Study IV: SD and ChAT/c-Fos Immunohistochemistry

CRH-COE-Cam mice (controls n=10; homozygous n=10) were single housed in transparent polycarbonate cages (type 2 – macrolone, 25.5 cm x 19.5 cm x 13.8 cm) and kept at the animal facility of the Max Planck Institute of Psychiatry in Munich, Germany.

4.2 Surgeries (study I-III)

Animals were anaesthetised using a custom-made inhalation narcosis device with an oxygen/sevoflurane mixture (Sevorane; Abbott, Wiesbaden, Germany), positioned into a stereotaxic apparatus (Stoelting Co., Wood Dale, USA) in order to maintain a stable head position, and placed on a heating pad to avoid a decrease of body temperature during the surgery. Before starting the surgery, mice received subcutaneously atropine sulfate (0.05mg/kg, Atropine; Braun Melsungen AG, Melsungen, Germany) to prevent bradycardia and meloxicam (0.5mg/kg, Metacam; Braun Melsungen AG, Melsungen, Germany) for postoperative pain reduction.

For the sleep study, after an incision was made on the scalp, connectivity tissues were carefully removed, and then tiny holes to implant EEG electrodes were drilled in the cranial bone using a dental drill (KaVo-5 Type EWL4970; Kaltenbach und Voigt Elektronisches Werk GmbH, Leutkirch, Germany). EEG electrodes made of 3 gold wires with ball-shaped ends (one in the frontal and two in the parietal field; Figure 10) were placed through the skull epidurally. Subsequently, two additional gold wires with ball-shaped ends were inserted in the neck muscle for EMG recordings. All electrodes were soldered to a 5-pin miniature-connector (BCP socket connector; Compona, Switzerland) and affixed with 2 anchoring screws to the skull with a dental acrylic resin (Paladur; Heraeus Kulzer, Hanau, Germany). Two cannulae made of microdialysis peek tubing (13 mm long, outsider diameter: 0.4 mm, inner diameter: 0.12 mm; Microbiotech, Stockholm, Sweden) were stereotaxically inserted into the bilateral CeA for atropine injections according to the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos, 1997), fixed to the skull with the resin and closed with a removable dummy cap. To further increase footing, all implants were fixed together with the resin.

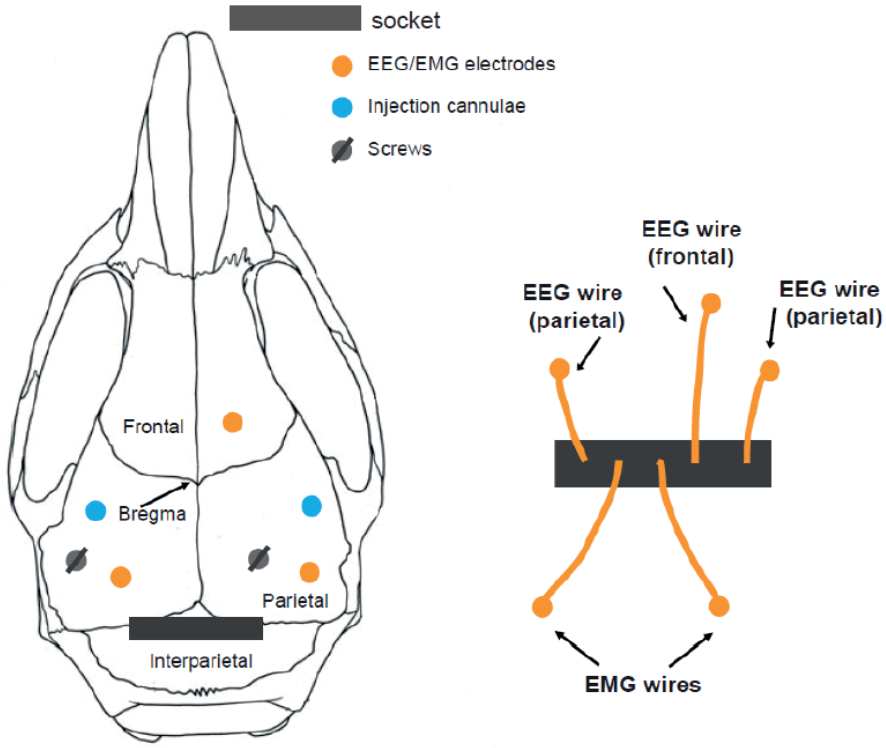

Figure 10: Dorsal scheme of the mouse skull and the five-pin connector.
On the left hand side are the locations of the holes for the EEG electrodes, the injection cannulae and the screws. On the right hand side a schematic draw of the 5-pin connector. Skull adapted from www.informatics.jax.org.

For the microdialysis study, mice were implanted with a guide cannula and two anchoring screws (Figure 11A). The custom-made sterile, stainless steel guide cannula (length: 13 mm; outsider diameter: 0.7 mm; insider diameter: 0.4 mm) was inserted slowly above the right CeA, (coordinates with bregma as reference: lateral -3.2 mm, posterior -1.2 mm, ventral -4.8 mm) and closed with a removable silicon plug. Additionally, a small peg was attached to the skull in order to connect a liquid swivel system during the microdialysis experiment. Both the guide cannula and the peg were first fixed to the skull using ethyl cyanocrylate glue, followed by the dental resin for a better fixation and stabilization.

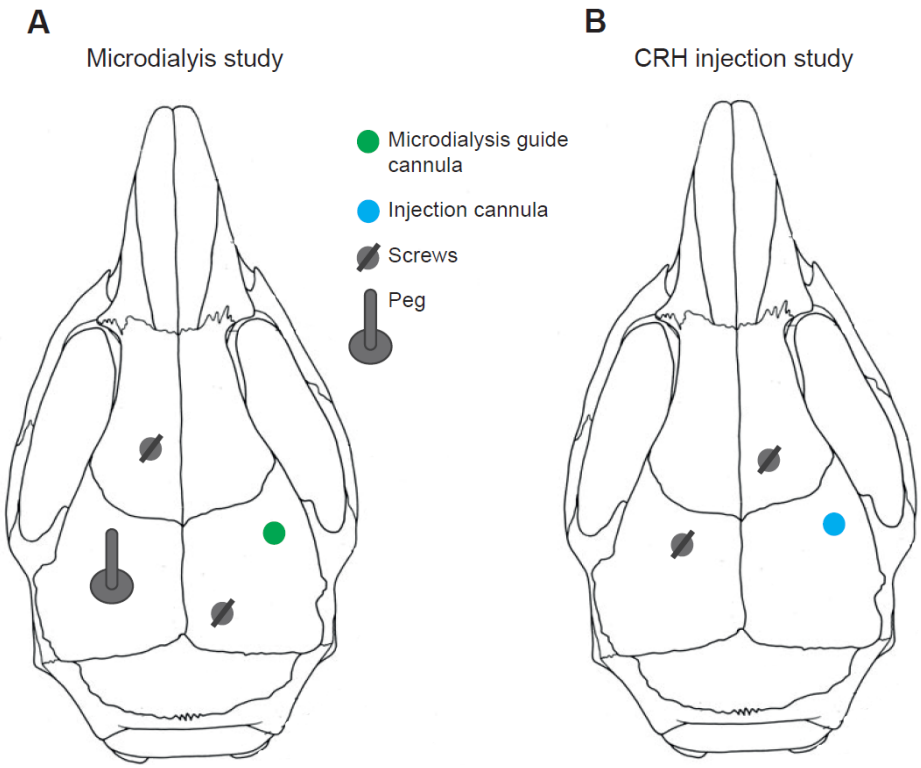

Figure 11: Dorsal scheme of mice skulls and locations of the holes in the microdialysis (A) and CRH injection study (B).

Skull adapted from www.informatics.jax.org

For the CRH injection study, C57BL/6J mice were implanted with a cannula made of microdialysis peek tubing (13 mm long, outsider diameter: 0.4 mm, inner diameter: 0.12 mm, Microbiotech, Stockholm, Sweden) flanked by two anchoring screws (Figure 11B). The cannula was inserted slowly into the right CeA, (coordinates with bregma as reference: lateral -3.1 mm, posterior -1.2 mm, ventral -5.3 mm) and closed with a removable dummy cap. Cannula and screws were affixed to the skull with the resin.

4.3 EEG/EMG recording and sleep data analysis (study I)

After 10 days of recovery from surgery, a 5-pole recording cable was plugged into the micro-socket and connected to an electric swivel (Type SW-921.18; Precisor Messtechnik, Munich, Germany) which was counterbalanced by a mechanical

device; thus, the animals could move almost freely and were easily adapted prior to the beginning of recording. The EEG and EMG recording signals were pre- and main amplified (1000 fold and 10 fold, respectively), filtered (EEG 0.5-29 Hz, 48 dB per octave; non-filtered EMG underwent root mean square rectification), transformed via an analogue-to-digital converter card at a sampling rate of 64 Hz (National Instrument, Austin, TX) and stored on a computer. Polysomnographic data were then analyzed offline by a LabVIEW-based acquisition system (EGEra Vigilanz; SEA, Cologne, Germany), in which a Fast Fourier Transform (FFT) algorithm served for spectral analysis of the EEG power across particular EEG frequency bands that are, delta (0.5-5 Hz), theta (6-9 Hz), sigma (10-15 Hz) and beta (16-29 Hz). The spectral analysis enabled semiautomatic classification of sleep-wake vigilance states by applying the FFT algorithm, adapted from a report by Louis et al. (Louis et al., 2004). Vigilance states were defined in 4-second epochs and classified as wake, NREMS or REMS. The defined semi-automatically scored data were further confirmed visually and corrected if necessary. In case of high amplitude, low frequency (delta bands) EEG, epochs were rescored as NREMS; whereas if the EMG signal was low or absent (muscle atonia) and EEG theta activity was dominant, they were rescored as REMS. All other cases were rescored as wake. EEG/EMG recordings were made for 23 hours per experimental day, allowing maintenance of the recording device and animal care during the remaining hour.

4.4 Microinjections (study I and III)

All microinjections for the atropine and CRH study were performed with the same apparatus and procedure. The microinjection apparatus consisted of a 50 cm long fluorethylenepolymer (FEP) microdialysis tubing (dead volume: 1.2 µL/10 cm length; outsider diameter: 0.4 mm; Microbiotech, Stockholm, Sweden), 2 FEP tubing adapters (no dead volume in the connections; Microbiotech, Stockholm, Sweden), a syringe needle (22 gauge, length: 51 mm, outsider diameter: 0.7 mm; Hamilton Company, Bonaduz, GR, Switzerland), a 10 µl Hamilton syringe (801 RN; Hamilton Company, Bonaduz, GR, Switzerland) and a plunger connected to a custom made control element (Figure 12). The control element consisted of a precision dial

allowing the delivery of the solution in steps of 0.17 µl/turn. An extra peek injection cannula, the tubing and the syringe were filled with distilled water, and a small air bubble drawn up into its distal end. This, separated the infused solution (drug dissolved in vehicle solution or vehicle solution only) from the water, and also acted as a suitable index of successful injection. During injection the animals were gently restraint, and the FEP tubing connected adapter was secured to the peek injection cannula. Solutions in a volume of 0.5 µl were slowly infused over 3 minutes, and the adapter was allowed to stay connected to the injection cannula 5 minutes after the microinjection was finished. Injections were always performed 10 days after surgery on maximum four mice at a time in order to complete the procedure within 1 hour.

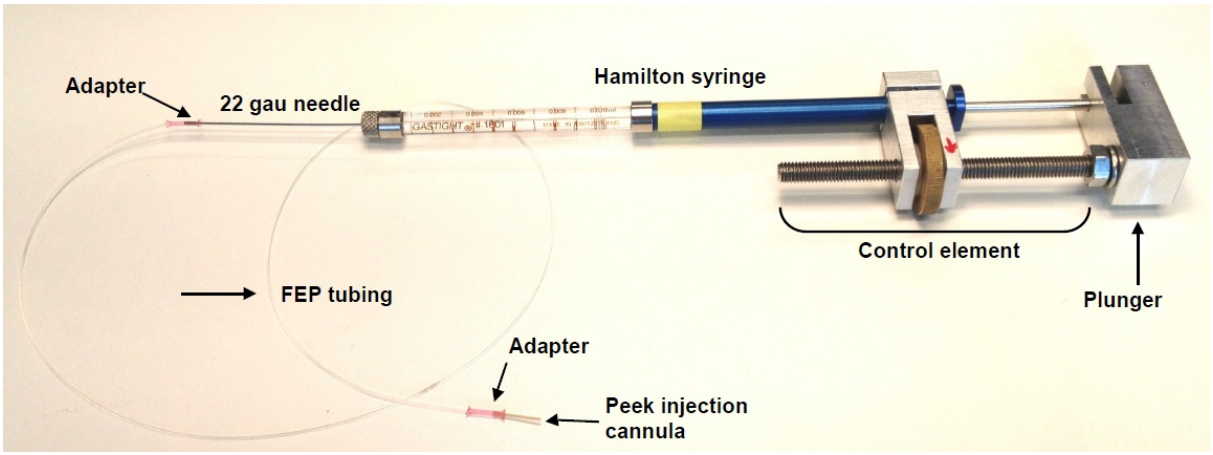

Figure 12: Microinjections apparatus

Experimental procedures for testing atropine (study I)

To test the hypothesis that cholinergic inhibition in the amygdala decreases REMS in CRH-COE-Cam mice, 14 mice (controls n=7; homozygous n=7) were bilaterally injected with a muscarinic antagonist (atropine) into the CeA and changes in REMS were analyzed. Each experimental animal was injected with a 10 µg dose of atropine (Braun Melsungen AG, Melsungen, Germany) whereas a different group of 14 mice (controls n = 7; homozygous n = 7) were used as a control and were injected with a saline solution (NaCl 0.9%; Berlin-Chemie AG, Berlin, Germany; Figure 13). All injections were completed before 15:00 (6 hours after the light onset).

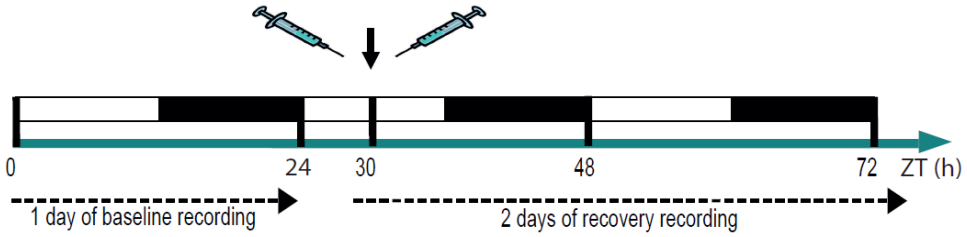

Figure 13: Schematic representation of the atropine injection schedule.
After 1 day of baseline EEG/EMG recording, all animals were bilaterally injected either with atropine or saline at zeitgeber time (ZT) 30. Recordings continued for 2 days (ZT 30 – ZT 72). Horizontal open bar, light period; horizontal filled bar, dark period.

Experimental procedures for testing CRH (study II)

In order to study the effects of limbic CRH on neuronal activity in REMS regulating brainstem structures, C57BL/6J mice were injected unilaterally with CRH (human/rat CRF, Bachem AG, Melsungen, Germany) into the CeA. A group of animal received a dose of 1 µg of CRH whereas a different group received a dose of 10 µg. All injections were completed before 13:00 (4 hours after the light onset). As control treatment, saline was injected at the same time to another group of mice. Sixty minutes after each treatment, animals were perfused.

4.5 Sleep deprivation (SD) (study II and IV)

To study the effects of SD on ACh release and on neuronal activity in REMS regulating structures, the animals were sleep-deprived for 6 hours from the beginning of the light period. SD was performed by gentle handling, which is a less stressful procedure than other SD approaches such as the rotating disk over water or “the flower pot” technique (Rechtschaffen et al., 1999). Whenever the animals appeared to be sleepy, examiners introduced novel objects into the home cage like cotton

swabs or tissue paper. Any direct contact with the animals was avoided. This method stimulates active wakefulness and results in increased sleep pressure (Jouvet et al., 1964, Borbély et al., 1984). The increased sleep pressure increases sleep propensity and leads to a rebound sleep during recovery. In study IV, CRH-COE-Cam mice were divided into 2 groups with 9 animals each (controls n=5, homozygous n=4). One group was sleep-deprived for 6 hours (starting at 9:00 and finishing at 15:00) and the other one was used as a non-sleep-deprived control. All animals were then anesthetized for perfusion at approximately 15:00. The experimental protocol for study II will be described in the following microdialysis section.

4.6 Immunohistochemistry (study III and IV)

After the CRH microinjections or SD experiments, immuhistochemistry was carried out for the detection of the c-Fos protein (as a marker for neuronal activation) together with ChAT labeling (as a marker for cholinergic neurons) within the brainstem region to examine whether CRH or SD activates of cholinergic neurons.

Animals were deeply anesthetized with an overdose of sodium pentobarbital (6,4 mg/kg, intraperitoneal), and perfused through the ascending aorta with 5 ml of saline followed by 5 ml of fixative containing 2% paraformaldehyde (PFA) in 1 M phosphate-buffered saline (PBS). Every perfusion was completed within 10 minutes from the injection of the anaesthetic. Brains were removed and stored at 4°C overnight in the fixative solution, after which they were submerged in a 30% sucrose solution at 4°C for three days for cryoprotection. After the brains were completely absorbed in the sucrose solution, they were rapidly frozen in methylbutane cooled with dry ice and stored at -80°C. Coronal sections were made through the entire brain at 30 µm thickness on a cryostat (Leica, Germany). Free-floating sections containing the amygdala, and brainstem structures were collected in cryoprotectant solution and stored at -20°C.

Study III: fluorescent labeling

To increase cell permeability the sections were incubated with 0.2 % Triton-X for 15 minutes, and then treated with a blocking solution (5% goat serum in PBS for 1 hour);

in between the sections were washed in PBS. The sections were incubated overnight with a rabbit anti c-fos (1:5000; Calbiochem, PC38) and a chicken anti ChAT (1:200; Chemicon, AB15468) primary antibody. The next day, after being washed in PBS, sections were incubated with the fluorophore tagged secondary antibodies: anti rabbit IgG (Alexa Fluor 568; 1:5000) and anti chicken IgG (Alexa Fluor 488; 1:200). Sections were then washed in PBS and incubated for 10 minutes with the fluorescent stain DAPI which was used for visualization of the nucleus since it labels DNA. After staining, the sections were mounted on microscope slides (Super Frost Plus; VWR International, Leuven, Belgium), dried, and coverslipped with prolong gold mounting medium (antifade reagent; Invitrogen, Germany).

The histochemical analysis was carried out under fluorescent microscopy. Dark-field photomicrographs were captured at a 20x magnification with Zeiss AxioCam MRm and AxioCam MRc5 digital cameras adapted to a Zeiss axioplan 2 imaging microscope and a stereomicroscope (Leica). Images were acquired simultaneously in three acquisition channels with the Axio Vision 4.5 and afterwards photomicrographs were integrated into plates using image-editing software. C-Fos positive cells were determined by a red punctuate nucleus whereas the cholinergic neurons were determined by a green cytoplasm. Single labeled c-Fos and double-labeled c-Fos/ChAT cells in different brainstem regions were counted manually from the digitalized pictures in three sections per animal. The cell counts of these three sections were averaged and compared between the CRH injection and saline group. Digitalized sections of the amygdala were used to verify the location of the cannula within this area.

Study IV: DAB/Nova Red labeling

After being washed in PBS, brain sections were first blocked in a normal goat serum for one hour, and then incubated overnight in rabbit anti-c-Fos antibody (1:5000; Calbiochem, PC38). On the next day, the sections were washed and incubated with the secondary antibody (biotinylated goat anti rabbit IgG, 1:300; Vector, BA1000) for one hour, followed by one hour in Avidin-Biotin Complex (ABC; Vector Elite Kit, Vector laboratories). In order to visualize c-Fos positive cells, sections were washed and placed for 40 seconds in a solution of 3,3′-diaminobenzidine (DAB; 0.06%) with a mixture of nickel-ammonium sulphate (0.01%) and hydrogen peroxide (0.02%; DAB kit, Vector Laboratories). The sections were then washed and incubated overnight

with a chicken-anti-ChAT (1:250; Chemicon, AB15468) primary antibody. The following day, the sections were rinsed, incubated in Avidin-Biotin Vector blocking kit (15 minutes in Avidin and 15 minutes in Biotin), and followed by one hour incubation with the secondary antibody (biotinylated goat anti chicken IgG, 1:300; Vector, BA9010). The sections were then washed, followed with one hour incubation with Avidin-Biotin Complex (already described). For the visualization of cholinergic neurons Nova Red (Vector, SK4800) was applied for 1 minute and 15 seconds. After staining, the sections were mounted onto microscope slides (Super Frost Plus; VWR International, Leuven, Belgium), dried, and coverslipped with Eukitt quick-hardening mounting medium (Fluka; Sigma-Aldrich).

Cholinergic cells that were also c-Fos positive (c-Fos/ChAT) were counted unilaterally at 120 µm intervals through the full rostrocaudal extend of the LDT using a light microscope (Leitz) at a 20x magnification; a 4x magnification was used to determine the outline of the structure. C-Fos/ChAT double-labeled cells were determined by the black punctuate nucleus (c-Fos positive) surrounded by brown/orange (ChAT-positive) cytoplasm.

Sections that were used for c-Fos counting were analyzed under a Zeiss microscope equipped with a CCD camera attached to a computer. C-Fos cells were more numerous than dual immunostained cells and were counted unilaterally in three sections (interval 120 µm). Photomicrographs of each selected section were captured at a 10 x and 5x magnification; the outline of the structure was delineated at a 4x magnification and c-Fos cells were counted using a computer-based image analysis system (Imagepro Plus, version 6.3).

4.7 In vivo brain microdialysis (study II)

4.7.1 The technique

We performed *in vivo* brain microdialysis to monitor the release of ACh in the extracellular space of the amygdala of CRH-COE-Cam mice. This technique is widely used in neuroscience to measure free, unbound neurotransmitter concentration in the

extrasynaptic space of freely moving animals (Ungerstedt and Pycock, 1974, Young, 1993, Westerink, 1995, Mas et al., 1996, Bradberry, 2000). A microdialysis system consists of three components: the microdialysis pump, the microdialysis probe and a refrigerated fraction collector (Figure 14).

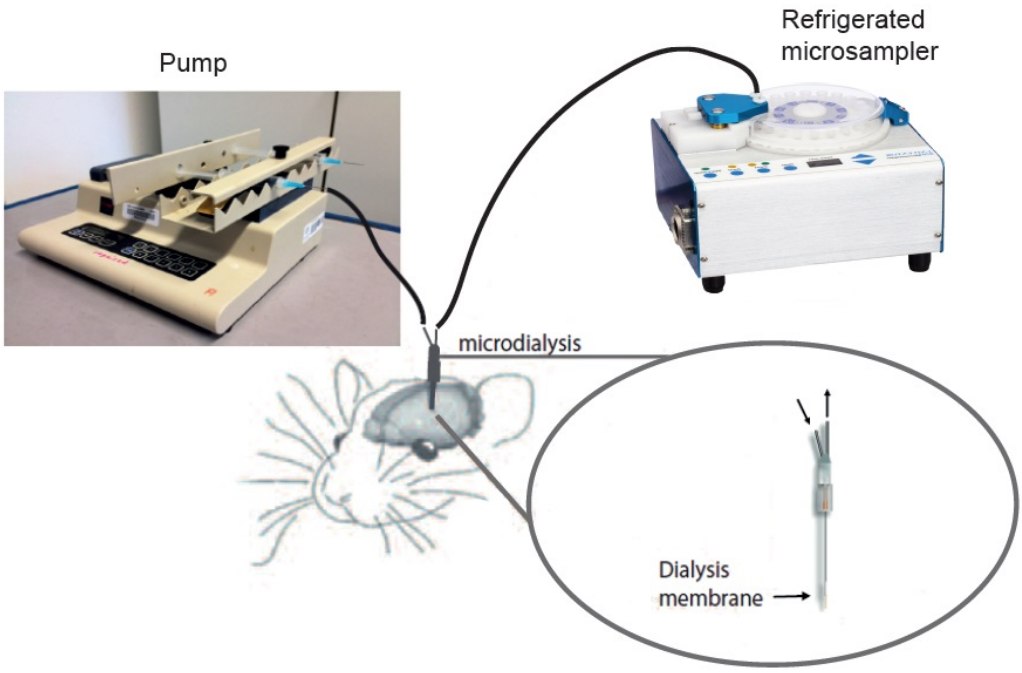

Figure 14: Schematic diagram illustrating the microdialysis setup

The technique requires brain insertion of a dialysis probe, which is designed to function mimicking a capillary blood vessel and consists of a semi permeable hollow membrane at its tip. The dialysis membrane is the key element of microdialysis. When it is perfused with a physiologically isotonic fluid, molecules are exchanged by diffusion in both directions along their concentration gradient, and generally, depending on filter size, the membrane excludes the transport of larger molecules which may interfere with the substances of interest (Hocht et al., 2007). Using the microdialysis pump set at a constant low flow rate, a perfusate solution goes into the probe through the inlet tubing, passes through the outlet tubing and is collected as a dialysate in a refrigerated fraction collector (Nirogi et al., 2010). Perfusion fluid, such as Ringer solution, is a solution that mimics the ionic constituents of the extracellular

fluid, and therefore circumvents the excessive migration of molecules into or out of the probe (Chefer et al., 2009). In general, the concentration of a neurotransmitter with one's particular interest in the dialysate is a fraction of its real concentration in the probe. In fact, the ratio between the concentration in the dialysate and the concentration in the probe is referred to as relative recovery. The relative recovery depends mostly on a flow rate and molecular weight (Plock and Kloft, 2005, Chefer et al., 2009, Nirogi et al., 2010). The *in vitro* recovery of ACh (with a flow rate of 2 µl/minute) is found to be 23 % (data not shown); however the data presented here are not corrected for recovery.

4.7.2 Experimental procedures

Following 14 recovery days after surgery, homozygous and control CRH-COE-Cam mice were lightly anaesthetized with sevoflurane, stereotaxically fixed and after removal of the silicon dummy tip, a 13 mm long concentric microdialysis probe (AZ-8-03; Eicom corp., Kyoto, Japan; membrane: artificial cellulose, length 1 mm, molecular weight cutoff 50,000 Da, outer diameter 0.22 mm) was slowly inserted through the guide cannula. In order to keep the probe in a stable location, it was fixed to the guide cannula with a drop of ethyl cyanocrylate glue followed by dental resin. Prior to this procedure, the probe was examined in order to prevent leakage. FEP tubing with a dead volume of 1.2 µL/10 cm length (Microbiotech, Stockholm, Sweden) was employed for all connections. The microdialysis probe was perfused with Ringer's solution (in mM: Na^+ 147; K^+ 4; Ca^{2+} 2.2; Cl^- 155.5; pH 7; Delta Select, Germany) at a flow rate of 2 µL/minute via a microinfusion pump. After the probe implantation, the animals, connected to a double channel swivels (Microbiotech) through the peg on their head and a counterbalancing system (Instech Laboratories, Plymouth Meeting, PA, USA) were allowed to move freely in the experimental cages without tangling the dialysis tubing. Moreover, the dual channel swivels connected the inlet and outlet tubing from the animals with a refrigerated auto sampler (820 microsampler, Univentor, Malta). Sample collection was always performed with four mice simultaneously on day 2 (first experimental day; baseline collection) and on day 4 (second experimental day: with SD) after the implantation of the microdialysis probe (Figure 15). To avoid rapid hydrolysis of ACh by AChE and to improve basal recovery of ACh, neostigmine bromide (2.5 µM; Research Biochemicals International, Natick,

MA, USA), an acetylcholinesterase inhibitor, was added to the perfusion fluid 12 hours before the start of the first experimental day and 3 hours before the start of the second experimental day.

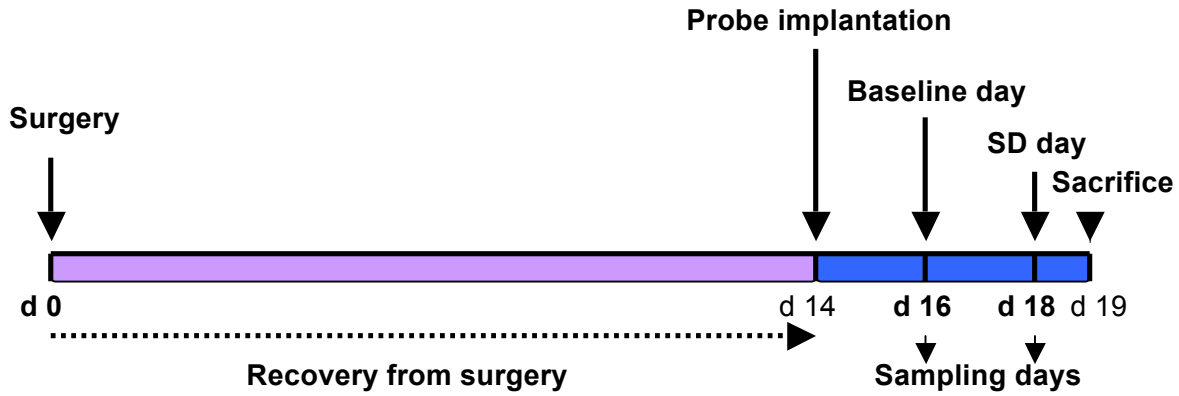

Figure 15: Schematic representation of the schedule for the microdialysis experiment

First experimental day: baseline

Two days after the implantation of the microdialysis probe, 1-hour dialysates samples were automatically collected for 24 hours (09:00-09:00) to monitor the diurnal changes in extracellular levels of ACh. During the baseline day, the animals (n=24) were allowed to sleep and wake undisturbed; therefore great attention was taken to avoid unexpected noise in the microdialysis room. The spontaneous locomotor activity of the mice in their cage was monitored and recorded on a video tape. The video recording equipment in our laboratory did not allow registering the behavior during the full 24 hours of the light/dark cycle. Therefore it was decided to monitor the locomotor activity only during two short periods; between 09:00 and 13:00 during the light period, and between 21:00 and 01:00 during the dark period.

Second experimental day: SD

Four days after inserting the probe, dialysates were automatically collected again every hour for 24 h (09:00-09:00). During the first 6 hours of the sampling time, the animals (n=12) were subjected to SD. At the end of SD, the animals were left undisturbed in their cage and spontaneous locomotor activity was monitored between

15:00 and 21:00 during the light period, and between 21:00 and 23:00 during the dark period.

During sampling, dialysates were collected in plastic vials which were cooled in refrigerated auto sampler and were then stored at -80 °C for further ACh quantification by high performance liquid chromatography with electrochemical detection (see below).

4.7.3 Quantification of ACh

The concentration of ACh was determined from the microdialysis samples using high-pressure liquid chromatography (HPLC) combined with electrochemical detection (EC). The HPLC setup consisted of an isocratic pump (Sunflow 100, Sunchrom, Friedrichsdorf, Germany), a mobile phase degasser (Sunchrom, Friedrichsdorf, Germany), a thermostat (Mistral column, Spark Holland Instruments, Emmen, The Netherlands), an ACh/Ch analytical column (UniJet microbore, Antec Leyden, Zoeterwoude, The Netherlands), an ACh/Ch post column immobilized enzyme reactor (IMER; UniJet microbore, Antec Leyden, Zoeterwoude, The Netherlands) and an electrochemical detector (Antec Leyden, Zoeterwoude, The Netherlands). The mobile phase containing 5 mM sodium phosphate (NaH_2PO_4), 12 mM potassium chloride (KCl) and 0, 5 mM EDTA (pH 8, 5) was filtered through a membrane (pore size: 0,22 µm; Durapore membrane filters, Millipore, Cork, Ireland). The eluent was delivered at a rate of 130 µl/minute, while the temperature in the column was maintained at 35°C. A volume of 10 µl of each sample was injected manually into the injector valve, which was directly connected to the stationary phase (a 530 mm long stainless steel tube with a 1/16 inch outside diameter and 1 mm inside diameter). After sample separation by ion-pairing mechanism in the analytical column (stationary phase), ACh and Ch were converted sequentially to betaine and hydrogen peroxide by the immobilized enzyme reactor (IMER, a 50 mm long stainless steel tube with a 1/16 inch outside diameter and 1 mm inside diameter, containing 2 covalently bounded enzymes: AChE and cholinoxidase [ChO]). The resultant hydrogen peroxide was oxidized on a platinum electrode, with the detector potential set at 550 mV with respect to an Ag/AgCl reference electrode (Figure 16).

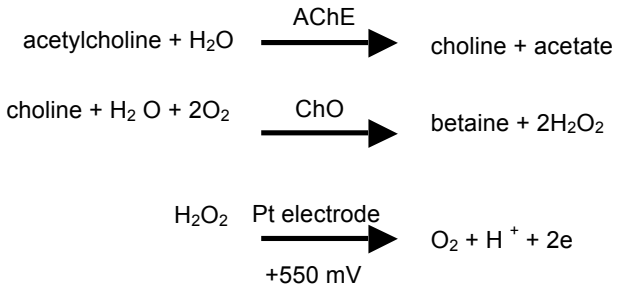

Figure 16: Enzymatic conversion of acetylcholine and choline and electrochemical detection of hydrogen peroxide.

For the ACh analysis, it was necessary to generate a calibration curve by injecting a set of 5 standards of known concentrations (in the range of femtomole) and computing response factors were based upon the linear regression of a plot of peak height vs. concentration (Figure 17). Every plot showed a good linearity with correlation coefficients of 0.998 (data not shown). The chromatograms were analyzed with the Clarity software (Data Apex, Prague, The Czech Republic), and ACh identification and peak quantification were achieved by comparison with the 5 reference standards.

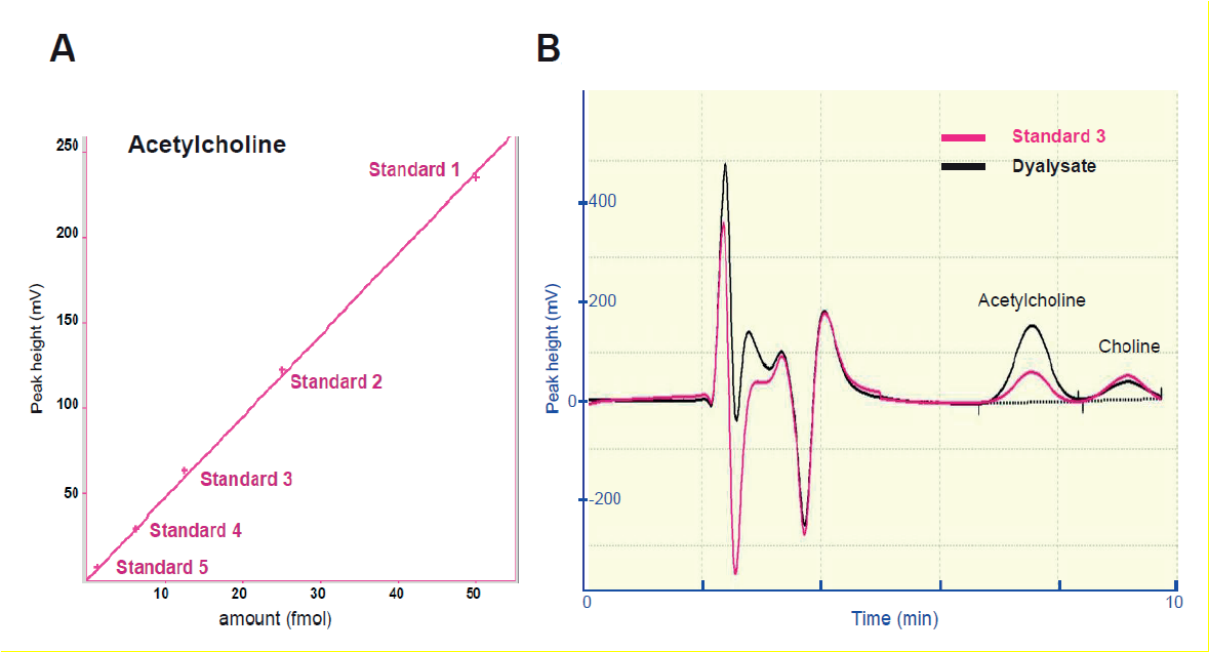

Figure 17: Example of calibration curve for acetylcholine (A) and separation of acetylcholine and choline (B)

(A) The linearity of the plot was obtained in the amount range of 1.25-50 fmol. The plot is showing a good linear detector response with correlation coefficients of 0.998 (data not shown). **(B)** Overlay of chromatograms of a dialysate and a reference standard 3.

4.8 Video monitoring of behavior (study II)

While measuring ACh release during the microdialysis study, the spontaneous locomotor activity of the animals in their cage was monitored with standard miniature infrared surveillance video cameras and recorded on a video tape for later scoring on a personal computer. The observer determined the spontaneous locomotor activity from the video tape by scoring as either “active” or “inactive” every 1 minute. Activity was defined as grooming, nest building, locomotion, climbing on the food rack, or activity along the separation wall while inactivity was defined as sleeping (lying with eyes closed), lying or sitting. The activity counts were then summed over 60 minutes intervals.

4.9 Verification of probe and cannula locations (study I and II)

After the experiments, animals received a lethal dose of sodium pentobarbital (0.1 ml, 160 mg/100 ml, intraperitoneal). The brains were removed, rapidly frozen in methylbutane cooled with dry ice, and stored at -80°C. Coronal sections were made at 20 µm thickness with a cryostat, stained with cresyl violet and visually inspected under a light microscope. Injection sites in CeA were verified by comparing sections to those in the stereotaxic atlas (Franklin and Paxinos, 1997).

4.10 Statistical analysis

All values are shown as a mean ± SEM. Statistical analyses were performed using GraphPad Prism (Version 6.01, GraphPad, San Diego, CA).

In study I the time spent in each vigilance state or only REMS was calculated in 1 or 2 hours averages. Differences in sleep-wake patterns during baseline were compared among the two different genotypes and analyzed by two-way ANOVA with factors ‘time’ and ‘genotype’. The effects of the muscarinic antagonist atropine on REMS were compared between vehicle and atropine treated mice and evaluated by two-way ANOVA with factors ‘treatment’ and ‘time’.

In study II two-way ANOVA with factors ‘genotype’ and ‘time’ was used to determine whether overall significant differences existed between absolute extracellular levels of ACh of the different genotypes during the baseline day, SD and recovery period. To compare ‘genotype’ and ‘light-dark’ effects on 12 hours averaged amounts of ACh release during SD, recovery, light and dark periods, two-way ANOVA was used. In order to reveal significant differences in ACh release between the two experimental days in both genotypes, two-way ANOVA was performed. Furthermore a paired t test was used to determine the differences in 6 hours averaged amounts of ACh between baseline and recovery for each line. Finally the correlation analysis between normalized ACh levels and spontaneous locomotor activity in both genotypes was performed by the Pearson Product Moment Correlation.

In study III one-way ANOVA with factor ‘treatment’ was performed on the number of c-Fos labeled neurons for each structure and each experimental condition (saline, 1 ng CRH, 10 ng CRH).

In study VI an unpaired t test was used to determine the differences in c-Fos or c-Fos/ChAT labeled cell numbers for each genotype across conditions (baseline and SD).

If the F values reached statistical significance, the Bonferroni’s multiple comparison test was further applied for *post-hoc* analysis. P values <0.05 were considered significant.

5 Results

5.1 REM sleep in CRH-COE-Cam mice (study I)

5.1.1 Spontaneous sleep-wake patterns in CRH-COE Cam homozygous and control littermate mice

Polysomnographic analysis of sleep-wake behavior in CRH-COE Cam mice during baseline recordings confirmed what has been previously demonstrated by our research group, namely characteristically upregulated REMS in homozygous mice (Kimura et al., 2010). As shown in Figure 18, both genotypes showed a clear circadian-dependent variation in distribution of each vigilance state, presenting a typical nocturnal sleep-wake cycle. Homozygous mice compared with their control littermates showed constantly elevated REMS levels, however significant differences were only found during the light period ($P<0.05$). In contrast, time course changes in NREMS and wakefulness were not significantly different between the two genotypes neither in the light period nor in the dark period.

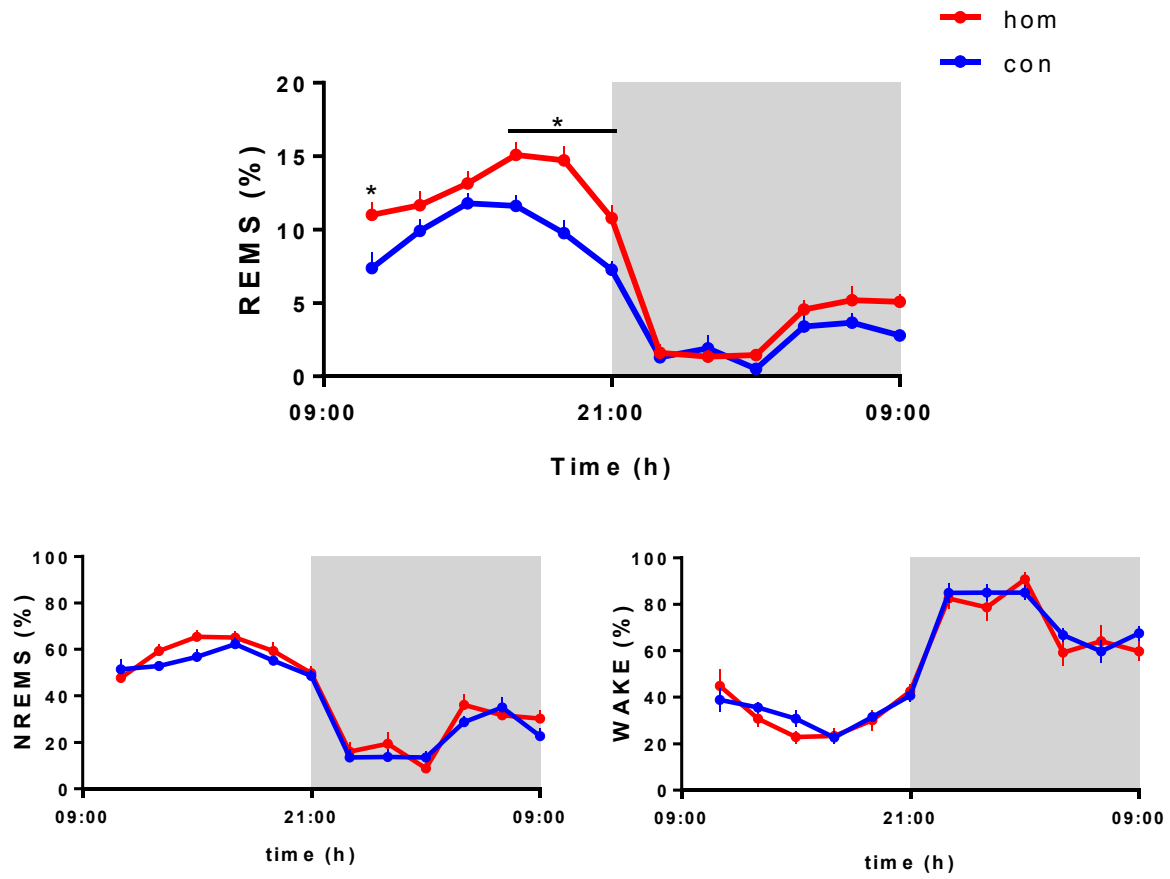

Figure 18: Sleep-wake distribution in homozygous (hom; n=15) and control littermate (con; n=15) CRH-COE-Cam mice under baseline conditions.

Data points represent 2 hour means ± SEM of time spent in REMS, NREMS and wake. The shaded areas indicate the dark period. Two-way ANOVA showed significant effects of 'genotype' for REMS across 24 hours ($P<0.0001$). Bonferroni's test applied for *post-hoc* analysis, showed that the significant differences between genotypes we present during the light phase; $^*P<0.05$. No statistical significance according to 'genotype' was found in respect to NREMS and wake.

5.1.2 The effect of atropine on REM sleep

Available evidences suggest that there is a major projection from the amygdala to REMS regulating brainstem areas (Amaral et al., 1992, Valentino et al., 1994, Gray and Bingaman, 1996, Quattrochi et al., 1998) and cholinergic activation of CeA may be important in REMS regulation (Calvo et al., 1996, Wiersma et al., 1998, Sanford et al., 2006). To determine whether enhanced REMS by limbic CRH overexpression is

mediated through the cholinergic system, atropine was microinjected into the CeA and the effects of locally applied atropine on sleep, especially on REMS were analyzed.

In homozygous CRH-COE Cam mice, significant interaction effects of treatment and time were found during the 6 hours light period ($P<0.0001$) but not in the dark period. In control mice, no statistical significant differences were found, neither during the light phase nor in the dark phase. In both genotypes, compared to the baseline recording, the injection itself even with saline induced a decrease in REMS during the first hour because the animals were still awake from the gentle restraint. Afterwards, REMS levels of saline treated animals returned to and remained at baseline levels (Figure 19A and B). Atropine application in homozygous CRH-COE Cam mice, however, caused a significant decrease of REMS by postinjection hour 2 and 3 in comparison to saline and thus baseline ($P<0.05$). REMS levels remained decreased during most of the light period in homozygous mice (Figure 19A). In control animals, REMS declined only for two hours after atropine treatment, then returned rapidly to the baseline similar to the level after saline (Figure 19B).

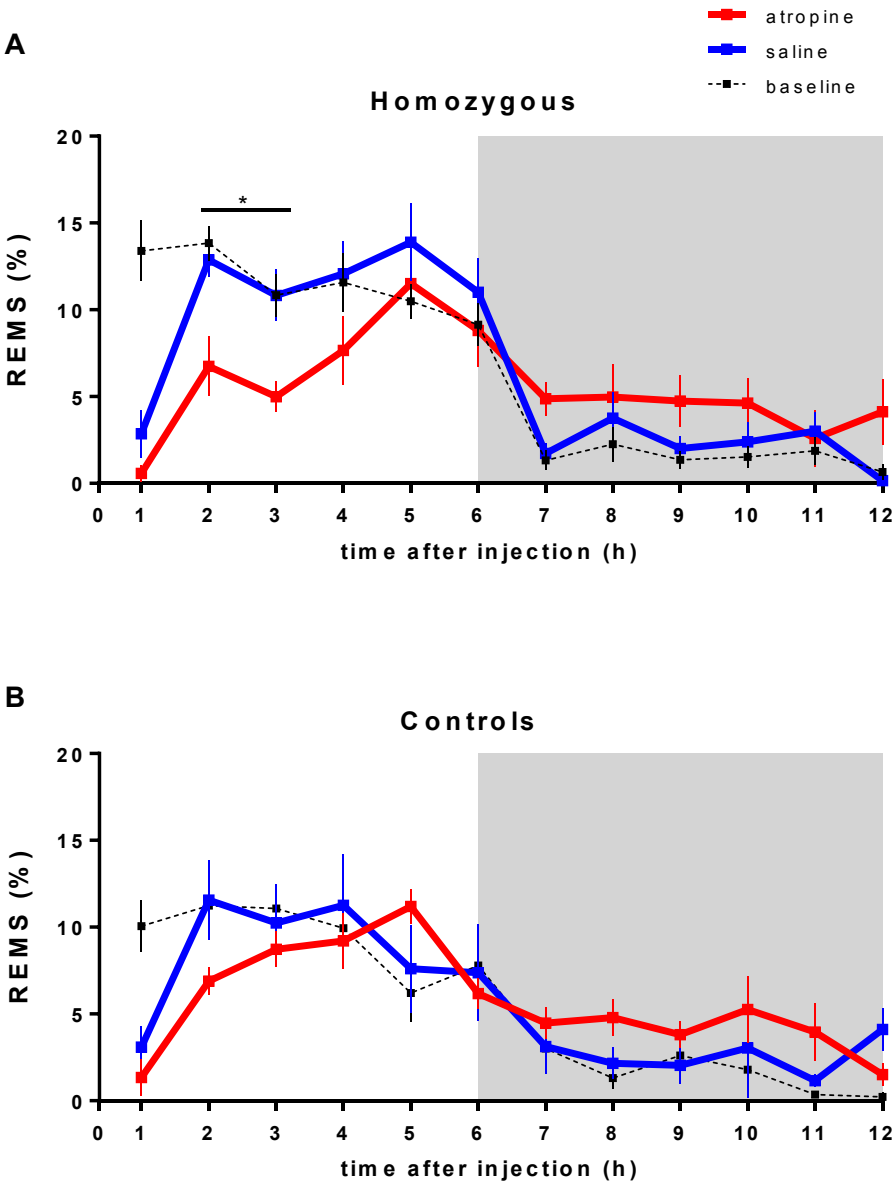

Figure 19: Effects of atropine microinjection into the CeA on REMS in homozygous (atropine n=7, saline n=7) and control littermate (atropine n=7, saline n=7) CRH-COE-Cam mice.

Data points represent 1 hour means ± SEM of time spent in REMS during 12 hours after injection. Animals received either saline or atropine treatment 6 hours after the light onset (clock time 15:00). The shaded areas indicate the dark period. (**A)** Two-way ANOVA showed significant interaction effects of 'treatment' and 'time' ($P<0.0001$) across 6 hours during the light phase. By comparing saline and baseline versus atropine treatment, significant differences assessed by Bonferroni's test for *post-hoc* analysis were found, $^*P<0.05$. During the subsequent 6 hours of dark period no statistical effects were found. (**B)** In control mice two-way ANOVA did not detect any statistical differences between treatments, neither during the first 6 hours nor in the second 6 hours recording periods.

Figure 20 shows the results from the histological validation of microinjection sites. Only CRH-COE-Cam mice that had the cannula positioned in the CeA were included in the following analysis. A series of three coronal diagrams indicates the atropine microinjection sites for each of the animals used for the analysis. Cannulae placements spanned along the rostro-caudal extend (1.22 to 1.70 posterior to bregma), though the locations were within the CeA. A representative coronal section is shown in Figure 20B.

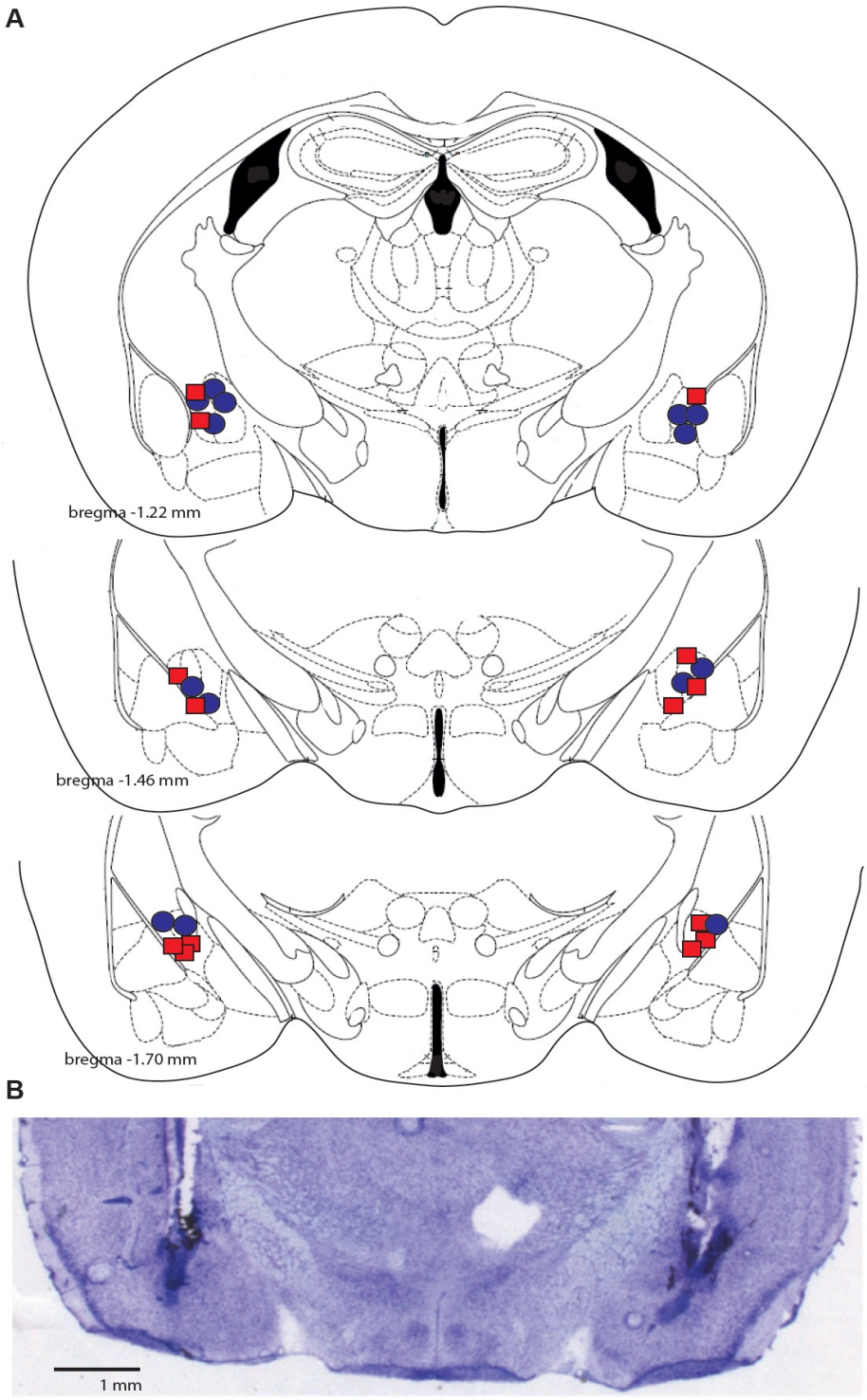

Figure 20: Histological confirmation of the microinjection sites.

(A) Coronal diagrams were modified from a mouse brain atlas (Franklin and Paxinos 1997) illustrating the placements of atropine-injected cannulae in 14 CRH-COE-Cam mice. Red squares and blue circles represent injection sites in homozygous and control animals, respectively. (**B)** A representative cresyl violet-stained coronal section.

5.2 Determination of extracellular ACh concentrations by quantitative microdialysis (study II)

5.2.1 Histological verification of targeted sites

Figure 21 shows the results from the histological analysis of microdialysis sites. Only CRH-COE-Cam mice that had the probe located within the CeA were included in the subsequent analysis. Probe locations spanned from 1.06 to 1.46 mm posterior to bregma. A series of three coronal diagrams indicates the location of microdialysis sites for each of the animals used for the ACh quantification (Figure 21A). A representative coronal section is shown in Figure 21B, with an arrow pointing to the tip of the microdialysis membrane.

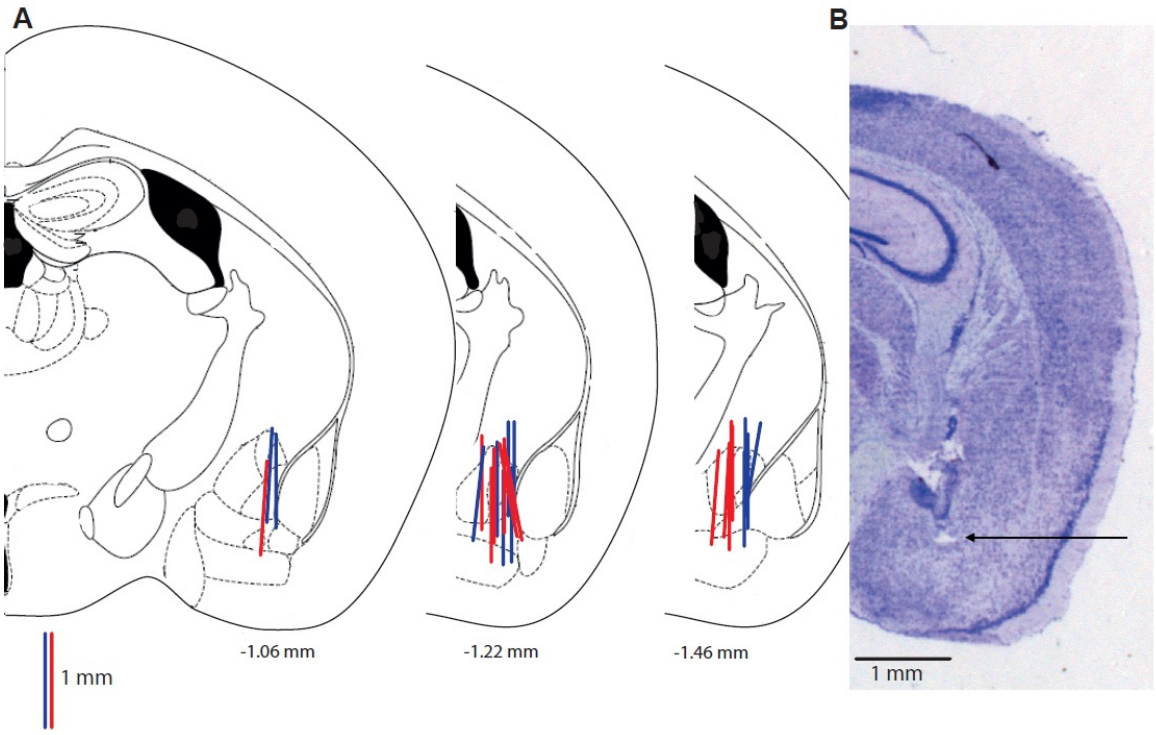

Figure 21: Histological confirmation of microdialysis sites.

(A) Coronal diagrams were modified from a mouse brain atlas (Franklin and Paxos 1997) to show the locations of microdialysis sites from 24 CRH-COE-Cam mice. Red and blue vertical lines represent microdialysis probe membranes in homozygous and control animals, respectively. Numbers below each coronal diagram indicate 1.06 mm to 1.46 posterior to bregma. Vertical lines are drawn to scale. (**B)** A representative cresyl violet-stained coronal section with a black arrow pointing at the tip of the microdialysis membrane.

5.2.2 ACh release in the CeA of CRH-COE Cam mice

To assess whether limbic ACh is differently released in homozygous CRH-COE Cam mice compared with control littermates, extracellular ACh levels were measured in the CeA during two experimental days.

On the first experimental day, extracellular levels of ACh in the CeA were analysed under baseline conditions in homozygous (hom) and control (con) CRH-COE-Cam mice. Figure 22A shows the time course of changes in ACh release across 24 hours.

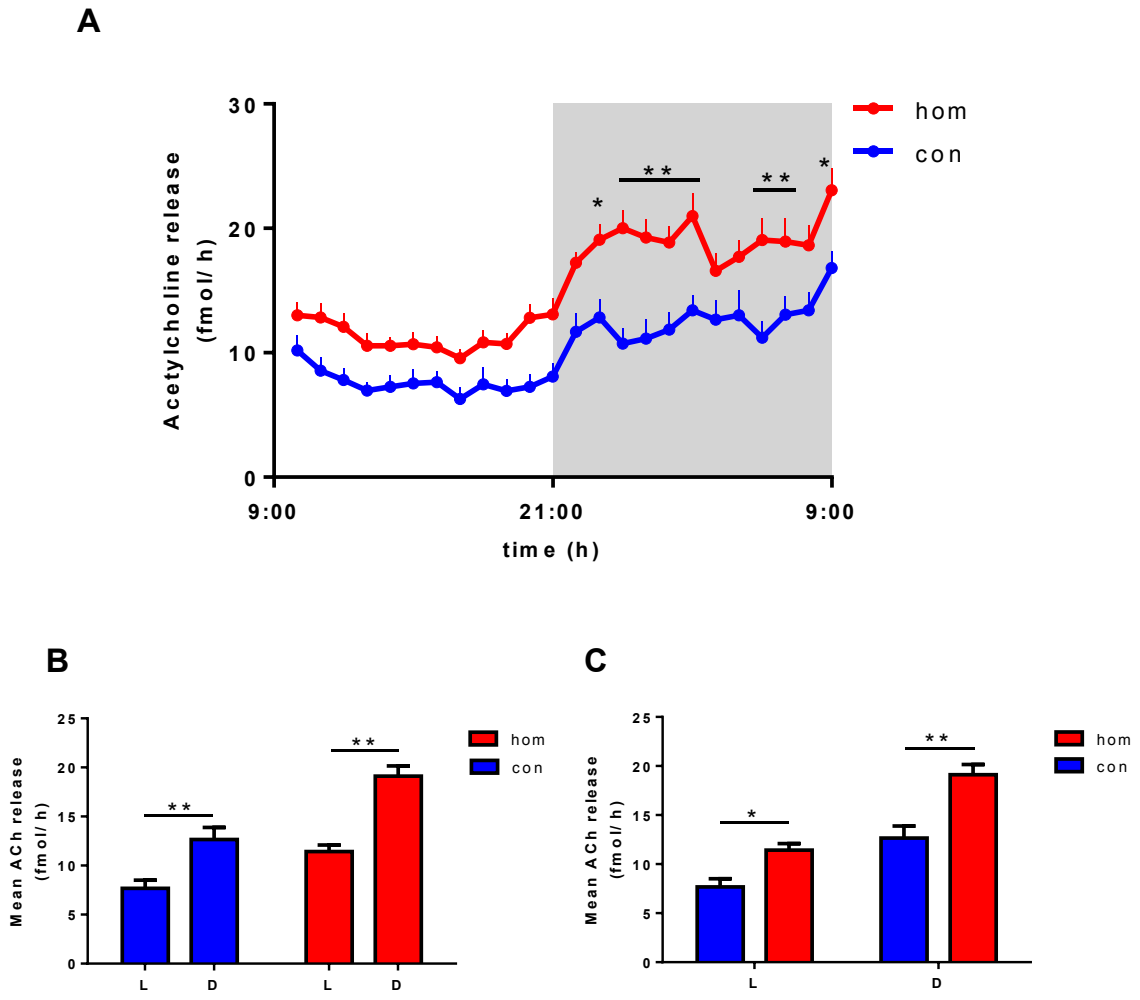

Figure 22: Basal ACh release from the CeA in homozygous (hom; n=12) and control littermate (con; n=12) CRH-COE-Cam mice.

(A) Time course changes in ACh levels measured for 24 hours. Data points represent 1 hour mean ± SEM and the shaded area indicates the dark period. Two-way ANOVA, revealed significant effects of 'time' ($P<0.0001$) and 'genotype' ($P=0.0005$) on ACh release and their interaction across 24 hours ($P=0.002$). Bonferroni's test, applied for *post-hoc* analysis, showed that the significant difference in ACh release between the genotypes was greater during the dark period; *$P<0.05$, **$P<0.01$. **(B)** and **(C)** Mean ACh release during the 12 hours light and dark period. Values are the 12 hour means ± SEM. L and D indicate the light and dark period, respectively. *$P<0.05$, **$P<0.0001$, assessed by two-way ANOVA followed Bonferroni's test.

In both genotypes, extracellular concentrations of ACh showed a clear diurnal rhythm with higher levels during the dark when compared to the light period of the 24 hours light-dark cycle ($P<0.0001$, Figure 22A and B). As shown in Figure 22A, ACh levels during the light period were constantly low with a slight increase towards the

beginning of the dark period. At dark onset, ACh levels increased rapidly during the first hours and remained at a constant high level until they further increased towards the end of the dark period. Extracellular levels of ACh in the pooled 12 hours sample from the light period significantly differed from the pooled ACh levels 12 hours sample obtained from the dark period (Figure 22B). Clearly, the ACh levels in the CeA were increased during the dark period in control and homozygous mice ($P<0.0001$). Even though the circadian rhythm was similarly represented in both groups of animals, significant differences were detected between lines ($P=0.0005$). In fact, homozygous mice showed constantly elevated ACh levels in comparison to controls with larger differences in the dark when compared to the light period (Figure 22A). As shown in Figure 22C homozygous mice exhibited a greater overall 12 hour means of ACh release compared to controls during the light period ($P<0.05$), a finding observed more prominently in the dark period ($P<0.001$).

On the second experimental day, the effects of SD on amygdala ACh release were analyzed in homozygous and control CRH-COE-Cam mice. SD immediately increased extracellular ACh levels within the first hour, which was sustained over the entire 6 hour procedure in both groups (Figure 23A). Significant differences were found in ACh release between SD and baseline in controls ($P<0.05$), which were even greater in homozygous mice ($P<0.01$). When SD ended, ACh release dropped immediately and in the subsequent 6 hours of recovery returned gradually to baseline levels. During the recovery period, only homozygous mice showed significantly higher ACh release when compared to baseline conditions ($P<0.05$). Paired t test revealed that 6 hours mean ACh release during the recovery period (15:00-21:00) was significantly greater than during baseline condition in homozygous mice ($P<0.05$) but not in controls ($P=0.5383$; Figure 23B). During the subsequent dark period, the SD effect was not detected and extracellular concentration of Ach returned to baseline levels in both control and homozygous mice (Figure 23A).

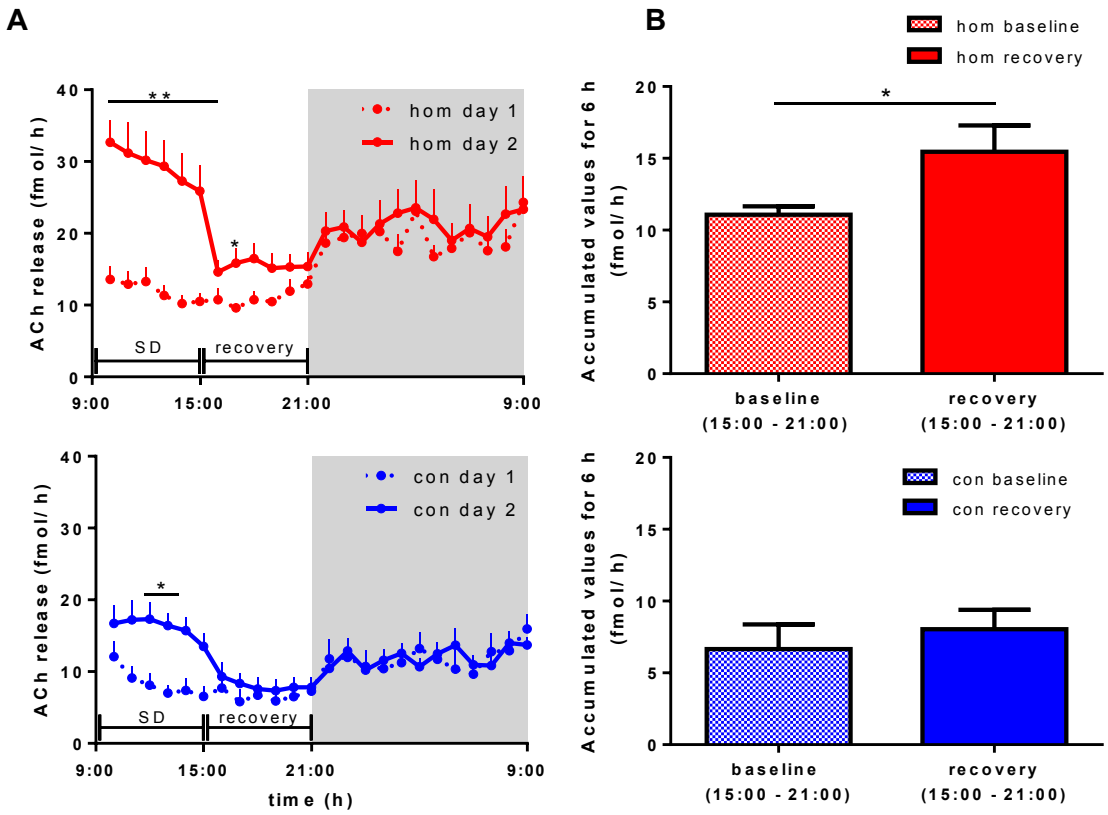

Figure 23: Comparison of amygdala ACh release between baseline and SD day in homozygous (hom; n=6) and control littermate (con; n=6) CRH-COE-Cam mice.

(A) Time course of changes in ACh levels measured for 24 hours. Data points represent 1 hour mean ± SEM and the shaded area indicates the dark period. Two-way ANOVA revealed significant differences in ACh release between day 1 and day 2 in control ($P<0.05$) and homozygous ($P<0.01$) mice during 6 hours of SD. Bonferroni's test showed that the significant difference in ACh release between day 1 and 2 was greater in homozygous than controls; *$P<0.05$, **$P<0.01$. During recovery and the subsequent dark period, two-way ANOVA did not detect significant differences in controls, however homozygous mice did show significantly differences during recovery ($P<0.05$) but not in the dark period. (**B)** Mean ACh release during the 6 hours of recovery period. Values are the 6 hour means ± SEM. *$P<0.01$, assessed by paired t test.

If compared with controls, homozygous CRH-COE-Cam mice showed constantly elevated ACh levels in comparison to controls during 6 hours of SD, recovery and the subsequent dark period ($P<0.05$). Furthermore, the significant difference between genotypes elicited by SD during the recovery period was larger than that during the baseline day (Figure 24A). In contrast to the baseline day, a further analysis on the SD experimental day revealed that the elevated ACh levels in homozygous mice

were greater during the light than the dark period. Moreover the mean values of the extracellular levels of ACh during SD (P<0.01) and recovery (P<0.05) in homozygous mice were significantly higher than in controls (Figure 24B).

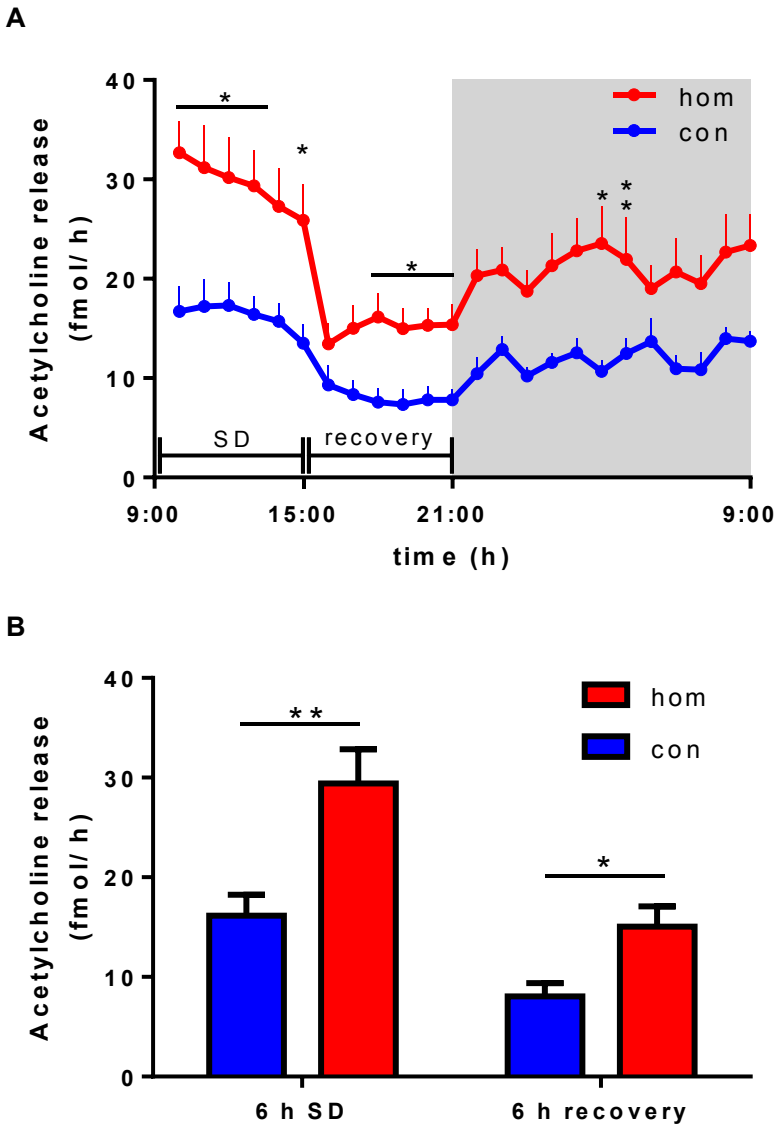

Figure 24: ACh release from the CeA on a SD day in homozygous (hom; n=6) and control littermate (con; n=6) CRH-COE-Cam mice.

(A) Time course changes in ACh levels measured on 24 hours. Data points represents 1 hour mean ± SEM, and the shaded area indicates the dark period. Two-way ANOVA revealed significant effects of 'genotype' on ACh release during SD, recovery and the subsequent dark period (P<0.05). Bonferroni's test, was applied for *post-hoc* analysis, *P<0.05, **P<0.01. (**B)** Mean ACh release during the SD and recovery period. Values are the 6 hour means ± SEM. *P<0.05, *P<0.01, assessed by two-way ANOVA followed by Bonferroni's test.

5.2.2 Correlation of ACh levels with spontaneous locomotor activity

In addition to ACh release measurements, spontaneous locomotor activity was monitored in the same subjects since locomotor activation appears to be associated with an increase in ACh levels (Pepeu and Giovannini, 2004)

To investigate whether ACh release in the amygdala correlates with locomotor activity as previously shown for the release from the cerebral cortex, hippocampus, and striatum (Day et al., 1991, Mizuno et al., 1991), ACh levels during the 8 hours post-SD (15:00-23:00) were compared with locomotor activity counts. ACh levels determined from a 60-minutes microdialysis sample were matched with locomotor activity counts at their corresponding 60-minutes time bin for homozygous and control animals. To adjust interindividual differences in absolute ACh levels in dialysates, the 8 hours data were normalized (2 hours pool of 6 animals divided by the 8 hours mean of each animal). Figure 25 shows the positive correlation between extracellular ACh levels and spontaneous locomotor activity in homozygous (r=0.36) and control (r=0.33) mice. A simple regression line is drawn across the plot for each genotype (hom and con, n=48; Pearson product moment correlation P<0.0001).

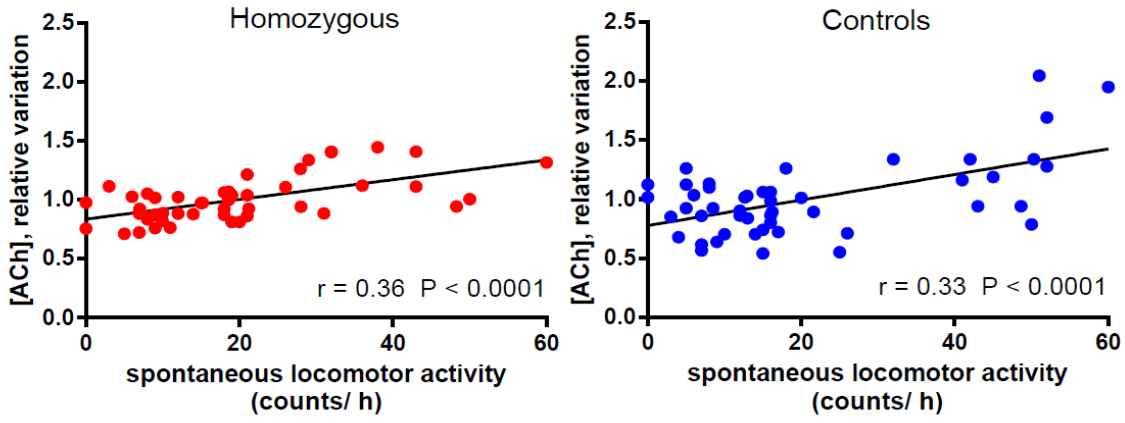

Figure 25: Graphical correlation between ACh levels and spontaneous locomotor activity in homozygous (n=6) and control littermate (n=6) CRH-COE-Cam mice.

Comparison between ACh levels during the 8 hours post-SD period with locomotor activity counts. ACh levels in CeA from a 60 minutes sample were matched with the activity counts in their corresponding 60 minutes bin. A simple regression line is drawn across the plot for each genotype (hom and con, n= 48; Pearson product moment correlation P<0.0001).

After confirming a positive correlation between ACh levels and spontaneous locomotor activity in both genotypes, tests were carried out to investigate whether homozygous mice display increased behavioural activity affecting the ACh release in the amygdala. As shown in Table 1 during both light and dark period homozygous and control mice showed a similar magnitude in behavioral activity. Furthermore, 6 hours of SD did not evoke any genotype effects on locomotor activity. Therefore, the elevated ACh measured in CRH-COE-Cam homozygous was not derived from the differences in locomotor activity.

Parameter	*CON*	*HOM*
Mean behavioral activity during the light period (counts/h)	14.6 ± 1.5 n=6	16.3 ± 2.3 n=6
Mean behavioral activity during the dark period (counts/h)	45.9 ± 3,8 n=6	46.2 ± 5 n=6
Mean behavioral activity after 6 h of SD (counts/h)	20.6 ± 0,9 n=6	19.7 ± 2 n=6

Table 1: Spontaneous locomotor activity during the light and dark period and after SD in homozygous (n=6) and controls littermates (n=6) CRH-COE-Cam mice.

Behavioral activity (light: 09:00-13:00; dark: 21:00-01:00; after SD: 15:00-23:00) was scored from video imaging in 1 minute intervals. Maximal numbers of counts are 60 counts/h. Data represent mean ± SEM.

5.3 Efferent CRH activation from the amygdala to the brainstem (study III and IV)

Previously neuroanatomical studies have described that CRH neurons from the central nucleus of the amygdala (CeA) have direct connections with REMS regulating brainstem areas (e.g. laterodorsal tegmental nucleus, parabrachial nucleus) (Amaral et al., 1992, Valentino et al., 1994, Gray and Bingaman, 1996, Quattrochi et al., 1998, Morrison et al., 2000). To further investigate the relationship between CRH and ACh upon REMS regulation, study III and IV examined how CRH in the limbic system influences neuronal activity in the brainstem where cholinergic neurons relevant for REMS regulation locate densely (Figure 26).

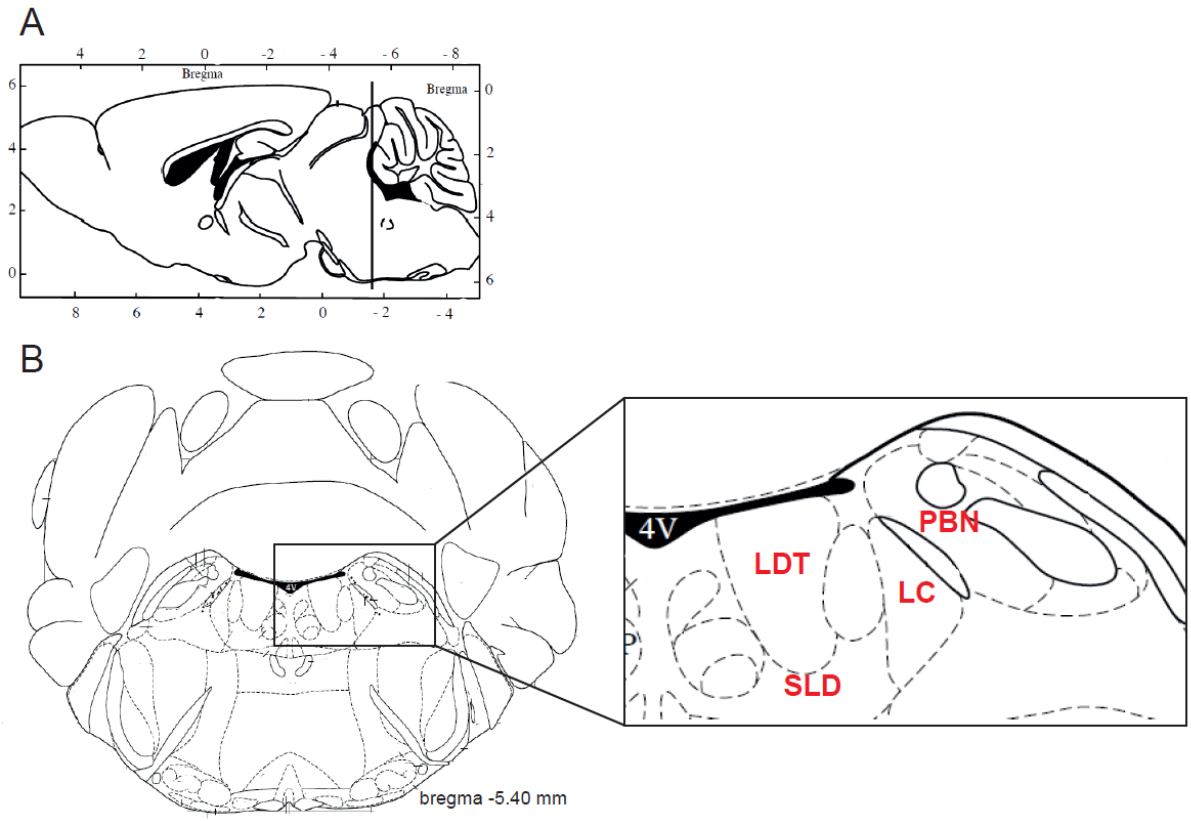

Figure 26: Location of important REM sleep regulating structures.
(A) A vertical line on the sagittal diagram of the mouse brain (Franklin and Paxos 1997) delineates the site of the REMS regulating structures within the brainstem. **(B)** A coronal diagram was modified from a mouse brain (Franklin and Paxos 1997) to show the locations of the laterodorsal tegmental nucleus (LDT), the sublaterodorsal tegmental nucleus (SLD), the locus coeruleus (LC) and the parabrachial nucleus (PBN).

5.3.1 The effect of CRH microinjection into the CeA on c-Fos expression

In this study performed in C57BL/6J mice (n=15), c-Fos expression, used as an indirect marker of neuronal activity, was examined in two cholinergic brainstem REMS regulating structures 1 hour following unilateral CRH injection (1 ng or 10 ng) into the CeA. Furthermore, c-Fos immunostaining was combined with immunostaining for ACh transferase (ChAT) in order to identify colocalization with cholinergic neurons in two specific structures, which are the LDT and the SLD.

As shown in figure 27 and 28, c-Fos immunoreactivity observed in saline injected animals was low in the LDT and SLD areas; nevertheless when animals were injected with CRH at either dose the number of c-Fos positive cells significantly increased in a dose dependent manner. Specifically, there were significant differences in the number of c-Fos cells induced by the lower dose of CRH (1 ng; $P<0.05$) or the higher dose of CRH (10 ng; $P<0.001$) compared to saline in both LDT and SLD areas. A significant increase in the number of c-Fos positive cells was also found in 10 ng injected animals compared to 1 ng injected animals ($P<0.01$) in both areas.

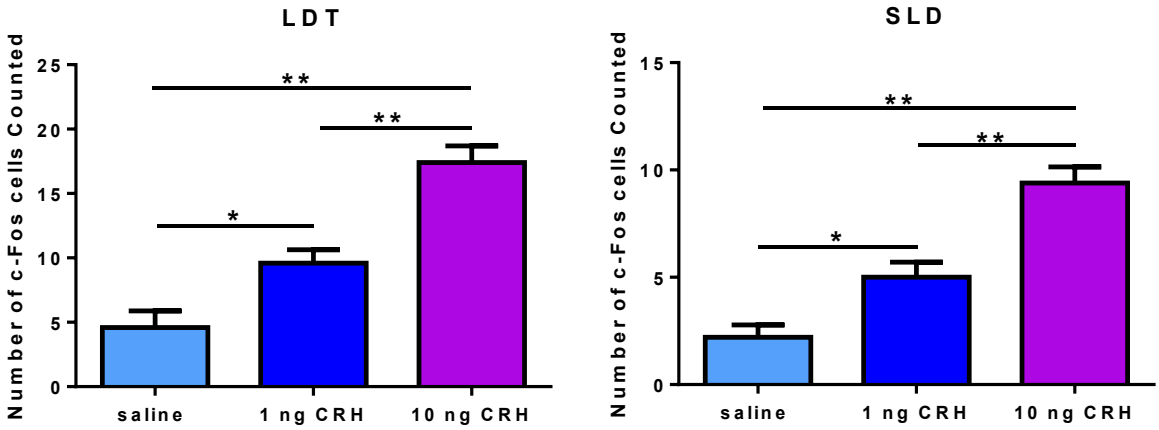

Figure 27: Effects of CRH microinjection into the CeA on c-Fos expression in C57BL/6J mice (n=15).

Height ± SEM of columns indicates number of c-Fos cells 1 hour after the injection in the laterodorsal tegmental nucleus (LDT) and in the sublaterodorsal tegmental nucleus (SLD). *P<0.05, **P<0.01, assessed by one-way ANOVA with the factor 'treatment' followed by Bonferroni's test.

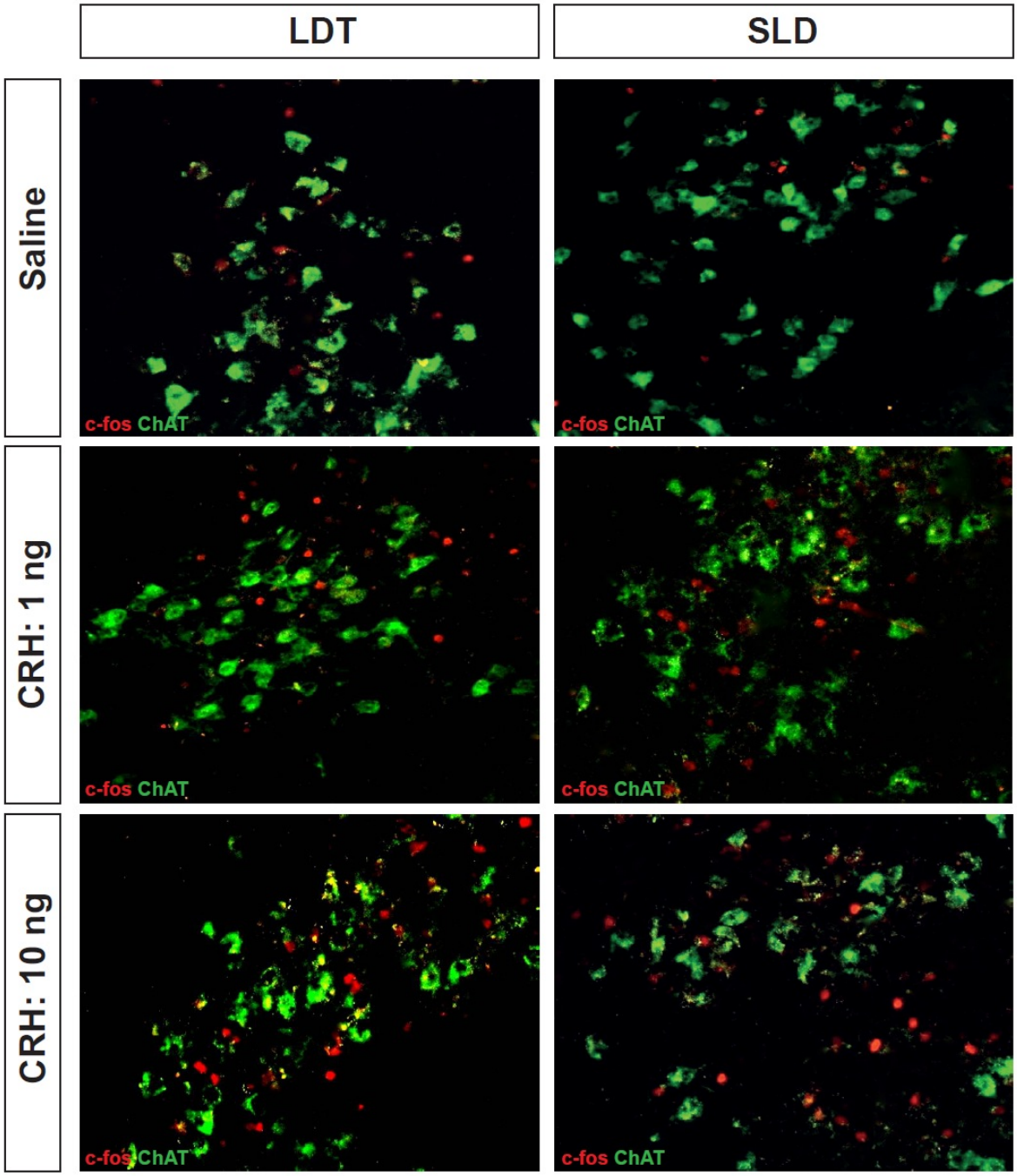

Figure 28: Example of c-Fos and ChAT positive neurons C57BL/6J mice.
Dark-field photomicrographs showing c-Fos and ChAT positive neurons in LDT and SLD after saline or CRH (1 ng and 10 ng) microinjection into the amygdala. Microinjections at either dose increased the number of c-Fos positive cells but none of them were identified as cholinergic. 20x magnification.

Even though CRH increased the number of c-Fos positive cells in the LDT and SLD, none of them were identified as cholinergic (Figure 27 and 28). As described in the Materials and Methods section, c-Fos positive cells were determined by a red

punctuate nucleus whereas the cholinergic neurons were determined by a green fluorescent cytoplasm (Figure 29). In case of double-labeled cells, the red punctuate nucleus should be surrounded by green fluorescent cytoplasm. However colocalization of c-Fos/ChAT positive neurons was not detected in any of the 15 mice tested in this study.

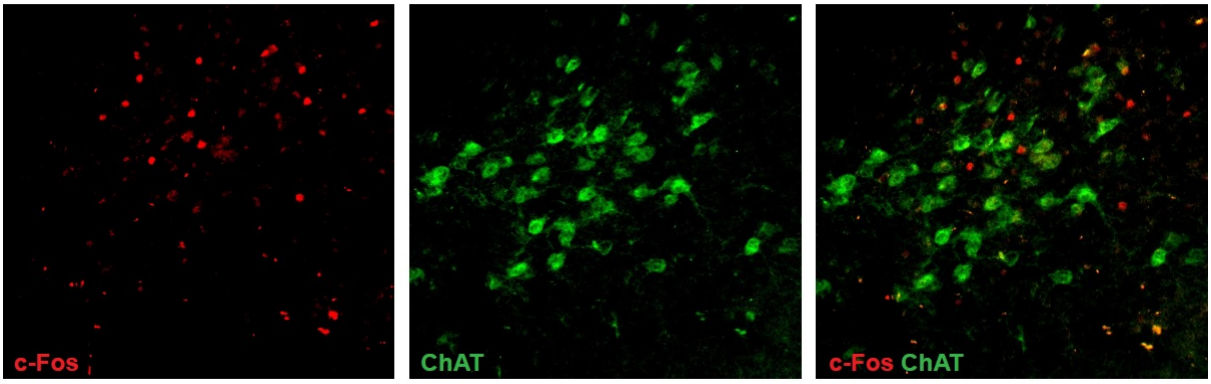

Figure 29: Example of C-Fos and ChAT positive neurons

Dark-field photomicrographs showing c-Fos and ChAT positive neurons at LDT level. C-fos/ChAT double-labeled neurons were not found in this study. 20x magnification.

5.3.2 C-Fos expression in CRH-COE Cam mice elicited by SD

The present study performed in CRH-COE Cam mice aimed to prove a different neuronal activation in cholinergic and non-cholinergic brainstem structures in response to 6 hours of SD across genotypes (controls con=5; homozygous hom=4). Differently from the previous study, c-Fos and ChAT cells were labeled with DAB and Nova Red, respectively (see Materials and Methods), with the intention to enhance the intensity of the staining. C-Fos expression was examined in the PBN, the LDT, the LC and in the amygdala as well. Furthermore, doubled-labeled c-Fos/Chat positive neurons were counted in the LDT.

During baseline condition c-Fos expression was in general low, and no differences were found across genotypes in respect to the number of c-Fos cells in all brainstem structures analyzed (LDT, PBN, LC; Figure 30). However, in response to SD both genotypes showed an increase in c-Fos positive neurons (Figures 30, 31, 32 and 33), in comparison to baseline. Specifically, in homozygous mice the number of c-Fos

cells was significantly increased in LDT, PBN ($P<0.01$) and LC ($P<0.05$) compared to baseline, while in controls the number of c-Fos labeled cells was also significantly increased in the same structures (LDT and LC, $P<0.01$; PBN, $P<0.05$).

Homozygous CRH-COE Cam mice with SD, however, showed significantly more c-Fos cells expression within the LDT and PBN ($P<0.01$) in comparison to their control littermates, whereas in the noradrenergic LC the c-Fos expression was similarly seen in both genotypes (Figure 30).

Within the cholinergic LDT, the number of c-Fos/ChAT positive neurons were undetectable in both genotypes during baseline condition but numerous in response to SD both genotypes showed a significant increase in comparison to baseline ($P<0.001$; Figure 34A). As illustrated in Figure 34, doubled-labeled cells were significantly increased after SD in homozygous mice as compared to their control littermates ($P<0.01$).

Additionally, c-Fos expression within the amygdala was analyzed (Figure 35). Similarly to the brainstem structures, in response to SD both genotypes showed an increase in single c-Fos positive neurons in comparison to baseline ($P<0.01$; Figure 35 and 36). However, homozygous mice showed higher c-Fos expression after SD when compared to their control littermates ($P<0.05$). No differences were found with respect to the number of c-Fos positive cells during baseline condition across genotypes.

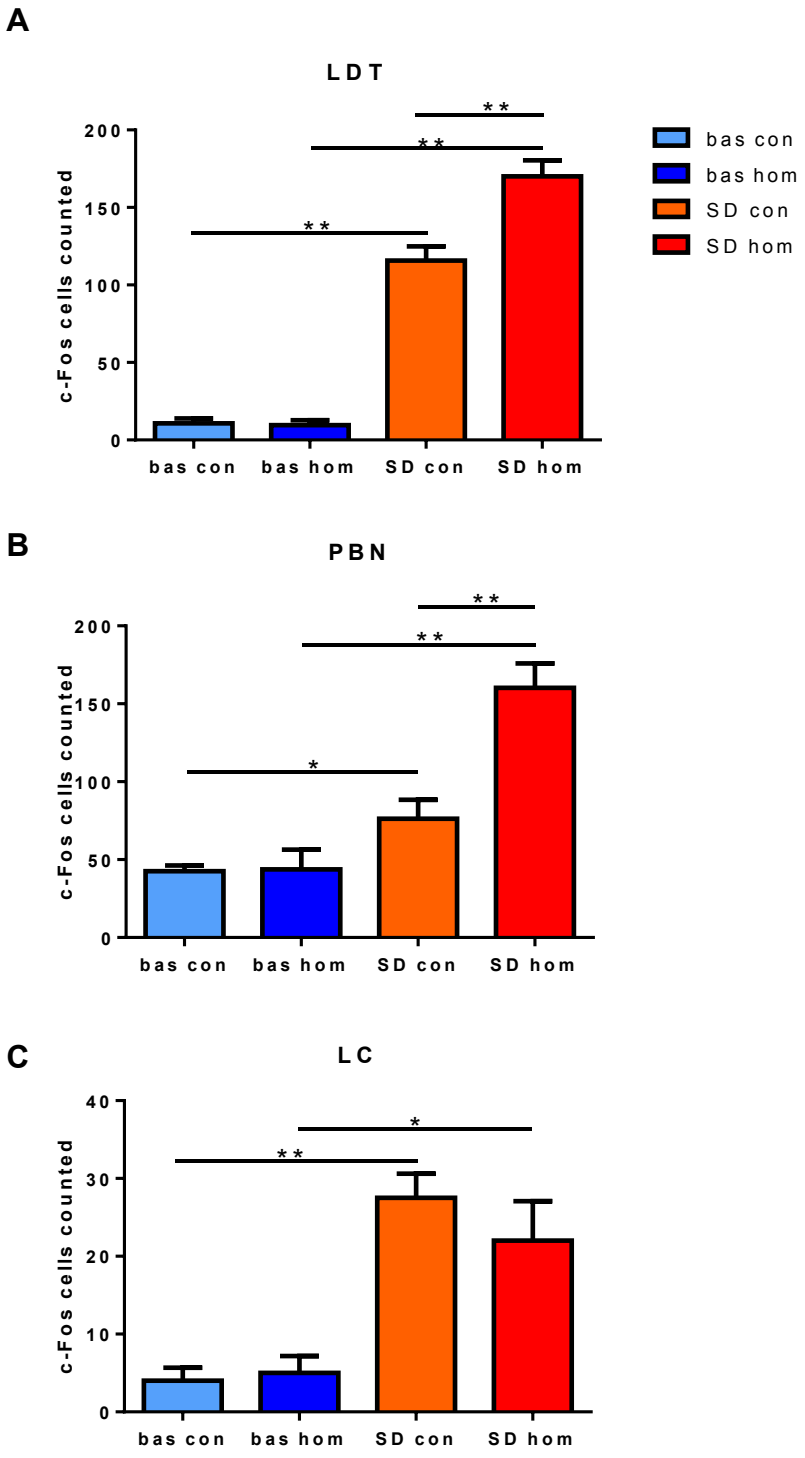

Figure 30: c-Fos positive neurons in the (A) laterodorsal tegmental nucleus (LDT), (B) parabrachial nucleus (PBN) and in the (C) locus coeruleus (LC) in CRH-COE Cam mice.

Number of c-Fos positive neurons counted in three sections in control (con; n=5) and homozygous (hom; n=4) mice during baseline (bas) and SD condition. Height ± SEM of columns indicates number of c-Fos cells. *P<0.05, **P<0.01 assessed by unpaired t-test.

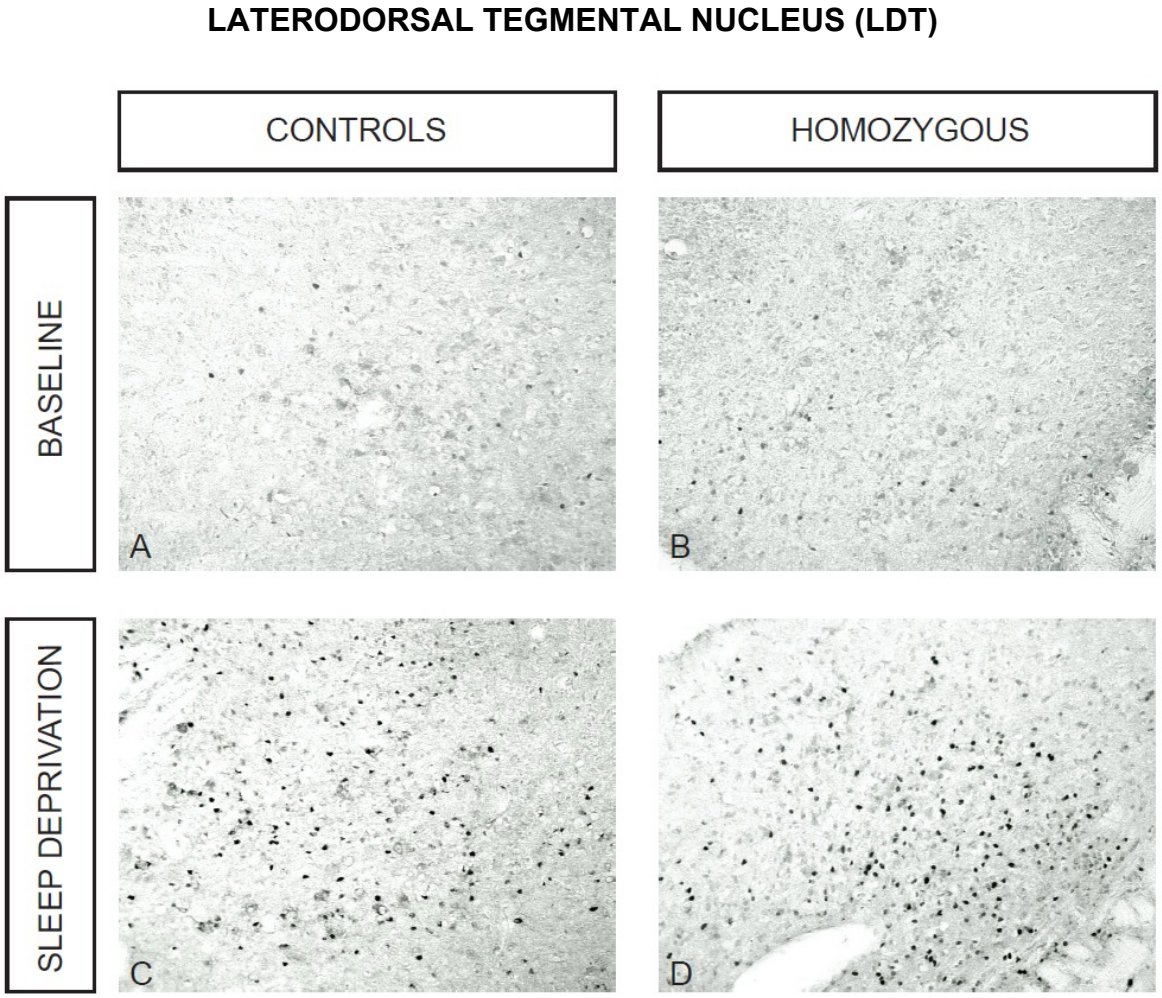

Figure 31: Photomicrographs of SD-induced or spontaneous c-Fos expression in homozygous and control CRH-COE-Cam mice within the LDT.

In non-SD (**A** and **B**) control and homozygous mice only few immunoreactive cells were detected, whereas abundant c-Fos positive cells were seen in both SD animals (**B** and **C**). 10x magnification.

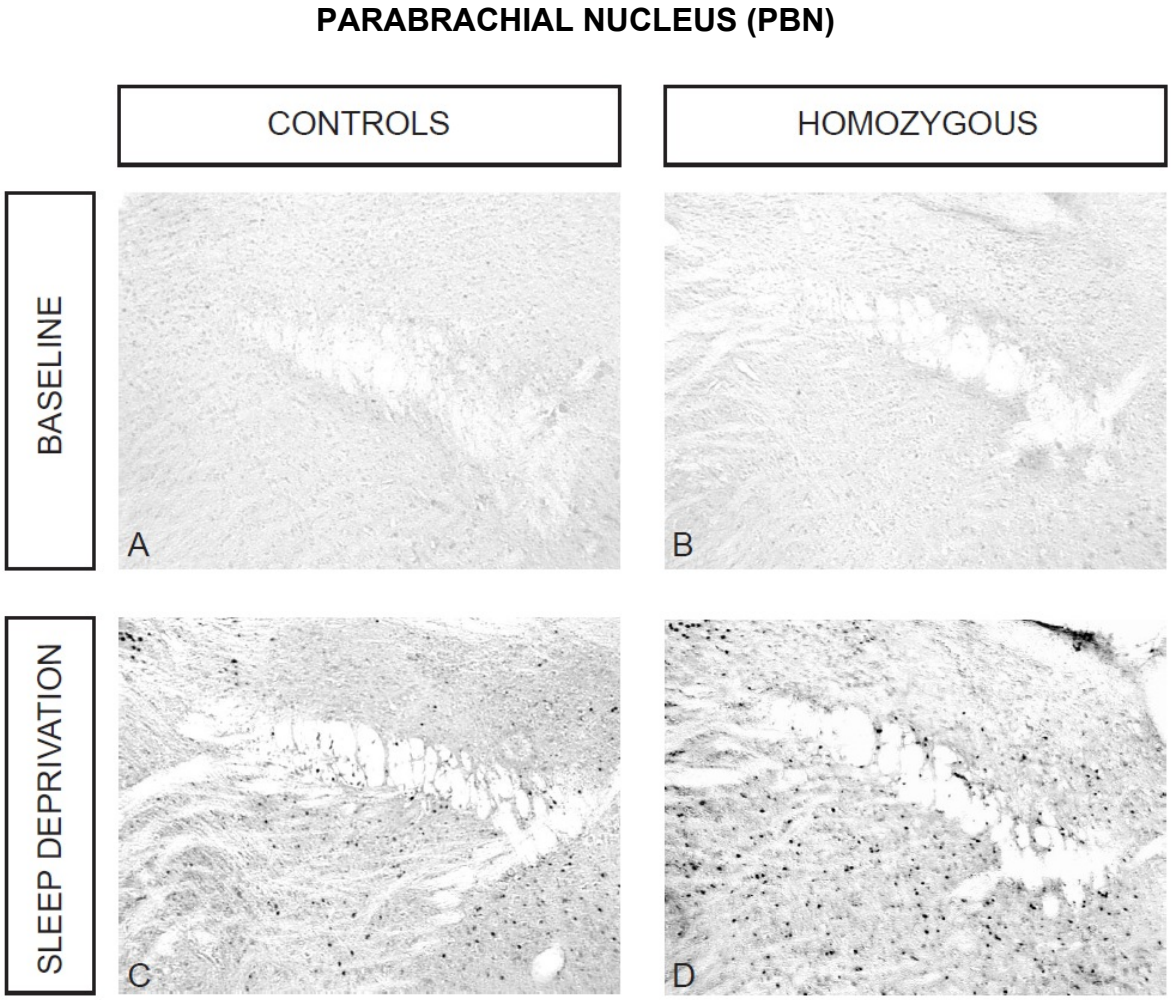

Figure 32: Photomicrographs of SD-induced or spontaneous c-Fos expression in homozygous and control CRH-COE-Cam mice within the PBN.

In non-SD (**A** and **B**) control and homozygous mice only few immunoreactive cells were detected, whereas abundant c-Fos positive cells were seen in both SD animals (**B** and **C**). 5x magnification.

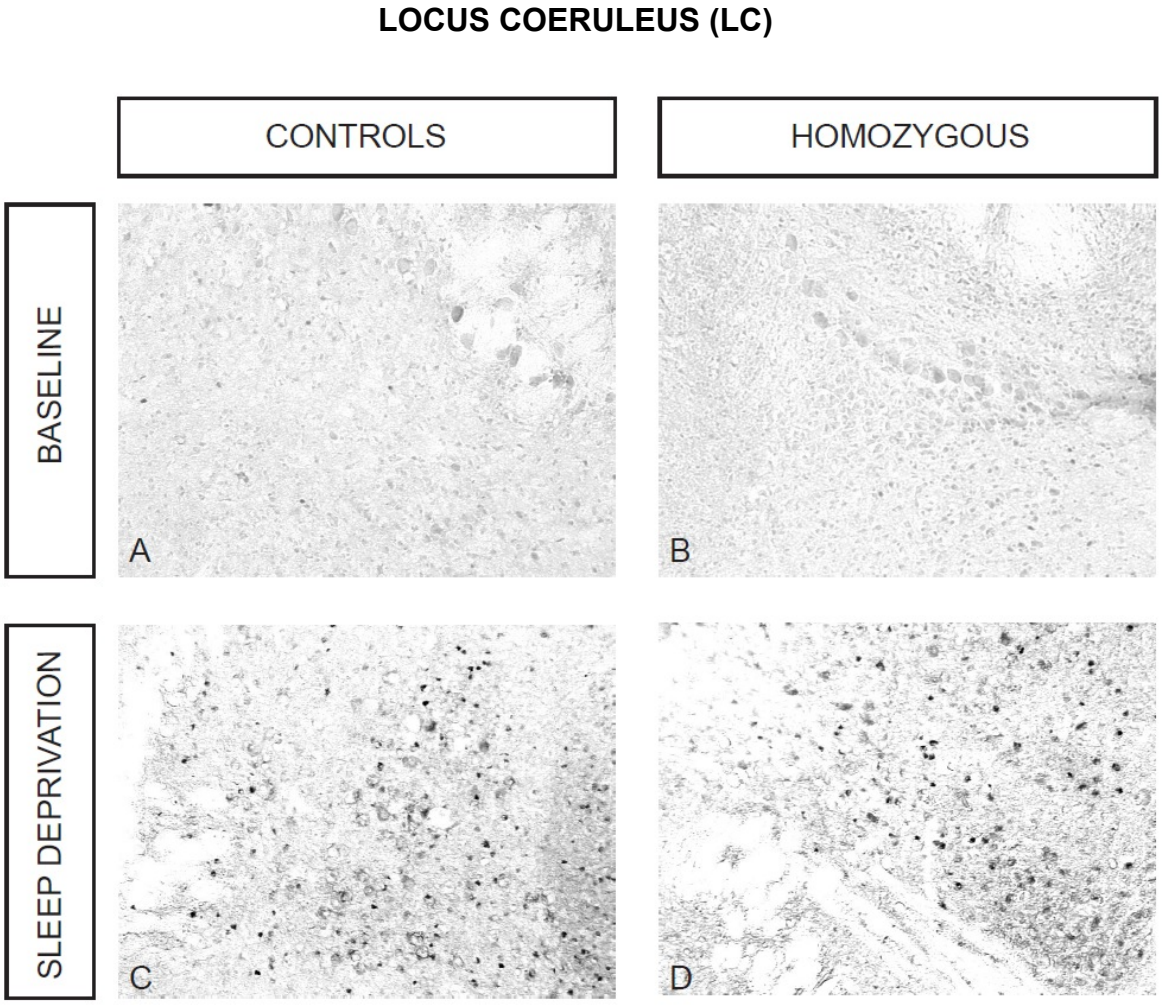

Figure 33: Photomicrographs of SD-induced or spontaneous c-Fos expression in homozygous and control CRH-COE-Cam mice within the LC.

In non-SD (**A** and **B**) control and homozygous mice few immunoreactive cells were seen, whereas abundant c-Fos positive cells are seen in both SD animals (**B** and **C**). 10x magnification.

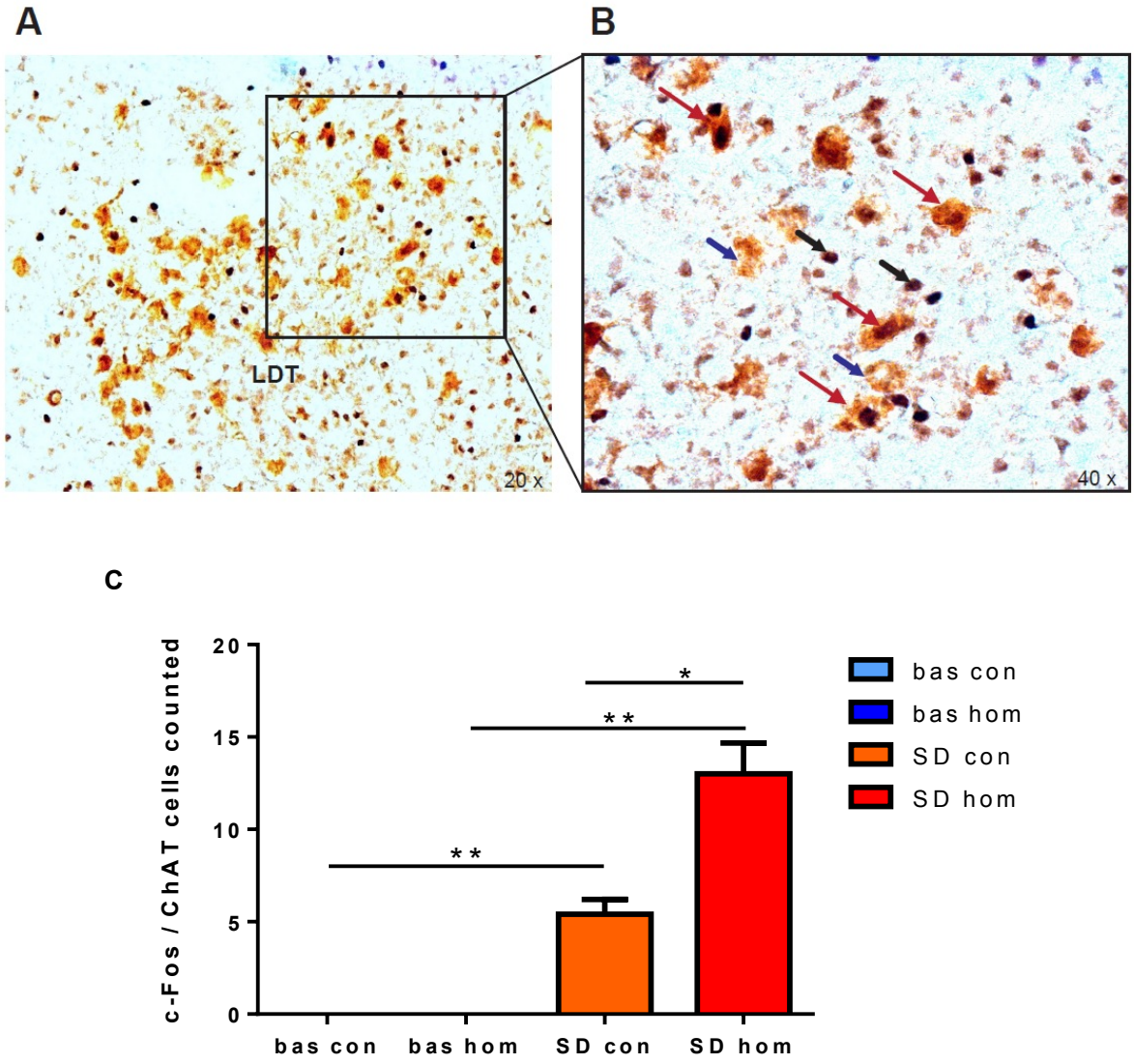

Figure 34: c-Fos/ChAT positive neurons in the laterodorsal tegmental nucleus (LDT) in CRH-COE Cam mice.

(A) and **(B)** are photomicrographs of a representative homozygous mouse after SD, showing c-Fos (black punctuate nucleus) and ChAT (brown/orange cytoplasmic staining) double staining at LDT level. **(B)** is a higher magnification of the rectangular box in **(A)**. Note the dense cluster of double-labeled neurons in the LDT of a homozygous mouse after SD. The black and the blue arrows indicate the single stained c-Fos and ChAT, respectively. The red arrows indicate the double-labeled neurons (c-Fos/ChAT). 20x magnification in **A** and 40x magnification in **B**. **(C)** Number of double-labeled c-Fos/ChAT positive neurons counted in 6 sections in control (con; n=5) and homozygous (hom; n=4) mice during baseline (bas) and SD condition. Height ± SEM of columns indicates number of c-Fos/Chat cells *P<0.01, **P<0.001 assessed by unpaired t-test.

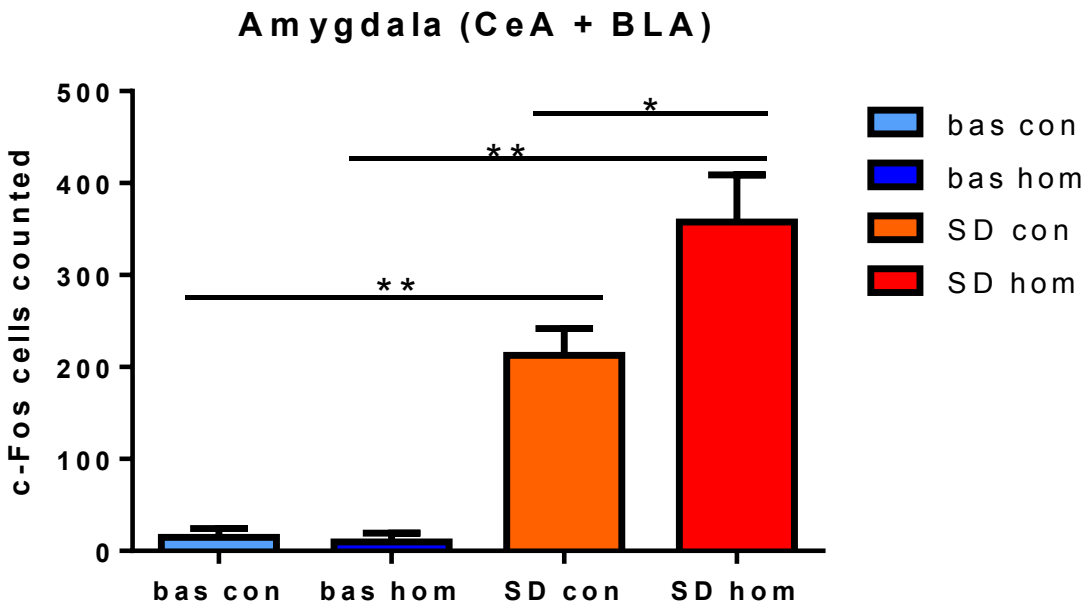

Figure 35: c-Fos positive neurons within the central nucleus (CeA) and the basolateral (BLA) amygdala in CRH-COE Cam mice.

Number of c-Fos positive neurons counted in three sections in control (con; n=5) and homozygous (hom; n=4) mice during baseline (bas) and SD condition. Height ± SEM of columns indicates number of c-Fos cells. *P<0.05, **P<0.001 assessed by unpaired t-test.

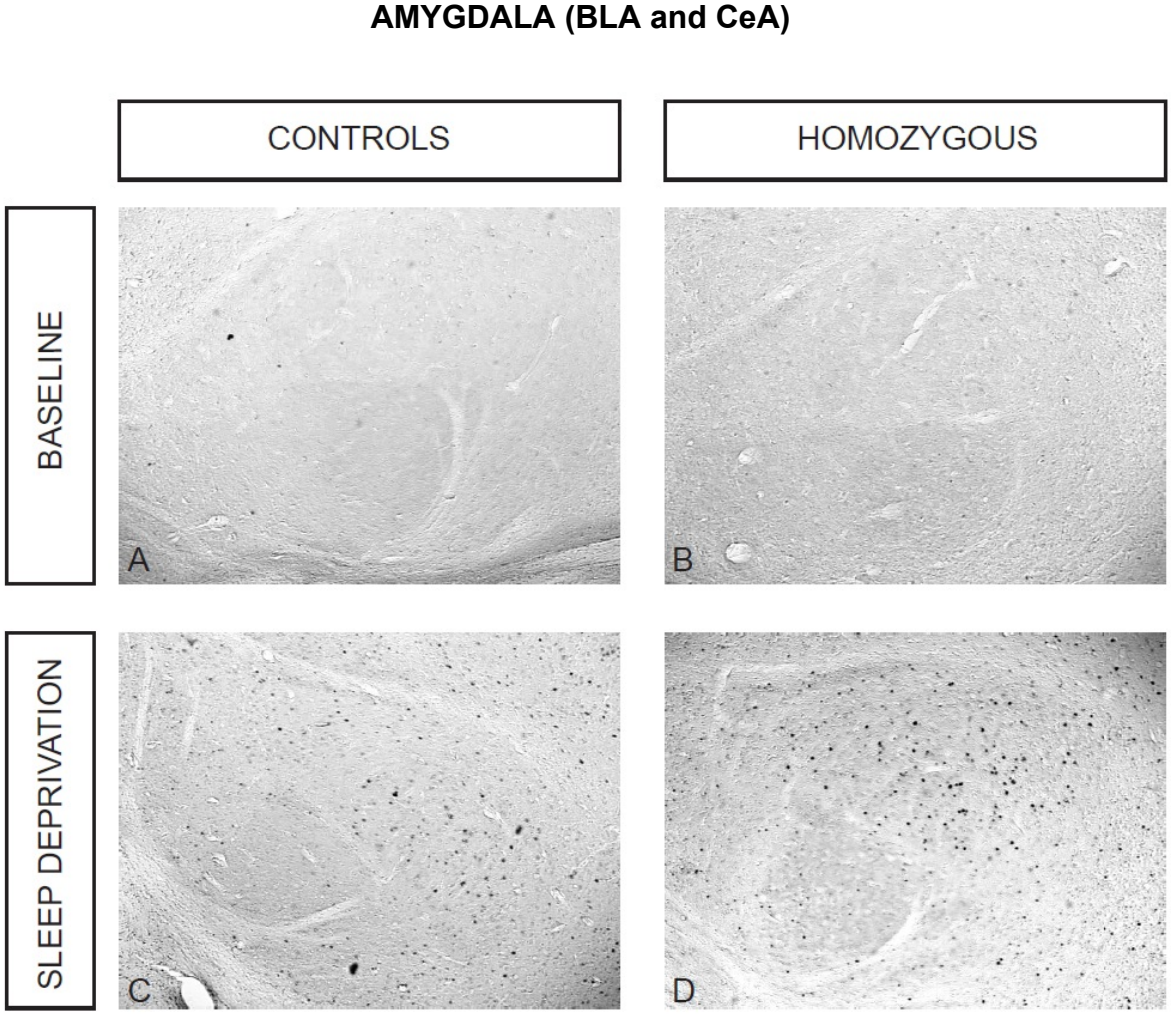

Figure 36: Photomicrographs of SD-induced or spontaneous c-Fos expression in homozygous and control CRH-COE-Cam mice within the central nucleus (CeA) and the basolateral (BLA) amygdala.

In non-SD (**A** and **B**) control and homozygous mice only few immunoreactive cells were detected, whereas abundant c-Fos positive cells were seen in both SD animals (**B** and **C**). 5x magnification.

6 Discussion

Previous data characterizing the significant contribution to enhanced REM sleep (REMS) of corticotropin-releasing hormone (CRH) specific to the limbic region (Kimura et al., 2010) were confirmed in the present study. To further explore the role of CRH in REMS regulation and to contribute to a better understanding of the underlying mechanism, three different approaches were conducted to examine the possible involvement of an altered cholinergic activity in sleep alteration occurring in CRH-COE Cam mice.

The main findings of the present study are that upregulated REMS in homozygous forebrain-specific CRH overexpressing mice can be decreased by injecting a muscarinic antagonist into the amygdala. Furthermore, these homozygous CRH-COE Cam mice possess higher extracellular levels of ACh in comparison to their control littermates, whereas spontaneous locomotor activity is similar in both genotypes, thus suggesting that higher ACh release is reflected in REMS enhancement. Finally cholinergic neurons within the brainstem REMS regulating structures become more active in homozygous CRH-COE-Cam mice in response to SD.

The results may indicate that CRH overexpression in the limbic system can lead to higher cholinergic activity and that it contributes to intensifying the mesopontine cholinergic system, which may at least in part result in upregulated REMS.

6.1 Characteristic sleep phenotype in forebrain-specific CRH overexpressing mice

6.1.1 Upregulated REM sleep

Homozygous mice overexpressing CRH within the forebrain (CRH-COE Cam) have a characteristic upregulated REMS compared with controls (Kimura et al., 2010), which could be confirmed in the present study. The corroborated REMS enhancement can

be taken as an endophenotype of increased CRH levels in the forebrain, including limbic structures, which coincides with altered REMS seen in patients with depressive symptoms. Specifically, these patients show an early occurrence of the first REMS period that is represented by a shorter latency and a higher density of eye movement (Figure 37A) (McCarley, 1982, Gottesmann and Gottesman, 2007). Parallels between increased REMS and appearance of depressive phenomena can be generated by common neurobiological control systems which were hypothesized by McCarley and supported by clinical data (Figure 37B) (McCarley, 1982). As in REMS regulation, the control of depressive phenomena involves a balance between the monoaminergic and cholinergic systems. Since the cholinergic system promotes both REMS and depression (Janowsky et al., 1980, Risch et al., 1980, Silberman et al., 1980, Brown, 2008, Lydic, 2008, Watson et al., 2010), the present study hypothesized that CRH overexpression in the forebrain including the limbic structure such as the amygdala affects REMS via an interaction with the cholinergic system.

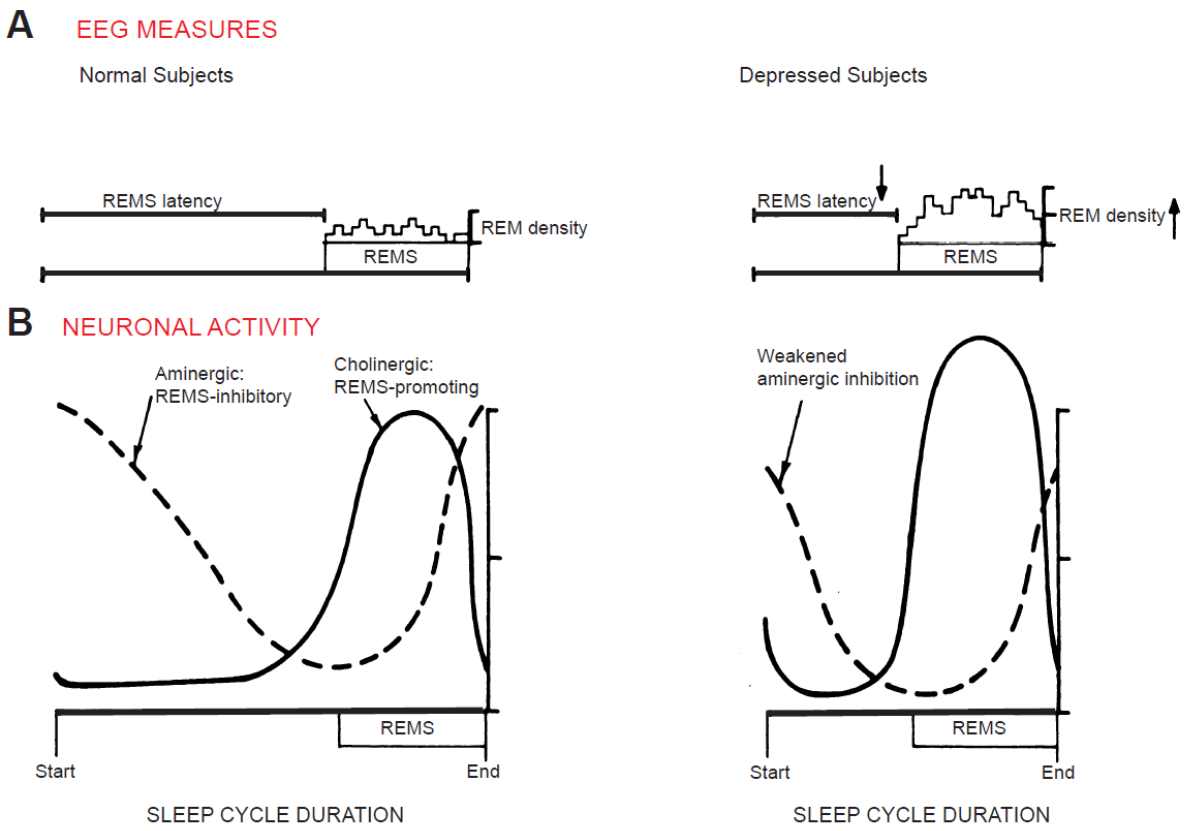

Figure 37: EEG measures and neuronal activity in the first cycle of normal and depressed subjects.

(A) Depressed patients show a first REMS period that has shorter latency and a higher density of eye movement. **(B)** REMS occurs when cholinergic activity becomes dominant with the gradual inhibition of the monoaminergic nuclei. The hypothesized weakened monoaminergic inhibition in depression produces a faster discharge from inhibition of the REMS-promoting cholinergic neurons and a resulting faster onset of REMS with stronger REM activity. Adapted from McCarley, 1982.

6.1.2 Effects of muscarinic antagonist on upregulated REM sleep

Blocking the muscarinic receptors by atropine injection into the amygdala decreased REMS in CRH-COE Cam mice. A similar result was obtained in a previous study performed on mice overexpressing CRH in the entire nervous system (CRH-COE Nes). In that study REMS was reduced by atropine injected intraperitoneally in both control and homozygous mice, but its effects lasted longer with a bigger magnitude in homozygous mice (own data, unpublished). This effect suggested that CRH in the brain may intensify cholinergic activity that results in elevated REMS and raised the question whether enhanced CRH expression in the amygdala of homozygous CRH-

COE Cam mice might similarly stimulate the cholinergic activity and would affect REMS. It is well known that the amygdala is implicated in emotional responses (Gray and Bingaman, 1996) that are closely related to REMS fluctuation. Several lines of evidence involve the amygdala, especially the central nucleus (CeA), in the regulation of REMS. For example inactivating the CeA with muscimol or TTX is able to produce a significant decrease in REMS in rats (Martin and Ghez, 1999, Sanford et al., 2002), whereas electric stimulation or cholinergic activation of the CeA promotes REMS in cats (Smith and Miskiman, 1975, Calvo et al., 1996). An association between amygdala activation and REMS is also proposed by an increase in the discharge rate of CeA neurons in cats during REMS (Frysinger et al., 1988), and by fMRI studies demonstrating that activity in the amygdala and appearance of REMS are correlated in humans (Maquet et al., 1996).

The reduction of REMS found after local microinjection into the CeA of atropine strongly suggests that CeA plays an important role in REMS regulation and support the findings of Calvo and colleagues showing increases in REMS after microinjections of the cholinergic agonist carbachol into CeA in cats (Calvo et al., 1996). A substrate for these effects could be provided by the brainstem REMS regulating areas (e.g. PPT, LDT, LC, PBN and SLD) since efferents from the amygdala are known to project to these areas (Krettek and Price, 1978, Moga and Gray, 1985, Rye et al., 1987, Semba and Fibiger, 1992).

The cholinergic input into the CeA arises from either the basal forebrain or the upper brainstem (Ottersen, 1981, Woolf and Butcher, 1982, Hecker and Mesulam, 1994); alternatively, intrinsic amygdaloid cholinergic neurons might also provide the CeA with cholinergic afferent projections (Nitecka and Frotscher, 1989) which in turn send fibers to the pontine nuclei. Moreover, muscarinic receptors are also concentrated in the CeA indicating that cholinergic/cholinoceptive neurons in the CeA might project to pontine nuclei and may participate in the modulation of REMS (Cortes and Palacios, 1986, Calvo et al., 1996). As shown in the present data, blocking the muscarinic receptor sites by atropine induces a reduction in REMS. Even though atropine has a high affinity for all 5 subtypes of muscarinic receptors (Rang, 2003), the amygdala contains only 3 subtypes, i.e., the inhibitory M_2 and excitatory M_1 and M_3 receptors (Cortes and Palacios, 1986, Mash and Potter, 1986, Spencer et al., 1986, Smith et al., 1991). Muscarinic receptors might modulate the excitatory output from the

amygdala to brainstem REMS regulating areas, however the exact interaction between the inhibitory (M_2) and excitatory (M_1 or M_3) muscarinic receptor types in the amygdala is not known.

In homozygous mice, atropine injection reduced REMS up to 6 hours, whereas in control animals REMS declined only for 2 hours during postinjection time. This may indicate that the number of muscarinic receptors blocked by atropine is greater in homozygous than control mice, suggesting that CRH overexpression in the forebrain may intensify the cholinergic system which in turn leads to a decrease in number of functional muscarinic cholinergic receptors in the amygdala.

6.2 Impacts of forebrain CRH overexpression on ACh release in the amygdala

Present microdialysis findings also support the hypothesis that cholinergic activity is higher in forebrain-specific CRH overexpressing mice than controls. Specifically, homozygous CRH-COE Cam mice showed constantly elevated ACh levels in the amygdala compared to controls. ACh release measured in the amygdala might correspond to the release from the terminals of neurons projecting from structures in the forebrain which are providing the main source of cholinergic input to the amygdala (Woolf and Butcher, 2011). In particular, the greatest number of cholinergic projecting neurons is found in the SI, nevertheless scattered cholinergic neurons projecting to the amygdala are also found in the diagonal band of Broca, medial septum and the NB (Mesulam et al., 1983, Woolf et al., 1984). With the exception of the NB, all of the cholinergic forebrain nuclei that are projecting to the amygdala are found to have cholinergic neurons coexpressing the CRHR1 (Sauvage and Steckler, 2001). This suggests that CRH is able to modulate ACh release in the CeA via stimulation of the CRHR1. In fact i.c.v. injections of CRH are reported to increase ACh release through CRHR1 receptor activation, although this was shown in the hippocampus. No directly confirming data are available regarding those effects in the amygdala (Day et al., 1998a, Day et al., 1998b).

To further clarify the effects of CRH on ACh release and to examine if CRH interacts with the cholinergic system through CRHR1, the present study examined the effects of a CRHR1 antagonist (DMP696) on ACh extracellular levels in the CeA in CRH-COE Cam mice (data not shown). Unexpectedly, three days of treatment with CRHR1 antagonist dissolved into drinking water did not show any effects on ACh release in homozygous mice and in controls. This result was in contrast to other findings showing that selective CRHR1 antagonists partly suppress the CRH-induced release of ACh in the hippocampus (Gully et al., 2002, Desvignes et al., 2003). Our result that DMP696 failed to affect ACh release could be explained by a different manner how CRH modulates ACh release levels in the amygdala. Besides acting directly on the cholinergic system via the CRHR1, CRH has also been reported to decrease high-affinity choline uptake, resulting in increased ACh release (Lai and Carino, 1990). On the other hand, the antagonist dose used in the present study might not have been sufficient to block the CRH signalling.

Present findings further demonstrated that both genotypes have a positive correlation with the levels of ACh in the CeA and spontaneous locomotor activity. As reported by Buzáki and colleagues, spontaneous movements are known to activate the cholinergic forebrain area in rodents; compared to the immobile condition neurons in this area are found to increase their firing frequency during spontaneous activity compared to the immobile condition (Buzsaki et al., 1988). Furthermore, behavioural arousal has been shown to induce increases in ACh release in the cerebral cortex, hippocampus and striatum (Day et al., 1991, Mizuno et al., 1991, Pepeu and Giovannini, 2004). Despite the considerable increase of ACh release in homozygous mice compared to their control littermates, both genotypes showed similar spontaneous locomotor activity. Therefore, enhanced ACh release in homozygous mice did not result from an increase in locomotor activity. Higher amount of ACh in homozygous mice might reflect their sleep phenotype of enhanced REMS but not locomotor activity. These results further emphasize that CRH overexpression in the amygdala may contribute to the enhanced affinity with the cholinergic system, resulting in a long-term enhancement of REMS.

6.3 Amygdaloid CRH and pontine cholinergic activation

The brainstem contains several key structures responsible for the initiation and maintenance of REMS (Jouvet, 1962), and is one of the targets of amygdaloid projections (Semba and Fibiger, 1992). Activation of CRH receptors (CRHR) by microinjection of CRH into CeA induced an increase of c-Fos expression in cholinergic structures such as the LDT and the sublaterodorsal tegmental nucleus (SLD) in normal C57BL/6J mice. This finding is in line with another study reporting an increase in single labeled c-Fos cells within the cholinergic brainstem after CRH infusion into the CeA of rats (Wiersma et al., 1998), supporting the hypothesis that limbic CRH projecting to brainstem REMS regulating structures is able to influence them. The CeA is known to project via the amygdalofugal pathway to several brainstem areas including the LDT area (Semba and Fibiger, 1992), the latter is known to coexpress CRH (Sauvage and Steckler, 2001). In addition, a major CRH pathway from the CeA projects to the cholinergic area within the brainstem (Amaral et al., 1992, Valentino et al., 1994). CRH is capable of activating these areas in the brainstem which results in muscle atonia that is one of the features accompanying REMS (Lai and Siegel, 1992).

The amygdala contains an abundance of CRH and its receptors (Merchenthaler, 1984, De Souza et al., 1985). Specifically, CRHR1 is the only subtype expressed in the CeA (Steckler and Holsboer, 1999), therefore the activation of brainstem structures in response to CRH injection could be mediated via this CRHR subtype.

Unexpectedly, none of the activated cells in the LDT and SLD were identified as cholinergic in response to CRH injection. Considering the high increase of c-Fos expression within the cholinergic cell area in comparison to saline injection, the lack of cholinergic neurons coexpressing c-Fos is somewhat unexpected. Both structures contain many cholinergic neurons (Sakai, 2012) which can coexpress c-Fos even in control conditions (Maloney et al., 1999). Therefore, this lack of cholinergic/c-Fos coexpressing neurons might have been caused by a low intensity of fluorescence labeling. In addition, the c-Fos staining method can also fail to reveal activated neurons (Kovacs, 1998). Another explanation could be that the neurons within the LDT and SLD are composed of other than cholinergic cells. In fact, there is evidence that a great part of the SLD neurons is glutamatergic and is able to trigger REMS

when activated (Clement et al., 2011, Luppi et al., 2011, Luppi et al., 2012). Moreover cholinergic LDT neurons are also known to be codistributed with the GABAergic neurons (Ford et al., 1995).

6.4 Effects of forebrain CRH overexpression on the cholinergic brainstem in response to SD

After confirming a significant influence of amygdaloid CRH on REMS regulating brainstem area in normal C57BL/6J mice, further findings in CRH-COE Cam mice proved that CRH overexpression in the forebrain is able to intensify the cholinergic activity within the brainstem in response of SD. Consistent with several studies examining c-Fos expression after physiological sleep and SD (Cirelli et al., 1995, Ledoux et al., 1996, Basheer et al., 1997), all brain structures of both genotypes examined in the present study showed low c-Fos expression reflecting a very low neuronal activation during baseline, whereas we observed an increase when the animals were kept awake for 6 hours. These results suggest that neurons in these areas are activated by forced wakefulness and might reflect a sleep need.

The low c-Fos staining after variable periods of sleep that included REMS episodes suggests that physiological REMS is not associated with c-Fos expression (Cirelli and Tononi, 2000). Nevertheless, a number of different non-pharmacological methods have been used to increase the duration of REMS periods in order to identify neuronal populations that are “activated” (Cirelli, 1999, Maloney et al., 1999, 2000, Verret et al., 2005). Specifically, these methods use REMS deprivation techniques in order to induce a long REMS rebound period. However, most previously applied selective REM SD methods such as the inverted “flower pot technique” or the “disk-over-water apparatus” are in fact stressful procedures that can induce a distinct activation of the HPA axis (Kovalzon and Tsibulsky, 1984, Coenen and van Luijtelaar, 1985, Suchecki et al., 1998). In order to avoid an additional stressor that could interfere with the neuronal activity of the examined structures, the present study used the gentle handling total SD method as a tool for building REMS need.

Interestingly homozygous mice showed significantly more c-Fos expression in the amygdala in response to SD when compared to control littermates. Since CRH overexpressing mice posses a higher REMS drive (Kimura et al., 2010), the increased neuronal activity seen in the amygdala in homozygous mice might reflect a REMS need and could contribute to initiate REMS after SD by its influence on brainstem structures. Similarly, SD increased c-Fos expression more in homozygous mice in the cholinergic LDT and PBN in comparison to controls, whereas c-Fos expression in the noradrenergic LC was comparable in both genotypes. The greater c-Fos induction after SD seen in homozygous mice suggests that the degree of activated cells in the REMS-related areas could provide an index of REMS need. On the contrary, as confirmed by the reciprocal interaction model (McCarley and Hobson, 1975), the noradrenergic LC in homozygous mice does not seem to play a major role in the regulation of REMS propensity and therefore no differences in genotype effects are observed after SD. Importantly, the higher number of c-Fos positive cells within the LDT in homozygous mice was found to be cholinergic, suggesting that the higher cholinergic activation could be responsible for the increased REMS drive found in the homozygous genotype.

In this model, CRH in the forebrain intensifies the cholinergic system within the brainstem, which may at least in part result in upregulated REMS after SD. This pathway, as limbic CRH activates cholinergic brainstem cells, may also apply to a mechanism of how stress increases REMS during recovery from SD.

7 General conclusions and outlook

The present study explored the role of CRH in REMS enhancement and provided a better understanding of the underlying mechanism. The confirmed REMS phenotype found in CRH-COE Cam mice suggested that overexpressed CRH in a specific structure of the limbic system could contribute to enhanced REMS by affecting a specific neurotransmitter activity known to play a role in REMS generation. In fact, CRH overexpression appears capable of stimulating the limbic cholinergic activity which in turn may lead to upregulated REMS. As seen in depressed patients, this animal model may possess hyper-cholinergic sensitivity that may contribute to REMS disinhibition. Furthermore, forebrain overexpressed CRH is also able to influence the neuronal activation in the brainstem where cholinergic and non-cholinergic neurons relevant for REMS regulation locate densely. Interestingly, in this animal model, CRH intensifies the mesopontine cholinergic system, whereas the monoaminergic system seems not to be affected, indicating the importance of ACh in mediating the effects of CRH on REMS-on cells. Increased activation of the cholinergic system by limbic CRH may thus be involved in REMS upregulation.

This thesis emphasizes that REMS upregulation seen in depressed patients might be the product of complex interactions between CRH and the cholinergic system. Further investigations are still necessary to complete the picture of the mechanism by which CRH influence REMS regulating structures.

According to the result, ACh release in the amygdala of CRH-COE Cam mice is increased, suggesting a higher cholinergic activity in the forebrain. A repetition of the microdialysis experiment targeting other cholinergic brain areas could confirm that CRH overexpression is able to induce ACh release. Since cholinergic neurons in the SI provide the major projections to the amygdala, immunohistochemistry for choline acetyltransferase could evidence a difference in the number of these neurons which contribute to the differential ACh release in homozygous amygdala.

The role of CRHR1 in mediating the interactions between CRH and ACh in CRH-COE Cam mice should be further explored. For instance, an i.p. pretreatment with the CRHR1 antagonist could reduce the CRH-mediated ACh release in the amygdala

and prove that this effect is CRHR1 mediated. Furthermore, a double-immunohistochemical procedure could detect differences between genotypes in the number of cholinergic neurons co-expressing CRHR1 in the cholinergic basal forebrain.

It was shown that the cholinergic system is affected by CRH overexpression; however further interactions of CRH and its receptors with other different neurotransmitter systems could exist in the CRH-COE Cam mouse model. Similarly to depression, a weakened monoamigergic system might also result in REMS disinhibition.

8 List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChRs	Acetylcholine receptors
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AVP	Arginin vasopressin
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of the amygdala
Ch	Choline
ChAT	Choline acetyltransferase
ChO	Choline oxidase
CNS	Central nervous system
Con	Control
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor type 1
CRHR2	Corticotropin-releasing hormone receptor type 2
CRHRs	Corticotropin-releasing hormone receptors
CSF	Cerebrospinal fluid
DR	Dorsal raphe
EEG	Electroencelography
EMG	Electromyography
GHRH	Growth-hormone-releasing hormone
GR	Glucocorticoid receptor
HDB	Horizontal limb of the diagonal band of Broca

Hom	Homozygous
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High pressure liquid chromatography
i.c.v.	Intracerebroventricular
i.v.	Intravenous
IS	Immobilization stress
LC	Locus coeruleus
LDT	Laterodorsal tegmental nucleus
LH	Lateral hypothalamus
LPT	Lateral pontine tegumentum
MnPO	Median preoptic area
MR	Mineralocorticoid receptor
MRN	Median raphe nucleus
mRNA	Messenger RNA
AChRs	Nicotinic acetylcholine receptors
NB	Nucleus basalis
NREMS	Non-rapid eye movement sleep
PAG	Periaqueductal grey
PBN	Parabrachial nucleus
PC	Precoeruleus
POMC	Pro-opiomelanocortin
PPT	Pedunculopontine tegmental nucleus
PVN	Paraventricular nucleus
REM	Rapid-eye movement
REMS	Rapid-eye movement sleep
RN	Raphe nucleus
SI	Substantia innominata
SLD	Sublaterodorsal tegmental nucleus

TMN	Tuberomammillary nucleus
TTX	Tetrodotoxin
VAChT	Vescicular acetylcholine transporter
VDB	Vertical limb of the diagonal band of broca
VLPO	Ventrolateral preoptic area
VPAG	Ventral periaqueductal gray

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11 Curriculum Vitae

Personal details

Last name:	Curzi
First names:	Maria Letizia
Date of Birth:	07.02.1984
Place of Birth:	Delft (NL)
Nationality:	Italian

Education

2009-2013 PhD student in the research group: Neurogenetics of Sleep

Max Planck Institute of Psychiatry, Munich, Germany

Thesis: “The role of corticotropin-releasing hormone in REM sleep regulation: A possible mechanism through the cholinergic system”

2002-2008 ALMA MATER STUDIORUM University of Bologna

Department of Biology

Bologna, Italy

2008 Master in Biological and Health Sciences

Thesis in Physiology: “The Influence of osmoregulation on wake-sleep cycle”.

2006 Bachelor in Biological Sciences

Thesis in Pharmacology: “Misuse of antimicrobial agents in five Italian hospitals”

2002 European School of Munich

Munich, Germany

Baccalaureate

Awards and Grants

2012 "Travel Grant for Young Researchers 2012" for a training visit at the institute of Biomedicine, University of Helsinki (FI) (sponsored by ESRS)

2011 World sleep 2011 Kyoto travel award (sponsored by the World Sleep Federation)

2010 Certification in "Sleep Research and Sleep Medicine Education Program European Sleep research Society" (awarded by the European Union Marie Curie Training Program)

Posters

2013 Kimura M, Curzi ML, Flachskamm C, Holsboer F, Deussing JM. Forebrain CRH overexpression facilitates activation of mesopontine cholinergic neurons in response to sleep deprivation. Neuroscience 2013, San Diego.

2012 Kimura M, Curzi ML, Flachskamm C, Holsboer F, Deussing JM. Cholinergic mediation of enhanced REM sleep in forebrain-specific CRH overexpressing mice. Neuroscience 2012, New Orleans.

2012 Curzi ML, Flachskamm C, Deussing JM, Kimura M. REM sleep and cholinergic hyperactivity in forebrain specific CRH-overexpressing mice. 21st Congress of the ESRS, Paris.

2011 Curzi M.L, Flachskamm C, Deussing J.M, Kimura M. Is enhanced REM sleep in conditional CRH-overexpressing mice due to cholinergic activation? World Sleep 2011, Kyoto.

2010 Curzi ML, Flachskamm C, Deussing JM, Kimura M. Cholinergic mediation of enhanced REM sleep in conditional CRH-overexpressing mice. 20th Congress of the ESRS, Lisbon.

Publications

2013 Jakubcakova V, Curzi ML, Flachskamm C, Hambsch B, Landgraf R, Kimura M. The glycolytic metabolite methylglyoxal induces changes in vigilance by generating low-amplitude non-REM sleep. *Journal of Psychopharmacology* 2013 Nov;27(11):1070-5

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13 Assertion / Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 30.Juli 2013