Sleep loss, brain vulnerability and psychopathology

Novati, Arianna

Sleep loss, brain vulnerability and psychopathology:

Experimental studies on the neurobiological consequences of chronic sleep restriction in rats

Arianna Novati

university of groningen

The studies described in the present thesis were carried out at the Department of Molecular Neurobiology and the Department of Behavioral Physiology of the University of Groningen and were financially supported by the Netherlands Organisation for Scientific Research (NWO-Vidi grant 864.04.002 to Peter Meerlo).

Nederlandse Organisatie voor Wetenschappelijk Onderzoek

The printing of this thesis was financially supported by the Graduate School of Science, Faculty of Mathematics and Natural Sciences and the University of Groningen.

Cover: Dick Visser

Printed by: Drukkerij van Denderen, Groningen

RIJKSUNIVERSITEIT GRONINGEN

Sleep loss, brain vulnerability and psychopathology:

Experimental studies on the neurobiological consequences of chronic sleep restriction in rats

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 3 februari 2012
om 16.15 uur

door

Arianna Novati

geboren op 25 augustus 1979

te Monfalcone, Italië

Promotor:	Prof dr J.M. Koolhaas
Copromotor:	Dr P. Meerlo
Beoordelingscommissie:	Prof dr P.J. Lucassen Prof dr T. Stenberg Prof dr A.J. Scheurink

ISBN: 978-90-367-5271-8
978-90-367-5272-5 (digital version)

TABLE OF CONTENTS

CHAPTER 1

General introduction

Background

Sleep is thought to be a process that serves neuronal recovery and synaptic plasticity, which in turn is crucial for brain function and performance (Benington and Frank 2003; Meerlo et al. 2009; Tononi and Cirelli 2006). However, the exact neurobiological processes at the molecular and cellular level underlying this important function of sleep are still unknown.

The expression of a wide variety of genes in the brain is regulated by sleep and altered by sleep deprivation (Cirelli 2002, 2006). Sleep loss can also influence the levels of brain neurotransmitters (Peñalva et al. 2003; Lopez- Rodriguez et al. 2003) and alter the expression and functionality of their receptors (Longordo et al. 2009). Several experiments show that restricted sleep affects different markers of adult neurogenesis as well (Meerlo et al. 2009). Through all these and other changes, sleep deprivation may ultimately impair the normal functionality of the brain and slowly increase the vulnerability to neuropathology.

If indeed sleep is of crucial importance for the brain, it seems likely that insufficient sleep may have deleterious consequences, especially when it becomes a chronic condition. This is an important issue since restricted and/or disrupted sleep loss is a common problem in our society (Bonnet and Arand 1995; Rajaratnam and Arendt 2001). A large number of people experience regular sleep loss as a consequence of our modern around-the-clock life style, shift work and an overall increase in work pressure (Bonnet and Arand 1995; Pilcher et al. 2000; Rajaratnam and Arendt 2001). In addition, staying up late for leisure activities is a common practice (Van den Bulck 2004; Basner and Dinges 2009). Importantly, sleep loss is not only a problem of adult people but is increasing among adolescents and young children as well (Meijer et al. 2000; Van den Bulck 2004; Bixler 2009). In young people, television, internet, and computer games are the most common causes for sleep shortage (Van de Bulk 2004; Dworak et al. 2007).

Sleep loss has various immediate effects, including tiredness, decreased attention, decreased motivation, and reduced cognitive function (Wolfson and Carskadon 2003; Durmer and Dinges 2005; Curcio et al. 2006; Banks and Dinges 2007). Moreover, some effects of sleep loss on cognitive functioning may accumulate over successive nights of sleep curtailment without being paralleled by equally increased feeling of sleepiness (Van Dongen et al. 2003). As consequence sleep loss is not always perceived as deleterious and therefore tends to become a habit more easily.

Whereas subjects may initially recover from the effects of sleep loss after subsequent sleep, frequent or chronic sleep loss could induce changes that are not immediately evident but accumulate over time, ultimately with serious health consequences. Short sleep alters several physiological parameters such as blood pressure, inflammatory markers and metabolic hormones and affects neurobehavioral aspects increasing also the risk to develop various types of diseases (Banks and Dinges 2007). Short sleep or sleep disorders are often associated with an increased risk of brain pathologies (Livingston et al. 1993; Chang et al. 1997; Malow 2004; Buysse et al. 2008; Germain et al. 2008; Roane and Taylor 2008).

Sleep loss and vulnerability to psychopathology

There is a complex and bidirectional relationship between sleep problems and mood disorders. Although disturbed sleep is often considered as a symptom and consequence of mood disorders such as depression, there is a rapidly growing amount of data suggesting that in some cases sleep disturbance may precede, predict and perhaps contribute to the development of psychiatric illnesses.

Subjects suffering from mood disturbances often complain of sleep problems. Many patients with depression experience insomnia (Lam 2006), have difficulty maintaining sleep, spend a shorter time in slow-wave sleep and have reduced REM sleep latency and increased REM sleep density compared to healthy individuals (Benca et al. 1992; Riemann et al. 2001). Interestingly, certain treatments administered for psychiatric disorders have a positive effect on sleep (Quera-Salva et al. 2010) and the other way around (Riemann et al. 2009). Moreover, insomniacs and other patients with sleep disorders often show depression and symptoms of anxiety (Taylor et al. 2005; Johnson et al. 2006; Macey et al. 2010). In fact, it has been suggested that insomnia in both young and adult subjects may be a risk factor to develop depression (Livingston et al. 1993; Chang et al. 1997; Buysse et al. 2008; Roane and Taylor 2008), the symptoms of which can appear also several years later (Chang et al. 1997).

Sleep disruption before or immediately after a traumatic experience is thought to increase the risk for post traumatic stress disorder (PTSD) and can exacerbate the symptoms of the disease (Krakow et al. 2001; Germain et al. 2008). Also, sleep loss has been proposed as risk factor for mania in patients with bipolar disorders (Wehr 1991) and in normal subjects (Jackson et al. 2003) and it has been associated with the development of puerperal psychosis (Sharma 2003). A relationship exists also between sleep disorders or insufficient sleep and suicidal tendency in both adult and young subjects with and without depression (Liu and Buysse 2006). Furthermore at young age, sleep problems seems to be linked to substance abuse (Shibley et al. 2008).

The cause effect-relation between sleep shortage and psychopathology is still unclear. Experiments in this thesis were based on the general hypothesis that sleep loss may affect brain vulnerability and sensitivity to psychopathologies, through a variety of different but interrelated pathways, including an increase in neurodegenerative processes and impairment of neuronal plasticity, both of which might be mediated by alterations in neurotransmitter systems (e.g., the serotonergic system) and changes in neuroendocrine regulation (e.g., the hypothalamic-pituitary-adrenal axis).

Neurodegeneration

Based on the idea that sleep serves neuronal recovery and synaptic plasticity, it is generally thought that lack of sleep has harmful effects that may impair neuronal integrity and perhaps contribute to neurodegeneration. Although several studies were devoted to this topic, all together

the results do not provide a clear picture of the relationship between sleep loss and neurodegeneration.

Different authors measured markers of neuronal degeneration after sleep deprivation in rat brain. Cirelli et al. (1999) showed no changes in markers of DNA damage and neuronal degeneration as measured by TUNEL and Fluor-Jade staining and no increase in the mRNA expression of apoptotic genes after total sleep deprivation for periods between 5 and 14 days. A study from Eiland et al. (2002) reported that 8-10 days of sleep deprivation did not induce cell damage, as assessed by silver staining, in most brain areas examined, except for a localized effect in the supraoptic nucleus of the hypothalamus. Other authors showed a temporary increase in the expression of apoptotic markers and an increase in the number of TUNEL- or silver-stained cells in several brain regions following rapid eye movement (REM) sleep deprivation lasting between 6 and 10 days (Biwas et al. 2006). In addition, various studies assessed measures that may indirectly indicate changes in neuronal viability. For example, some studies reported changes in measures of oxidative stress (D' Almeida et al. 1997, 1998; Ramanathan et al. 2002) while others showed altered expression of structural proteins, cell size and nuclear volume in sleep deprived animals (Majumdar and Mallick 2005; Biwas et al. 2006).

Establishing a link between sleep deprivation and neurodegeneration from the data of all these studies is complicated by several factors. First, most of the available data are based on animal studies that have used a variety of different sleep deprivation methods, including tread mills, rotating drums and platforms over water, each of which may be associated with different confounding factors and side effects that may not be related to sleep loss per se. In addition, the different methods have been applied to deprive animals of different sleep stages, i.e., total sleep deprivation or specific REM sleep deprivation. Second, the studies applied a wide variety of methods and measures to determine neurodegeneration. Some of the markers used to assess cell degeneration in these experiments, are only expressed for a short time. For example TUNEL staining can recognize dying cells only during DNA degradation and therefore for a period of time of hours, while amino cupric silver staining can detect neurodegeneration over a couple of days. Therefore, many of these measures are not suitable to detect gradually accumulating neurodegeneration in the course of prolonged periods of restricted or disrupted sleep as it often occurs in society.

Sleep loss might potentially increase the sensitivity to neurodegenerative processes through a variety of different routes, including changes in neuroprotective mechanisms or by influencing the sensitivity to excitatory pathways in the brain. Recent studies suggested that wakefulness and prolonged wakefulness by sleep deprivation are associated with synaptic potentiation and this is in part the result of enhanced expression of glutamate receptors (Vyazovskiy et al. 2008). The stimulation of glutamate receptors on neurons induces the influx of calcium that has important signaling functions inside the cell but may also lead to neuronal damage through different pathways when concentrations become too high (Ankarcrona et al. 1995; Sattler and Tymianski 2000). There is evidence that the expression of molecules that take

part in calcium dependent neurodegenerative and neuroprotective pathways such as Ca2+/calmodulin-dependent protein kinase (CAMKII) and calcineurin (Xifrò et al. 2008; Ashpole and Hudmon 2011) are altered in sleep deprived animals (Guzman-Marin et al. 2006; Wang et al. 2009; Alhaider et al. 2010). Moreover molecules having a function in neuroprotection against calcium toxicity like cAMP response element-binding (CREB), brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Kume et al. 2000; Gratacòs et al. 2001; Valera et al. 2008; Kim et al. 2009) show different expression during sleep deprivation (Sei et al. 2000; Guzma-Marin et al. 2006; Alhaider et al. 2011). It is not known whether very long periods of sleep loss increase the expression of glutamate receptors to a level that is toxic for neurons and changes the calcium signaling pathways into a direction that may enhance neurodegeneration and contribute to neuropathology. In support of a causal role of sleep loss as a process that may contribute to neurodegeneration is the occurrence of sleep disturbances in neurodegenerative disorders like Alzheimer or Parkinson's diseases and its association with the molecular and functional alterations in some of these diseases (Lauterbach et al. 2010).

Neuronal plasticity and neurogenesis

Irrespective of neurodegenerative processes, insufficient sleep may compromise brain function and contribute to neuropathology in more subtle ways by altering structural plasticity. The generation of new neurons in the adult brain has been associated with hippocampal functioning, memory formation (Deng et al. 2010) and emotional regulation (Perera et al. 2008). Reduced levels of such adult neurogenesis have been proposed as cause of mood alteration in depression (Sahay and Hen 2007; Perera et al. 2008). Animal models of depression based on exposure to stress have shown decreased neurogenesis in the dentate gyrus of the hippocampus (Czeh et al. 2001; Pham et al. 2003; Malberg and Duman 2003; Alonso et al. 2004) and a normalization of neurogenesis with administration of antidepressants (Malberg et al. 2000; Malberg and Duman 2003).

Sleep loss can inhibit cell proliferation and neurogenesis in the dentate gyrus of the hippocampus (for a review, see Meerlo et al. 2009). In rodents, these negative effects have been demonstrated with different methods of sleep deprivation (Guzman-Marin 2003, 2005; Tung et al. 2005; Guzman-Marin 2008; Mueller et al. 2008; Junek et al. 2010; Garcìa-Garcìa et al. 2011), sleep restriction (Hairston et al. 2005; Roman et al. 2005a) and sleep fragmentation (Guzman-Marin et al. 2007; Sportiche et al. 2010) and generally only occur following multiple days of restricted or disrupted sleep (Meerlo et al. 2009).The latter confirms that certain effects of insufficient sleep may accumulate over time. Sleep disturbance for periods shorter than 24 hours do not appear to affect cell proliferation rates (Roman et al. 2005a; Mirescu et al. 2006; Van der Borght et al. 2006; Guzman-Marin et al. 2007) or even increases cell proliferation (Grassi Zucconi et al. 2006). Only few sleep deprivation studies considered the survival of new cells. While one of

them has shown that sleep loss reduces survival of newly generated cells (Hairston et al. 2005), other studies found no effect on survival (Roman et al. 2005a; Garcìa-Garcìa et al. 2011).

The mechanisms by which sleep loss modulates neurogenesis are not known. The levels of neurogenesis in the hippocampus are regulated by a wide variety of molecules among which neurotransmitters, hormones, cytokines and growth factors (Abrous et al. 2005; Ming and Song 2005; Pathania et al. 2010; Platel et al. 2010) and are sensitive to various factors including stress, inflammation, and aging (Abrous et al. 2005; Lucassen et al. 2010).

Sleep loss could decrease neurogenesis levels by activating stress systems. The results of one study suggested that sleep deprivation decreases neurogenesis by increasing circulating levels of the adrenal stress hormone corticosterone (Mirescu et al. 2006). However, other studies showed that the negative effects of sleep loss on neurogenesis do not depend on corticosterone levels (Guzman-Marin et al. 2007; Mueller et al. 2008). Other potential mechanisms underlying the effect of sleep loss may involve changes in neurotransmitter systems, inflammatory processes, neurotrophic factors and cell signals that regulate neurogenesis (Meerlo et al. 2009; Lucassen et al. 2010; Pathania et al. 2010).

Another link between sleep loss, brain plasticity and neuropathology comes from data on changes in volume of the hippocampus and other brain regions. Neuroimaging studies in patients with mood disorders demonstrated reduced volume of the hippocampus (Sapolsky 2000; Czeh and Lucassen 2007; Perera et al. 2008, Boldrini et al. 2009, Lucassen et al. 2010) and decreased grey matter in some cortical regions (Bora et al. 2011). Decreased hippocampal volume and reduced cortical gray matter have been reported also in patients with chronic insomnia and obstructive sleep apnea (OSA) (Morrell et al. 2003; Riemann et al. 2007; Altena et al. 2010; Neylan et al. 2010; Torelli et al. 2011). These last findings may suggest that the sleep disturbances often present in patients with mood disorders contribute to the volume reduction in the examined brain areas.

Neurotransmitter systems: Serotonergic system

Insufficient sleep may also affect brain vulnerability through changes in neurotransmitter systems, e.g., the serotonergic system. Serotonergic neurons are located mostly in the raphe nuclei of the brainstem and send their innervation to the whole nervous system and in particular to the cerebral cortex, limbic areas, forebrain, basal ganglia, brainstem and grey matter in the spinal cord (Tork 1990). Through a complex system of different receptor subtypes (Martin and Humphrey 1994), serotonin is involved in the regulation of emotional, neuroendocrine, cognitive and motor functions in the central nervous system (CNS) (Dinan 1996; Monti and Jantos 2008). The serotonergic system plays an important role in the regulation of the sleep wake cycle (Portas et al. 2000). The extracellular concentration of serotonin in the brain stem and in cortical and sub-cortical areas receiving serotonergic projections, is highest during waking, decreases during slow wave sleep and reaches lowest values during REM sleep. This reflects the pattern of discharge of the

serotonergic neurons in the dorsal raphe nucleus which show the highest activity during the waking period, decrease their firing in slow wave sleep and are silent in the phase of REM sleep (Portas et al. 2000; Sakai and Crochet 2001).

The serotonergic system is sensitive to sleep loss. Studies in rodents have shown that sleep deprivation increases the firing rate of serotonergic neurons in the dorsal raphe (Prevot et al. 1996; Gardner et al. 1997) and increases the release and concentration of serotonin in some of the projection areas such as the hippocampus (Lopez-Rodriguez et al. 2003; Peñalva et al. 2003). It also enhances the serotonin turnover (Asikainen et al. 1995, 1997; Senthilvelan et al. 2006) and decreases serotonin transporter binding in some brain areas (Hipòlide et al. 2005). Together these results indicate a potentiated serotonergic signaling following sleep deprivation. The effects of chronic sleep loss on the serotonergic neurons are not well known; however, chronic sleep restriction in animals has been shown to cause a gradually developing desensitization of the serotonin-1A receptors (5-HT_{1A}) (Roman et al. 2005b, 2006). It has been suggested that this effect could be the result of the repeated stimulation of these receptors due to an enhanced serotonin release.

Reduced serotonergic transmission and reduced sensitivity of the 5-HT_{1A} receptor system represent a potential pathway through which sleep loss may alter neuronal plasticity and enhance the sensitivity to neurodegeneration. Agonists of the 5-HT_{1A} receptors can prevent neuronal apoptosis caused by different factors (Ahlemeyer and Krieglstein 1997; Semkova et al. 1998; Suchanek et al. 1998; Ahlemeyer et al. 1999) and activation of the 5-HT_{1A} receptors have neuroprotective effects in several models of brain damage such as excitotoxicity (Oosterink et al. 2003), ischemia (Semkova et al. 1998; Alessandri et al. 1999; Kukley et al. 2001) and traumatic brain injury (Kline et al. 2001; Cheng et al. 2007). The mechanisms responsible for the 5-HT_{1A} - mediated neuroprotective action include membrane hyperpolarization and reduced excitability (Kruger et al. 1999), reduced release of glutamate (Mauler et al. 2001) and blockade of voltage-sensitive Na+ channels (Melena et al. 2000). A desensitization of the 5-HT_{1A} receptors by chronically insufficient sleep might result in a decrease neuroprotection and therefore higher sensitivity to neurodegenerative processes.

In the long run, changes in the serotonergic system resulting from sleep loss might also contribute to psychopathology. Serotonergic neurotransmission is impaired in depression and other mood disorders. In postmortem brain tissue of depressed patients, the concentration of serotonin as well as the number of serotonin transporter binding sites is decreased, while the density of serotonin 2 receptor (5-HT_2) binding sites is increased. In depressed patients the concentrations of tryptophan in the plasma and the major metabolite of serotonin, 5-hydroxyindoleacetic acid, in the cerebrospinal fluid (CSF) are decreased. Moreover pharmacological studies have shown that the 5-HT_{1A} receptor is desensitized in depressed subjects (Lesch 1991; Mann et al. 1995) similarly to sleep deprived animals (Roman et al. 2005b, 2006). The latter is consistent with the idea that sleep disturbance by itself might be able to induce alterations that predispose individuals to mood disorders.

Neuroendocrine regulation: HPA axis

Another pathway through which restricted and disrupted sleep might affect brain vulnerability is by changes in neuroendocrine regulation, particularly changes in the HPA axis. The HPA axis is one of the major neuroendocrine systems involved in stress responses (de Kloet et al. 1993, 2005). A stressful stimulus in the organism induces the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus. CRH is transported through the portal system to the anterior pituitary where it induces the secretion of adrenocorticotropin hormone (ACTH) into the bloodstream. In turn, ACTH stimulates the release of glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal cortex. Glucocorticoids have to two types of receptors, the high affinity mineralcorticoid receptors (MR) or type 1 receptors and the low affinity glucocorticoids receptors (GR) or type 2 receptors. Through these receptors, glucocorticoids regulate a wide variety of processes in brain and body from energy homeostasis to cognitive function (de Kloet et al. 2005) and send feedback inhibition to the PVN and pituitary controlling the synthesis and release of CRH and ACTH (de Kloet et al. 1993; Yudt and Cidlowsky 2002). The HPA axis receives important regulatory inputs from the hippocampus, amygdala, bed nucleus of the stria terminalis, and the brain stem (Ulrich-Lay and Herman, 2009). Also the raphe serotonergic system regulates the HPA axis at multiple sites, mostly through the 5-HT_{1A} receptors (Dinan 1996).

Under normal baseline conditions, HPA axis activity displays a clear daily rhythm that is controlled by the suprachiasmatic nucleus of the hypothalamus (Moore and Eichler 1972) and is characterized by parallel changes in the levels of corticosterone and ACTH (Weitzman et al. 1983; Gudmundsson and Carnes 1997; Pruessner et al. 1997). In both diurnal and nocturnal species, glucocorticoid concentrations are lowest early in the resting phases and peak around or just before the start of the main activity phase (Weitzman et al. 1983; Gudmundsson and Carnes 1997; Pruessner et al. 1997).

Several studies indicate that sleep loss affects the HPA axis (for review, see Meerlo et al. 2008). Sleep deprivation in humans have been shown to induce activation of the HPA axis, increasing cortisol levels (Von Treuer et al. 1996; Leproult et al. 1997; Spiegel et al. 1999). Increased HPA axis activity occurs also in sleep deprived animals although the level of such activation can in part result from the sleep deprivation method used (Suchecki et al. 1998; Meerlo et al. 2002; Hipolide et al. 2006). This increased HPA axis activity during sleep deprivation may be a consequence of increased expression and release of CRH by PVN (Koban et al. 2006). Interestingly, sleep disorders such as insomnia and OSA have been associated with HPA axis dysfunction (Bratel et al. 1999; Vgontzas et al. 2001).

In addition to a direct activating effect of sleep deprivation on the HPA axis, chronically insufficient sleep may gradually alter the regulation of the HPA axis in more fundamental ways and change the reactivity of this system to subsequent new stressors. A number of studies have shown an altered HPA reactivity in sleep deprived animals that displayed blunted ACTH response

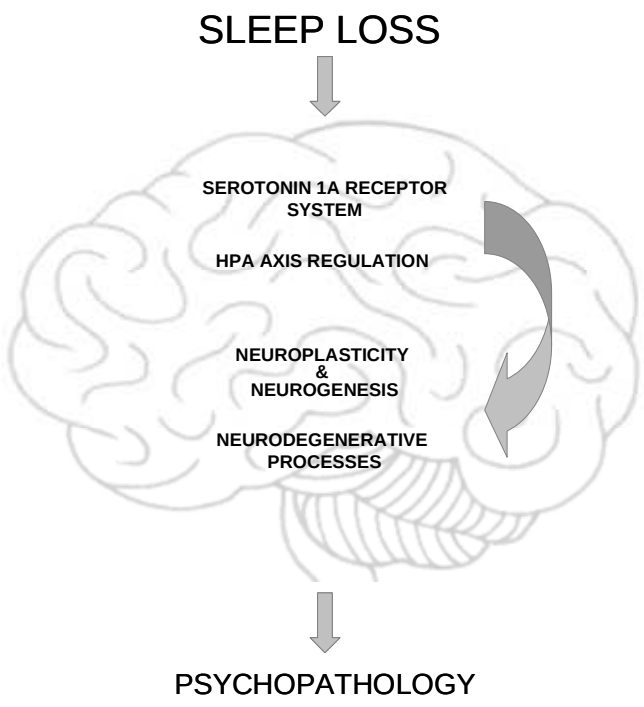
to a stressor (Meerlo et al. 2002; Sgoifo et al. 2006). The mechanisms mediating these effects of chronic sleep loss on HPA axis regulation are not known but could involve the HPA axis itself, as well as its regulatory input systems, including the serotonergic system (Prevot et al. 1996; Gardner et al. 1997; Lopez-Rodriguez et al. 2003; Peñalva et al. 2003; Hipòlide et al. 2005; Roman et al. 2005b; 2006; Alfaro-Rodriguez et al. 2006).

Regular sleep may contribute to a normal HPA axis function and maintenance of the glucocorticoid concentrations in a range that is protective for the brain, while chronic sleep loss, by altering their concentration, could have harmful consequences for the brain and contribute to pathology. Indeed, while optimal levels of glucocorticoids in the brain have neuroprotective effects (Rasmussen and Gulati 1962; De Courten-Myers et al. 1994; Abraham et al. 1997, 2000; Felszeghy et al. 2004; Schmitt et al. 2006), frequent exposure to high concentrations of glucocorticoids are thought to promote neuronal damage and cell loss, particularly in the hippocampus (Sapolsky et al. 1985, Sousa et al. 1999, Watanable et al. 1992). Elevated doses of glucocorticoids can also exacerbate the damage induced by several types of insults in the hippocampus, cortex and striatum (Sapolsky et al. 1985; Sapolsky and Pulsinelli 1985; Koide et al. 1986; Sapolsky 1986; Supko and Johnston 1994; Abraham et al. 2000; Roy and Sapolsky 2003). Moreover, different conditions with high glucocorticoids levels are associated with smaller hippocampal volume and cognitive impairment (Lupien et al. 1998; Sapolsky 2000, Huang et al. 2009).

In psychiatric disorders and especially in depression, the HPA axis shows abnormal activity. Depressed patients display elevated levels of CRF and cortisol (Nemeroff et al. 1984; Gillespie and Nemeroff 2005), reduced GR function (Pariante 2004), and in some cases a blunted ACTH response to CRF (Holsboer et al. 1984) that can be accompanied by increased volume of the pituitary and adrenal glands (Kessing et al. 2011).Considering that sleep loss affects the HPA axis function and that dysfunctional HPA axis is one of the most consistent findings in depressed subjects, it might be that sleep loss represents an important factor predisposing to depression through its influence on the HPA axis.

Aims and outline of the thesis

This thesis presents a series of studies on the consequences of chronic sleep restriction on brain vulnerability, with special emphasis on systems that have been implicated in psychopathology. Evidence suggests that sleep loss may increase the risk to develop psychopathology, although the mechanisms underlying this effect are largely unknown. Loss of sleep could increase the risk for psychopathology acting on various neurobiological systems. The work in this thesis focuses on the effects of sleep loss on serotonergic system (5-HT_{1A} receptor functionality) and neuroendocrine regulation (HPA axis activity and reactivity) that in turn may affect neurogenesis, neuroplasticity and neurodegenerative pathways (see scheme below). All these systems are influenced by sleep loss and altered in depression or other mood disorders.



The experiments of this thesis used an established rat model of chronic sleep restriction to mimic sleep loss in humans (Meerlo et al. 2002; Roman et al. 2005a, 2005b). Animals were subjected to a protocol of sleep restriction with only four daily hours of sleep, which presumably is not enough to recover from the period of wakefulness.

Based on the hypotheses that chronically insufficient sleep may increase brain vulnerability and sensitivity to neurodegenerative processes, in chapter 2 we examined the degree of neuronal damage induced by an acute neurotoxic insult in control animals and in animals that had been sleep restricted for 1 month. Animals received an injection with a neurotoxic concentration of NMDA in the cholinergic nucleus basalis magnocellularis (NBM) and established the degree of cholinergic cell and fiber loss.

In subsequent chapters, we assessed the effects of chronic sleep restriction on serotonergic function and HPA axis regulation. Chapter 3 describes how chronic sleep restriction alters subsequent HPA responses to acute stress challenges. The HPA axis response was studied measuring the plasma levels of ACTH and corticosterone. To assess potential mechanisms that might be underlying sleep-restriction induced changes in HPA axis regulation, we directly stimulated the HPA axis of sleep restricted and control animals with either CRH or a 5-HT_{1A} receptor agonist. Since sleep deprivation affects metabolism and in rats most often leads to a decrease in body weight, which in turn may affect serotonergic function, in chapter 4 we

examined whether sleep-restriction induced changes in 5-HT_{1A} sensitivity would persist independently of body weight loss. To prevent the decrease in body weight during sleep restriction, rats received a medium fat diet. In chapter 5, we studied the effect of chronic sleep loss on the HPA axis feedback system. In particular, we examined if the expression of MR and GR receptors is affected by chronic sleep restriction and chronic sleep restriction combined with repeated stress. In chapter 6, we extended our studies on the consequences of insufficient sleep to younger, adolescent animals and evaluated the effects of chronic sleep restriction on hippocampal plasticity, HPA axis activity and behavioral parameters of anxiety and anhedonia. The results of all the experimental chapters are finally discussed in chapter 7.

CHAPTER 2

Chronic partial sleep deprivation reduces brain sensitivity to glutamate N-methyl-D-aspartate receptor-mediated neurotoxicity

Arianna Novati, Henriëtte J Hulshof, Ivica Granic, Peter Meerlo

Journal of Sleep Research (2011), ahead of print

Abstract

It has been hypothesized that insufficient sleep may compromise neuronal function and contribute to neurodegenerative processes. While sleep loss by itself may not lead to cell death directly, it may affect the sensitivity to a subsequent neurodegenerative insult. Here we examined the effects of chronic sleep restriction (SR) on the vulnerability of the brain to N-methyl-d-aspartate (NMDA)-induced excitotoxicity. Animals were kept awake 20 h per day and were only allowed to rest during the first 4 h of the light phase, i.e. their normal circadian resting phase. After 30 days of SR all rats received a unilateral injection with a neurotoxic dose of NMDA into the nucleus basalis magnocellularis (NBM). Brains were collected for assessment of damage. In the intact non-injected hemisphere, the number of cholinergic cells in the NBM and the density of their projections in the cortex were not affected by SR. In the injected hemisphere, NMDA caused a significant loss of cholinergic NBM cells and cortical fibers in all animals. However, the loss of cholinergic cells was attenuated in the SR group as compared with the controls. These data suggest that, if anything, SR reduces the sensitivity to a subsequent excitotoxic insult. Chronic SR may constitute a mild threat to the brain that does not lead to neurodegeneration by itself but prepares the brain for subsequent neurotoxic challenges. These results do not support the hypothesis that sleep loss increases the sensitivity to neurodegenerative processes.

Introduction

Sleep is considered to be a process that serves neuronal recovery and synaptic plasticity, which in turn is crucial for brain function and performance (Benington and Frank 2003; Meerlo et al. 2009; Tononi and Cirelli 2006). Along these lines, it is generally thought that insufficient sleep has detrimental effects that may threaten neuronal integrity and perhaps contribute to neurodegeneration. Several studies have been devoted to this issue, but no clear picture on the relationship between sleep loss and neurodegeneration has emerged.

Several studies have examined this issue directly by staining for markers of dying neurons in brain tissue of sleep-deprived laboratory rats. One study reported that prolonged total sleep deprivation for 5–14 days had no effect on expression of apoptotic genes and did not increase staining for TUNEL and Fluor-Jade as markers of DNA damage and neuronal degeneration (Cirelli et al. 1999). Another study showed that sleep deprivation for 8–10 days did not increase cell damage in most brain regions as assessed by silver staining, except for a localized increase in the supraoptic nucleus of the hypothalamus (Eiland et al. 2002). A third study showed a temporary increase in the expression of apoptotic markers, and an increase in the number of TUNEL- or silver-stained degenerating cells in several brain areas of rats deprived of rapid eye movement (REM) sleep for 6–10 days (Biwas et al. 2006).

Other experiments have assessed effects of sleep deprivation on indirect measures that may indicate neurodegenerative processes and cell damage. Yet, these measurements do not actually prove there is an increased neurodegeneration. For example, a number of papers have reported on changes in the expression of structural proteins and other structural measures such as cell size and nuclear volume (Biwas et al. 2006; Majumdar and Mallick 2005), or on measures of oxidative stress that could potentially lead to cellular damage (D'Almeida et al. 1998; Ramanathan et al. 2002).

Interpretation of the data on sleep loss and neurodegeneration is complicated by the wide variety of experimental approaches used with respect to both duration (from days to weeks) and method of sleep deprivation (treadmills, rotating drums, platforms over water), as well as the sleep state most affected by it (non-REM or REM sleep). In addition, interpretation is further complicated by the variety of markers used to assess neurodegeneration and the fact that some of these are only expressed for a very brief period of time, which makes them unsuitable to establish cumulative cell loss over longer periods of sleep deprivation.

Collectively, the available literature does not appear to make a convincing case for sleep loss-induced neurodegeneration. However, even when sleep deprivation has little or no direct neurodegenerative effect by itself, it might still change the integrity and function of neurons in such a way that they become more sensitive to other neurodegenerative inputs and processes (e.g. stress, stroke, ageing).

Recent studies suggested that wakefulness and prolonged wakefulness by sleep deprivation are associated with synaptic potentiation, partly mediated by increased expression of

glutamate receptors (Vyazovskiy et al. 2008). A sleep loss-induced increase in glutamate receptor expression might increase the sensitivity of neurons to neurotoxic insults, which often involve glutamate signaling and calcium overload (Ankarcrona et al. 1995; Sattler and Tymianski 2000, 2001). Therefore, rather than measuring the direct neurodegenerative effect of sleep loss itself, one might assess the sensitivity of sleep-deprived animals to an acute glutamate receptor-mediated neurotoxic stimulus.

In the present study, we aimed to test whether chronic sleep restriction (SR) increases the subsequent sensitivity to a neurotoxic challenge by applying a well-validated neurodegeneration model. Particularly, we experimentally induced local damage of the nucleus basalis magnocellularis (NBM) in laboratory rats by overstimulation of the N-methyl-d-aspartate (NMDA) glutamate receptors (Luiten et al. 1995; Stewart et al. 1986). Overstimulation of these receptors induces a cascade of events that leads to cell damage and eventually to cell death (Choi 1988; Stewart et al. 1986). As a measure of brain vulnerability in sleep-restricted and control rats, we assessed the loss of NBM cholinergic neurons and their cortical fiber projections in the cortex.

Materials and methods

Animals and housing

This study was performed with adult male Wistar rats, weighing 200–300 g at the beginning of the experiment. Animals were individually housed in Plexiglas cages and kept in a room with a 12 : 12 h light : dark cycle (lights on 9AM – 9PM) and a temperature of 21±1℃. Standard laboratory chow food and water were provided ad libitum. The experiment was approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Sleep restriction and controls

A total of 30 animals were assigned to one of the following three groups (n = 10 in each): chronic SR, forced activity (FA) control or home cage control (HC). SR and control procedures were performed following the protocol used in previous studies (Novati et al. 2008; Roman et al. 2005a, 2005b). To assess the effects of insufficient sleep on the subsequent sensitivity to a neurodegenerative insult, rats were subjected to chronic SR for 30 days. SR was achieved by placing the animals in 40 cm diameter drums, rotating at the speed of 0.4 m/min. Rats were sleep deprived 20 h per day (1PM – 9AM) and were left undisturbed for the remaining 4 h at the beginning of the light phase (9AM – 1PM). To examine whether effects of the treatment were caused by forced locomotion rather than SR, a FA group was included as control. Animals of the FA group were housed in the same type of drums, but rotating at double speed for half the time (0.8 m/min for 10 h). The FA animals therefore walked the same distance as sleep-restricted animals, but had sufficient time to sleep. The 10-h FA was done in the dark phase, the main

activity phase of the rats (11PM – 9AM). In addition to the FA control, we also used undisturbed controls that remained in their home cage throughout the experiment (HC).

NMDA-induced damage to the NBM

After 30 days of restricted sleep or control procedures, all rats received a unilateral injection with a neurotoxic dose of NMDA into the NBM (see Figure 1). This is a well-validated model to study neurodegenerative processes and treatments that modulate the degree of neurodegeneration (Dolga et al. 2009; Horvath et al. 2000; Luiten et al. 1995; Stewart et al. 1986). The NBM contains NMDA receptor-expressing cholinergic neurons that project to the neocortex (Mesulam et al. 1983; Wenk et al. 1980). These cortical projections are exclusively unilateral (Semba et al. 1988). Therefore, with unilateral NMDA injection and selective damage to the NBM in one hemisphere, one can use the other hemisphere as an intact, within-individual control to assess loss of cholinergic cells and projections (Horvath et al. 2000; Luiten et al. 1995). Each rat received a unilateral injection of NMDA (60 nmol in 1 µl of phosphate buffer saline at pH 7.4; Sigma, St Louis, MO, USA) at the level of the NBM (coordinates: AP –1.5 mm, L 3.2 mm from bregma; V – 6.2 mm and –7 mm from the dura; Paxinos and Watson, 1986). The concentration of NMDA was chosen to produce submaximal damage (Luiten et al., 1995). At each of the two dorso-ventral injection sites, 0.5 µl NMDA was infused over a period of 5 min. After surgery, the animals were returned to their home cage and were allowed to recover for the next 8 days.

Brain collection

Eight days after the NMDA injection, the brains of the rats were collected for immunocytochemical analysis. Animals were injected with 2 ml/kg pentobarbital and transcardially perfused, first with saline and then with a 4% solution of paraformaldehyde in 0.1 m phosphate buffer. Brains were removed from the skull, postfixated in 4% paraformaldehyde for 24 h and immersed in 30% sucrose for 48 h for cryoprotection. Eight series of 20 µm sections were cut with a cryostat and preserved in 0.01 M PBS with 0.1% sodium azide for further processing.

Immunocytochemistry

The number of cholinergic cells in the NBM and the density of cholinergic fiber projections in the cortex were assessed on the basis of immunostaining for choline acetyltransferase (ChAT). This enzyme for the synthesis of acetylcholine is highly expressed in cholinergic cell bodies and terminals (Wu and Hersh 1994), and is commonly used as a marker to estimate the number of cholinergic cells and fibers (Dolga et al. 2009; Horvath et al. 2000; Luiten et al. 1995); for an illustration see Fig. 1. Immunostaining for ChAT was performed following previously published methods (Dolga et al. 2009; Horvath et al. 2000). Sections were pretreated for 30 min with 0.3% H_2O_2 and 1 h with 5% normal rabbit serum to prevent non-specific staining before incubation with 1 : 400 goat anti-ChAT primary antibody (Chemicon International, Temecula, CA, USA) for 48 h at 4 ℃. Tissue was exposed to 1 : 500 rabbit anti-goa t secondary antibody for 2 h (Jackson

ImmunoResearch, Suffolk, UK). Sections were then incubated for 2 h with 1 : 500 avidine-biotine complex (Elite Vector Laboratories, Burlingame, CA, USA). The staining was developed through reaction with 0.2 mg/ml diaminobenzidine and 0.003% H_2O_2.

Quantification

Cholinergic cells were counted in the NBM of both brain hemispheres, in three sections per animal between bregma −1.2 and −1.8, with about 280–300 µm intersectional distance (Paxinos and Watson 1986). Cell counting was carried out using a microscope at a 400x final magnification. The number of cells in the examined sections was divided by the NBM surface (mm^2) as measured in the same sections with a computerized image analysis system (Leica Qwin, Rijswijk, the Netherlands). The resulting value was expressed as number of cells per mm^2. Quantification of the cholinergic fiber density was performed in the same sections with the same image analysis system. The coverage area of ChAT-positive fibers was measured in layer V of the somatosensory cortex, with three measurements per hemisphere, per section for each animal. After background subtraction and grey-scale threshold determination, the surface area of skeletonized ChAT-positive fibers was computed (the area covered by ChAT-positive fibers/total sampling area). The loss of cholinergic cells and fibers induced by the NMDA injection was calculated for each animal by comparing the intact hemisphere with the damaged side and was expressed as percentage difference.

Statistics

To establish if the NMDA injection effectively induced neurodegeneration in each of the three treatment groups, ChAT-positive cell number and fiber density in the intact and damaged hemisphere were compared with a paired t-test. To establish if SR by itself had an effect on the NBM cholinergic cell number and projection density, independent of the NMDA injection, cell number and fiber density in the intact hemisphere of the different treatment groups were compared by one-way analysis of variance (ANOVA). To assess if SR altered the sensitivity to NMDA-induced damage, the percentage cell loss and fiber loss in the three treatment groups were also compared by one-way anova. When anova revealed a significant treatment effect, a post hoc Fisher's LSD test was applied to determine differences between specific groups. The level of significance for all tests was set at $P < 0.05$. All data in text and figures are expressed as average per group ± SEM.

Results

Figure 1 shows representative photographs of the ChAT-positive cells in the NBM as well as ChAT-positive fiber projections in the cortex. The photographs also illustrate the effect of the NMDA injection into the NBM.

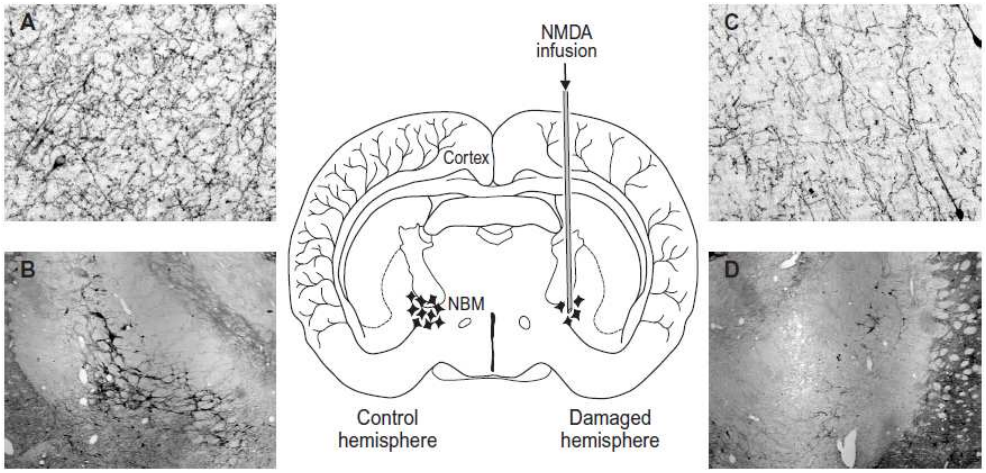


Fig.1. Unilateral injection of N-methyl-D-aspartate (NMDA) in the nucleus basalis magnocellularis (NBM). NMDA infused in the NBM of one hemisphere induces degeneration of the resident cholinergic cells and their projection to the cortex. As the cholinergic projections to the cortex are exclusively unilateral, the loss of cholinergic cells and fibers occur only in the injected hemisphere. A, B) Photographs of ChAt-positive fibers in the cortex and ChAt-positive cells in the NBM in the intact hemisphere. C, D) Photographs of ChAt-positive fibers in the cortex and ChAt-positive cells in the NBM in the injected hemisphere.

From the 30 animals used (n = 10 in each groups), four were excluded from the analysis because the NMDA injection was not centered in the NBM (2 HC, 2 SR). Due to damaged section and staining artifacts, four additional animals had to be excluded from the cortical fiber density analysis (1 HC, 2 FA, 1SR).

Chronic SR by itself had no significant effect on ChAT-positive cell number and fiber density in the intact, non-damaged hemisphere (cell number: $F_{2,23}$=0.99, P=0.39; Fig. 2a; fiber density: $F_{2,19}$=0.64, P=0.54; Fig. 3a).

In the injected hemisphere, NMDA caused a highly significant loss of ChAT-positive cholinergic neurons in the NBM as compared with the number of cells in intact hemisphere in all three treatment groups (HC: t=11.80, P<0.001; FA: t=17.57, P<0.001; SR: t=8.35, P<0.001; Fig. 2b). It also caused a highly significant loss of ChAT-positive fibers in the cortex in all three groups (HC: t=8.67, P<0.001; FA: t=10.75, P<0.001; SR: t=11.32, P<0.001; Fig. 3b). Importantly, the NMDA-induced loss of cholinergic cells in the NBM differed among the experimental groups ($F_{2,23}$=8.17, P=0.002). On average, cell loss in the SR rats was significantly smaller than in FA and HC control groups (post hoc LSD test: SR versus FA P=0.032; SR versus HC P<0.001; FA versus HC P=0.061; Fig. 2b). Also the loss of ChAT-positive fibers in the cortex was affected by the experimental treatment ($F_{2,19}$=7.98, P=0.003), and was lower in SR and FA rats as compared with the HC group (post hoc LSD test: SR versus HC P=0.019; FA versus HC P<0.001; Fig. 3b).

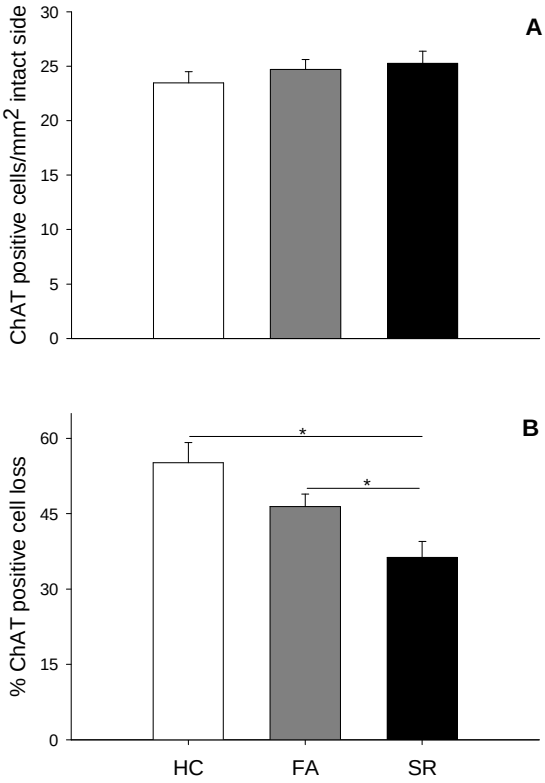


Fig.2. Chronic sleep restriction (SR) and cholinergic cell numbers in the NBM. A) The number of choline acetyltransferase (ChAT)-positive cells in the intact side of the NBM was not significantly different in the three groups of rats. B) The percentage of ChAT-positive cells lost in SR animals was significantly lower than in forced activity (FA) and home cage (HC) animals. * $P < 0.05$

Discussion

The aim of this study was to test the hypothesis that chronically insufficient sleep sensitizes the brain to neurodegenerative processes, specifically glutamate receptor-mediated neurotoxicity. After 30 days of SR, rats received an injection with a neurotoxic dose of NMDA unilaterally into the NBM of the basal forebrain, which caused a significant loss of cholinergic neurons and fiber projections to the cortex. However, contrary to the hypothesis, this effect was smaller in sleep-restricted animals as compared with non-sleep-restricted controls.

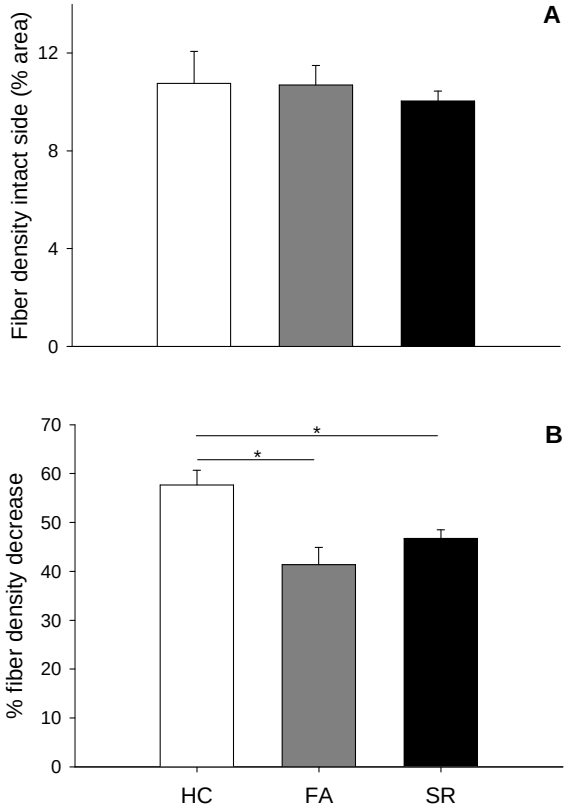


Fig.3. Chronic sleep restriction (SR) and cholinergic fiber density in the cortex. A) The area covered by fibers in the fourth layer of the somato-sensory cortex of the intact side was comparable among experimental groups. B) The loss of ChAt positive fibers in the cortex was significantly lower in both SR and forced activity (FA) groups compared with home cage (HC) controls. * $P < 0.05$

Rats in the present study received a unilateral injection of NMDA in the NBM, which allows for a comparison of the damaged hemisphere with the intact hemisphere. Analysis of the intact side showed similar cholinergic cell numbers and cortical fiber densities in all groups, indicating that chronic SR by itself had no effect on the cell numbers and fiber projections. This finding is in agreement with earlier studies suggesting that prolonged sleep deprivation per se does not lead to widespread neuronal damage and death (Cirelli et al. 1999; Eiland et al. 2002).

While SR had no direct neurodegenerative effect, it attenuated the damage induced by the NMDA injection into the NBM. This attenuated NMDA-induced neurodegeneration in sleep-restricted rats was to some extent observed in the FA controls as well. These animals were housed in the same rotating drums as the sleep-restricted animals, but walked for half the time at double speed. Thus, they covered the same distance, but had sufficient time to sleep. The

cholinergic cell loss of these animals was in between that of sleep-restricted rats and home cage controls (significantly different from SR and a trend towards a difference with HC). Therefore, while the mild FA involved in our sleep deprivation procedure may have been partly responsible for the attenuated cell loss, it does not explain all of it.

Although the NMDA-induced cholinergic cell loss in the NBM was stronger in the FA control rats than in the SR rats, the reduction in fiber density in the cortex was similar. The reason for this is unclear but it may be that in sleep-restricted rats more cells were preserved, but these surviving cells had fewer projecting fibers.

All together, while part of the effect of chronic SR may have been a non-specific effect of FA, sleep loss per se may have further attenuated cell loss. Importantly, the direction of this effect directly opposes the hypothesis that sleep loss increases the sensitivity to neurodegenerative processes. Yet, our results are in agreement with a number of other publications. Studies from at least two different laboratories reported attenuated damage in sleep-deprived rats subjected to cerebral artery occlusion, an experimental model of stroke (Hsu et al. 2003; Moldovan et al. 2010). In one of these studies (Hsu et al. 2003), rats were sleep deprived for 5 days before undergoing cerebral ischaemia. The neuronal damage on the pyramidal cells in the CA1 of hippocampus and the glial reactivity following the ischaemic event was lower in sleep-deprived animals compared with control animals. The second study (Moldovan et al. 2010) assessed the damage induced by cerebral ischaemia after a brief episode of 6 h sleep deprivation. The authors reported reduced neuronal injury and better sensorimotor performance in sleep-deprived animals compared with control animals during the first week of recovery. In agreement with our findings, these experiments suggest that sleep deprivation prior to a neurodegenerative insult may reduce subsequent brain damage. In contrast, one recent study reported that 12 h sleep deprivation or 3 days of sleep disturbance increased cell death and infarct volume in a rat model of focal cerebral ischaemia (Gao et al. 2010). In this latter study, rats were subjected to sleep deprivation or disturbance after the ischaemic insult, instead of before, as was the case in the other papers. Further studies are required to assess whether this indeed is a crucial difference leading to opposite results. Together these findings might indicate that sleep deprivation before a neurodegenerative insult attenuates the damage, whereas sleep deprivation after an insult increases the damage.

The mechanism through which SR in our study attenuated experimental neurodegeneration remains to be established. Alterations in glutamate receptor expression and glutamate signaling might be one possibility as that is the system targeted in our model. In fact, we anticipated that SR might sensitize the brain to neurodegeneration on the basis of reports suggesting increased expression of glutamate receptors with prolonged wakefulness (Vyazovskiy et al. 2008). On the other hand, other studies have shown that sleep deprivation may decrease expression and functionality of glutamate NMDA and AMPA receptors (Hagewoud et al. 2010a; Kopp et al. 2006; McDermott et al. 2006). Such a decrease in glutamate receptor expression may not only reduce the functional excitability but also reduce the sensitivity to overstimulation and

excitotoxicity, which would be in line with our current findings. Yet, all of these studies applied relatively short sleep deprivation periods, and it remains to be established how glutamate receptor signaling is affected when SR becomes a chronic condition.

An alternative pathway that might be involved in the attenuated neurodegeneration in sleep-restricted rats is the adenosine receptor system. Besides regulating sleep homeostasis in the basal forebrain (Bjorness et al. 2009; Porkka-Heiskanen et al. 1997; Thakkar et al. 2003), adenosine is known to exert neuroprotective functions that are mostly dependent on the adenosine A1 receptor (Cunha 2005). Acute sleep deprivation was found to increase the expression of adenosine A1 receptors (Basheer et al. 2007; Elmenhorst et al. 2007; Yanik and Radulovacki 1987), which would be in line with our findings of a reduced sensitivity to a neurotoxic insult. Again, it remains to be established how adenosine turnover and receptor expression are affected following SR over more prolonged periods of time.

Recently it was shown that a brief 6 h sleep deprivation increases the activity of certain antioxidant enzymes and levels of antioxidants in various brain regions in the rat (Ramanathan et al. 2010). On the basis of this finding, one might argue that an increase in antioxidant defense responses constitutes another potential mechanism through which chronic SR might protect the brain against damage caused by an acute insult. However, the literature on sleep deprivation and antioxidant defense mechanisms is rather inconsistent. In fact, the majority of studies suggest that prolonged deprivation or disturbance of sleep either has no effect (D'Almeida et al. 1997; Gopalakrishnan and Cirelli 2004) or it reduces the antioxidant responses and increases measures of oxidative stress (D'Almeida et al. 1998; Ramanathan et al. 2002). The latter would not be in line with our findings.

One way or another, sleep deprivation might constitute a threat to neuronal integrity that in itself is not sufficient to cause neurodegeneration but gradually activates compensatory neuroprotective mechanisms. As such, sleep deprivation might act as a pre-conditioning stimulus, which, when it precedes an acute insult, may attenuate the subsequent damage (Hsu et al. 2003; Moldovan et al. 2010).

In conclusion, the present study shows that chronic SR results in an attenuation of the damage caused by an acute neurotoxic insult. The mechanism of this effect remains to be established, but this finding clearly opposes the hypothesis that insufficient sleep sensitizes the brain to neurodegeneration.

Acknowledgments

The authors thank Jan Keijser, Folkert Postema and Csaba Nyakas for their assistance with the immunohistochemical procedures and analysis. We also thank Dick Visser for preparing Figure 1. This study was supported by the Nederlands Organization for Scientific Research (VIDI grant 864.04.002 to P. Meerlo).

CHAPTER 3

Chronically restricted sleep leads to depression-like changes in neurotransmitter receptor sensitivity and neuroendocrine stress reactivity in rats

Arianna Novati, Viktor Roman, Timur Cetin, Roelina Hagewoud, Johan A. den Boer, Paul G.M. Luiten, Peter Meerlo

Sleep (2008), 31: 1579-1585

Abstract

Frequently disrupted and restricted sleep is a common problem for many people in our Western society. In the long run, insufficient sleep may have repercussions for health and may sensitize individuals to psychiatric diseases. In this context, we applied an animal model of chronic sleep restriction to study effects of sleep loss on neurobiological and neuroendocrine systems that have been implied in the pathophysiology of depression, particularly the serotonergic system and the hypothalamic-pituitary-adrenal (HPA) axis.

Adult rats were exposed to a schedule of chronic partial sleep deprivation allowing them only 4 h of sleep per day. Sleep restriction was achieved by placing the animals in slowly rotating drums. To examine the regulation and reactivity of the HPA axis, blood samples were collected to measure adrenocorticotropin (ACTH) and corticosterone (CORT) responses.

While one day of restricted sleep had no significant effect on HPA axis stress reactivity, sleep restriction for a week caused a blunted pituitary ACTH response in a conditioned fear paradigm. Despite this lower ACTH response, adrenal CORT release was normal. The blunted pituitary response may be related to reduced sensitivity of serotonin-1A receptors and/or receptors for corticotrophin releasing hormone (CRH), since sleep restricted rats showed similar reductions in ACTH release to direct pharmacological stimulation with a serotonin-1A agonist or CRH.

Chronic sleep restriction may lead to changes in neurotransmitter receptor systems and neuroendocrine reactivity in a manner similar to that seen in depression. This experimental study thus supports the hypothesis that disrupted and restricted sleep may contribute to the symptomatology of psychiatric disorders.

Introduction

Restricted or disrupted sleep is a widespread and serious problem in our western society (Bonnet and Arand 1995; Rajaratnam and Arendt 2001). Many people experience regular sleep loss due to our modern around-the-clock lifestyle, increased work pressure, and psychosocial stress. In the long run, insufficient sleep may have many yet unknown repercussions for health and well being. Controlled studies have shown that acute sleep deprivation affects cognitive performance and emotionality (Pilcher and Huffcutt 1996). Recent experimental studies in healthy subjects show that successive nights of restricted sleep result in a gradually accumulating decline in cognitive function (Dinges et al. 1997; Van Dongen et al. 2003). Whereas subjects may initially recover from these effects after subsequent sleep, frequent or chronic sleep loss may induce neurobiological changes that are not immediately evident but accumulate over time, ultimately with serious health consequences. Indeed, sleep complaints and restricted sleep have been identified as risk factors for various diseases including psychiatric disorders (Breslau et al. 1996; Chang et al. 1997, Ford and Kamerow 1989, Koren et al. 2002; Neckelmann et al. 2007; Buysse et al. 2008).

Although sleep disturbances associated with psychiatric disorders are traditionally considered as a symptom of the disease, several studies suggest that the relationship between sleep changes and mood disorders is more complex and may work in the other direction as well. Instead of being a symptom, disrupted and restricted sleep may also be a causal factor that sensitizes individuals and contributes to the development of mood disorders (Riemann et al. 2003; Taylor at al. 2008). Consistent with this, primary insomnia often precedes and predicts the onset of a new depressive episode (Ford and Kamerow 1989; Breslau et al. 1996; Chang et al. 1997; Neckelmann et al. 2007; Buysse et al. 2008). However, in a clinical setting, cause and consequence are often difficult to separate, and the mechanisms by which disrupted sleep might contribute to the development of mood disorders are unknown.

In this context, we applied an animal model to establish the consequences of chronically disrupted and restricted sleep. We focused our attention on neurobiological and neuroendocrine systems that have been implicated in the pathophysiology of depression, particularly the serotonergic system and the hypothalamic-pituitary-adrenal (HPA) axis (Meerlo et al. 2002; Roman et al. 2005b, 2006).

The HPA axis is an important neuroendocrine stress system, and depression is often described as a condition with HPA axis overactivity on the basis of elevated CRH and cortisol levels (Nemeroff et al. 1984; Gillepsie and Nemeroff 2005). On the other hand, depressed patients often display a blunted pituitary ACTH response (Holsboer et al. 1984, 1987). Perhaps the chronically elevated CRH levels gradually desensitize the CRH receptors, which in turn may be responsible for the attenuated pituitary responsiveness. Alternatively, the attenuated pituitary ACTH response may also be a result of reduced sensitivity of serotonin receptors and reduced serotonergic neurotransmission. The serotonin-1A receptors in particular are involved in

regulating ACTH release, not only directly at the level of the pituitary, but also at the level of the paraventricular nucleus of the hypothalamus (Fuller 1992; Dinan 1996). Several lines of evidence indicate that serotonergic neurotransmission is impaired in depression (Cryan and Leonard 2000; Sobczak at al. 2002; Stockmeier 2003). A decrease in serotonin-1A receptor-mediated signaling in depressed patients has been shown by pharmacological challenges (Lesch et al. 1990; Mann et al. 1995; Shapira et al. 2000) and positron emission tomography (PET) studies (Drevets et al. 1999; Sargent et al. 2000; Drevets et al. 2007; Hirvonen et al. 2008). Although postmortem studies have yielded various results, some of them are consistent with a decrease in serotonin-1A receptor function in depression (Stockmeier 2003).

The altered HPA axis regulation in depressed patients is often taken as an indication that depression is a disorder of stress. However, the question of whether changes in regulation and responsivity of the HPA axis are related to disrupted sleep has received little attention (Meerlo et al. 2008).

The first aim of this study was to establish the effects of restricted sleep on neuroendocrine stress reactivity, particularly the reactivity of the HPA axis. Rats were subjected to chronic partial sleep deprivation for 7 d, after which they were exposed to a stressor to measure pituitary ACTH and adrenal CORT responses. To examine effects of sleep restriction on the HPA axis response to different kinds of stressors, the animals were subjected to a fear conditioning protocol, which consists of a stressor with a clear physical component (footshocks) and a more emotional stressor (re-exposure to the shock box, which is associated with a conditioned fear response).

The second aim was to establish potential neurobiological mechanisms underlying sleep restriction-induced changes in HPA axis stress reactivity. In a previous study we showed that restricted sleep causes a gradual desensitization of the serotonin- 1A receptor system (Roman et al. 2005b). Similar to depressed patients, chronically sleep restricted rats had a blunted temperature response to direct stimulation of serotonin-1A receptors with a 1A agonist (Roman et al. 2005b, 2006). In the present experiment we studied whether changes in HPA axis reactivity might be related to this serotonin-1A receptor desensitization. In addition, we examined whether changes in HPA axis reactivity might be directly related to altered CRH sensitivity. We therefore injected sleep restricted and control rats with a serotonin-1A agonist or CRH and measured their ACTH and CORT responses.

Material and methods

Animals and Housing

We used adult male Wistar rats (Harlan, Horst, The Netherlands) weighing approximately 350 g at the start of the experiments. Animals were housed under a 12 h light/12 h dark cycle, with lights on from 9AM to 9PM. Temperature in the room was maintained at 21±1℃. Rats were provided

with food and water ad libitum in all experiments. Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Experimental Design

Two experiments were performed with different groups of animals. In both experiments, animals were subjected to a protocol of sleep restriction, allowing them 4 h of sleep each day. Since rats normally sleep about 10 to 12 h each day, 4 h of sleep may not be sufficient to fully recover from 20 h of wakefulness. In previous studies we have shown that rats survive well on this protocol but it does result in gradual neuroendocrine and neurobiological changes (Meerlo et al. 2002; Roman et al. 2005b). For example, although one day of sleep restriction had no significant effects on the systems that were studied, 8 days of restricted sleep caused a significant reduction in serotonin-1A receptor sensitivity. In the first experiment of the present study, we examined the effect of sleep restriction on HPA axis stress responsivity. Rats were subjected to a fear conditioning protocol after 1 or after 7 days of sleep restriction. In the second experiment, we examined the effects of sleep restriction on serotonin-1A and CRH receptor sensitivity, particularly in relation to HPA axis responsivity. The HPA axis response to direct stimulation of serotonin-1A receptors and CRH receptors was measured after 7 and 8 days of restricted sleep, respectively.

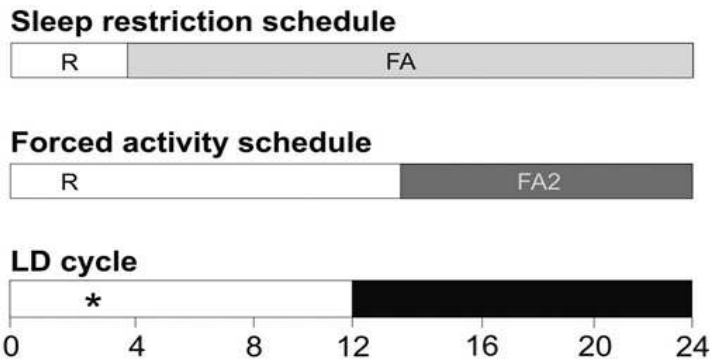


Fig.1. Experimental set-up of sleep restriction protocol and forced activity control. Top bar: rats were sleep restricted by forced locomotion (FA) for 20 h each day (grey section of the bar) and were allowed 4 h of rest (R) in their home cage (first 4 h of the light phase). Middle bar: rats were subjected to a protocol of forced activity at double speed (FA2) for half the time. Animals were subjected to the 10 h of forced activity in one block (dark grey section of the bar) which coincided with the last 10 h of the dark phase of the light-dark cycle. Lower bar depicts the 24-h light-dark cycle. As indicated by (*), the stress exposure in the first experiment (fear conditioning on day 1 / 2 or day 7 / 8) and the pharmacological challenges in the second experiment (8-OH-DPAT and CRH on day 7 and 8, respectively) took place between the third and fourth hour of the light phase.

Sleep Restriction and Forced Activity

Rats were subjected to a protocol of repeated partial sleep deprivation for 8 days, allowing them to sleep 4 h per day at the beginning of the light phase (9AM–1PM) in their home cage (Meerlo et

al. 2002; Roman et al. 2005, 2006). The remainder of the time, animals were kept awake by placing them in slowly rotating wheels (40 cm in diameter) driven by an engine at constant speed (0.4 m/min). Since the sleep deprivation procedure includes mild forced locomotion, we used forced activity control rats to test whether effects of sleep restriction might be caused by forced activity rather than sleep loss per se. Animals of the forced activity group were placed in the same plastic drums as the ones that were used for sleep restriction. However, these wheels rotated at double speed (0.8 m/min) for half the time (10 h). Therefore, the rats of the forced activity control group walked the same distance as sleep restricted ones, but had sufficient time for sleep (14 h). Animals were subjected to forced activity during the last 10 h of the dark phase, i.e., their circadian activity phase (Figure 1). Before starting the experiments, all rats were habituated to the experimental apparatus by placing them in the wheels for 1-2 h on 3 successive days.

Blood Sampling and Hormone Assays

In the first experiment on HPA axis reactivity in a fear conditioning paradigm, blood samples were collected by making a small incision at the end of the tail (Meerlo et al. 2002). Although brief handling was required for this blood sampling procedure, it likely did not interfere with neuroendocrine responses, since it coincided with the beginning and end of the stress sessions. Any effect of the mild handling stress would be obscured by the more severe stress of the footshock conditioning protocol. In the second experiment, we aimed to measure the HPA axis response after direct stimulation of CRH and serotonin-1A receptors without stress exposure. In this case, we made use of permanent heart catheters that allowed stress-free and frequent blood sampling in unrestrained and freely moving animals (Steffens 1969). Rats were provided with a polyethylene catheter in the right atrium of the heart under isoflurane/N2O/O2 inhalation anaesthesia. The catheter was inserted through the right jugular vein and externalized on top of the head according to techniques described earlier (Steffens 1969). After surgery, rats were allowed ≥10 days of recovery before the start of experiments. During this period, animals were habituated to handling and blood sampling procedures. In all experiments, blood was collected in chilled centrifuge tubes (0℃) containing EDTA. Blo od samples were centrifuged at 4℃ for 15 min at 2600 g, and the supernatant was stored at –80℃ for later analysis. ACTH and CORT concentrations were determined by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA).

Conditioned Fear Challenge

To establish the effect of restricted sleep on HPA axis stress reactivity, rats were subjected to a fear conditioning paradigm after 1 or after 7 days of sleep restriction. The experiments after 1 and 7 days were done in separate groups of rats (in each experiment: 8 sleep restriction, 8 forced activity control, 8 home cage control). On these days, rats returned to their home cage after the daily sleep deprivation session and were exposed to a fearful environment approximately 3 h later. Rats were placed in a shock box (25×25×15 cm) for a half-hour, during which they received

3 shocks at t = 5, 15, and 25 min (2 sec, 0.8 mA each). After the shock session, rats were returned to their home cage. The next day, approximately 24 h later, the rats were re-exposed to the shock box for another 30-min period, this time without shocks, to establish HPA axis response to an emotional stressor. Sleep deprivation was not continued between the initial shock session and the shock box re-exposure. Importantly, we have previously shown that some effects of chronic sleep restriction persist for many days, even with unrestricted recovery sleep (Roman et al. 2005b). Therefore, we anticipated that changes in HPA axis reactivity would persist as well and not normalize with a single day of recovery sleep. Blood samples were collected to measure plasma levels of ACTH and CORT at the beginning of the stress session, the end of the stress session, and after 45 min of recovery in the home cage (t = 0, 30 and 75 min). On both days, the shock box was thoroughly cleaned and dried between the tests of successive animals.

Serotonergic Challenge

In order to examine the effect of sleep restriction on serotonin- 1A receptor sensitivity and serotonergic modulation of HPA function, the rats were pharmacologically challenged with the serotonergic 1A receptor agonist (±)-8-hydroxy-2-(di-n-propyl-amino) tetralin hydrobromide (8-OH-DPAT; Sigma, St. Louis, MO). The experiment was done with a total of 30 rats (10 sleep restriction, 10 forced activity control, 10 home cage control). The challenge test took place after 7 days of sleep restriction, during the 4-h rest period in the home cage, between the third and fourth hour of the light phase. Each rat received a tube for blood sampling and infusion of the agonist, which was connected to the permanent heart catheter that externalized on top of the head. After ≥1.5 h, when any handling effect would have disappeared, rats received an intravenous injection of 8-OH-DPAT through the catheter (0.1 mg/kg body weight, dissolved in saline). The concentration of 8-OH-DPAT was based on earlier studies and was chosen to cause intermediate hormone responses (Korte et al. 1995). To measure plasma levels of ACTH and CORT in response to serotonin-1A receptor activation, blood samples were taken shortly before as well as 5, 15, and 60 min after the 8-OH-DPAT injection. After the last blood sample, the rats were placed back in the rotating wheels to continue the sleep restriction regime.

CRH Challenge

In order to investigate whether sleep restriction alters CRH control of the HPA-axis, rats received an injection of ovine CRH (oCRH; American Peptide Company, Sunnyvale, CA). After 8 days of sleep restriction, i.e., one day after the 8-OH-DPAT challenge, the same rats were again connected to the sampling tubes while in their home cage during the daily 4-h resting phase. Between the third and fourth hour of the light phase, the rats received an intravenous injection of CRH through the jugular vein catheter (0.5 µg/kg body weight, dissolved in saline). The concentration of CRH was based on earlier studies and was known to induce intermediate ACTH and CORT responses (Buwalda et al. 1999). Blood samples were taken to assess the sensitivity

of the pituitary gland to CRH. Blood sampling and hormone measurements for ACTH and CORT were carried out as described for the serotonin-1A challenge.

Data Analysis and Statistics

To test for effects of sleep restriction on the HPA axis responses to fear conditioning and to injections of 8-OH-DPAT and CRH, hormone data were subjected to analysis of variance (ANOVA) with repeated measures. When appropriate, post hoc Tukey test was applied to establish at which time points after stress exposure or pharmacological challenge the ACTH or CORT levels differed between experimental and control groups.

Results

Stress and Conditioned Fear Response

Both the 30-min footshock session and the re-exposure to the shock box next day induced a pronounced HPA axis response. This response was not significantly altered after one day of sleep restriction (data not shown). However, 7 days of sleep restriction caused significant alterations in the HPA axis response (Figure 2). For the ACTH response to footshock, ANOVA revealed a significant treatment effect ($F_{2,21}$=5.42, P=0.013) and a significant treatment × time interaction ($F_{4,42}$=2.99, P=0.029). On average, the ACTH response was lower in sleep restricted animals than it was in forced activity controls and home cage controls (Figure 2A). This difference only reached statistical significance for the comparison with home cage controls (post hoc Tukey test: P<0.05 for t=30 min and t=75 min). The plasma levels of CORT after the footshock session were not different between the groups (Figure 2B).

Upon re-exposure to the shock box next day, chronically sleep restricted animals had a significantly blunted ACTH response compared to both control groups (Figure 2C; ANOVA: treatment effect $F_{2,21}$=10.73, P<0.001 and treatment × time interaction $F_{4,42}$=5.77, P<0.001; post hoc Tukey test: P < 0.01 versus home cage controls at t=30 min and t=75 min, and P<0.05 versus forced activity controls at t=30 min). Also the CORT response upon re-exposure to the shock box was slightly but significantly lower in sleep restricted animals (Figure 2D; ANOVA: treatment effect $F_{2,21}$=5.54, P=0.012 and treatment × time interaction $F_{4,42}$=3.43, P=0.016; post hoc Tukey test: P < 0.05 versus home cage controls at t = 30 min and t = 75 min, and P<0.05 versus forced activity controls at t = 30 min).

Serotonergic Challenge

Due to partially blocked catheters, blood samples could not be drawn from 2 of 30 animals (both were sleep restricted rats). An IV injection of the serotonin-1A agonist 8-OH-DPAT induced a clear HPA axis response in all animals. On average, the ACTH response of the sleep restricted rats was lower than that of the home cage control animals, which in turn was lower than that of the forced activity control rats (Figure 3A). Repeated measures ANOVA revealed a significant

treatment effect ($F_{2,25}$=6.16, P=0.007) and a significant treatment × time interaction ($F_{6,75}$=4.32, P=0.001).

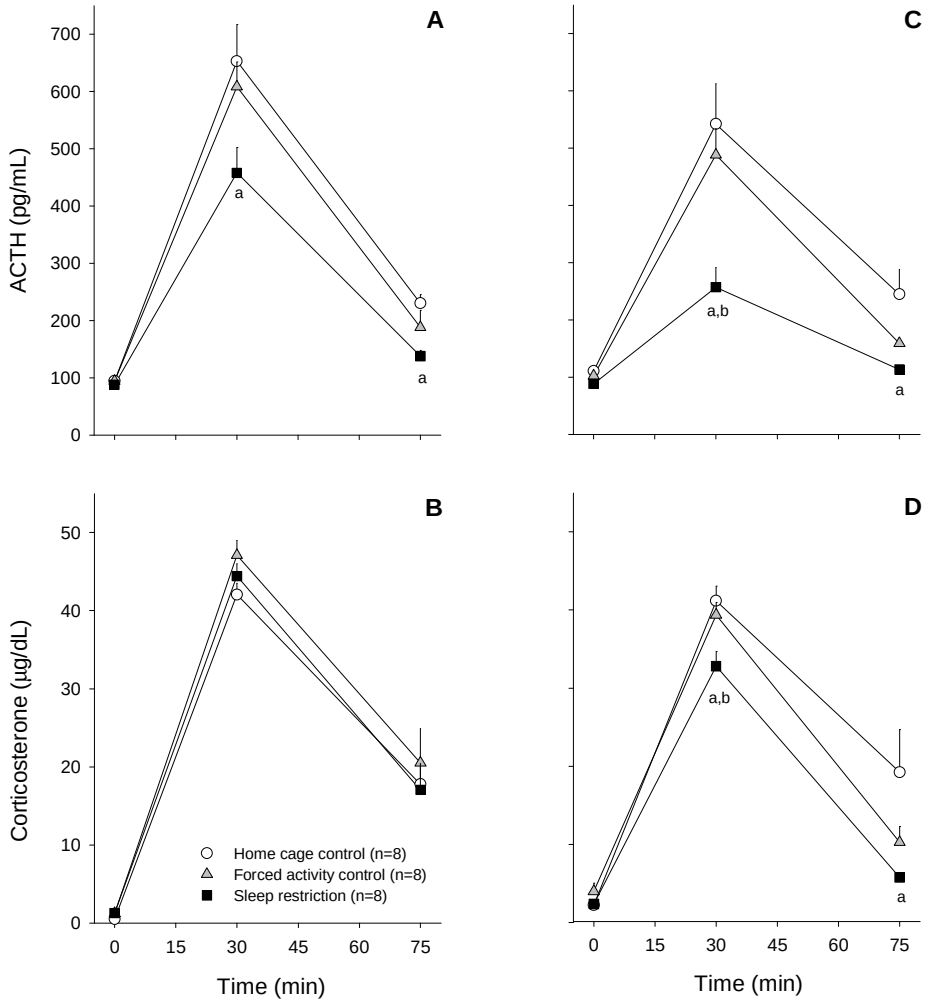


Fig.2. Effect of sleep restriction on the HPA axis response to stress. Sleep restricted and control rats were exposed to a 30-min session of footshocks after 7 days (left panels, A and B) and a 30-min re-exposure to the shock box on day 8 (right panels, C and D). Significant differences in ACTH and CORT responses between sleep restricted and control animals: a = p<0.05 compared to home cage control, b = p<0.05 compared to forced activity control. All data are expressed as average ± SEM.

Post hoc Tukey test indicated that the response of sleep restricted animals was significantly lower than that of forced activity control animals (P<0.01 for t = 15 min and P<0.05 for t = 60 min). In contrast, the CORT response to 8-OH-DPAT was not significantly different between sleep restricted and control rats (Figure 3B).

CRH Challenge

Because of blocked catheters, blood samples could not be taken from 2 animals (1 home cage control and 1 sleep restricted rat). The IV injection of CRH resulted in a clear ACTH and CORT response. However, the magnitude of the ACTH response differed between the groups (Figure 4A).

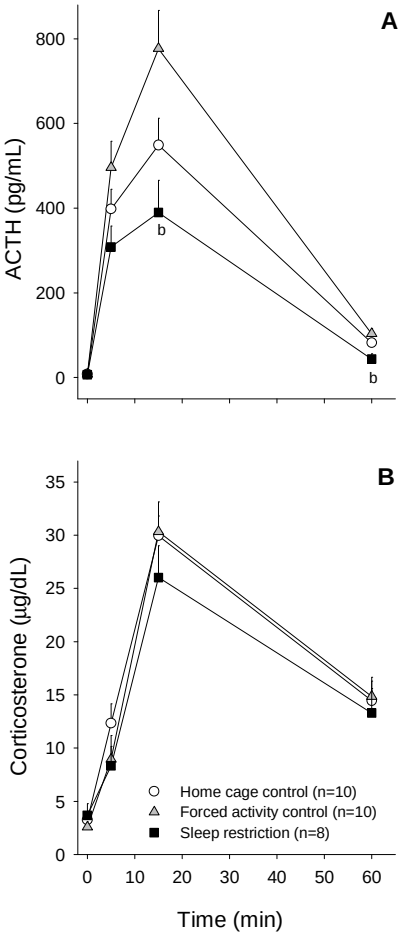


Fig.3. Effect of sleep restriction on serotonin-1A mediated HPA axis responsivity. After 7 days of sleep restriction, rats received an IV injection of the serotonin-1A receptor agonist 8-OH-DPAT and blood samples were taken to establish ACTH and CORT responses (A and B respectively). Significant differences between sleep restricted and control animals, b = p<0.05 compared to forced activity control. All data are expressed as average ± SEM.

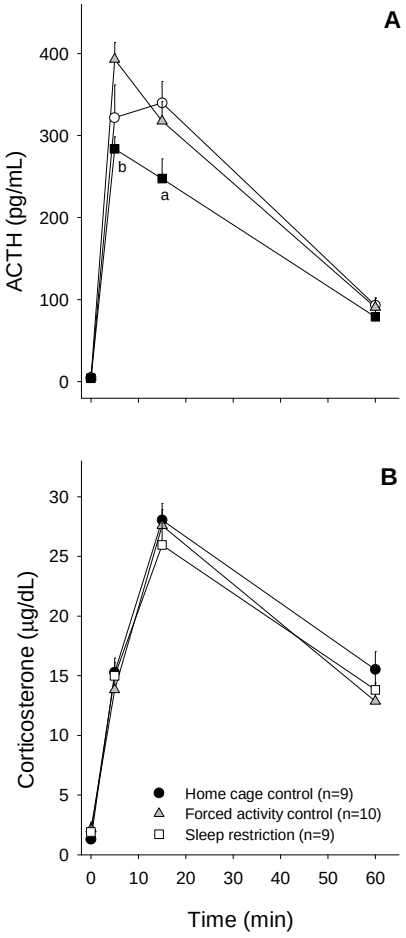


Fig.4. Effect of sleep restriction on CRH-induced HPA axis responses. After 8 days of sleep restriction, rats received an IV injection of CRH and blood samples were taken to establish ACTH and CORT responses (A and B respectively). Significant differences between sleep restricted and control animals: a = p<0.05 compared to home cage control, b = p<0.05 compared to forced activity control. All data are expressed as average ± SEM.

Repeated measures ANOVA indicated an overall treatment effect ($F_{2,25}$=3.54, P=0.044) and a treatment × time interaction ($F_{6,75}$=4.03, P=0.001). Post hoc Tukey test showed that ACTH release in sleep restricted animals was significantly lower than that in forced activity controls (P<0.05 for t = 5 min) and lower than that in the home cage controls (P<0.05 for t = 15 min). Yet, despite a lower ACTH response, the adrenal CORT response was not significantly different between the treatment groups (Figure 4B).

Discussion

This experimental study in rats shows that chronic sleep restriction may lead to alterations in neurotransmitter receptor systems (the serotonin-1A receptor and CRH receptor system) and neuroendocrine stress systems (the HPA axis), which are quite similar to changes that have been reported for major depression. While one day of restricted sleep had no significant effect on the HPA axis response to stress, sleep restriction for a week caused a blunted pituitary ACTH response in a conditioned fear paradigm. This blunted pituitary response may, in part, be related to a reduced sensitivity of the CRH and/or serotonin-1A receptors since sleep restricted rats showed a similar reduction in ACTH release to direct pharmacological stimulation with CRH and the serotonin-1A agonist 8-OH-DPAT.

The finding of a blunted pituitary ACTH response in a fear conditioning paradigm is consistent with an earlier study showing a chronic sleep restriction-induced attenuation of the pituitary response to restraint stress (Meerlo et al. 2002). In the present study, pituitary ACTH release was significantly reduced upon re-exposure to the fearful environment in which the rats had previously received a series of shocks. On average, the ACTH response of sleep restricted rats to the initial footshock session itself was lower as well, but this reduction was smaller and statistically significant only in comparison with the home cage controls (but not the forced activity controls). It cannot be ruled out that the actual footshocks represented a stronger stressor, whereby a ceiling effect in the HPA axis response may have prevented larger differences between sleep-restricted animals and control groups. However, another explanation for the more pronounced effect of sleep restriction on the response to shock box re-exposure versus the weaker effect on the response to the actual shock session may lie in the nature of the stressor. The footshocks are a direct and physical stimulus whereas re-exposure to the shock box constitutes a more psychological stress. Perhaps effects of sleep loss on stress reactivity are more pronounced in case of psychological stressors (Meerlo et al. 2008). Although most stressors, both physical and emotional, are associated with the acute and typical increase in HPA axis activity, the magnitude of this response is regulated and modified by different brain circuits (Lopez et al. 1999; Herman et al. 2003). Many brain regions are activated regardless of the nature of the stressor; for other regions there is some specificity in the activation depending on the stimulus. It may be that some of the brain regions and circuits that are specifically involved in the regulation and modulation of

emotional stress responses are more sensitive to sleep loss than the circuits involved in physical stress (Meerlo et al. 2008).

The second experiment of this study suggests that the blunted pituitary ACTH stress response of sleep restricted rats may at least partly be the result of a reduced sensitivity to serotonergic and CRH input. When serotonin-1A receptors or CRH receptors were stimulated directly, via an intravenous injection of an agonist, a similar attenuation of the ACTH response was found. However, for the serotonin-1A mediated ACTH response, this attenuation in sleep-restricted rats was statistically significant relative only to the forced activity controls but not the home cage controls. Interestingly, the ACTH response in sleep-restricted animals to both CRH and the serotonin-1A agonist 8-OH-DPAT was lower than that of the home cage control animals; whereas the response in the forced activity controls, if anything, was higher than that of home cage animals. In other words, restricted sleep and forced activity appeared to have opposite effects. The increase in ACTH responsivity in the forced activity control group may have been the result of mild stress experienced by these animals (Roman et al. 2005a). Indeed, a similar increase in ACTH response to CRH was found in a model of social stress (Buwalda et al. 1999). Thus, mild forced activity involved in our sleep restriction procedure may have partly counteracted the effects of sleep loss per se. Sleep loss without forced activity might result in an even stronger attenuation of the pituitary ACTH response.

The attenuated ACTH release upon injection of CRH in sleep-restricted rats suggests a desensitization of CRH receptors in the pituitary gland. Such desensitization might be the result of prolonged activation of the CRH system itself. Although information is limited, a number of animal studies suggest that sleep deprivation is indeed associated with elevated expression and release of CRH (Fadda and Fratta 1997; Fujihara et al. 2003; Koban et al. 2006). Especially in cases where disrupted and restricted sleep are a chronic condition, the corresponding overstimulation of CRH receptors by their own ligand might result in a downregulation of these receptors.

The attenuated ACTH response to serotonin-1A stimulation in sleep-restricted animals may be the result of changes at the level of pituitary or changes in other brain areas that provide input to the pituitary. Part of the attenuated serotonin-1A response may be an indirect effect related to the attenuated sensitivity to CRH discussed above. As injection of 8-OH-DPAT stimulates release of CRH from the paraventricular nucleus of the hypothalamus (Dinan 1996), the reduced ACTH response to 8-OH-DPAT may in part be a result of reduced sensitivity to CRH. It is also possible that the attenuated response to 8-OH-DPAT is a consequence of reduced sensitivity of the serotonin-1A receptors themselves at the level of the pituitary and the hypothalamus or even in other brain areas that innervate the HPA axis, such as the amygdala (Calogero 1990; Dinan 1996). In accordance with this, bilateral lesions of the central amygdaloid nucleus lead to a marked decrease in the ACTH release during stress (Beaulieu et al. 1986; Herman et al. 2005). A reduction in serotonin- 1A receptor sensitivity in sleep-restricted rats is supported by our earlier studies, showing that not only 1A receptor-induced ACTH release but also other 1A-receptor mediated responses are decreased. In reaction to 8-OH-DPAT, chronically sleep-restricted rats

displayed a significantly attenuated hypothermic response (Roman et al. 2005b; Roman et al. 2006). The finding of a reduction in multiple physiological responses makes it likely that this attenuation is, at least partly, a consequence of a reduced sensitivity of the serotonin- 1A receptor system itself. The mechanism underlying such a sleep-restriction induced serotonin-1A desensitization may be similar to the one proposed for a desensitization of the CRH receptors. Since the levels of serotonin are higher during wakefulness and sleep deprivation than they are during sleep (Portas et al. 2000), chronic sleep restriction and prolonged wakefulness may lead to overstimulation and ultimately downregulation of the serotonin receptors (Roman et al. 2005b).

In both experiments on HPA axis reactivity, despite reduced pituitary ACTH release, the adrenal glucocorticoid response was not affected or only mildly affected. Upon re-exposure to the shock box in the conditioned fear paradigm, the ACTH response of sleep-restricted animals was reduced by more than 60%, whereas there was only a minor, albeit significant, reduction of the CORT response. In reaction to pharmacological stimulation of the CRH or serotonin-1A receptors, the ACTH response of sleep restricted animals was attenuated, whereas the CORT response was not different from that of the control rats. An unchanged CORT response in the face of blunted ACTH levels can be explained by increased ACTH sensitivity in the adrenal cortex. This would imply that sleep restriction alters regulation of the HPA axis at multiple levels (Meerlo et al. 2002).

It is noteworthy that the pharmacological CRH and serotonin-1A challenge tests that we applied in the present study have been used many times in clinical settings to investigate alterations in receptor sensitivity in psychiatric disorders such as depression. Very much like chronically sleep-restricted rats, depressed patients show blunted ACTH but normal cortisol responses to CRH injections (Holsboer et al. 1984, 1987). Also in response to serotonin- 1A agonists, depressed subjects show blunted physiological responses (Lesch 1991; Mann et al. 1995; Shapira et al. 2000). A reduction in serotonin-1A receptor binding capacity in depressed patients has been confirmed by several PET studies (Drevets et al. 1999; Sargent et al. 2000; Drevets et al. 2007; Hirvonen et al. 2008). In one of these imaging studies, decreased 1A receptor binding potential was associated with the occurrence of insomnia, underscoring the complex relationship between sleep disturbance, depression, and changes in serotonergic neurotransmission (Hirvonen et al. 2008).

In summary, chronic experimental reduction of sleep in laboratory rats causes gradual changes in neurotransmitter receptor systems and HPA axis regulation - changes similar to those seen in human depression. These data suggest that several important symptoms of depression, i.e., disturbed sleep, altered serotonergic neurotransmission, and changes in HPA axis regulation, may be interrelated. In fact, whereas changes in serotonin-1A receptor sensitivity may in part explain the alterations in HPA axis responsivity, sleep disturbance may be causal to both the neurobiological and neuroendocrine changes. On the basis of changes in the regulation and activity of neuroendocrine stress systems, depression is often considered a disorder of stress. However, our study suggests that some of the symptoms traditionally ascribed to stress may also be a result of insufficient sleep. This experimental study thus provides support for the hypothesis

that sleep disturbance and insomnia may contribute to the symptomatology of psychiatric disorders. Chronically disrupted and restricted sleep may lead to alterations in neurobiological systems and altered regulation of stress systems, which may eventually sensitize individuals to stress-related disorders such as depression.

Acknowledgments

The authors thank Jan Bruggink for his assistance with the surgeries, blood sampling and hormone analyses and Paulien Barf for her assistance with the experiments. This work was supported by the School of Behavioral and Cognitive Neurosciences and the Netherlands Organization for Scientific Research (VIDI grant 864.04.002 to P. Meerlo).

CHAPTER 4

Changes in serotonin-1A receptor sensitivity following sleep restriction are not dependent on diet and body weight changes

R Paulien Barf; Arianna Novati; Henriëtte J Hulshof; Anton J Scheurink; Peter Meerlo

Abstract

Short and disrupted sleep has been linked to the development of a wide variety of diseases, including mood disorders such as depression. Insufficient sleep might contribute to these disorders by altering serotonergic neurotransmission. In support of this, experimental studies in rodents show that chronic sleep curtailment leads to desensitization of the serotonin-1A receptor system. According to the literature, such changes in serotonergic signaling may in part be a consequence of changes in metabolism and reductions in body weight. Therefore, in the present study, we assessed whether a medium fat food diet could protect against these effects of insufficient sleep.

Adult male rats were subjected to a schedule of chronic sleep restriction only allowing them 4h of undisturbed sleep per day. Half of the sleep restricted animals were maintained on a medium fat diet to prevent the decrease in body weight during sleep restriction. To study the serotonin-1A receptor sensitivity, we measured body temperature and endocrine responses to injections with the serotonin-1A agonist 8-OHDPAT after 8 days of sleep restriction.

Providing sleep restricted rats with a medium fat diet strongly attenuated the drop in body weight seen in sleep restricted animals on a standard Chow diet. However, this did not prevent desensitization of the serotonin-1A system. Sleep restricted animals on both medium fat and standard Chow food showed a similar blunted body temperature response and pituitary ACTH response upon stimulation of the serotonin-1A receptor system. Interestingly, insulin and leptin levels were equally decreased in both sleep restricted groups on different diets despite the differences in body weight.

In conclusion, the present study shows that changes in serotonin-1A receptor sensitivity following sleep restriction are not dependent on diet and body weight changes. The mechanism through which chronically restricted sleep gradually desensitizes the serotonin-1A system may be complex and multifactorial. It might be the result of altered regulation of physiological and endocrine factors such as insulin and it may in part be the consequence of a cumulative effect of sleep loss directly acting on the brain.

Introduction

Restricted and disrupted sleep is a common problem in our Western society. Epidemiological and clinical studies suggest that chronically disrupted sleep may contribute to the development of various diseases, including mood disorders such as depression (Ford and Kamerow 1989; Breslau et al. 1996; Chang et al. 1997). Impairment of serotonergic neurotransmission might be one of the pathways through which insufficient sleep contributes to the onset of depressive symptoms. This idea is supported by experimental studies in laboratory rodents showing that chronic sleep restriction causes a gradually developing desensitization of the serotonin-1A receptor system and an attenuation of 1A-mediated functions (Roman et al. 2005b, 2006; Novati et al. 2008). Yet, it remains an important question what aspect of sleep disturbance is causing these alterations in serotonergic function.

One factor that deserves attention in this context is metabolism. Controlled studies in humans have shown that sleep restriction is associated with changes in metabolic regulation, alterations in glucose homeostasis, and increases in appetite (Spiegel et al. 1999; Spiegel et al. 2004; Donga et al. 2010). Also, numerous studies on prolonged sleep deprivation in rodents have reported increased food intake and reduced body weight (e.g. Rechtschaffen and Bergmann 1995; Everson 1995; Hipolide et al. 2006). In recent studies, we focused on the metabolic consequences of insufficient sleep in our model of chronic sleep restriction in rats. Partial sleep deprivation for 8 days resulted in weight loss and reduced insulin responses, accompanied with glucose intolerance and hyperglycemia (Barf et al. 2010).

Based on the data above, one could argue that changes in serotonergic neurotransmission resulting from chronically restricted sleep might be secondary to the effects on energy metabolism and body weight. Indeed, literatures shows that weight loss affects serotonin levels (Brewerton 1995; Bailer et al. 2005; Kaye 2005; Haleem 2009) and a decrease in body weight due to food restriction is associated with a desensitization of the serotonin-1A receptor in rats (Li et al. 2008; Li et al. 2009). Furthermore, preventing a decrease in body weight in socially stressed rats by providing them with a fat food diet also prevents the stress-induced serononin-1A desensitization (Buwalda et al. 2001).

Therefore, in the present study we investigated whether sleep restriction induced changes in serotonergic signaling might be a consequence of changes in metabolism and body weight and we assessed if a fat food diet could protect against these effects of insufficient sleep. We performed a study during which half of the sleep restricted rats received a medium fat (MF) diet with a higher caloric density compared to standard Chow food. We hypothesized that the MF diet during the sleep restriction protocol would prevent at least partly the decrease in body weight compared to animals on standard Chow. To assess whether this would also prevent the changes in serotonin-1A receptor sensitivity, we measured body temperature and endocrine responses to injections with the serotonin-1A receptor agonist (±)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OHDPAT) (Roman et al. 2005b; Novati et al. 2008).

Material and methods

Animals and housing

Male Wistar rats (weight ± 320g; Harlan Netherlands BV, Horst, The Netherlands) were individually housed in Plexiglas cages in a climate-controlled room (21℃±1) under a 12h:12h light-dark cycle (lights on at 10:00h). Water and food were available ad libitum throughout the study. Different experimental groups were fed with standard Chow food or MF food (respectively 5.5% vs. 45.0% fat; Arie Blok Diervoeding B.V., Woerden, The Netherlands). Body weight and food intake were measured daily. Energy intake was calculated on the basis of the caloric density for both food types (Chow: 3.7 kCal/g; MF: 4.8 kCal/g). Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Chronic sleep restriction

Animals were subjected to chronic partial sleep deprivation according to previously published method (Roman et al. 2005a, 2005b; Novati et al. 2008; Barf et al. 2010). Sleep restriction groups were allowed to sleep in their home cage for 4 hours per day at the beginning of the light phase. During the remaining 20 hours, rats were kept awake by placing them in drums rotating at a constant speed of 0.4 m/min. Animals were subjected to this schedule of sleep restriction for 8 days during which they had free access to food and water inside the drums. All rats were habituated to the experimental conditions by placing them in the drums for 1-2h on 3 consecutive days before the onset of the sleep restriction protocol. Control animals were left undisturbed in their home cage throughout the experiment.

Experiment 1: Radio telemetry and serotonin-1A mediated temperature responses

In the first experiment we assessed the effects of MF food during the sleep restriction protocol on the sensitivity of the serotonin-1A receptor by measuring the acute hypothermic response to an 8-OHDPAT challenge. All animals were equipped with radio telemetry transmitters in the abdominal cavity to measure body temperature (model TA10TA-F40; Data Sciences, St. Paul, MN, USA). Surgeries were carried out under general 2% isoflurane inhalation anesthesia. The transmitters measured core body temperature and transformed temperature values into frequency coded radio signals. These radio signals were relayed to a PC by receivers placed underneath the home cages (model RPC-1; Data Sciences, St. Paul, MN, USA). Body temperature was sampled for 10 seconds every 10 minutes and processed with Dataquest LabproTM (Data Sciences). After 10 days of recovery from surgery, the animals were divided over three groups (n=8 in each group): home cage control on standard Chow food (Control-Chow), sleep restriction on standard Chow food (SR-Chow), and sleep restriction on medium fat food (SR-MF). In this first experiment we did not include a control group on MF food since previous studies indicated that MF per se does not affect serotonin-1A mediated body temperature responses (Buwalda et al. 2001). After the last sleep restriction session on day 8, during the fourth hour of the light phase, all rats received a

subcutaneous injection of the serotonin-1A receptor agonist 8-OHDPAT (Sigma, St. Louis, MO, USA) at a concentration of 0.25 mg/kg body weight (Roman et al. 2005b, 2006).

Experiment 2: Blood sampling and serotonin-1A mediated HPA axis responses

In the second experiment we studied the effects of MF food and sleep restriction on the serotonin-1A receptor mediated hypothalamic-pituitary-adrenal (HPA) axis responses. All animals were equipped with a chronic heart catheter in the jugular vein allowing repeated and stress free blood sampling according to the method described by Steffens (Steffens 1969). Under 2% isoflurane inhalation anesthesia, a silicon heart catheter (0.95 mm OD, 0.50 mm ID) was inserted into the right jugular vein and kept in place with a ligament. The other end of the catheter was subcutaneously directed to the top of the head were it was fixed with dental cement and could be used to connect the animals to sampling tubes. After 10 days of recovery from surgery, the animals were divided over four groups (n=8 in each group): home cage control animals on standard Chow food (Control-Chow) or medium fat food (Control-MF), and sleep restriction animals on standard Chow food (SR-Chow) or medium fat food (SR-MF). After 8 days of sleep restriction, animals were prepared for blood sampling by connecting them to sampling tubes. After 1h, when any handling effect should have disappeared, rats received an intravenous injection of 8-OHDPAT through the catheter at a concentration of 0.1 mg/kg body weight (Novati et al. 2008). To measure plasma levels of ACTH and corticosterone in response to serotonin-1A receptor stimulation, blood samples were taken shortly before as well as 10, 20, 30 and 60 min after the 8-OHDPAT injection. All samples had a volume of 250 µl, except the first one, which had a volume of 500 µl to allow analysis of plasma levels of insulin and leptin. The blood samples were collected in pre-cooled tubes containing EDTA (20 µL/ml blood). The samples were centrifuged at 2600g for 10 min and plasma was stored at −20 ℃ un til analysis. Plasma ACTH levels were measured by ImmuChem 125I ACTH Radioimmunoassay and plasma corticosterone levels were measured by ImmuChem 125I Corticosterone Radioimmoassay (MP Biomedicals, Orangeburg, NY, USA). Plasma insulin levels were measured by Linco Research Rat Insulin Radioimmoassay and plasma leptin levels were measured by Linco Research Rat leptin Radioimmoassay (Linco Research, St Charles, MO, USA).

Statistical analysis

Body weight and energy intake data were subjected to an analysis of variance (ANOVA) with repeated measures. Effects of sleep restriction and diet on the 8-OHDPAT induced temperature response in experiment 1 and endocrine responses in experiment 2 were assessed with repeated measures ANOVA as well. Effects of the treatments on plasma insulin and leptin levels were analyzed with two way ANOVA. When appropriate, posthoc Tukey test was applied to establish differences between specific groups. $P<0.05$ was considered statistically significant. All data in text and figures are expressed as averages ± SEM.

Results

Experiment 1: serotonin-1A induced temperature responses

The change in body weight during the first experiment is shown in figure 1A. During the 8-day experiment, sleep restricted animals on standard Chow food significantly decreased in body weight compared to control animals on Chow (Repeated Measures ANOVA, sleep restriction x time: $F_{11,154} = 33.58$, $P < 0.001$). Sleep restricted animals on Chow also lost significantly more weight than sleep restricted animals on MF food (Repeated Measures ANOVA, diet x time: $F_{11,154} = 7.19$, $P < 0.001$). During the same time period, energy intake did not differ between the three groups of animals. Despite the gradually developing differences in body weight, all groups maintained a stable and similar energy intake (average energy intake per day: Control-Chow: 88.6 ± 2.1 kCal; SR-Chow: 85.4 ± 2.7 kCal; SR-MF: 85.2 ± 1.8 kCal).

Figure 1B depicts the body temperature responses to the 8-OHDPAT injection after 8 days of sleep restriction. The subcutaneous injection of the serotonin-1A agonist caused an immediate hypothermia that reached lowest levels around 20-30 min post-injection. Body temperature returned to control values after 80 min. Sleep restricted animals on the standard Chow diet displayed a significantly attenuated temperature response compared to control animals on Chow (Repeated Measures ANOVA, sleep restriction x time: $F_{12,144} = 3.60$, $P < 0.001$). Sleep restricted animals on MF showed the same attenuated response and did not differ from the sleep restricted animals on Chow (Repeated Measures ANOVA, diet x time: $F_{12,144} = 0.70$, $P > 0.5$).

Experiment 2: serotonin-1A induced endocrine responses

The effects of sleep restriction and diet on body weight in the second experiment are shown in Figure 2A. Two Way Repeated Measures ANOVA revealed a significant effect of diet (diet x time: $F_{11,308} = 4.07$, $P < 0.001$), sleep restriction (sleep restriction x time: $F_{11,308} = 58.97$, $P < 0.001$), and an interaction between the two (sleep restriction x diet x time: $F_{11,308} = 3.00$, $P < 0.01$). The home cage control groups on the two different diets did not differ from each other. Sleep restriction caused a decrease in body weight but, as in the first experiment, this effect was strongly attenuated in the animals on MF food. Again, energy intake was not different between groups (average energy intake per day: Control-Chow: 88.6 ± 2.1 kCal; Control-MF: 87.6 ± 1.9 kCal; SR-Chow: 89.4 ± 2.0 kCal; SR-MF: 88.9 ± 2.0 kCal).

The injection of 8-OHDPAT induced a clear HPA axis response in all animals and treatment groups (ACTH: Figure 2B, corticosterone: Figure 2C).

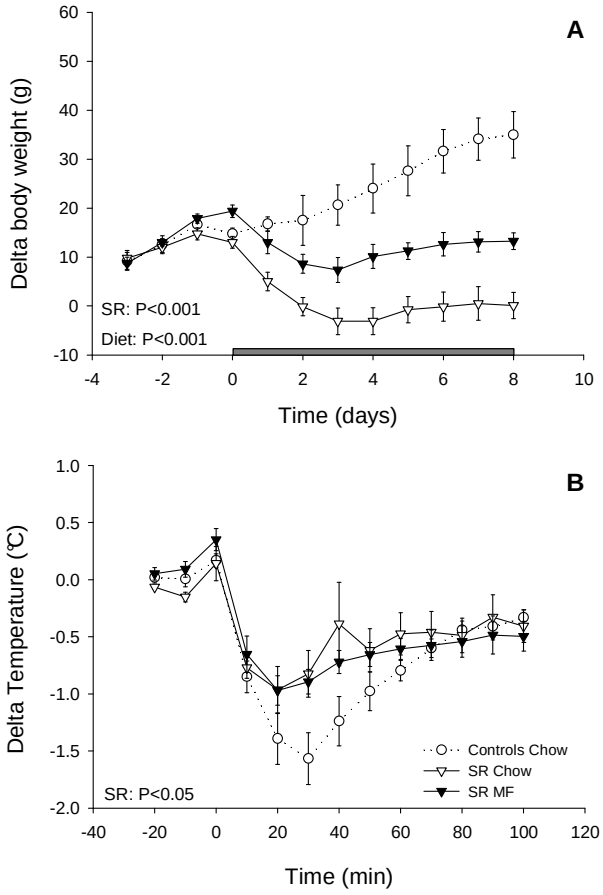


Fig.1. The effects of 8 days of sleep restriction on body weight and serotonin-1A sensitivity in rats receiving a standard Chow diet or a MF diet: (A) body weight changes in the course of the experiment; (B) body temperature responses to an injection with the serotonin-1A agonist 8-OHDPAT (0.25 mg/kg) after 8 days of restricted sleep. The horizontal grey bar at the bottom of graph 1A represents the 8-day period of sleep restriction. N=8 in each group. Data are presented as average values ± SEM. See text for details on statistics.

The ACTH response was not affected by diet but ANOVA revealed a significant overall effect of the 8 days sleep restriction ($F_{1,26} = 4.70$, $P < 0.05$). Sleep restricted animals on average had a slightly attenuated ACTH response as compared to the control animals. In contrast, the corticosterone response to 8-OHDPAT was not affected by prior sleep restriction but, instead, was significantly altered by diet (diet x time: $F_{4,108} = 12.07$, $P < 0.001$). The animals on the MF diet showed a significantly stronger corticosterone response compared to the animals on a standard Chow diet.

Plasma levels of insulin and leptin after 8 days of sleep restriction are shown in Figure 3. Eight days of sleep restriction decreased both leptin and insulin levels compared to control animals (Two Way ANOVA: Leptin: $F_{1,27} = 21.32$, $P < 0.01$; Insulin: $F_{1,27} = 14.63$, $P < 0.001$), while a MF diet increased both leptin and insulin levels compared to animals on a Chow diet (Two Way ANOVA: Leptin: $F_{1,27} = 12.19$, $P < 0.001$; Insulin: $F_{1,27} = 4.58$, $P < 0.05$), but no interaction effects were found.

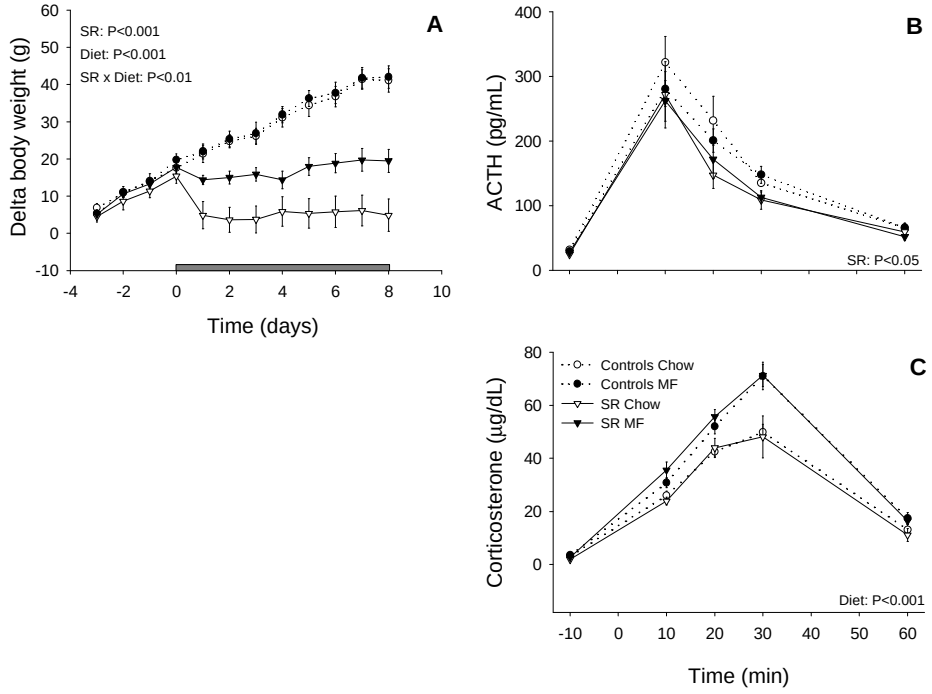


Fig.2. The effects of 8 days of sleep restriction on body weight and serotonin-1A sensitivity in rats receiving a standard Chow diet or a MF diet: (A) body weight changes in the course of the experiment; (B) ACTH and (C) corticosterone responses to an injection with the serotonin-1A agonist 8-OHDPAT (0.1 mg/kg) after 8 days of restricted sleep. The horizontal grey bar at the bottom of graph 2A represents the 8-day period of sleep restriction. N=8 in each group. Data are presented as average values ± SEM. See text for details on statistics.

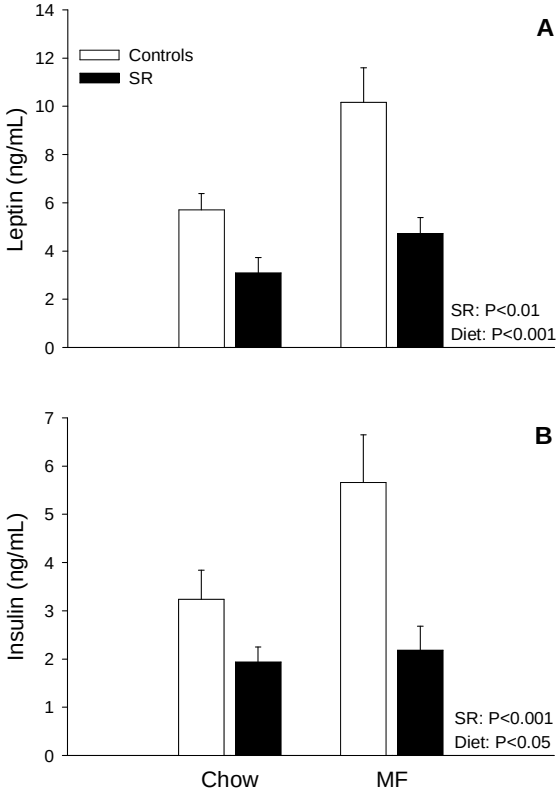


Fig.3. The effects of 8 days of sleep restriction on plasma levels of leptin (A) and insulin (B) in rats receiving a standard Chow diet or a MF diet. N=8 in each group. Data are presented as average values ± SEM. See text for details on statistics.

Discussion

Previous studies in our laboratory showed that chronic sleep restriction is associated with a gradually developing desensitization of the serotonin-1A system (Roman et al. 2005b, 2006; Novati et al. 2008). In the present study we aimed to assess whether these changes are a secondary consequence of changes in metabolism and body weight. Providing sleep restricted rats with a MF diet strongly attenuated the drop in body weight that is normally seen in sleep restricted animals on a standard Chow diet. However, this did not prevent the desensitization of the serotonin-1A system. Sleep restricted animals on both MF and standard Chow food showed a similar blunted body temperature response and pituitary ACTH response upon stimulation of the serotonin-1A receptor system with the agonist 8-OHDPAT.

Interestingly, the MF diet caused a significant increase in the adrenal corticosterone response, independent of sleep restriction, suggesting that fat food per se may increase adrenal sensitivity. This finding appears to be in line with other published studies on the consequences of a chronic high fat diet. Indeed, animals on a high fat diet have enlarged adrenals, increased basal corticosterone levels, and increased adrenal responses to challenges during the first months on the diet (Tannenbaum et al. 1997). Even though in our study basal corticosterone levels were not affected, the corticosterone response to the 8-OHDPAT challenge was. Perhaps the MF food used in this experiment had the same effect on the adrenals compared to a high fat diet. Future experiments should therefore take adrenal size into account.

The MF diet in the present study was administered to prevent the decrease in body weight that is normally seen during sleep restriction. As expected, the animals on the MF diet did not lose as much weight as the animals on regular Chow did. Nevertheless, the MF diet did not prevent the changes in serotonin-1A sensitivity seen after sleep restriction. In contrast, a previous study on the consequences of social stress showed that a high fat diet was able to prevent not only the stress-induced drop in body weight, but the stress-induced desensitization of the serotonin-1A receptor as well (Buwalda et al. 2001). Thus, whereas fat food ameliorates the effects of stress on the serotonergic system, it does not protect against the effects of sleep restriction. Apparently, the mechanisms through which stress and sleep disturbance affect the serotonin-1A sensitivity are different.

Intriguingly, although the sleep restricted groups on control and MF diet differed in body weight, they had a similar energy intake as calculated from their food intake and the caloric content of their respective diets. Possible explanations for this discrepancy are that animals on a fat diet increase the actual absorption of energy in the intestinal system or a fat diet somehow lowers energy expenditure (Buwalda et al. 2001; Morens et al. 2006; Koolman et al. 2010). Literature shows that sleep restriction increases energy expenditure (Everson 1995; Rechtschaffen and Bergmann 1995) and a MF diet might influence this in such a way that it attenuates weight loss compared to animals on a standard Chow diet.

Both sleep restriction and diet had specific and independent effects on basal levels of insulin and leptin. Sleep restriction decreased the levels of these hormones whereas a MF diet increased them. These changes may in part reflect the changes in body weight and fat content in the different groups (Picarel-Blanchot et al. 1995; Redman and Ravussin 2009). It remains unclear though why sleep restricted rats on a MF diet did not have significantly higher insulin and leptin levels than the sleep restricted animals on a standard Chow diet. Yet, the changes in the levels of these metabolic hormones may be relevant in the context of the changes in serotonin-1A sensitivity that we found. Particularly changes in the regulation of insulin may be associated with altered serotonin receptor sensitivity (Li et al. 2009). Rats receiving an intraperitoneal injection with streptozotocin, which is extremely toxic for the β-cells in the pancreas, have a decreased insulin production similar to what is seen in diabetic patients (Rerup 1970). After the injection, rats become hyperglycemic and hyperphagic but have decreased body weights, which is all

normalized after insulin treatment (Friedman 1977, 1978; Friedman and Ramirez 1994). Li and colleagues (2009) showed that the body temperature response to an 8-OHDPAT challenge was decreased 7 days after a streptozotocin injection, indicating reduced serotonin-1A sensitivity very much like in our sleep restricted rats. Again, the effect was reversible after 10 days of insulin treatment. It might thus be that an altered insulin regulation contributes to a desensitization of the serotonin-1A system in sleep restricted rats.

Clearly, the exact mechanism through which chronically restricted sleep gradually desensitizes the serotonin-1A system may be complex and multifactorial. It may in part be the result of altered regulation of physiological and endocrine factors such as insulin and it may in part be the consequence of a cumulative effect of sleep loss directly acting on the brain.

In conclusion, a MF food diet partially prevents the drop in body weight but not the desensitization of the serotonin-1A receptor system in sleep restricted rats. Chronically sleep restricted rats on a standard Chow diet and a MF diet showed a similar blunted temperature response and blunted pituitary ACTH response to stimulation of the 1A receptors with the agonist 8-OHDPAT. The physiological and molecular mechanism through which sleep restriction gradually alters the serotonin-1A sensitivity remains to be established.

Acknowledgements

The authors thank Jan Bruggink for technical assistance and radioimmunoassay. This work was supported by the Netherlands Organization for Scientific Research (VIDI grant 864.04.002 to PM).

CHAPTER 5

Chronic sleep restriction in rats does not affect adrenal corticosterone response and mRNA expression of brain glucocorticoid and mineralocorticoid receptors

Arianna Novati, Henriëtte J Hulshof, R Paulien Barf, Tim De Jager, Onno Meijer, Peter Meerlo

Abstract

Since disrupted and restricted sleep is a major problem in our society, the question of whether sleep loss affects neuroendocrine stress systems and their brain receptor systems is important. Chronically restricted sleep is known to affect central systems involved in regulating the hypothalamic-pituitary-adrenal (HPA) stress response system. However, little is known on the effects of insufficient sleep on the expression of brain receptors for glucocorticoids, the final output of the HPA axis. In this study we examined if chronic sleep restriction alters the expression of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in hippocampus and hypothalamus. Adult male rats were subjected to a schedule of chronic sleep restriction only allowing them 4h of sleep at the beginning of the light phase, i.e., their normal resting phase. The animals were sleep deprived for 20h each day by placing them in slowly rotating drums. Effects on MR and GR expression in the brain were established by in situ hybridization. In the first experiment, MR/GR expression was measured after 14 days of sleep restriction. In the second experiment, MR/GR expression was measured after 14 days of sleep restriction combined with a 30-min daily restraint stress on day 9 - 13. On the first and last day of restraint stress, blood samples were collected to assess the corticosterone response and changes therein. The results did not show significant changes in GR and MR mRNA expression in the areas considered, neither after sleep restriction alone nor after sleep restriction combined with repeated restraint stress. Restraint stress induced a pronounced corticosterone response, which was not different between experimental groups and did not habituate with repeated stress exposure. All together, these results do not support the hypothesis that chronically restricted sleep leads to altered corticoid receptor expression in the brain.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is one of the main neuroendocrine systems involved in the regulation of stress responses (de Kloet et al. 2005). The paraventricular nucleus of hypothalamus (PVN) produces corticotropin releasing hormone (CRH) that stimulates the release of adrenocorticotropin hormone (ATCH) from the anterior pituitary, which in turn stimulates the adrenal cortex to release glucocorticoids (CORT, cortisol in humans or corticosterone in rats). Glucocorticoids together with the other HPA axis hormones serve a wide variety of functions (de Kloet et al. 2005). They not only support metabolic processes and physical activity under acute stress but also affect brain function, cognition and mood (de Kloet et al. 2005).

Glucocorticoids accomplish their tasks through two types of receptors, the mineralocorticoid receptors (MR) and the glucocorticoid receptors (GR), which have different patterns of expression in the brain (de Kloet et al. 1993, 2005). While GR are widely distributed in the brain, the expression of MR receptors is largely restricted to hippocampus, lateral septum, amygdale, olfactory nucleus, layer II of the cortex and brain stem (de Kloet et al. 1993). The MR has a higher affinity for glucocorticoids than the GR, which is activated only when glucocorticoids reach high concentrations, such as during stress (Datson et al. 2008). Through these receptors, glucocorticoids exert both rapid non-genomic effects and slow, long-lasting genomic effects and influence a wide variety of processes in the brain and body (Datson et al. 2008; de Kloet et al. 2008, 2009). Also, by activating these receptors, glucocorticoids provide negative feedback to the HPA axis system and regulate their own release (Miller et al. 1992; de Kloet et al. 1993; Feldman and Weidenfeld 1999). It has been suggested that due to the difference in affinity for glucocorticoids, MR control the basal activity of the HPA axis, whereas GR regulate the HPA axis mostly under conditions of stress (de Kloet et al. 1998; Datson et al. 2008).

While changes in MR/GR expression in the brain and alterations in HPA axis regulation under conditions of chronic stress have been topic of numerous studies (e.g., Sapolsky et al. 1984; Chao et al. 1993; Herman et al. 1995; Makino et al. 1995; Gomez et al. 1996; Yau et al. 2001), little is known on the consequences of disrupted and restricted sleep, although the latter in itself is sometimes considered as a stressful condition (for review and discussion, see Meerlo et al. 2008). Since disrupted and restricted sleep is a major problem in our society, the question of whether sleep loss affects the neuroendocrine stress systems and their brain receptor systems is important. Given the wide range of actions of CORT in the brain, effects of sleep loss on these receptor systems might have direct functional consequences for the way we perform and deal with everyday challenges. In fact, chronically disrupted and restricted sleep is considered to be an important factor contributing to the development of mood disorders (Meerlo et al. 2008; Riemann and Voderholzer 2003). Changes in HPA axis regulation and corticoid receptor expression might be one potential pathway underlying this.

Studies in both humans and rodents have shown that sleep deprivation and sleep restriction are conditions sometimes associated with mild, temporary increases in the activity of the major neuroendocrine stress systems, including the HPA axis (for review, see Meerlo et al. 2008). Most of the experiments considered mainly changes in HPA axis hormone levels, while the effects of sleep loss on HPA axis negative feedback and expression of corticoid receptors in the brain are largely unknown. It is noteworthy that the expression of MR and GR in the brain is regulated by a variety of neurotransmitters and hormones such as serotonin, noradrenaline, CRH, AVP and CORT (Sapolsky et al. 1984; Maccari et al. 1992, Alema et al. 1995; Hügin-Flores et al. 2003; Robertson et al. 2005), some of which are also influenced by sleep deprivation (Fadda and Fratta. 1997; Spiegel et al. 1999; Lac and Chamoux 2003; Hipolide et al. 2005; Roman et al. 2005b).

In the present study, we applied an animal model of sleep restriction and sleep restriction in combination with repeated restraint stress to examine whether chronic sleep restriction alters the expression of corticoid receptors in the brain.

Material and methods

Animals and housing

Forty-eight male Wistar rats weighing 400-450 g at the beginning of the treatment were used in this study. Animals were housed in a room with a 12h light - 12h dark cycle (lights on 9AM – 9PM) and constant temperature of 21 ±1 ℃. Standard labo ratory chow and water were provided ad libitum. Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Experimental design

We examined the effects of chronic sleep restriction on MR/GR expression and adrenal CORT responses in two different experiments. In the first experiment, rats were subjected to a sleep restriction protocol for 14 days and brains were collected for analysis of MR/GR expression by in situ hybridization. In the second experiment, rats were again sleep restricted for 14 days but this time they were also exposed to a daily 30-min restraint from day 9 until day 13. Blood samples were taken on the first and fifth day of restraint stress to assess the adrenal CORT response. After the last sleep restriction session on day 14 brains were again collected to assess possible changes in MR/GR receptor expression when sleep restriction was combined with stress exposure. Each of the two experiments included three groups of rats: sleep restriction (SR), forced activity controls (FA) and home cage controls (HC), with n=8 per group.

Sleep restriction and forced activity controls

In both experiments, animals were subjected to a protocol of sleep restriction allowing them 4h of sleep each day at the beginning of the light phase (9 AM – 1PM) (Roman et al. 2005b, Novati et

al. 2008). The remainder of the time, animals were kept awake by placing them in slowly rotating drums (40 cm in diameter) driven by an engine at constant speed (0.4 m/min). Since the sleep deprivation procedure includes mild forced locomotion, we used forced activity control rats to test whether effects of sleep restriction might be due to forced activity rather than sleep loss per se. Animals of the forced activity group were placed in the same drums as the ones that were used for sleep restriction. However, in this case the wheels rotated at double speed (0.8 m/min) for half the time (10h). Therefore, the rats of the forced activity control group walked the same distance as sleep restricted ones but had sufficient time for sleep (14h). Animals were subjected to forced activity during the last 10h of the dark phase, i.e., their circadian activity phase.

Restraint stress

In the second experiment, rats were subjected to restraint on 5 consecutive days, from day 9 until 13 of the sleep restriction protocol. Each time, at the end of the daily 4h resting phase, the rats were placed in cylindrical wire-mesh restrainers for 30 min (Meerlo et al. 2002).

Blood sampling and CORT assay

For repeated blood sampling, all rats in experiment 2 received permanent polyethylene heart catheters according to procedures previously described (Steffens 1969). Under 2% isoflurane inhalation anaesthesia, one end of a silicon heart catheter (0.95 mm OD, 0.50 mm ID) was inserted in the right atrium of the heart through the jugular vein and kept in place with a ligament. The other end of the catheter was subcutaneously directed to the top of the head, where it was fixed with dental cement and could be used to connect the rats to sampling tubes. After surgery, rats were allowed at least 10 days of recovery before the start of experiments. During this period, animals were habituated to handling and blood sampling procedures. To asses adrenal CORT responses, blood samples were taken on the first and last restraint stress day at the beginning of the 30-min restraint session, at the end of the restraint session, and after 45 and 65 min of recovery in the home cage (t = 0, 30, 75, 100 min). All blood samples were collected in chilled Eppendorf tubes containing EDTA. The samples were centrifuged at 4℃ for 15 min at 2600g and the supernatant was used to measure the concentration of CORT by radioimmunoassay (MP Biomedicals, Orangeburg, NY, USA).

Brain collection and tissue preparation

At the end of the last sleep restriction session on day 14, after two hours of rest, animals were anaesthetized with dry ice CO2 and fresh brains were extracted from the skull, frozen in isopenthane on dry ice and preserved at -80 ºC until sectioning. 14 µm coronal sections through the entire PVN and dorsal hippocampus were cut with a microtome cryostat. The sections were mounted on poly-L-lysine-coated slides and then stored at -80 ºC until further processing for in situ hybridization.

In situ hybridization

To measure the expression of MR and GR mRNA, in situ hybridization was performed using ^{35}S labeled ribonucleotide probes, following a slightly modified version of published methods (Meijer et al. 2000, 2005). The MR probe was generated from a 500-bp fragment (EcoRI, Hind III fragment of rat MR cDNA) coding for rat MR exon two in pGEM4. A 510bp fragment of the original full-length GR clone (Sal 1-Hind III fragment of rat GR from exon 2), coding for the N terminus of the receptor, inserted in pGEM3, was used for the GR probe. Linearized plasmids were transcribed with RNA polymerase. ^{35}S-labelled UTP were used to obtain antisense and sense probes (negative control). Mounted sections were fixed by placing the slides in 4 % paraformaldehyde with 0.5 % glutaraldehyde and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine / HCl. Dehydration followed in increasing concentrations of ethanol. Tissue sections were saturated with 120 µl hybridisation buffer containing 25 mM Tris-HCl at pH 7.4, 1.2 mM EDTA (ph 8.0), 350 mM NaCl, 50% formamide, 10% dextran sulfate, 100 mM dithiothreitol, 1x Denhardt's in DEPC- H2O, RNA mix (0.1 mg/ml transfer RNA and 0.1 mg/ml hsDNA) and 2 x 106 cpm of the labeled MR or GR probe. Following over night incubation at 55℃, the tissue was rinsed in 20x SSC, treated with 10 mg/ml RNAse A (30', 37℃), washed in decreasing concentrations of SSC (65℃ and RT) and dehydrated with increasing concentrations of ethanol. Air dried sections were placed on film (X-OMAT AR; Kodak, Rochester, NY) and developed (4 days for MR and 12 days for GR). The mRNA expression was estimated by quantifying autoradiograph optical density with imaging software (Leica Qwink, NL). Measured values were corrected for film background.

Statistics

One-way analysis of variance (ANOVA) was used to analyze difference in MR/GR expression among the three animal groups, in selected brain areas. Repeated measures ANOVA was used for the analysis of corticosterone data. The level of significance was set at $p<0.05$. Data are expressed as average per group ±SEM.

Results

Adrenal CORT response

The adrenal CORT response to repeated restraint stress was not significantly different among groups in day 1 or in day 5 (Figure 1). Rats in all groups displayed a robust CORT response to restraint, which, contrary to the expectation, was as high on the fifth restraint day as it was on the first.

Table 1. MR mRNA expression (in arbitrary units) in hippocampus and PVN and GR mRNA expression in hippocampus following two weeks of sleep restriction and two weeks of sleep restriction combined with 5 days restraint stress

		2wk sleep restriction			2 wk sleep restriction + restraint		
		HC	FA	SR	HC	FA	SR
MR	CA1	153±15	143±16	164±17	286±25	251±18	266±21
	CA2	262±34	216±26	238±27	425±40	381±37	424±26
	CA3	121±14	121±12	138±16	187±17	164±15	165±11
	DG	207±22	198±21	236±22	365±35	291±26	317±17
GR	CA1	724±38	665±22	730±34	762±39	737±40	707±58
	CA2	200±16	196±13	171±12	191±21	181±15	173±25
	CA3	230±12	207±11	242±15	242±16	238±17	237±30
	DG	874±38	786±28	858±28	868±19	920±22	863±40
	PVN	687±41	670±25	618±11	685±25	694±15	685±28

HC = home cage; FA = forced activity; SR = sleep restricted; MR = mineralcorticoid receptor; GR = glucocorticoid receptor; MR and GR expression in arbitrary units, values are group averages ± SEM.

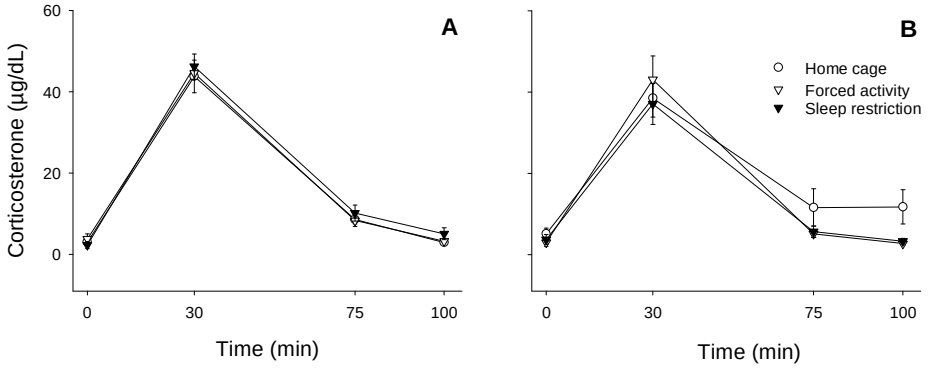


Fig.1. Effect of sleep restriction on the adrenal CORT response to repeated restraint stress. Sleep restricted and control rats were exposed to a 30-min session of restraint. Blood samples were collected before the restraint (baseline, t = 0), immediately after (t = 30) and during recovery (t = 75, t = 100). The CORT response did not differ among the three experimental groups and was comparable between day 1 (A) and day 5 (B).

MR and GR expression

The expression of MR and GR receptor mRNA was analyzed after two weeks of sleep restriction (Exp. 1) and after two weeks of sleep restriction combined with repeated restraint (Exp. 2). For both the conditions, one-way ANOVA did not reveal any differences in the expression of MR and GR among experimental groups for the areas considered (Table 1, all $p>0.05$).

Discussion

This study examined the effects of chronic sleep restriction or sleep restriction, alone or combination with repeated stress, on the expression of MR and GR in the brain. While 2 weeks of sleep restriction appeared to have no effect on CORT receptor mRNA in the brain, there is reason to consider these data with care. We measured MR and GR mRNA expression in brains collected two hours after the last sleep deprivation session on day 14 (in both experiments) and one full day after the last restraint session (in experiment 2). Changes in GR mRNA levels induced by acute stress were in some cases shown to normalize within a few hours following stress (Paskitti et al. 2000). Therefore, we cannot exclude that restraint, perhaps in combination with sleep restriction, has short-lasting effects on MR/GR expression that went unnoticed in our experiment.

Another potential complication is that we measured levels of MR and GR mRNA and not the actual receptor protein or the receptor functionality. The regulation of at least GR may occur at several post-transcriptional levels, through mechanisms of translational repression, post-translational modification and alternative translation variants (Yudt and Cidlowsky 2002; Duma et al. 2006; de Kloet et al. 2009). Regulation of the receptor expression at post-transcriptional level could lead to changes in receptor protein expression that are not visible in mRNA analysis. Therefore, additional quantification of protein expression would provide a more complete overview of the effects of sleep loss on the corticoid receptors.

Also, in the present study we did not find adaptation of the adrenal CORT response to repeated restraint stress, neither in the control animals nor in the sleep restricted rats. This is not in line with the fact that repeated restraint has been extensively used as a model of adaptation to stress (Glavin et al. 1994) and several studies have reported a rapid decrease of the HPA axis response after few sessions (Melia et al. 1994; Ma and Lightman 1998; Cole et al. 2000; Pace et al. 2001; Barnum et al. 2007), although such decrease did not occur with all the experimental protocols used (Kant et al. 1983; Pitman et al. 1988). Our control animals even on the fifth exposure displayed a high CORT response that may have been near ceiling levels. One may speculate that, with a milder stress protocol, we might have found habituation and perhaps even differences in habituation between SR and non SR animals.

Notwithstanding the complications and limitations discussed above, the present study does not provide evidence for a strong effect of chronically restricted sleep on glucocorticoid sensitivity in the brain. In contrast to this lack of effect on downstream elements of the HPA axis, previous studies have shown that chronic sleep restriction does alter central systems that provide input to the HPA axis. Studies in rats have shown that chronic sleep restriction reduces sensitivity of the serotonin-1A receptors system and the CRH-receptor system (Roman et al. 2005b; Novati et al. 2008). Both of these systems play important roles not only within the brain but also provide input to the HPA axis. Besides stimulating the release of CRH and AVP from the paraventricular nucleus of the hypothalamus (Calogero et al. 1989, 1993), serotonin also regulate the release of ACTH and CORT at the level of the anterior pituitary and the adrenals respectively (Johns et al.

1982; Alper 1990; Calogero et al. 1993), while CRH activates the HPA axis inducing release of ACTH (Tsigos and Chrousos 2002) and facilitates the integration of the stress response (Bale and Vale 2004). The alterations of these systems reported in earlier sleep restriction studies, using the same protocol as in the present study, do not appear to be paralleled by changes of MR/GR expression.

In summary, chronic sleep restriction alone or combined to repeated restraint stress, as applied in these experiments, did not change the expression of MR and GR mRNA in the hippocampal and hypothalamic areas measured and did not influence the CORT response to repeated restraint stress.

Acknowledgements

The authors thank Jan Bruggink for his assistance with the surgeries and CORT analyses and Servane Lachize for performing the GR/MR in situ hybridizations. This work was supported by the School of Behavioral and Cognitive Neurosciences and the Netherlands Organization for Scientific Research (VIDI grant 864.04.002 to P. Meerlo).

CHAPTER 6

Chronic sleep restriction causes a decrease in hippocampal volume in adolescent rats, which is not explained by changes in glucocorticoid levels or neurogenesis

Arianna Novati, Henriëtte J Hulshof, Jaap M Koolhaas,
Paul J Lucassen, Peter Meerlo

Neuroscience (2011), 190: 145-155

Abstract

Sleep loss strongly affects brain function and may even predispose susceptible individuals to psychiatric disorders. Since a recurrent lack of sleep frequently occurs during adolescence, it has been implicated in the rise in depression incidence during this particular period of life. One mechanism through which sleep loss may contribute to depressive symptomatology is by affecting hippocampal function. In this study, we examined the effects of sleep loss on hippocampal integrity at young age by subjecting adolescent male rats to chronic sleep restriction (SR) for 1 month from postnatal day 30 to 61. They were placed in slowly rotating drums for 20 h per day and were allowed 4 h of rest per day at the beginning of the light phase. Anxiety was measured using an open field and elevated plus maze test, while saccharine preference was used as an indication of anhedonia. All tests were performed after 1 and 4 weeks of SR. We further studied effects of SR on hypothalamic-pituitary-adrenal (HPA) axis activity, and at the end of the experiment, brains were collected to measure hippocampal volume and neurogenesis. Behavior of the SR animals was not affected, except for a transient suppression of saccharine preference after 1 week of SR. Hippocampal volume was significantly reduced in SR rats compared to home cage and forced activity controls. This volume reduction was not paralleled by reduced levels of hippocampal neurogenesis and could neither be explained by elevated levels of glucocorticoids. Thus, our results indicate that insufficient sleep may be a causal factor in the reductions of hippocampal volume that have been reported in human sleep disorders and mood disorders. Since changes in HPA activity or neurogenesis are not causally implicated, sleep disturbance may affect hippocampal volume by other, possibly more direct mechanisms.

Introduction

Restricted sleep in our society is a problem that not only affects adults but is increasingly common among children and adolescents as well (Meijer et al. 2000; Van den Bulck 2004; Bixler 2009). One contributing factor is that adolescence is characterized by spontaneous changes in circadian organization resulting in a stronger tendency for evening activities and delayed sleep timing (Crowley et al. 2007; Roenneberg et al. 2007; Hagenauer et al. 2009). Subsequently, the combination of late evening activities and early morning school or work obligations prevents a large number of young people from getting sufficient sleep (Meijer et al. 2000; Van den Bulck 2004). Lack of sleep at this age, as in adulthood, has various immediate effects, including tiredness, decreased attention, decreased motivation, reduced cognitive function, and decreased academic performance (Wolfson and Carskadon 2003; Curcio et al. 2006). In addition, it might be that reduced sleep time at a younger age also affects ongoing brain development, perhaps leading to more persistent effects at later ages.

Many systems in the adolescent brain are still maturing and the morphology of several brain areas go through prominent changes in, for example, grey and white matter ratio (Sowell et al. 2002). The maturation of neurobehavioral systems in this early phase of life requires a high level of plasticity and is associated with strong emotional alterations that may increase the vulnerability to psychopathologies (Dahl and Gunnar 2009). Indeed, the prevalence of mood disorders such as depression seems to increase from childhood to adolescence (Birmaher et al. 1996; Costello et al. 2002). This increase most likely is a complex interaction between endogenous developmental processes and external factors, one of which may be a recurrent lack of sleep. In agreement with this are various studies in young subjects that have linked the onset of anxiety and depression to short and disrupted sleep (Chang et al. 1997; Gregory et al. 2005; Buysse et al. 2008). In some studies, sleep problems preceded the onset of psychopathology with several years (Chang et al. 1997; Gregory et al. 2005).

One of the brain regions that appears to be particularly sensitive to sleep disruption is the hippocampus (Graves et al. 2003; McDermott et al. 2003; Ruskin et al. 2004; Van der Werf et al. 2009). The hippocampus plays an important role in cognition and emotional regulation (Bannerman et al. 2004; Bast 2007) and is one of the few brain regions that displays neurogenesis continuing from adolescence into adulthood (Abrous et al. 2005; Ming and Song 2005). Lower levels of hippocampal neurogenesis and reduced hippocampal volume have been implicated in the etiology and symptomatology of emotional and depressive disorders (Sapolsky 2000; Czéh and Lucassen 2007; Perera et al. 2008; Boldrini et al. 2009; Lucassen et al. 2010). Moreover, experimental studies show that hippocampal integrity can be affected by prolonged restriction or disruption of sleep (McDermott et al. 2003; Roman et al. 2005a; Guzman-Marin et al. 2006, 2007) while clinical studies have reported a reduction in hippocampal volume in primary insomnia and sleep apnea (Morrell et al. 2003; Riemann et al. 2007). However, most of these data are based on studies in adult animals or humans. The impact of chronically disrupted sleep

on hippocampal integrity at a young age, when the brain might be particularly sensitive, has so far received little attention.

In the present study, we applied an animal model of chronic sleep restriction that is aimed at mimicking chronically insufficient sleep as it often occurs in human society. Thus, rather than total sleep deprivation, rats were allowed to sleep part of the day but, presumably, not enough to fully recover (Roman et al. 2005b; Novati et al. 2008). We used the model in the adolescent period to study whether insufficient sleep (i) changes hypothalamic-pituitary-adrenal (HPA) axis activity, (ii) affects hippocampal volume and neurogenesis, and (iii) alters anxiety and anhedonic behavior.

Material and methods

Animals and housing

This study was performed with 48 male Wistar rats, 25 or 28 days old at the start of the experiments. Animals were housed in pairs in a room with a 12 h: 12 h light-dark cycle (lights on 9AM – 9PM) and temperature of 21±1 ℃. Standard lab oratory chow and water were provided ad libitum. Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Experimental design

Two experiments were performed in this study. In the first one, we examined effects of sleep restriction on anxiety and depression like behavior measured in an open field test, an elevated plus maze and a saccharine preference test. Rats were sleep restricted throughout adolescence, from postnatal day (PD) 28 to 61. All behavioral tests were performed on consecutive days, after 1 and 4 weeks of sleep restriction. In the second experiment, rats were sleep restricted from PD 30 to 61. Blood samples were collected after 7 and 25 days of sleep restriction to assess plasma levels of stress hormones and brains were collected after 4 weeks of sleep restriction to measure hippocampal volume and examine hippocampal neurogenesis. To quantify survival of newly generated hippocampal cells, all rats received an intraperitoneal injection with the thymidine analogue 5-bromodeoxiuridine (BrdU) at PD 25, 5 days before the start of the experiment (100 mg/kg BrdU in saline, pH = 7.0, Sigma, St Louis, MO, USA). As BrdU is incorporated into the DNA of cells in S phase of the cell cycle, it is used to label newborn cells (Kee et al. 2002). Within 1–4 days after injection, BrdU labeled cells stop dividing and any change in the number of labeled cells thereafter indicates a change in survival (Dayer et al. 2003). Five days after the BrdU injection, at PD 30, the sleep restriction treatment started and continued until PD 61.

Sleep restriction and forced activity controls

For both experiments, 24 animals were assigned to one of the following groups (n = 8 in each): chronic sleep restriction (SR), forced activity control (FA), and home cage control (HC). Details on

our sleep restriction and control procedures have been reported before (Roman et al. 2005a, 2005b; Novati et al. 2008). Briefly, SR was performed by placing rats in drums of 40 cm diameter, rotating at a speed of 0.4 m/min. Animals were kept awake 20h per day (1PM – 9AM) and were left undisturbed for the remaining 4 h at the beginning of the light phase (9AM – 1PM). Electroencephalographic (EEG) recordings have shown that rats in the wheels have occasional brief sleep bouts. Since this adds up to no more than 10% of the time, the animals are severely sleep deprived on a daily basis, which is confirmed by a sleep rebound during the 4 h daily rest periods (Barf and Meerlo, unpublished results). To examine whether consequences of the treatment were caused by forced locomotion rather than sleep loss per se, a forced activity (FA) group was included as control. Animals of the FA group were housed in the same type of drums which were rotating at double speed for half the time (0.8 m/min for 10 h). As a result, the forced activity animals walked the same distance as sleep restricted animals, but had sufficient time to sleep. The 10 h forced activity took place during the last 10 h of the dark phase (11PM – 9AM), that is, during the main activity phase of the rats. In addition to the FA controls, we also used undisturbed, naïve controls that remained in their home cage throughout the experiment (HC).

Open field test

An open field test was performed to assess effects of SR on general explorative activity and anxiety (Meerlo et al. 1996). The animals were subjected to a 5 min test twice, on days 7 and 31 of the SR protocol, between the third and fourth hour of the daily rest period, before the SR animals were returned to the rotating drums. The open field consisted of a round arena (120 cm in diameter and 20 cm high walls) with a central zone and an outer zone (two imaginary concentric circles with diameters of 60 and 120 cm, respectively). Before the test of each new animal, the arena was thoroughly cleaned with water and soap to eliminate odor cues. Animals were transported in their home cage to the experimental room and placed in the outer zone of the arena. The behavior of the animals was recorded by a camera and analyzed with a computerized imaging analysis system (Ethovision, Noldus Information Technology, Wageningen, The Netherland). The time spent in the central and outer zone and the total distance covered in each of the two zones were calculated.

Elevated plus maze

On days 8 and 32 of the SR period, during the last two hours of the daily rest phase, animals were subjected to an elevated plus maze test, a widely used non conditioned anxiety test (Pellow and File 1986). The plus maze consisted of a black wooden apparatus with two open and two closed arms, 55 cm above the floor. Each arm was 45 cm long and 10 cm wide and the closed arms had 50 cm high walls. Before the test of each new animal, the maze was thoroughly cleaned to eliminate odor cues. At the start of the test, the animals were placed in the centre of the plus facing a corner between a closed and an open arm. The test lasted 5 min and the behavior of the

animals was recorded on video for later analysis. Time spent in open and closed arms as well as time in the centre between the arms was scored and expressed as percentage of the total time.

Saccharine preference

To examine the effects of SR on depression-like behavior, we performed a saccharine preference test, which is often used to measure anhedonic behavior (Moreau 1997). On days 8 and 32 of the SR protocol, following the elevated plus maze test, rats were water deprived for 20 h (1 PM – 9 AM) and then subjected to a two bottle choice preference task during the 4 h of rest (9 AM – 1 PM). One bottle contained the regular tap water and the other one a sweet 0.05% saccharin solution. The bottles were placed in central position on the top of the cage to avoid position preference. The liquid intake was measured by comparing the weight of the bottles before and immediately after the test. Water or saccharine solution intake was expressed as percentage of the total fluid intake.

Blood samples and hormones analysis

To measure effects of chronic SR on plasma levels of stress hormones, 0.5 ml blood samples were taken from the tail of the animals at the end of the daily sleep deprivation phase (9AM) and at the end of the resting phase (1PM), after 7 and 25 days of SR. Samples were collected within 1–2 min in cold Eppendorf tubes containing EDTA and centrifuged (4 ℃, 2600 g, 15 min). The supernatant was stored at -80 ℃ for later analysis . Plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) were determined by radioimmunoassay according to the manufacturer's instructions (MP Biomedicals, Orangeburg, NY, USA).

Brain collection

At PD, 61 brains of the rats from the second experiment were collected for immunocytochemical analysis. After injection with 2 ml/kg pentobarbital, the animals were transcardially perfused with 0.9% saline followed by a 4% solution of paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed from the skull and post-fixed in 4% PFA for another 24 hours. Then they were kept in 0.01 M PBS overnight and subsequently cryoprotected by 30% sucrose for 48 h before freezing. With a cryostat, eight series of 30 µm sections were cut and collected in 0.01 M PBS with 0.1% sodium azide until further processing.

Immunohistochemistry

Immunohistochemical staining for the neuronal marker NeuN was used to measure hippocampal volume. Differentiation of new hippocampal cells into neurons was examined by staining for doublecortin (DCX), a microtubule-associated protein that is found in immature neurons (Rao and Shetty, 2004; Couillard-Despres et al., 2005). Survival of newborn cells was assessed by staining for BrdU, which had been injected five days before the start of the SR protocol.

For BrdU immunostaining, first, DNA was denatured with 50% formamide in 2x saline sodium citrate (30 min, 65 ℃), followed by repeate d rinsing in saline sodium citrate. Sections were subsequently placed in a 2 M HCl solution (30 min, 37 ℃) and then in 0.1 M borate buffer (pH=8.5). After treatment with 0.3% H_2O_2 (30 min, RT), the sections were first incubated in 3% normal serum and 0.1% Triton-X-100 in 0.01 M TBS and then in primary antibody (rat anti-BrdU, 1:800, Serotec, Oxford, UK) for 48 h at 4 ℃. The s ections were then incubated in 3% normal serum and 0.01% Triton-X100 before the secondary antibody (donkey anti-rat 1:400, Jackson ImmunoResearch, Suffolk, UK) was applied (2 h, RT). Reaction with avidine biotin complex (1:500, ABC Elite Vector Laboratories, Burlingame, CA, USA) was done for 2 h at room temperature before development in 0.2 mg/ml diaminobenzidine and 0.003% H_2O_2.

For DCX and NeuN immunostaining, sections were pretreated for 30 min with 0.3% and 0.6% H_2O_2, respectively. After blocking of aspecific staining with normal serum (3% for DCX and 0.3% for NeuN), sections were incubated with primary antibody (goat anti-DCX at 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA; or mouse anti-NeuN at 1:700; Chemicon, Temecula, CA, USA) for 48 h at 4℃. Following 40-min exposure to a secondary antibody (Rabbit anti-Goat at 1:400 for DCX and goat anti-mouse at 1:400 for NeuN; Jackson ImmunoResearch, Suffolk, UK), sections were incubated with avidine-biotin complex (1:400 for DCX and 1:500 for NeuN; Elite Vector Laboratories, Burlingame, CA, USA) and the cell labeling was visualized through reaction with 0.2 mg/ml diaminobenzidine and 0.003% H_2O_2.

Quantification

All quantifications were conducted by an investigator blind to the experimental conditions. The number of BrdU positive cells was quantified in the granular cell layer (GCL) and subgranular zone (SGZ) of the dentate gyrus (DG) in every eighth section of the dorsal hippocampus (bregma –1.80 to –4.08; Paxinos and Watson, 1986) at a 400x magnification. Immunopositive cells less than one cell diameter away from the SGZ inner border were also included in the analysis, whereas other cells present in the hilus were excluded. In each section, the number of cells was divided by the length of the GCL in that section. The total number of cells per animal was expressed per mm GCL.

DCX immunoreactivity in the DG was quantified by measuring optical density (OD) with a computerized image analysis system (Leica Qwin, Rijswijk, The Netherlands) according to previously published methods (Dagyte et al., 2009). The OD of DCX expression was measured in the GCL and SGZ and corrected for nonspecific background labeling measured in the corpus callosum. For each animal, DCX immunoreactivity was measured by delineating the entire GCL and SGZ in both hemispheres of three dorsal hippocampal sections (around bregma – 2.50, – 3.20, – 4.00; Paxinos and Watson, 1986). OD values were expressed in arbitrary units corresponding to grey levels measured by the analysis system.

To estimate the total volume of the dorsal hippocampus and the volume of its cellular subregions, every eighth section was stained with NeuN antibody (11 sections total). We

performed the volume measurements in the dorsal hippocampal area of 11 sections per animal (bregma – 1.80 to – 4.08; Paxinos and Watson, 1986) using the Leica Qwin image software (Rijswijk, The Netherlands). In each of these sections, the complete hippocampal area was outlined as indicated in Fig. 4A. The areas of the cellular subregions, particularly the GCL of the DG and the pyramidal cell layer of the CA1 and the CA2/3, were outlined as indicated in Fig. 4C. The volume estimate for the total hippocampus and the cellular subregions was based on the Cavalieri's method and was obtained by multiplying the sum of the section areas per animal, by section thickness and number of series (Walker et al. 2002; Czéh et al. 2010).

Statistics

Immunohistochemical and behavioral data were statistically tested with a one-way analysis of variance (ANOVA). Repeated measures ANOVA was used for the analysis of ACTH and corticosterone data. When treatment effects were detected with ANOVA, a post hoc Tukey test was used to assess differences between specific treatment groups. A paired t-test was applied to assess differences in preference between water and saccharin in each group of animals. The level of significance was set to P=0.05. Data in text, tables, and figures are expressed as average per group ± SEM.

Results

Behavior

The rats in the present study coped with the protocol of 1-month SR without visible signs of deterioration or illness. Growth was slightly suppressed in both the sleep-restricted rats and forced-activity controls as compared to the home cage controls, resulting in a significantly lower body weight by the end of the experimental period (SR: 234.6±8.1g, FA: 222.6±7.1g, HC: 260.1±6.4g; treatment effect $F_{2,21}$=7.01, P=0.005; post hoc Tukey test P>0.05 for both SR and FA vs. HC).

No significant effect of SR was found on explorative behavior in the open field test (Table 1). In each of the two tests, after 1 and 4 weeks of treatment, all groups spent most of their time in the outer zone of the arena. Time spent in the central area and the distance traveled in the central area were small and did not differ between the groups (day 7: time $F_{2,21}$=0.95, P=0.401; distance $F_{2,21}$=1.91, P=0.174; and day 31: time $F_{2,21}$=0.35, P=0.711; distance $F_{2,21}$=1.68, P=0.209).

Also, SR did not affect anxiety-related behavior in the elevated plus maze test (Fig. 1). Rats in all three groups spent a large part of the time in the closed arms and no difference was found between groups in the percentage of time spent in open arms (day 8: $F_{2,21}$=1.06, P=0.363 and day 32: $F_{2,20}$=0.083, P=0.921).

Table 1. Behavior in the open field test after 7 and 31 days of sleep restriction. Data show percentages of time spent and distance covered in the central zone of the arena

	Day	Home cage	Forced activity	Sleep restricted
Time (%)	7	3.9 ± 1.0	2.3 ± 0.4	2.9 ± 0.8
	31	10.6 ± 1.5	8.9 ± 1.7	9.2 ± 1.5
Distance (%)	7	11.3 ± 4.9	3.8 ± 0.9	5.9 ± 0.8
	31	17.0 ± 3.1	12.9 ± 2.4	20.2 ± 2.9

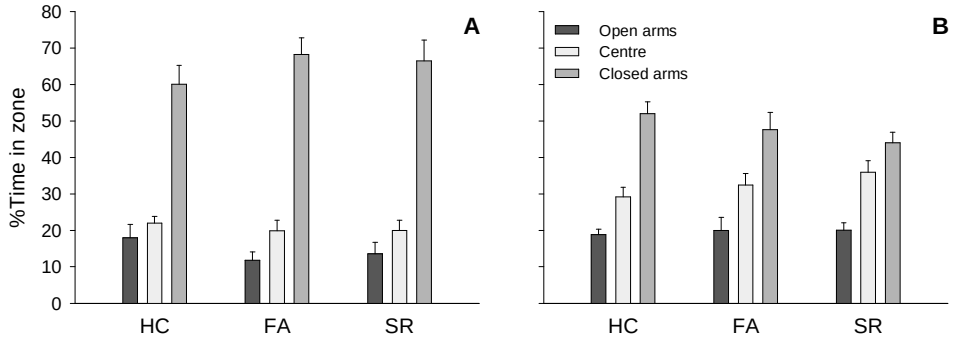


Fig 1. Anxiety behavior in the elevated plus maze test on day 8 and 32 of the sleep restriction protocol (panel A and B, respectively). All rats had showed preference for the closed arms of the plus maze. The percentage of time spent in open arms was similar for all experimental groups on both test days.

A saccharine preference test was used to measure anhedonia (Fig. 2). On day 9 of the experiment, HC animals displayed a clear and significant preference for saccharine over water ($t_{1,7}$=4.13, P=0.004), which was less clear and not significant in the FA rats ($t_{1,7}$=1.48, P=0.181) and completely absent in the SR rats ($t_{1,7}$=-0.34, P=0.742). One-way ANOVA indicated a significant effect of treatment on saccharine intake ($F_{2,21}$=6.07, P=0.008), but post hoc Tukey test only showed a difference between the HC and SR rats (P=0.007). After 4 weeks of treatment, all three groups displayed a clear preference for the saccharine solution over water (HC: $t_{1,7}$=20.16, P=0.001; FA: $t_{1,7}$=24.34, P=0.001; SR: $t_{1,7}$=2.89, P=0.023). While the preference of the SR rats was on average still lower, ANOVA no longer indicated any significant treatment effect at this time point ($F_{2,21}$=2.40, P=0.115).

Stress hormones

To examine the level of HPA axis activation in response to SR, blood samples were collected after 7 and 25 days of treatment (Table 2). Both immediately after the daily 20-h sleep deprivation and at the end of the daily 4-h resting phase, plasma ACTH and CORT levels in the SR animals were low and not different from FA or HC controls. Statistical analysis did not show significant differences in ACTH and CORT levels between experimental groups for any time point (P>0.05 in all cases).

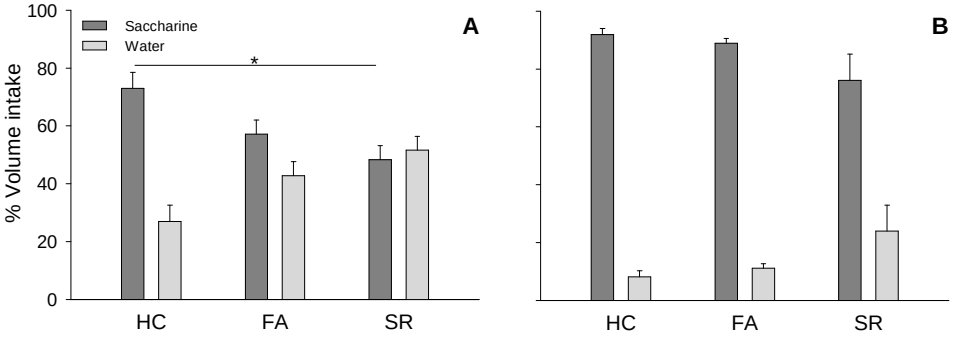


Fig 2. Saccharine preference test on days 9 and 33 of the sleep restriction protocol (panels A and B, respectively). Rats were water deprived for 20 h and then subjected to a two-bottle choice preference task during the daily 4-h rest period. On day 9, home cage control rats displayed a clear preference for saccharine over water. In both sleep restricted and forced activity rats saccharine intake was comparable to water intake showing a decreased preference for sweet taste. Saccharine intake in sleep restricted rats was significantly lower than in home cage controls (* $P < 0.05$). On day 33 all groups displayed a strong preference for the saccharine over water and there was no longer any difference between the treatments.

Table 2. Stress hormones concentrations after 7 and 25 days of sleep restriction. Plasma samples collected at the end of the daily 20h sleep deprivation session (9 AM) and after 4 hours rest (1 PM)

	ACTH (pg/ml)				Corticosterone (µg/dl)			
	Day 7		*Day 25*		*Day 7*		*Day 25*	
	9 AM	*1 PM*	*9 AM*	*1 PM*	*9 AM*	*1 PM*	*9 AM*	*1 PM*
HC	42.8±6.7	35.8±2.6	35.9±2.8	33.7±2.6	1.2±0.4	0.8±0.1	1.1±0.6	1.6±0.4
FA	3.8±2.0	33.7±3.3	33.6±2.0	27.3±1.7	1.4±0.3	7.0±3.9	3.2±1.1	1.3±0.4
SR	47.0±6.6	32.5±3.8	38.8±4.0	26.0±2.5	10.5±6.9	2.2±1. 0	3.7±0.9	1.1±0.1

Hippocampal neurogenesis

To investigate effects of chronic SR on survival of newly produced cells in the hippocampus, BrdU was injected in the rats 5 days before the beginning of the treatment. The number of BrdU-positive cells determined at the end of the experiment, that is, at 36 days after BrdU injection, was not different between the groups (Fig. 3B, $F_{2,21}$=0.77, P=0.478), suggesting that 4 weeks of SR or FA treatment had not affected survival of the newly formed cells in the dorsal hippocampus. Also the differentiation of new cells into neurons did not appear to be affected by the treatment as the optical density of DCX labeling in the GCL and SGZ of the DG did not differ between the groups (Fig. 3D, $F_{2,21}$=1.32, P=0.288).

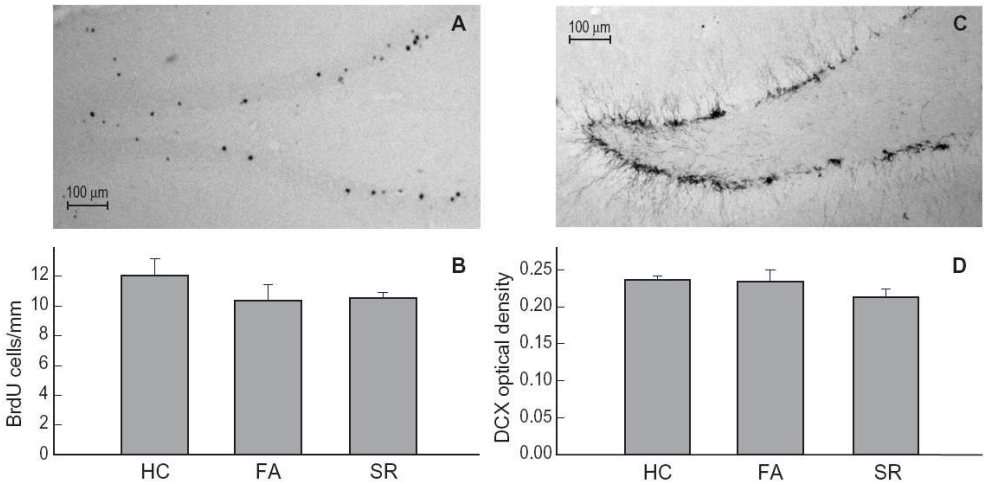


Fig.3. Sleep restriction and hippocampal neurogenesis. (A) Photograph of BrdU-positive cells in the granular layer of the dentate gyrus. (B) Chronic sleep restriction did not affect the survival of newly formed cells in the dentate gyrus of the dorsal hippocampus. (C) Photograph of DCX expressing neurons in the dentate gyrus. (D) Chronic sleep restriction did not affect the optical density of DCX expressing new neurons in dentate gyrus of dorsal hippocampus.

Hippocampal volume

The volume of the dorsal hippocampus was estimated in NeuN stained sections (Fig. 4A). A number of animals had to be excluded from the volume analysis because of missing and/or damaged sections (one SR, two FA, and two HC). Chronic SR in adolescent rats caused a reduction in volume of the dorsal hippocampus of about 10% compared to controls (Fig. 4B). One-way ANOVA revealed a significant treatment effect ($F_{2,16}$=9.63, P<0.002) and post-hoc Tukey test indicated a lower volume in SR rats as compared to HC controls (P=0.009) and FA controls (P=0.003). Hippocampal volume in FA rats was not changed compared to HC controls (P=0.871).

The effect of SR did not appear to be restricted to a specific hippocampal subregion (Fig. 4D). One-way ANOVA revealed a trend toward a treatment effect on the volume of the GCL of the DG ($F_{2,16}$=2.95, P=0.085) and also a trend toward a treatment effect on the volume of the pyramidal cell layer of the CA1 region ($F_{2,16}$=2.75, P=0.096). Moreover, ANOVA revealed a significant treatment effect on the volume of the pyramidal cell layer of the CA2/3 region ($F_{2,16}$=5.91, P=0.014) with a significant reduction in SR rats compared to FA animals but not compared to HC rats (post-hoc Tukey P=0.011 and P=0.023, respectively).

To assess if the overall decrease in hippocampal volume might reflect a more global change in brain size, we determined the thickness of the neocortex in the sections used for the hippocampal measurements. On average the cortical thickness was slightly lower in SR rats but this did not reach statistical significance.

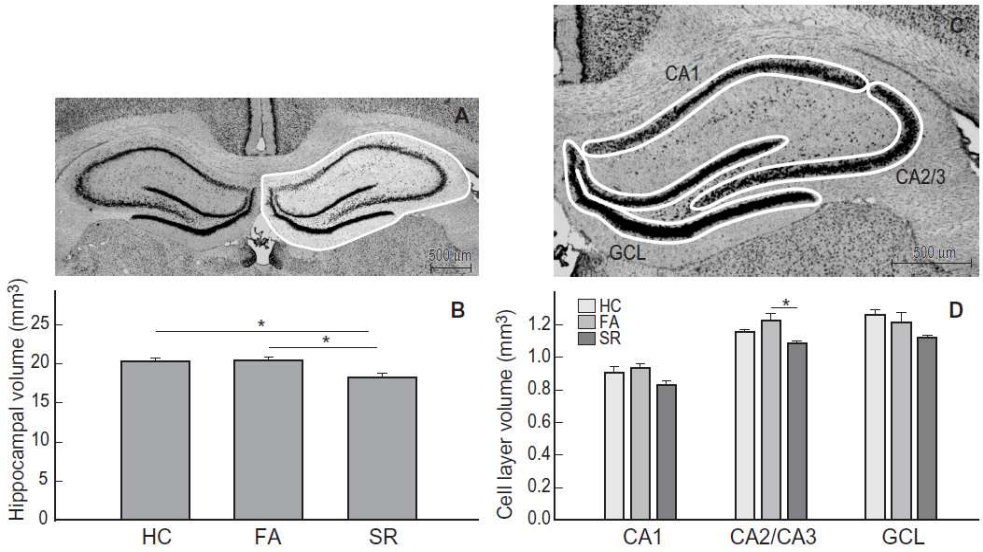


Fig.4. Sleep restriction and hippocampal volume. (A) Photograph of NeuN staining in the dorsal hippocampus. The hippocampal area measured in each brain section is indicated on the right. (B) Volume of dorsal hippocampus was significantly reduced in sleep restricted rats compared to home cage and forced activity control groups. (C) Photograph indicating the cellular subregions that were measured. (D) On average the volume of the cellular subregions was lower in the sleep restricted rats than in control rats but this difference only reached statistical significance for the CA2/3 subregion in comparison with the forced activity control rats. * $P < 0.05$, see text for details.

Discussion

The main finding of this study is that rats subjected to chronic SR during adolescence displayed a 10% reduction in dorsal hippocampal volume. This reduction in size of the hippocampus was not associated with significant changes in survival of newly generated BrdU-labeled cells or changes in DCX expression as a marker of young neurons. Therefore, the volume reduction is not likely explained by a reduction in neurogenesis. During the extended period of SR, the young rats displayed a temporary anhedonia as reflected in reduced saccharine preference, but this effect of SR had normalized at the end of the experiment. SR did not affect anxiety-like behavior in the open field test and elevated plus maze test.

In the current study, we did not quantify the exact amount of SR, although it may very well be that changes or lack of changes in some of the measures we took critically depend on the amount of sleep that is lost. Rats were subjected to a protocol of SR that only allowed them to sleep undisturbed for 4 h every day at the beginning of the light phase but it is not excluded that animals also had microsleeps even in the rotating wheels. Indeed, EEG recordings have shown that rats in the wheels have occasional brief sleep bouts that may add up to 10% of the time (Barf and Meerlo, unpublished observation). Furthermore, a recent study shows that after a long period without sleep, local clusters of cortical neurons may go offline while the rest of the brain is apparently awake (Vyazovskiy et al. 2011). The latter finding indicates that in general an exact

quantification of sleep loss may not be as straight forward as usually thought. Importantly, rather than trying to achieve total sleep deprivation or an exact amount of sleep deprivation, with our approach, we aimed to mimic chronically insufficient sleep as it often occurs in human society. While rats cope with our protocol of SR for prolonged periods of time without visible signs of illness, earlier studies had already shown that it gradually leads to neurobiological and neuroendocrine changes similar to what has been reported for depressed patients (Roman et al. 2005b; Novati et al. 2008).

In the present study, our analysis of SR effects on brain integrity was largely focused on the hippocampus and we only performed restricted control measurements in the cortex. The finding that cortical thickness was not significantly affected by SR suggests that the reduction in hippocampal volume was at least partly specific and did not simply reflect a more global change in brain morphology. On the other hand, this study certainly does not rule out the possibility that chronic SR affects other areas as well. Indeed, while the hippocampus appears to be particularly sensitive to sleep loss (Meerlo et al. 2009), other sensitive brain regions have been suggested, for example, the prefrontal cortex (Horne 1993; Muzur et al. 2002).

Reductions in hippocampal volume have been found before in human sleep disorders such as primary insomnia (Riemann et al. 2007) and sleep apnea (Morrell et al. 2003). Also in psychopathologies such as major depression, reductions in hippocampal volume are commonly observed and have been implicated in specific symptoms of the disorders (Sapolsky 2000; Czéh and Lucassen 2007; Perera et al. 2008; Boldrini et al. 2009; Lucassen et al. 2010). Intriguingly, psychopathologies are often associated with disturbed sleep-wake patterns that may contribute to the development and aggravation of the disease (Tsuno et al. 2005; Riemann and Voderholzer 2003). Along these lines, also the reduction in hippocampal volume in psychopathologies might be partly a consequence of the sleep-wake disturbance. Indeed, the present reduction in hippocampal volume after experimental SR under controlled conditions in rats suggests that the hippocampal volume reduction observed in human sleep disorders and mood disorders may be a direct consequence of disrupted sleep rather than a nonspecific side-effect.

A variety of explanations have been proposed for a decrease in hippocampal volume in disease including neuronal death, neuronal shrinkage, lower dendritic arborization, reductions in neurogenesis, or decreases in glia numbers and production (Czéh and Lucassen 2007). The lack of effects of SR on different markers of neurogenesis in the present study does not support the hypothesis that the smaller hippocampal size was related to a reduction in neurogenesis. In fact, even if SR would have fully suppressed neurogenesis it still could not have explained the magnitude of the hippocampal volume reduction we found. Moreover, measurements of the cellular subregions suggest that the volume reduction was not limited to the DG but may have included the non-neurogenic CA regions as well. Since there is little to no evidence for massive neuronal death even after prolonged total sleep deprivation (Cirelli et al. 1999; Eiland et al. 2002), it seems more likely that part of the volume reduction was caused by a decrease in the size of

neuronal cell bodies and dendritic arborizations or by changes in the number and size of glia cells.

In the present study, we focused our analysis of neurogenesis on survival of new BrdU-labeled cells and differentiation of new cells into young DCX-expressing neurons. While we did not specifically assess effects of SR on cell proliferation, a strong reduction in proliferation in the later part of the experiment most likely would have shown up in a reduced DCX expression as well since these DCX positive cells were born in the last 1 or 2 weeks of the experiment. This was clearly not the case. However, we cannot exclude that SR may have suppressed cell proliferation in the first half of the experiment, which would not be visible in DCX expression. Another limitation of our study is that we only assessed cell survival and differentiation in the dorsal limb of the hippocampus. One might argue therefore that SR possibly had an effect on neurogenesis in the ventral hippocampus that went unnoticed in our analysis. Yet, although one study on sleep deprivation in adult rats indeed showed a stronger suppression of cell proliferation in the ventral part of the hippocampus (Tung et al. 2005), most studies report a reduction of neurogenic measures in the dorsal limb as well (Guzman-Marin et al. 2007; Roman et al. 2005a). All together, the fact that our measures of neurogenesis were not affected by SR in young animals was somewhat unexpected. One explanation may lie in the fact that we sleep restricted rats during the transition from adolescence to adulthood, when neurogenesis rapidly declines toward the low levels that then persist in the mature brain throughout middle age and senescence (Heine et al. 2004; He and Crews 2007; Cowen et al. 2008). Perhaps the additional impact of SR on neurogenesis is modest during a phase where neurogenesis already shows a strong and spontaneous decrease. Alternatively, the lack of an SR effect may be related to the social housing conditions in the current experiment. We chose to house the adolescent rats, two per cage or per SR drum because in this phase of their life social contact and play behavior is important for normal development. However, it might be that the enriched social housing condition has compensated for the adverse effects of SR. In support of this explanation are studies showing that environmental enrichment or exercise promote hippocampal neurogenesis (Kempermann et al. 1997; Van Praag et al. 2000) and may even decrease or reverse earlier brain deficits (Twiggs et al. 1978; Francis et al. 2002; Bredy et al. 2003; Nithianantharajah and Hannan 2006; Naylor et al. 2008). Importantly, even if the present housing conditions modulated the consequences of SR and counteracted putative effects on neurogenesis, it clearly did not prevent the reduction in hippocampal volume.

Hippocampal atrophy as observed in various pathologies is often proposed to be a result of elevated concentrations of glucocorticoid stress hormones (Sapolsky 2000; Czéh and Lucassen 2007). However, smaller hippocampal size and cortisol levels do not always correlate (O'Brien et al. 2004). In the present study, SR did not lead to a major activation of the HPA axis. ACTH and CORT concentrations measured after 1 and 4 weeks of SR were similar to the levels in FA and HC controls. Obviously, given the restricted number of samples and time points, these data need to be considered with care. One might argue that samples collected at the end of the

daily 20-h sleep deprivation session do not necessarily reflect HPA axis activity during the initial hours of sleep deprivation. Yet, previous studies with our model have shown that, if anything, CORT levels are low at the beginning of sleep deprivation and gradually increase towards the end (Meerlo et al. 2002). Moreover, the results are in line with various other studies showing only mild effects of sleep deprivation on HPA axis activity (see Meerlo et al. 2008 for review), which often do not explain sleep deprivation-induced changes in hippocampal integrity and function (Guzman-Marin et al. 2007; Mueller et al. 2008; Tiba et al. 2008; Hagewoud et al. 2010b). Together the data indicate that the reduction in hippocampal volume in our study is not easily explained by elevated HPA axis activity and glucocorticoid release.

Alternatively, a smaller hippocampus may have resulted from a reduction in trophic factors or a dysregulation in their signaling. One of the possible mechanisms involves altered expression of brain-derived neurotrophic factor (BDNF), which is highly expressed in the adult hippocampus (Schmidt-Kastner et al. 1996) and stimulates dendritic arborization (McAllister et al. 1995). Evidence suggests that low levels of BDNF may play a role in the hippocampal atrophy associated with depressive disorders (Shimizu et al. 2003). Literature further suggests BDNF levels can be affected by sleep deprivation, although the direction of the effect differs between studies and is so far difficult to interpret (Adrien 2002; Fujihara et al. 2003; Cirelli 2006; Guzman-Marin et al. 2006). In one study, 48 h of sleep deprivation resulted in decreased levels of BDNF in hippocampus (Guzman-Marin et al. 2006) while effects of longer periods of sleep deprivation or restriction on BDNF expression have not been reported. Should SR have a negative effect on BDNF expression, this may underlie hippocampal dendrite atrophy, which in turn could account for the reduction in hippocampal volume observed in this experiment. Future studies are needed to investigate the relationship between sleep, BDNF expression, and hippocampal integrity.

The reduction in hippocampal volume in our study was not associated with obvious changes in explorative activity or anxiety in an open field and elevated plus maze test. This is consistent with other studies in our laboratory that failed to find clear anxiety effects of acute and short sleep deprivation on the elevated plus maze test (Hagewoud et al. 2010b). In fact, with a few exceptions (Silva et al. 2004), the majority of experimental studies show no effect or even indicate decreased anxiety in sleep-deprived rodents (Hicks and Moore 1979; Moore et al. 1979; Suchecki et al. 2002; Martinez-Gonzalez et al. 2004; Tartar et al. 2009). While there is some inconsistency in the literature, this may be the result of a complex interaction between the duration and method of sleep deprivation, the anxiety test involved, species and strain differences, and perhaps other factors. Also in humans, some studies report no relationship between sleep loss and anxiety (Bonnet and Arand 1998), while others suggest an association between lack of sleep and self-reported anxiety (Peeke et al. 1980; Sagaspe et al. 2006), and a correlation between sleep disturbance and the risk for anxiety disorders (Gregory et al. 2005; Roth et al. 2006). Apparently, the relationship between restricted or disrupted sleep and anxiety is complex and requires further study.

While chronic SR in adolescent rats did not affect specific measures of anxiety, the treatment did have a temporary effect on the preference for a sweet saccharine solution, a commonly used measure of anhedonia (Willner et al. 1992; Moreau 1997). Anhedonia refers to an inability to experience pleasure or to the loss of interest for normal aspects of life and is considered a psychological marker of depression (Schrader 1997). After 1 week, sleep restricted rats did not display the normal preference for the saccharine solution that was seen in HC control animals. However, this lack of preference also occurred in the FA controls and therefore we cannot distinguish between effects of chronic sleep loss and forced locomotion. Also, this anhedonia did not persist but gradually disappeared in the course of the experiment and was no longer noticeable after 1 month, when all groups exhibited a clear preference for saccharine solution over water. Few other published records exist on chronic SR and anhedonic behavior. In several unpublished experiments, we have examined the effect of prolonged SR on saccharine or sucrose preference in adult animals with the same protocol as we used in the present study but the results have been inconsistent (Novati et al. unpublished observation). It could be that the preference test is not sophisticated enough to really detect anhedonic behavior or, alternatively, SR may only have a weak effect that is easily modulated or overruled by other factors we are yet unaware of.

In summary, the results of this experimental study suggest that lack of sleep during adolescence may reduce hippocampal volume, without affecting survival and differentiation of new neurons in the DG. The reduction in hippocampal volume was not associated with obvious changes in anxiety or persistent changes in anhedonic behavior. The mechanisms underlying the effect of SR on hippocampal volume require further investigation.

Acknowledgments

The authors thank Josè Vidal for helpful suggestions concerning the behavioral tests, Jan Bruggink for performing the radioimmunoassays, Folkert Postema for assistance with the immunochemistry, Jan Keijser for contributing to the immunostaining quantification and Dick Visser for his help with the figures. This study was supported by the Nederlands Organization for Scientific Research (VIDI grant 864.04.002 to P.M.). P.J.L. is supported by the EU (NEURAD), ISAO and the Dutch Brain foundation.

CHAPTER 7

General Discussion

The consequences of chronic sleep restriction

This thesis describes a series of experiments on the consequences of chronic sleep restriction and how this affects brain vulnerability with special emphasis on neurobiological and neuroendocrine systems involved in psychopathology.

In chapter 2 we directly assessed the sensitivity of the sleep restricted brain to neurodegenerative processes by experimentally inducing local brain damage. Contrary to the expectation, the damage caused by an injection of NMDA into the NBM was lower in sleep restricted rats than in control rats. This result directly opposes the hypothesis that sleep loss sensitizes the brain to neurodegeneration. On the other hand, while insufficient sleep may not directly contribute to neurodegeneration, it may affect brain integrity and brain function in other, more subtle ways. Chapters 3 and 4 in this thesis show that chronic sleep restriction may gradually lead to changes in neurotransmitter systems (i.e., the serotonergic system) and neuroendocrine regulation (i.e., HPA axis) that are similar to what has been described for depression. In the long run, chronic sleep restriction may even cause morphological changes such as a decrease in volume of the hippocampus (Chapter 6), which is another frequently reported characteristic of depression. Together, the findings in this thesis strongly support the hypothesis that insufficient sleep may contribute to the development of psychopathology and provide clues to the potential underlying mechanisms.

The experiments in chapter 3 show that chronic sleep restriction in rats affects the HPA axis reactivity in a fear conditioning paradigm. The ACTH response to foot shock stress and to subsequent re-exposure to the foot shock environment was blunted in sleep restricted rats. This effect was evident following 1 week, but not 1 day of treatment, which confirms the important notion that restricted sleep may have effects that are not immediately noticeable but gradually accumulate over time.

The release of pituitary ACTH is under control of various neurochemical inputs, including CRH and serotonin. Direct stimulation of the 5-HT_{1A} and the CRH receptors with the 5-HT_{1A} receptor agonist 8-OH-DPAT and CRH respectively produced a similar blunted ACTH response in sleep restricted animals as found under real stress conditions. This suggests that mechanism underlying the sleep-restriction induced blunted ACTH response is caused by a desensitization of CRH and/or 5-HT_{1A} receptors. Despite the changes in ACTH response, sleep loss did not have a strong influence on the adrenal CORT response to stress or to direct 5-HT_{1A} and CRH receptors stimulation, suggesting an increased sensitivity to ACTH in the adrenals.

Long term sleep deprivation in rodents has been shown to affect food intake and body weight, which in turn can influence the sensitivity of the 5-HT_{1A} receptor systems. In chapter 4, we therefore performed an additional experiment to assess whether the sleep restriction-induced 5-HT_{1A} receptor desensitization reported in chapter 3 was the result of decreased body weight. The experiment examined the temperature and HPA axis response to stimulation with the 5-HT_{1A} agonist 8-OH-DPAT comparing control and sleep restricted animals fed with a normal diet or a

medium fat diet that was meant to prevent the sleep restriction-induced decrease in body weight. The results show that the desensitization of the 5-HT_{1A} receptors in sleep restricted rats persists even when the decrease in body weight is partially prevented. Since body weight in these animals was still lower than in controls, it cannot be completely excluded that such difference in body weight may have contributed to the changes in the 5-HT_{1A} receptor system.

Chapter 5 reports the effects of sleep loss, alone or in combination with repeated restrain stress, on the expression of corticoid receptors in the brain. The expression of these receptors is sensitive to stress and regulated by a variety of systems that in turn may be influenced by sleep loss. Yet, the results in this chapter do not show an effect of sleep restriction and/or restrain stress on the GR/MR mRNA expression. Further studies are required to assess if sleep restriction does not affect protein levels of the receptor or sensitivity of the receptor at a functional level.

Data in chapter 6 show a reduced dorsal hippocampal volume in sleep restricted adolescent animals. The volume decrease was not accompanied by changes in HPA axis activity or in neurogenesis indicating that the mechanisms that mediated the volume reduction were independent of these factors. Chronic sleep restriction did not increase anxiety levels in these animals as assessed in an open field test and elevated plus maze test. Sleep restriction caused a temporary suppression in the normal preference of sweet solution over water, which might indicate a temporary increase in anhedonic or depression-like behavior. However, we could not rule out that this effect was related to forced activity associated with the sleep restriction method rather than insufficient sleep per se.

Chronic sleep restriction and the sensitivity to neurodegeneration

Sleep is thought to be of primary importance for the brain and it has been proposed as a phenomenon necessary to maintain brain homeostasis favoring restoration, energy regulation and neuronal detoxification (Walker and Berger 1980; Bennington and Heller 1995; Inuoè et al. 1995; Scharf et al. 2008). Along this line, the common view is that sleep loss is bad for the brain and may in the long run increase neurodegenerative processes. The results in this thesis, showing that chronic sleep restriction attenuates the subsequent damage induced by an excitotoxic injection of NMDA, clearly do not fit with this simple view. Our findings, however, are in line with the results of several other studies combining sleep deprivation with a subsequent brain ischemia (Hsu et al. 2003; Moldovan et al. 2010) and with studies that did not show a strong neurodegenerative effect of sleep loss directly (Cirelli et al. 1999; Eiland et al. 2002).

An important factor determining a protective effect in these studies may be that sleep loss was applied before the brain insult. Indeed, a recent study suggests that opposite results may be found when sleep deprivation follows an insult (Gao et al. 2010). To clarify whether the effect of sleep loss may go in opposite directions based on the order of the events in the experiment,

further studies should directly compare sleep deprivation pre- or post brain insult, with the same sleep deprivation or restriction method.

A possible explanation for the neuroprotective effect of sleep restriction may be that sleep loss is a challenge to the brain that by itself does not cause any immediate damage but is sufficient to activate neuroprotective pathways and defense mechanisms that would subsequently facilitate the response to more severe insults. In this direction, it has been suggested earlier that the neuroprotection induced by sleep loss is the result of a preconditioning effect (Hsu et al. 2003). In other words, if sleep loss occurs before an acute insult, the resulting damage would be attenuated. Potential factors determining the decrease in sensitivity to neurotoxicity are changes in antioxidative stress mechanisms, altered inflammatory pathways as well as alterations in the expression, composition and signaling of the glutamate receptors since all these elements participate in neurodegenerative phenomena and appears to be sensitive to sleep loss (D'Almeida et al. 1998; Ramanathan et al. 2002; Guzman-Marin et al. 2006; Kopp et al. 2006; McDermott et al. 2006; Simpson and Dinges. 2007; Wang et al. 2009; Alhaider et al. 2010; Hagewoud et al. 2010a).

Chronic sleep restriction and systems implicated in mood disorders

While sleep loss did not increase the sensitivity for a neurotoxic insult, it did lead to changes in HPA axis reactivity, serotonergic system and hippocampal structure that are similar to changes reported for depression.

Postsynaptic serotonergic 1A receptor functionality appears to be impaired in depression. Imaging studies in depressed subjects show decreased binding potential of 5-HT_{1A} receptors in various brain regions including the hippocampus and amygdala (Drevets et al. 1999, 2007). Also, pharmacological challenge studies demonstrated attenuated decrease of the body temperature and smaller increase in plasma ACTH and cortisol in response to 5-HT_{1A} receptors agonists in depressed patients (Lesch et al. 1990; Shapira et al. 2000). Our observations after chronic sleep restriction are in line with these findings. Chronic sleep restriction reduced the postsynaptic 5-HT_{1A} receptor sensitivity as indicated by a reduced hypothermic response and a blunted ACTH in response to 5-HT_{1A} receptors agonists. Similarly to depressed patients, the injection of CRH in sleep restricted rats resulted in blunted ACTH and normal cortisol responses (Holsboer et al. 1984, 1987). Blunted ACTH levels in response to CRH and 5-HT_{1A} stimulation suggest altered regulation at the levels of both CRH and serotonergic 1A receptor systems. Quantification of the CRH and 5-HT_{1A} receptors and study of their signaling pathways in distinguished brain areas regulating the HPA axis may help to understand the changes responsible for an altered HPA regulation axis regulation. The mechanisms accounting for normal corticosterone response despite a blunted ACTH response in sleep restricted rats, may be related to increased sensitivity of the ACTH receptors at the level of the adrenals and should be examined in new experiments.

Another finding that links sleep loss to the risk to develop psychopathology is the decreased hippocampal volume in sleep restricted rats. The hippocampus is thought to be an important structure for the etiology of mood disorders especially depression and to mediate the action of antidepressants (Samuels and Hen 2011). A smaller hippocampal volume is a characteristic often present in subjects with depression and other mood disorders and seems to be related to the severity of the symptoms (Sapolsky 2000).
Interestingly, the smaller hippocampal volume in sleep restricted animals does not appear to be a consequence of high glucocorticoid levels as it has been proposed for depressed patients (Sapolsky 2000; Czeh and Lucassen 2007). As depressed patients often complain of sleep disturbances, it could be that hippocampal atrophy in these subjects is in part a consequence of chronically insufficient sleep and not entirely caused by increased glucocorticoid levels.

It has been suggested that the decreased hippocampal volume in depression could be the reflection of structural plasticity such as decreased dendritic branches, decreased cellular volume, decreased number of neurons or astroglia, and perhaps lower levels of neurogenesis. In fact, it is generally believed that decreased levels of cell proliferation in the DG play a role in the pathology of depression while increased neurogenesis is necessary for antidepressant action (Perera et al. 2008; Samuels and Hen 2011). In chapter 6, survival of newly generated cells and their differentiation into new neurons was not affected in sleep restricted rats that had a reduced hippocampal volume. This seems to be consistent with our observation that sleep deprivation does not affect the process of neurodegeneration. We did not assess the levels of cell proliferation directly and this should perhaps be examined in future analysis. However, other studies in the literature show an effect of sleep deprivation on cell proliferation (Guzman-Marin et al. 2003, 2005; Roman et al. 2005a; Tung et al. 2005; Mirescu et al. 2006; Guzman-Marin et al. 2007, 2008; Mueller et al. 2008) providing a link between sleep loss, decreased neurogenesis, and reduced hippocampal volume. At the same time, even a severe suppression of neurogenesis may not be sufficient to explain the reduction in hippocampal volume that we found in our study.

One remaining question that deserves attention is whether the reduction in hippocampal volume and other possible alterations persist throughout adult age or are reversed by subsequent regular sleep. Longitudinal studies could be done in human subjects affected by sleep disturbances only in the course of their childhood or adolescence. In addition, experimental studies in laboratory rodents should study the temporal dynamics and underlying molecular mechanisms of hippocampal volume changes in more detail during both sleep restriction and recovery. Furthermore changes in other brain structures like amygdala and prefrontal cortex that are known to be affected in mood disorders and are still developing during the adolescent period (Gogtay et al. 2004; Toga et al. 2006) could be considered as well.

Sleep restriction and behavior

Notwithstanding the effects of sleep loss on the brain, the results in this thesis do not show convincing behavioral changes in sleep restricted rats. Our assessment of behavior was limited to the experiments with adolescent rats described in Chapter 6 and a number of unreported experiments in adult rats.

To measure anxious behavior, we subjected the animals to open field and an elevated plus maze test that are based on the natural conflict between the tendency of rodents to explore a new place and the fear for a potentially dangerous environment (Ramos 2008). These tests are generally considered reliable to study anxious behavior in animals and are frequently used in this context. As we did not detect any change in open field or EPM that should detect changes in different parameters of anxiety, we feel confident to conclude that chronic sleep restriction does not affect trait anxiety.

This lack of effect of chronic sleep restriction on anxiety was not unexpected and in line with most of the available data from other rodent studies showing that (short) sleep deprivation does not cause major changes in behavioral measures of anxiety (Hicks and Moore 1979; Moore et al. 1979; Suchecki et al. 2002; Martinez-Gonzales et al. 2004; Tartar et al. 2009, Hagewoud et al. 2010b). We now confirm that even with prolonged chronic sleep restriction, rats do not become more anxious. Also in humans, the literature on restricted or disrupted sleep and anxiety shows contradictory results (Peeke et al. 1980; Bonnet and Arand 1998; Sagaspe et al. 2006).

To test depressive behavior in our animals, we used the saccharin preference test that is commonly used to measure anhedonia in animal models (Moreau 1997). Anhedonia is defined as a strong reduction of interest and pleasure for all or most of activities (Moreau 1997) and is considered one of the core symptoms of depression (Moreau 1997; Schrader 1997). Sleep restriction in our animals affected the preference for a sweet saccharin solution, but this effect was only temporary and it was to a lesser extent present also in control animals subjected to forced locomotion. We also applied this test in several other, unpublished studies on sleep restriction in adult animals and the results were inconsistent. While in some experiments there was a tendency towards reduced saccharin preference, in others there was not. Together these data may indicate that the effect of restricted sleep on saccharine preference is weak or the saccharin preference test may not be sensitive enough to detect anhedonic behavior in our experimental model.

One reason for the minimal behavioral effects of sleep restriction may thus lie in the sensitivity of our tests and the crude behavioral measures that were used. Another consideration is that some of the neurobiological consequences of sleep restriction that we observed in the brain, such as the hippocampal volume reduction, may not actually indicate a state of depression but, instead, a state of increased sensitivity to depression that is not yet associated with functional behavioral changes. In real life, besides experiencing sleep restriction, individuals are often exposed to other challenges such as stress and traumatic events that, by affecting similar

neurobiological systems, can cause mood disorders. Even though sleep loss by itself may not be sufficient to cause psychopathology, it may still increase the risk to develop it in subjects that have a certain predisposition. For example, literature in humans suggests that the risk to develop post traumatic stress disorder (PTSD) is higher in subjects interested by chronic sleep loss before or after a trauma (Krakow et al. 2001). Future experiment should therefore aim at the hypothesis that sleep restricted animals may more readily fail to cope with traumatic events than controls.

Sleep deprivation therapy

While several findings indicate that chronic sleep disruption is depressogenic, it is well established that one night of sleep deprivation can significantly improve mood in 50-60% of depressed patients (Pflug and Tolle 1971). Differently from other antidepressant treatments that require few weeks before inducing a therapeutic response, sleep deprivation for one night exerts its effect already the following day, although a considerable part of the patients responding to it relapses after the next night of recovery sleep (Southmayd and David 1990). Besides the clinical data on sleep deprivation therapy, a number of animal experiments have shown that acute total sleep deprivation for a single rest phase can reverse stress-induced depression-like behavior in rats as well (Koolhaas et al. 1990, Meerlo et al. 1996).

It may appear paradoxical that sleep deprivation is capable of improving mood in depressed subjects that already experience insufficient and irregular sleep, which in turn is even believed to contribute to the depressive symptomatology. The mechanisms underlying the antidepressant effect of sleep deprivation are not clear, although hypothesis have been made about physiological mechanisms (Beersma and Van den Hoofdakker 1992; Van den Burg et al. 1992) and neurobiological systems, among which monoaminergic pathways, glutamate neurotransmission and hormonal signaling (Adrien 2002; Payne et al. 2002; Benedetti and Smeraldi 2009). There is also evidence suggesting that the antidepressant effect of sleep deprivation may be the effect of changes at neuroendocrine level (Schüle et al. 2001). Furthermore neuroimaging studies show that the antidepressant action of sleep deprivation may involve very specific brain areas such as the orbital medial prefrontal cortex and the ventral areas of the anterior cingulate cortex (Gillin et al. 2001).

One of the mechanisms proposed for the antidepressant action of sleep deprivation involves the serotonergic system. Subjects homozygous for the long variant of the serotonin transporter gene, compared to the ones with the short variant, respond better to both sleep deprivation therapy and SSRI (Smeraldi et al. 1998; Benedetti et al. 1999). Experiments in rats showed that 24 h sleep deprivation produces desensitization of the 5-HT_{1A} mediated firing response of NRD neurons, similarly to the effect induced by antidepressant treatment (Prevot et al. 1996). Similarly to antidepressants that work on the serotonergic system, sleep deprivation therapy would induce a desensitization of the 5-HT_{1A} somatodendritic autoreceptors, which would

reduce the inhibitory feedback of serotonin on its own cells and, consequently, increase the release of serotonin in the projection areas (Adrien 2002).

While these data may seem at odds with the findings presented in this thesis, it is important to realize that our experiments show that chronic sleep restriction most likely reduces 5-HT_{1A} sensitivity post-synaptically in the projection areas. We have shown blunted ACTH and body temperature responses to a 5-HT_{1A} receptor agonist after 8 days of sleep restriction, which are mediated by postsynaptic 1A receptors (Fuller et al. 1996; Blier et al. 2002). In other words, while chronic sleep restriction decreases postsynaptic 1A sensitivity and may thereby reduce serotonergic neurotransmission in projection areas, sleep deprivation therapy reduces the sensitivity of auto-receptors on the raphe cells and through that ultimately enhances serotonergic neurotransmission. Acute sleep deprivation therapy and chronic sleep disturbance may thus exert their action at different levels of the serotonergic system and or differentially affect the sensitivity of the different 5-HT_{1A} receptors resulting in opposite effects.

An alternative hypothesis about the mechanisms of the antidepressant effect of sleep deprivation considers the expression of plasticity molecules such as pCREB, BDNF and TrkB through regulation of the noradrenergic system (Payne et al. 2002). This theory is based on the finding that the expression of these factors is enhanced by short term sleep deprivation and that such increase in expression is abolished by lesion of the noradrenergic system (Cirelli et al. 2000).

With all the potential mechanisms that have been proposed for the therapeutic effect of sleep deprivation, it is important to realize that the therapy perhaps only works in a depressed brain by reversing changes in a compromised system. In other words, sleep deprivation may have effects in depressed patients that are normally not seen in healthy subjects.

Clearly, understanding the bases of the sleep deprivation therapy could help to clarify the pathways of this complex relation between sleep and mood disorders. Considering that sleep deprivation exerts a faster effect and has fewer side effects compared to antidepressants, knowing how it works would also be useful to explain the mechanism of action of antidepressants and to design new medications to treat depression.

Concluding remarks

The experiments in this thesis applied an animal model of chronic sleep restriction as it often occurs in our society to study the consequences of insufficient sleep in terms of brain vulnerability and susceptibility to psychopathology. The data in this thesis do not provide evidence for the hypothesis that chronic sleep loss induces neurodegeneration but they do show that chronic sleep restriction gradually leads to more subtle neurobiological and neuroendocrine changes that are very similar to what is seen in depressed patients, particularly, a desensitization of the postsynaptic 5-HT_{1A} receptor system, an altered regulation and reactivity of the HPA axis and a reduction in hippocampal volume. These findings provide strong support for the hypothesis that

chronically insufficient sleep may increase the sensitivity and contribute to the development of mood disorders. This is important considering that an increasing number of people in our society are chronically sleep restricted. Supporting sleep hygiene education in order to avoid sleep loss as a habit together with an early diagnose and a proper therapy of sleep disorders could prevent serious consequences on health and wellbeing. Although sleep loss could increase the risk for psychopathology, in many cases it may not be a sufficient condition. A first interesting point is to clarify if and how sleep loss effectively increase the predisposition to psychopathology by studying its interaction with other elements that can cause mood disorders, in animal models. Second it is important to understand better which predisposing factors may make certain individuals more sensitive or more resistant than others against sleep loss in the context of psychopathology. These factors may be genetically determined and may affect the neurobiological pathways that sleep disturbances share with mood disorders. A deeper knowledge of these pathways will also help the interpretation of the complex relationship between sleep loss and mood disorders.

References

Abraham I, Veenema AH, Nyakas C, Harkany T, Bohus BGJ, Luiten PGM (1997). Effect of corticosterone and adrenalectomy on NMDA-induced cholinergic cell death in rat magnocellular nucleus basalis. J Neuroendocrinol, 9: 713-720

Abraham I, Harkany T, Horvath KM, Veenema AH, Penke B, Nyakas C, Luiten PGM (2000). Chronic corticosterone administration dose-dependently modulates A beta((1-42))- and NMDA-induced neurodegeneration in rat magnocellular nucleus basalis. J Neuroendocrinol, 12: 486-494

Abrous DN, Koehl M, Le Moal M (2005). Adult neurogenesis: from precursors to network and physiology. Physiol Rev, 85: 523-569.

Adrien J (2002) Neurobiological bases for the relation between sleep and depression. Sleep Med Rev 6: 341–351

Ahlemeyer B, Krieglstein J (1997). Stimulation of 5-HT1A receptor inhibits apoptosis induced by serum deprivation in cultured neurons from chick embryo. Brain Res, 777: 179-186

Ahlemeyer B, Glaser A, Schaper C, Semkova I, Krieglstein J (1999). The 5-HT1A receptor agonist Bay x 3702 inhibits apoptosis induced by serum deprivation in cultured neurons. Eur J Pharmacol, 370: 211-216

Alema GS, Casolini P, Patacchioli FR, Angelucci L (1995). Rat brain corticosteroid receptors are modulated by septo-hippocampal cholinergic innervation. Neuroreport, 6: 2461-2464

Alessandri B, Tsuchida E, Bullock RM (1999). The neuroprotective effect of a new serotonin receptor agonist, BAY X3702, upon focal ischemic brain damage caused by acute subdural hematoma in the rat. Brain Res, 845: 232-235

Alfaro-Rodriguez A, Gonzalez-Pina R, Gonzalez-Maciel A, Arch Tirado E (2006). Serotonin and 5-hydroxy-indole-acetic acid contents in dorsal raphe and suprachiasmatic nuclei in normal, malnourished and rehabilitated rats under 24 h of sleep deprivation. Brain Res, 1110: 95-101

Alhaider IA, Aleisa AM, Tran TT, Alkadhi KA (2010). Caffeine prevents sleep loss-induced deficits in long-term potentiation and related signaling molecules in the dentate gyrus. Eur J Neurosci, 31: 1368-1376

Alhaider IA, Aleisa AM, Tran TT, Alkadh KA (2011). Sleep deprivation prevents stimulation-induced increases of levels of P-CREB and BDNF: Protection by caffeine. Mol Cell Neurosci, 46: 742-741

Alonso R, Griebel G, Pavone G, Stemmelin J, Le Fur G, Soubrié P (2004). Blockade of CRF(1) or V(1b) receptors reverses stress-induced suppression of neurogenesis in a mouse model of depression. Mol Psychiatry, 9: 278-286

Alper RH (1990). Evidence for central and peripheral serotonergic control of corticosterone secretion in the conscious rat. Neuroendocrinology, 51: 255-260

Altena E, Vrenken H, Van Der Werf YD, van den Heuvel OA, Van Someren EJW (2010). Reduced orbitofrontal and parietal gray matter in chronic insomnia: a voxel-based morphometric study. Biol Psychiatry, 67: 182-185

Ankarcrona M, Dipbukt JM, Bonfoco E,Zhivotovsky B, Orrenius S, Liptn SA, Nicotera P (1995). Glutamate-induced neuronal death: a succession of necrosis and apoptosis depending on mitochondrial function. Neuron, 15: 961-973.

Asikainen M, Deboer T, Porkka-Heiskanen T, Stenberg D, Tobler I (1995). Sleep deprivation increases brain serotonin turnover in the Djungarian hamster. Neurosci Lett, 198: 21-2Asikainen M, Toppila J, Alanko L, Ward DJ, Stenberg D, Porkka-Heiskanen T (1997). Sleep deprivation increases brain serotonin turnover in the rat. Neuroreport 8: 1577-1582

Ashpole NM, Hudmon A (2011). Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. Mol Cel Neurosci, 46: 720-730

Bailer UF, Frank GK, Henry SE, Price JC, Meltzer CC, Weissfeld L, Mathis CA, Drevets WC, Wagner A, Hoge J, Ziolko SK, McConaha CW, Kaye WH (2005). Altered brain serotonin 5-HT1A receptor binding after recovery from anorexia nervosa measured by positron emission tomography and [carbonyl11C]WAY-100635. Arch Gen Psychiatry, 62: 1032-1041

Bale TL, Vale WW (2004). CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol, 44: 525-557

Banks S, Dinges DF (2007). Behavioral and physiological consequences of sleep restriction. J Clinical Sleep Med, 3: 519-528

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast, T, Zhang WN, Pothuizen HH, Feldon J (2004). Regional dissociations within the hippocampus–memory and anxiety. Neurosci Biobehav Rev, 28: 273-283.

Barf RP, Meerlo P, Scheurink AJW (2010). Chronic sleep disturbance impairs glucose homeostasis in rats. Int J Endocrinology, 2010: 14-20

Barnum CJ, Blandino P Jr, Deak T (2007). Adaptation in the corticosterone and hyperthermic responses to stress following repeated stressor exposure. J Neuroendocrinol, 19: 632-642

Basheer, R, Bauer A, Elmenhorst D, Ramesh V, McCarley RW (2007). Sleep deprivation upregulates A1 adenosine receptors in the rat basal forebrain. Neuroreport, 18: 1895-1899

Basner M, Dinges DF (2009). Dubious bargain: trading sleep for Leno and Letterman. Sleep, 32: 747-752

Bast T (2007). Towards an integrative encoding on hippocampal function: from the rapid encoding of experience to adaptive behaviour. Rev Neurosci, 18: 253-281

Beaulieu S, Di Paolo T, Barden N (1986). Control of ACTH secretion by the central nucleus of the amygdala: implication of the serotonergic system and its relevance to the glucocorticoid delayed negative feedback mechanism. Neuroendocrinology, 44: 247-254

Beersma BGM, Van den Hoofdakker RH (1992). Can non-REM sleep be depressogenic. Journal of affective disorders, 24: 101-108

Benca RM, Obermeyer WH, Thisted RA, Gillin JC (1992). Sleep and psychiatric disorders. A meta-analysis. Arch Gen Psychiatry, 49: 651-670

Benedetti F, Serretti A, Colombo C, Campori E, Barbini B, di Bella D, Smeraldi E (1999). Influence of a functional polymorphism within the promoter of the serotonin transporter gene on the effects of total sleep deprivation in bipolar depression. Am J Psychiatry, 156: 1450-1452

Benedetti F, Smeraldi E (2009). Neuroimaging and genetics of antidepressant response to sleep deprivation: implication for drug development. Cur Pharm design, 15: 2637-2649

Benington JH, Frank MG (2003). Cellular and molecular connections between sleep and synaptic plasticity. Prog Neurobiol, 69: 77-101

Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW (2009). Control and function of the homeostatic sleep response by adenosine A1 receptors. J Neurosci, 29: 1267-1276

Birmaher B, Ryan ND, Williamson DE, Brent DA, Kaufman J, Dahl RE, Perel J, Nelson B (1996) Childhood and adolescent depression: a review of the past 10 years. Part I. J Am Acad Child Adolesc Psychiatry 35:1427-1439.

Biwas S, Mishra P, Mallick BN (2006). Increased apoptosis in rat brain after rapid eye movement sleep loss. Neuroscience, 142: 315-331

Bixler E (2009). Sleep and society: an epidemiological perspective. Sleep med, 10 Suppl 1: s3-6

Blier P, Seletti B, Gilbert F, Young SN, Benkelfat C (2002). Serotonin $_{1A}$ receptor activation and hypothermia in humans: lack of evidence for a presynaptic mediation. Neuropsychopharmacology, 27: 301-308

Boldrini M, Underwood MD, Hen R, Rosoklija GB, Dwork AJ, John Mann J, Arango V (2009). Antidepressants increase neural progenitor cells in the human hippocampus. Neuropsychopharmacology 34: 2376-2389

Bonnet MH, Arand DL (1995). We are chronically sleep deprived. Sleep, 18: 908-911

Bonnet MH, Arand DL (1998). The consequence of a week of insomnia, II: Patients with insomnia. Sleep, 21: 359-368

Bora E, Fornito A, Pantelis C, Yücel M (2011). Gray matter abnormalities in Major Depressive Disorder: A meta-analysis of voxel based morphometry studies. Journal of affective disorders, in press

Bratel T, Wennlund A, Carlstrom K (1999). Pituitary reactivity, androgens and catecholamines in obstructive sleep apnoea. Effects of continuous positive airway pressure treatment (CPAP). Respir Med, 93: 1-7

Bredy TW, Humpartzoomian RA, Cain DP, Meaney MJ (2003). Partial reversal of the effect of maternal care on cognitive function through environmental enrichment. Neuroscience, 118: 571-576

Breslau N, Roth T, Rosenthal L, Andreski P (1996). Sleep disturbance and psychiatric disorders: longitudinal epidemiological study of young adults. Biol Psychiatry, 39: 411-418

Brewerton TD (1995). Toward a unified theory of serotonin dysregulation in eating and related disorders. Psychoneuroendocrinology, 20: 561-590

Buwalda B, De Boer SF, Schmidt ED, Felszeghy K, Nyakes C, Sgoifo A, Van der Vegt BJ, Tilders FJH, Bohus B, Koolhaas Jm (1999). Long-lasting deficient dexamethasone suppression of hypothalamic-pituitary-adrenocortical activation following peripheral CRF challenge in socially defeated rats. J Neuroendocrinol, 11: 513-20

Buwalda B, Blom WA, Koolhaas JM, van Dijk G (2001). Behavioral and physiological responses to stress are affected by high-fat feeding in male rats. Physiol Behav, 73: 371-377

Buysse DJ, Angst J, Gamma A, Ajdacic V, Eich D, Rossler W (2008). Prevalence, course, and comorbidity of insomnia and depression in young adults. Sleep, 31: 473-480

Calogero AE, Bernardini R, Margioris AN, Bagdy G, Gallucci WT, Munson PJ, Tamarkin L, Tomai TP, Brady L, Gold PW, Chrousos GP (1989). Effects of serotonergic agonists and antagonists on corticotropin-releasing hormone secretion by explanted rat hypothalami. Peptides, 10: 189-200

Calogero AE, Bagdy G, Szemeredi K, Tartaglia ME, Gold PW, Chrousos GP (1990). Mechanisms of serotonin receptor agonist-induced activation of the hypothalamic-pituitary-adrenal axis in the rat. Endocrinology, 126: 1888-1894

Calogero AE, Bagdy G, Moncada ML, D'Agata R (1993). Effect of selective serotonin agonists on basal, corticotrophin-releasing hormone- and vasopressin–induced ACTH release in vitro from rat pituitary cells. J Endocrinol, 136: 381-387

Chang PP, Ford DE, Mead LA, Cooper-Patrick L, Klag MJ (1997) Insomnia in young men and subsequent depression. Am J Epidemiol, 146: 105-114

Chao HM, Blanchard DC, Blanchard RJ, McEwen BS, Sakai RR (1993). The effect of social stress on hippocampal gene expression. Mol Cell Neurosci, 4: 543-548

Cheng JP, Aslam HA, Hoffman AN, Zafonte RD, Kline AE (2007). The neurobehavioral benefit conferred by a single systemic administration of 8-OH-DPAT after brain trauma is confined to a narrow therapeutic window. Neurosci Lett, 416: 165-168

Choi DW (1988). Glutamate neurotoxicity and diseases of the nervous system. Neuron, 1: 623–634

Cirelli, C Saw PJ, Rechtschaffen A, Tononi G (1999). No evidence of brain cell degeneration after long-term sleep deprivation in rats. Brain Res, 840: 184-193

Cirelli C, Tononi G (2000). Differential expression of plasticity related genes in waking and sleep and their regulation by the noradrenergic system. J Neurosci, 20: 9187-9194

Cirelli C (2002). How sleep deprivation affects gene expression in the brain: a review of recent findings. J Appl Physiol, 92: 394-400

Cirelli C (2006). Cellular consequences of sleep deprivation in the brain. Sleep Med Rev, 10: 307-321

Cole MA, Kalman BA, Pace TWW, Topzewski F, Lowrey MJ, Spencer RL (2000). Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. J Neuroendocrinol, 12: 1034-1042

Costello EJ, Pine DS, Hammen C, March JS, Plotsky PM, Weissman MM, Biederman J, Goldsmith HH, Kaufman J, Lewinsohn PM, Hellander M, Hoagwood K, Koretz DS, Nelson CA, Leckman JF (2002). Development and natural history of mood disorders. Biol Psychiatry, 52: 529-542

Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, Aigner L (2005). Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci, 21: 1-14

Cowen DS, Takase LF, Fornal CA, Jacobs BL (2008). Age-dependent decline in hippocampal neurogenesis is not altered by chronic treatment with fluoxetine. Brain Res, 1228: 14-19

Crowley SJ, Acebo C, Carskadon MA (2007). Sleep, circadian rhythms and delayed phase in adolescence. Sleep Med, 8: 602-612

Cunha, RA (2005). Neuroprotection by adenosine in the brain: from A1 receptor activation to A2 receptor blockade. Purinergic Signal, 1: 111-134

Curcio G, Ferrara M, De Gennaro L (2006). Sleep loss, learning capacity and academic performance. Sleep Med Rev, 10: 323-337

Cryan JF, Leonard BE (2000). 5-HT1A and beyond: the role of serotonin and its receptors in depression and the antidepressant response. Hum Psychopharmacol Clin Exp, 15: 113-135

Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A Fuchs E (2001). Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci U S A, 98: 12796-12801

Czeh B, Lucassen PJ, 2007. What causes the hippocampal volume decrease in depression? Are neurogenesis, glial changes and apoptosis implicated? Eur Arch Psychiatry Clin Neurosci, 257: 250-260

Czeh B, Abumaria N, Rygula R, Fuchs E (2010) Quantitative changes in hippocampal microvasculature of chronically stressed rats: no effect of fluoxetine treatment. Hippocampus, 20: 174-185

Dagyte G, Van der Zee EA, Postema F, Luiten PG, Den Boer JA, Trentani A, Meerlo P (2009). Chronic but not acute foot-shock stress leads to temporary suppression of cell proliferation in rat hippocampus. Neuroscience, 162: 904-913

Dahl RE, Gunnar MR (2009) Heightened stress responsiveness and emotional reactivity during pubertal maturation: implications for psychopathology. Dev Psychopathol, 21: 1-6

D'Almeida V, Hipòlide DC, Azzalis LA, Lobo LL, Junqueira,VBC, Tufik S (1997). Absence of oxidative stress following paradoxical sleep deprivation in rats. Neurosci Lett, 235: 25-28

D'Almeida V, Lobo LL, Hipólide DC, de Oliveira AC, Nobrega JN, Tufik S (1998). Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport, 9: 2853-2856

Datson NA, Morsink MC, Meijer OC, de Kloet ER (2008). Central corticosteroid actions: Search for gene targets. Eur J Pharmacol, 583: 272-289

Dayer AG, Ford AA, Cleaver KM, Yassaee M, Cameron HA (2003). Short-term and long-term survival of new neurons in the rat dentate gyrus. J Comp Neurol, 460: 563-572

de Courten-Myers GM, Kleinholz M, Wagner KR, Xi G, Myers RE (1994). Efficacious experimental stroke treatment with high-dose methylprednisolone. Stroke, 25: 487-493

de Kloet ER, Oitzl MS, Joëls M (1993). Functional implications of brain corticosteroid receptor diversity. Cell mol Neurobiol, 13: 433-455

de Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M (1998). Brain corticosteroid receptor balance in health and disease. Endocr Rev, 19: 269-301

de Kloet ER, Joels M, Holsboer F (2005). Stress and the brain: From adaptation to disease. Nat rev Neurosci, 6: 463-475

de Kloet ER, Karst H, Joëls M (2008). Corticosteroid hormones in the central stress response: quick-and-slow. Front Neuroendocrinol, 29: 268-272

de Kloet ER, Fitzsimons CP, Datson NA, Meijer OC, Vreugdenhil E (2009). Glucocorticoid signaling and stress-related limbic susceptibility pathway: about receptors, transcription machinery and microRNA. Brain Res, 1293: 129-141

Deng W, Aimone JB Gage FH (2010). New neurons and new memo- ries: how does adult hippocampal neu- rogenesis affect learning and memory? Nat Rev Neurosci 11, 339-350

Dinan TG (1996). Serotonin and the regulation of hypothalamic-pituitary-adrenal axis function. Life Sci, 58: 1683-1694

Dinges DF, Pack F, Williams K, Gillen KA, Powell JW, Ott GE, Aptowicz, Pack AI (1997). Cumulative sleepiness, mood disturbances and psychomotor vigilance performance decrements during a week of sleep restricted to 4-5 hours per night. Sleep, 20: 267-277

Dolga AM, Granic I, Nijholt IM, Nyakas C, van der Zee EA, Luiten PGM, Eisel ULM (2009). Pretreatment with lovastatin prevents N-methyl-d-aspartate-induced neurodegeneration in the magnocellular nucleus basalis and behavioral dysfunction. J Alzheimer's Dis, 17: 327-336

Donga E, van Dijk M, van Dijk JG, Biermasz NR, Lammers GJ, van Kralingen KW, Corssmit EP, Romijn JA, (2010). A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects. J Clin Endocrinol Metab, 95: 2963-2968

Drevets WC, Frank E, Price JC, Kupfer DJ, Holt D, Greer PJ, Huang YY, Gualtier C, Mathis C (1999). PET imaging of serotonin 1A receptor binding in depression. Biol Psychiatry, 46: 1375-1387

Drevets WC, Thase ME, Moses-Kolko EL, Price J, Frank E, Kupfer DJ, Mathis C (2007). Serotonin- 1A receptor imaging in recurrent depression: replication and literature review. Nucl Med Biol, 34: 865-877

Duma D, Jewell CM, Cidlowski JA (2006). Multiple glucocorticoid isoforms and mecchanims of post-transational modification. J Steroid Biochem Mol Biol, 102: 11-21

Durmer JS, Dinges DF (2005). Neurocognitive consequences of sleep deprivation. Seminar Neurol, 25: 117-129

Dworak M, Schierl T, Bruns T, Struder HK (2007). Impact of singular excessive computer game and television exposure on sleep patterns and memory performance of school-aged children. Pediatrics, 120: 978-985

Eiland MM, Ramanathan L, Gulyani S, Gilliland M, Bergmann BM, Rechtscaffen A, Siegel JM (2002). Increases in amino-cupric-silver staining of the supraoptic nucleus after sleep deprivation. Brain Res, 945: 1-8

Elmenhorst D, Meyer PT, Winz OH, Matusch A, Ermert J, Coenen HH, Basheer R, Haas HL, Zilles K, Bauer A (2007). Sleep deprivation increases A1 adenosine receptor binding in the human brain: a positron emission tomography study. J Neurosci, 27: 2410-2415

Everson CA (1995). Functional consequences of sustained sleep deprivation in the rat. Behav Brain Res, 69: 43-54

Fadda P, Fratta W (1997). Stress-induced sleep deprivation modifies corticotropin releasing factor (CRF) levels and CRF binding in rat brain and pituitary. Pharmacol Res, 35: 443-446

Feldman S, Weidenfeld J (1999). Glucocorticoid receptor antagonists in the hippocampus modify the negative feedback following neural stimuli. Brain Res, 821: 33-37

Felszeghy K, Banisadr G, Rostène W, Nyakas C, Haour F (2004). Dexamethasone downregulates chemokine receptor CXCR4 and exerts neuroprotection against hypoxia/ischemia-induced brain injury in neonatal rats. Neuroimmunomodulation, 11: 404-413

Ford DE, Kamerow DB (1989). Epidemiologic study of sleep disturbances and psychiatric disorders: An opportunity for prevention? J Am Med Assoc, 262: 1479-1484

Francis DD, Diorio J, Plotsky PM, Meaney MJ (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. J Neurosci, 22: 7840-7843

Friedman MI (1977). Insulin-induced hyperphagia in alloxan-diabetic rats fed a high-fat diet. Physiol Behav 19: 597-599

Friedman MI (1978). Hyperphagia in rats with experimental diabetes mellitus: A response to a decreased supply of utilizable fuels. J Comp Physiol Psychol, 92: 109-117

Friedman MI, Ramirez I (1994). Food intake in diabetic rats: relationship to metabolic effects of insulin treatment. Physiol Behav, 56: 373-378

Fujihara H, Sei H, Morita Y, Ueta Y, Morita K (2003). Short-term sleep disturbance enhances brain-derived neurotrophic factor gene expression in rat hippocampus by acting as internal stressor. J Mol Neurosci, 21: 223-232

Fuller RW (1992). The involvement of serotonin in regulation of pituitary-adrenocortical function. Front Neuroendocrinol, 13: 250-270

Fuller RW (1996). Serotonin receptors involved in regulation of pituitary-adrenocortical function in rats. Behav Brain Res, 73: 215-219

Gao B, Cam E, Jaeger H, Zunzunegui C, Sarnthein J, Bassetti CL (2010). Sleep disruption aggravates focal cerebral ischemia in the rat. Sleep, 33: 879-887

Gardner JP, Fornal CA, Jacobs BL (1997). Effects of sleep deprivation on serotonergic neuronal activity in the dorsal raphe nucleus of the freely moving cat. Neuropsychopharmacology, 17: 72-81

Germain A, Buysse DJ, Nofzinger E (2008). Sleep-specific mechanisms underlying posttraumatic stress disorder: integrative review and neurobiological hypotheses. Sleep Med Rev, 12: 185-195

Gillespie CF, Nemeroff CB (2005). Hypercortisolemia and depression. Psychosom Med, 67: S26-S28

Gillin JC, Buchsbaum M, Wu J, Clark C, Bunney W (2001). Sleep deprivation as a model experimental antidepressant trretment: findings from functional brain imaging. Depress Anxiety, 14: 37-49

Glavin GB, Pare WP, Sandbak T, Bakke HK, Murison R (1994). Restraint stress in biomedical research: an update. Neurosci Biobehav Rev, 18: 223-249

Gogtay N, Giedd JN, Lusk L, Hayashi KM, Greenstein D, Vaituzis AC, Nugent III TF, Herman DH, Clasen LS, Toga AW, Rapoport JL, Thompson PM (2004). Dynamic mapping of human cortical development during childhood through early adulthood. Proc Natl Acad Sci USA 101: 8174–8179

Gomez F, Lahmame A, de Kloet ER, Armario A (1996). Hypothalamic-pituitary adrenal response to chronic stress in five inbred rat strains: differential responses are mainly located at the adrenocortical level. Neuroendocrinology, 63: 327-337

Gopalakrishnan A, Ji LL Cirelli, C (2004). Sleep deprivation and cellular response to oxidative stress. Sleep, 27: 27-35

Grassi-Zucconi G, Cipriani S, Balgkouranidou I, Scattoni R (2006). 'One night' sleep deprivation stimulates hippocampal neurogenesis. Brain Res Bull, 69: 375-381

Gratacos E, Perez-Navarro E, Tolosa E, Arenas E, Alberch J (2001). Neuroprotection of striatal neurons against kainate excitotoxicity by neurotrophins and GDNF family members. J Neurochem, 78: 1287-1296

Graves LA, Heller EA, Pack AI, Abel T (2003). Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. Learn Mem, 10: 168-176.

Gregory AM, Caspi A, Eley TC, Moffitt TE, Oconnor TG, Poulton R (2005). Prospective longitudinal associations between persistent sleep problems in childhood and anxiety and depression disorders in adulthood. J Abnorm Child Psychol, 33: 157-163

Gudmundsson A, Carnes M (1997). Pulsatile adrenocorticotropic hormone: An overview. Biol Psychiatry, 41: 342-365

Guzman-Marin R, Suntsova N, Stewart DR, Gong H, Szymusiak R, McGinty D (2003). Sleep deprivation reduces proliferation of cells in the dentate gyrus of the hippocampus in rats. J Physiol, 549: 563-571

Guzman-Marin R, Suntsova N, Methippara M, Greiffenstein R, Szymusiak R, McGinty D (2005). Sleep deprivation suppresses neurogenesis in the adult hippocampus of rats. Eur J Neurosci, 22: 2111-2116

Guzman-Marin R, Ying Z, Suntsova N, Methippara M, Bashir T, Szymusiak R, Gomez-Pinilla F, McGinty D, (2006). Suppression of hippocampal plasticity-related gene expression by sleep deprivation in rats. J Physiol, 575: 807-819

Guzman-Marin R, Bashir T, Suntsova N, Szymusiak R, McGinty D (2007). Hippocampal neurogenesis is reduced by sleep fragmentation in the adult rat. Neuroscience, 148: 325-333

Guzman-Marin R, Suntsova N, Bashir T, Nienhuis R, Szymusiak R, McGinty D (2008). Rapid eye movement sleep deprivation contributes to reduction of neurogenesis in the hippocampal dentate gyrus of the adult rat. Sleep, 31: 167-175

Hagenauer MH, Perryman JI, Lee TM, Carskadon MA (2009). Adolescent changes in homeostatic and circadian regulation of sleep. Dev Neurosci, 31: 276-284

Hagewoud R, Havekes R, Novati A, Keijser JN, Van der Zee EA, Meerlo P (2010a). Sleep deprivation impairs spatial working memory and reduces hippocampal AMPA receptor phosphorylation. J Sleep Res, 19: 280-288

Hagewoud R, Havekes R, Tiba P, Novati A, Hogenelst K, Weinreder P, Van der Zee EA, Meerlo P (2010b) Coping with sleep deprivation: shifts in regional brain activity and learning strategy. Sleep, 19: 280-288

Hairston IS, Little MTM, Scanlon MD, Barakat MT, Palmer TD, Sapolsky RM, Heller HC (2005). Sleep Restriction Suppresses Neurogenesis Induced by Hippocampus-Dependent Learning. J Neurophysiol, 94: 4224-4233

Haleem DJ (2009). Exaggerated feedback control decreases brain serotonin concentration and elicits hyperactivity in a rat model of diet-restriction-induced anorexia nervosa. Appetite, 52: 44-50

He S, Crews FT (2007). Neurogenesis decreases during brain maturation from adolescence to adulthood. Pharmachol Biochem Bev, 86: 327-333

Heine VM, Maslam S, Joëls M, Lucassen PJ (2004). Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. Neurobiol Aging, 25: 361-375

Herman JP, Adams D, Prewitt C (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology, 61: 180-190

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol, 24: 151-180

Herman JP, Ostrander MM, Mueller NK, Figueiredo H (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. Prog Neuro-Psychopharm Biol Psychiatry, 29: 1201-1213

Hicks RA, Moore JD (1979). REM sleep deprivation diminishes fear in rats. Physiol Behav, 22: 689-692.

Hipólide DC, Moreira KM, Barlow KB, Wilson AA, Nobrega JN, Tufik S (2005). Distinct effects of sleep deprivation on binding to norepinephrine and serotonin transporters in rat brain. Prog Neuropsychopharmacol Biol Psychiatry, 29: 297-303

Hipolide DC, Suchecki D, Pinto APD, Faria EC, Tufik S, Luz J (2006). Paradoxical sleep deprivation and sleep recovery: Effects on the hypothalamic-pituitary-adrenal axis activity, energy balance and body composition of rats . Journal of neuroendocrinology, 18: 231-238

Hirvonen J, Karlsson H, Kajander J, Leopola A, MArkkula J, RAsi-HAkala H, Nagren K, Salminen JK, Hietala J (2008). Decreased brain serotonin 5-HT1A receptor availability in medication-naïve patients with major depressive disorder: an in-vivo imaging study using PET and [carbonyl-C]WAY-100635. Int J Neuropsychopharmacol, 11: 465-476

Holsboer F, Vonbardeleben U, Gerken A , Stalla GK, Müller OA (1984). Blunted corticotrophin and normal corticol response to human corticotrophin releasing factor in depression. New Engl J Med, 311: 1127

Holsboer F, Gerken A, Stalla GK, Muller OA (1987). Blunted aldosteron and ACTH response after human CRH administration in depressed patients. Am J Psychiatry, 144: 229-231

Horne JA (1993). Human sleep, sleep loss and behaviour. Implications for the prefrotnal cortex and psychiatric disorder. Brit J Psychiat, 162: 413-419

Horvath KM, Abrahám IM, Harkany T, Meerlo P, Bohus BGJ, Nyakas C, Luiten PGM (2000). Postnatal treatment with ACTH-(4-9) analog ORG 2766 attenuates N-methyl-d-aspartate-induced excitotoxicity in rat nucleus basalis in adulthood. Eur J Pharmacol, 405: 33-42

Hsu JC, Lee YS, Chang CN, Ling EA, Lan CT (2003). Sleep deprivation prior to transient global cerebral ischemia attenuates glial reaction in the rat hippocampal formation. Brain Res, 984: 170-181

Huang CW, Lui CC, Chang WN, Lu CH, Wang YL Chang CC (2009). Elevated basal cortisol level predicts lower hippocampal volume and cognitive decline in Alzheimer's disease. J Clin Neurosci, 16: 1283-1286

Hügin-Flores ME, Steimer T, Schulz P, Vallotton MB, Aubert ML (2003). Chronic corticotropin-releasing hormone and vasopressin regulate corticosteroid receptors in rat hippocampus and anterior pituitary. Brain Res, 976: 159-170

Inuoé S, Honda K, Komoda Y (1995). Sleep as neuronal detoxification and restitution. Behav Brain Res, 69: 91-96

Jackson A, Cavanagh J, Scott J (2003). A systematic review of manic and depressive prodromes. J Affect Disord, 74: 209-217

Johns MA, Azmitia EC, Krieger DT (1982). Specific in vitro uptake of serotonin by cells in the anterior pituitary of the rat. Endocrinology, 110: 754-760

Johnson EO, Roth T, Breslau N (2006). The association of insomnia with anxiety disorders and depression: exploration of the direction of risk. J Psychiatr Res, 40: 700-708

Junek A, Rusak B, Semba K (2010). Short-term sleep deprivation may alter the dynalics of hippocampal cell proliferation in adult rats. Neurosci, 170: 1140-1152

Kant GJ, Bunnell BN, Mougey EH, Pennington LL, Meyerhoff JL (1983). Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats. Pharmacol Biochem Behav, 18: 967-971

Kaye WH, Frank GK, Bailer UF, Henry SE (2005). Neurobiology of anorexia nervosa: clinical implications of alterations of the function of serotonin and other neuronal systems. Int J Eat Disord, 37: S15-S19

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods, 115: 97-105

Kempermann G, Kuhn HG, Gage FH (1997). More hippocampal neurons in adult mice living in an enriched environment. Nature, 386: 493-495

Kessing LV, Willer IS, Knorr U (2011). Volume of the adrenal and pituitary glands in depression. Psychoneuroendocrinology, 36: 19-27

Kim HW, Chang YC, Chen M, Rapoport SI, Rao JS (2009). Chronic NMDA administration to rats increases brain pro-apoptotic factors while decreasing anti-apoptotic factors and causes cell death. BMC Neurosci, 10: 123-130

Kline AE, Yu J, Horvath E, Marion DW, Dixon CE (2001). The selective 5-HT(1A) receptor agonist repinotan HCl attenuates histopathology and spatial learning deficits following traumatic brain injury in rats. Neuroscience, 106: 547-555

Koban M, Le WW, Hoffman GE (2006). Changes in hypothalamic corticotropin-releasing hormone, neuropeptide Y, and proopiomelanocortin gene expression during chronic rapid eye movement sleep deprivation of rats. Endocrinology, 147: 421-431

Koide T, Wieloch TW, Siesjö BK (1986). Chronic dexamethasone pretreatment aggravates ischemic neuronal necrosis. J Cereb Blood Flow Metab, 6: 395-404

Koolhaas JM, Hermann PM, Kemperman C, Bohus B, Van den Hoofdakker RH, Beersma DGM (1990). Single social defeat in male rats induces a gradual but long-lasting behavioural change: a model of depression? Neurosci Res Commun, 7: 35-41

Koolman AH, Bloks VW, Oosterveer MH, Jonas I, Kuipers F, Sauer PJJ, vanDijk G (2010). Metabolic responses to long-term pharmacological inhibition of CB1-receptor activity in mice in relation to dietary fat composition. Int J Obesity, 34: 374-384

Kopp C, Longordo F, Nicholson JR, Lüthi A (2006). Insufficient sleep reversibly alters bidirectional synaptic plasticity and NMDA receptor function. J Neurosci, 26: 12456-12465

Koren D, Arnon I, Lavie P, Klein E (2002). Sleep complaints as early predictors of posttraumatic stress disorder: a 1-year prospective study of injured survivors of motor vehicle accidents. Am J Psychiatry, 159: 855-857

Korte SM, Buwalda B, Meijer O, de Kloet ER, Bohus B (1995). Socially defeated male rats display a blunted adrenocortical response to a low dose of 8-OH-DPAT. Eur J Pharmacol, 272: 45-50

Kukley M, Schaper C, Becker A, Rose K, Krieglstein J (2001). Effect of 5-hydroxytryptamine 1A receptor agonist BAY X 3702 on BCL-2 and BAX proteins level in the ipsilateral cerebral cortex of rats after transient focal ischaemia. Neuroscience, 107: 405-413

Krakow B, Germain A, Warner TD, Schrader R, Koss M, Hollifield M, Tandberg D, Melendrez D, Johnston L, (2001). The relationship of sleep quality and posttraumatic stress to potential sleep disorders in sexual assault survivors with nightmares, insomnia, and PTSD. J Trauma Stress, 14: 647-665

Kruger H, Heineman U, Luhmann HJ (1999). Effects of ionotropic glutamate receptor blockade and 5-HT1A receptor activation on spreading depression in rat neocortical slices. NeuroReport, 10: 2651-2656

Kume T, Nishikawa H, Tomioka H, Katsuki H, Akaike A, Kaneko S, Maeda T, Kihara T, Shimohama S (2000). p75-mediated neuroprotection by NGF against glutamate cytotoxicity in cortical cultures. Brain Res, 852: 279-289

Lac G, Chamoux A (2003). Elevated salivary cortisol levels as a result of sleep deprivation in a shift worker. Occup Med (Lond), 53 : 143-145

Lam RW (2006). Sleep disturbances and depression: a challenge for antidepressants. Int Clin Psychopharmacol, 21: S25-S29

Lauterbach EC, Shillcutt SD, Victoroff J, Coburn KL, Mendez MF (2010). Psychopharmacological neuroprotection in neurodegenerative disease: heuristic clinical applications. J Neuropsychiatry Clin Neurosci, 22: 130-154

Leproult R, Copinschi G, Buxton O, VanCauter E (1997). Sleep loss results in an elevation of cortisol levels the next evening. Sleep, 20: 865-870

Lesch KP, Mayer S, Disselkamp-Tietze J, Hoh A, Wiesmann M, Osterheider M, Schulte HM, (1990). 5-HT1A receptor responsivity in unipolar depression: evaluation of ipsapirone-induced ACTH and cortisol secretion in patients and controls. Biol Psychiatry, 28: 620-628

Lesch KP (1991). 5-HT1A receptor responsivity in anxiety disorders and depression. Prog Neuropsychoph, 15: 723-733

Li JX, France CP (2008). Food restriction and streptozotocin treatment decrease 5- HT1A and 5-HT2A receptor-mediated behavioral effects in rats. Behav Pharmacol, 19: 292-297

Li JX, Koek W, France CP (2009). Food restriction and streptozotocin differentially modify sensitivity to the hypothermic effects of direct- and indirect-acting serotonin receptor agonists in rats. Eur J Pharmacol, 613: 60-63

Liu X, Buysse DJ (2006). Sleep and youth suicidal behavior: a neglected field. Curr Opin Psychiatry, 19: 288-293

Livingston G, Blizard B, Mann A (1993). Does sleep disturbance predict depression in elderly people? Br J Gen Pract, 43: 445-448

Longordo F, Kopp C, Lüthi A (2009). Consequences of sleep deprivation on neurotransmitter receptor expression and function. Eur J Neurosci, 29: 1810-1819

Lopez JF, Akil H, Watson SJ (1999). Neural circuits mediating stress. Biol Psychiatry, 46: 1461-1471

Lopez-Rodriguez F, Wilson CL, Maidment NT, Poland RE, Engel J (2003). Total sleep deprivation increases extracellular serotonin in the rat hippocampus. Neuroscience, 121: 523-530

Lucassen PJ, Meerlo P, Naylor AS, van Dam AM, Dayer AG, Fuchs E, OOmen CA, Czeh B (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. Eu Neuropsychopharmacol, 20: 1-17

Luiten PG, Douma BR, Van der Zee EA, Nyakas C (1995). Neuroprotection against NMDA induced cell death in rat nucleus basalis by Ca2 + antagonist nimodipine, influence of aging and developmental drug treatment. Neurodegeneration, 4: 307-314

Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NPV, Thakur M, McEwen BS, Hauger RL, Meaney MJ (1998). Cortisol levels during human aging predict hippocampal atrophy and memory deficits. Nature Neurosci, 1: 69-73

Ma XM, Lightman SL (1998). The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. J Physiol, 510: 605-614

Maccari S, Mormede P, Piazza PV, Simon H, Angelucci L, Le Moal M (1992). Hippocampal type I and type II corticosteroid receptors are modulated by central noradrenergic systems. Psychoneuroendocrinology, 17: 103-112

Macey PM, Woo MA, Kumar R, Cross RL, Harper RM (2010). Relationship between obstructive sleep apnea severity and sleep, depression and anxiety symptoms in newly-diagnosed patients. PLoS One, 5: e10211

Makino S, Smith MA, Gold PW (1995). Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: Association with reduction in glucocorticoid receptor mRNA levels. Endocrinology, 136: 3299-3309

Majumdar S., Mallick BN (2005). Cytomorphometric changes in rat brain neurons after rapid eye movement sleep deprivation. Neuroscience, 135: 679-690

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci, 20: 9104-9110

Malberg JE, Duman RS (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology, 28: 1562-1571

Malow BA (2004). Sleep Deprivation and Epilepsy. Epilepsy Curr, 4: 193-195

Mann JJ, McBride PA, Malone KM, DeMeo M, Keilp J (1995). Blunted serotonergic responsivity in depressed inpatients. Neuropsychopharmacology, 13: 53-64

Martin GR, Humphrey PP, 1994. Receptors for 5-hydroxytryptamine: current perspectives on classification and nomenclature. Neuropharmacology, 33: 261-73

Martinez-Gonzalez D, Obermeyer W, Fahy JL, Riboh M, Kalin NH, Benca RM (2004). REM sleep deprivation induces changes in coping responses that are not reversed by amphetamine. Sleep, 27: 609-617

Mauler F, Fahrig T, Horvath E, Jork R (2001). Inhibition of evoked glutamate release by the neuroprotective 5-HT(1A) receptor agonist BAY x 3702 in vitro and in vivo. Brain Res, 888: 150-157

McAllister AK, Lo DC, Katz LC (1995). Neurotrophins regulate dendritic growth in developing visual cortex. Neuron, 15: 791-803

McDermott CM, LaHoste GJ, Chen C, Musto A, Bazan NG, Magee JC (2003). Sleep deprivation causes behavioral, synaptic, and membrane excitability alterations in hippocampal neurons. J Neurosci, 23: 9687-9695

McDermott CM, Hardy MN, Bazan NG Magee JC (2006). Sleep deprivation-induced alterations in excitatory synaptic transmission in the CA1 region of the rat hippocampus. J Physiol, 570: 553-565

Meerlo P, Overkamp GJ, Benning MA, Koolhaas JM, van den Hoofdakker RH (1996). Long-term changes in open field behaviour following a single social defeat in rats can be reversed by sleep deprivation. Physiol Behav, 60: 115-119.

Meerlo P, Koehl M, van der Borght K, Turek FW (2002). Sleep restriction alters the hypothalamic-pituitary-adrenal response to stress. J Neuroendocrinol, 14: 397-402

Meerlo P, Sgoifo A, Suchecki D (2008). Restricted and disrupted sleep: effects on autonomic function, neuroendocrine stress systems and stress responsivity. Sleep Med Rev, 12: 197-210

Meerlo P, Mistlberger RE, Jacobs BL, Heller HC, McGinty D (2009). New neurons in the adult brain: the role of sleep and consequences of sleep loss. Sleep Med Rev, 13: 187-194

Meijer AM, Habekothe HT, Van-Den-Wittenboer GL (2000). Time in bed, quality of sleep and school functioning of children. J Sleep Res, 9: 145-153

Meijer OC, Steenbergen PJ, de Kloet ER (2000). Differential expression and regional distribution of steroid receptor coactivators SRC-1 and SRC-2 in brain and pituitary. Endocrinology, 141: 2192-2199

Meijer OC, Topic B, Steenbergen PJ, Jocham G, Huston JP, Oitzl MS (2005). Correlations between hypothalamus-pituitary-adrenal axis parameters depend on age and learning capacity. Endocrinology, 46: 1372-1381

Melena J, Chidlow G, Osborne NN (2000). Blockade of voltage-sensitive Na+ channels by the 5-HT1A receptor agonist 8-OH-DPAT: possible significance for neuroprotection. Eur J Pharmacol, 406: 319-324

Melia KR, Ryabinin AE, Schroeder R, Bloom FE, Wilson MC (1994). Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. J Neurosci, 14: 5929-5938

Mesulam MM, Mufson EJ, Wainer BH, Levey AI (1983). Central cholinergic pathways in the rat: on overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience, 10: 1185-1201

Miller AH, Spencer RL, Pulera M, Kang S, McEwen B.S, Stein M (1992). Adrenal steroid receptor activation in rat brain and pituitary following dexamethasone: implications for the dexamethasone suppression test. Biol Psychiatry, 32: 850-869

Ming G, Song H (2005). Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci, 28: 223-250

Mirescu C, Peters JD, Noiman L, Gould E (2006). Sleep deprivation inhibits adult neurogenesis in the hippocampus by elevating glucocorticoids. Proc Natl Acad Sci U S A, 103: 19170-19175

Moldovan M, Constantinescu AO, Balseanu A, Prescu N, Zagrean L, Popa-Wagner A (2010). Sleep deprivation attenuates experimental stroke severity in rats. Exp Neurol, 222: 135-143

Monti JM, Jantos H (2008). The roles of dopamine and serotonin, and of their receptors, in regulating sleep and waking. Prog Brain Res, 172: 625-646

Moore RJ, Eichler VB (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res, 42: 201-206

Moore JD, Hayes C, Hicks RA (1979). REM sleep deprivation increases preference for novelty in rats. Physiol Behav, 23: 975-976

Moreau JL (1997). Validation of an animal model of anhedonia, a major symptom of depression. Encephale, 23: 280-289

Morens C, Sirot V, Scheurink AJW, van Dijk G (2006). Low-carbohydrate diets affect energy balance and fuel homeostasis differentially in lean and obese rats. Am J Physiol, 291: R1622-R1629

Morrell MJ, McRobbie DW, Quest RA, Cummin AR, Ghiassi R, Corfield DR (2003). Changes in brain morphology associated with obstructive sleep apnea. Sleep Med, 4: 451-454

Mueller A, Pollock MS, Lieblich SE, Epp J, Galea LAM, Mistlberger RE (2008). REM sleep deprivation can inhibit adult hippocampal neurogenesis independent of adrenal stress hormones. Am J Physiol, 294: R1693-R1703

Muzur A, Pace-Schott EF, Hobson JA (2002). The prefrontal cortex in sleep. Trends Cogn Neurosci, 6: 475-481

Naylor AS, Bull C, Nilsson MK, Zhu C, Björk-Eriksson T, Eriksson PS, Blomgren K, Kuhn HG (2008). Voluntary running rescues adult hippocampal neurogenesis after irradiation of the young mouse brain. Proc Natl Acad Sci USA, 105: 14632-14637

Neckelmann D, Mykletun A, Dahl AA (2007). Chronic insomnia as a risk factor for developing anxiety and depression. Sleep, 30: 873-380

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W (1984). Elevated concentrations of CSF corticotrophin-releasing factor-like immunoreactivity in depressed-patients. Science, 226: 1342-1344

Neylan T, Mueller S, Wang Z, Metzler T, Lenoci M, Truran D, Marmar C, Weiner MW, Schuff N (2010). Insomnia severity is associated with a decreased volume of the CA3/dentate gyrus hippocampal subfield. Biol Psychiatry, 68: 494-496

Nithianantharajah J, Hannan AJ (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci, 7: 697-709

Novati A, Roman V, Cetin TR, Den Boer JA, Luiten PG, Meerlo P (2008). Chronically restricted sleep leads to depression-like changes in neurotransmitter receptor sensitivity and neuroendocrine stress reactivity in rats. Sleep, 31: 1579-1585

Nithianantharajah J, Hannan AJ (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci, 7: 697-709

O'Brien JT, Lloyd A, McKeith I, Gholkar A, Ferrier N (2004). A longitudinal study of hippocampal volume, cortisol levels, and cognition in older depressed subjects. Am J Psychiatry, 161: 2081-2090

Oosterink BJ, Harkany T, Luiten PG (2003). Post-lesion administration of 5-HT1A receptor agonist 8-OH-DPAT protects cholinergic nucleus basalis neurons against NMDA excitotoxicity. NeuroReport, 14: 57-60

Pace TWW, Cole MA, Ward G, Kalman BA, Spencer RL (2001). Acute exposure to a novel stressor further reduces the habituated corticosterone response to restraint in rats. Stress, 4: 319-331

Pariante CM (2004). Glucocorticoid receptor function in vitro in patients with major depression. Stress, 7: 209-219

Paskitti ME, McCreary BJ, Herman JP (2000). Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. Mol Brain Res, 80: 142-152

Pathania M, Yan LD, Bordey A (2010). A symphony of signals conducts early and late stages of adult neurogenesis. Neuropharmacology, 58: 865-876

Paxinos G, Watson C (1986). The Rat Brain in Stereotaxic Coordinates, 2nd edn. Academic Press, Sydney,

Payne JL, Quiroz JA, Zarate Jr CA, Manji HK (2002). Timing is everything: does the robust upregulation of noradrenergically regulated plasticity genes underlie the rapid antidepressant effects of sleep deprivation? Biol Psychiatry, 52: 921-926

Peeke SC, Callaway E, Jones RT, Stone GC, Doyle J (1980). Combined effects of alcohol and sleep deprivation in normal young adults. Psychopharmacology, 67: 279-287

Pellow S, File S (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav, 24: 525-529

Peñalva RG, Lancel M, Flachskamm C, Reul JM, Holsboer F, Linthorst AC (2003). Effect of sleep and sleep deprivation on serotonergic neurotransmission in the hippocampus: a combined in vivo microdialysis/EEG study in rats. Eur J Neurosci, 17: 1896-1906

Perera TD, Park S, Nemirovskaya Y (2008). Cognitive role of neurogenesis in depression and antidepressant treatment. Neuroscientist, 14: 326-338

Pflug B, Tölle R (1971). Disturbance of 24-hour rhythm, endogenous depression and the treatment of endogenous depression by sleep deprivation. Int Pharmacopsychiat, 6: 187-196

Pham K, Nacher J, Hof PR, McEwen BS (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur J Neurosci, 17: 879-886

Picarel-Blanchot F, Alvarez C, Bailbe D, Pascual-Leone AM, Portha B (1995). Changes in insulin action and insulin secretion in the rat after dietary restriction early in life: influence of food restriction versus low-protein food restriction. Metabolism, 44: 1519-1526

Pilcher JJ, Huffcutt AI (1996). Effects of sleep deprivation on performance: a meta analysis. Sleep, 19: 318-326

Pilcher JJ, Lambert BJ, Huffcutt AI (2000). Differential effects of permanent and rotating shift on self-report sleep length: a meta-analytic review. Sleep, 23: 155-163

Pitman DL, Ottenweller JE, Natelson BH (1988). Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: chronic stress and habituation. Physiol Behav, 43: 47-55

Platel JC, Stamboulian S, Nyugen I, Bordey A (2010). Neurotransmitter signalling in postnatal neurogenesis: The first leg. Brain Res Rev, 63: 60-71

Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW (1997). Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. Science, 276: 1265-1268

Portas CM, Bjorvatn B, Ursin R (2000). Serotonin and the sleep/wake cycle: special emphasis on microdialysis studies. Prog Neurobiol, 60: 13-35

Prevot E, Maudhuit C, LePoul E, Hamon M, Adrien J (1996). Sleep deprivation reduces the citalopram-induced inhibition of serotoninergic neuronal firing in the nucleus raphe dorsalis of the rat . J Sleep Res, 5: 238-245

Pruessner JC, Wolf OT, Hellhammer DH, Buske-Kirschbaum A, von Auer K, Jobst S, Kaspers F, Kirschbaum C (1997). Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. Life Sci, 61: 2539-2549

Quera-Salva MA, Lemoine P, Guilleminault C (2010). Impact of the novel antidepressant agomelatine on disturbed sleep-wake cycles in depressed patients. Hum Psychopharmacol, 25: 222-229

Rajaratnam SM, Arendt J (2001). Health in a 24-h society. Lancet, 358: 999-1005

Ramanathan L, Gulyani S, Nienhuis R, Siegel JM (2002). Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport, 13: 1387-1390

Ramanathan L, Shuxin H, Frautschy SA Siegel JM (2010). Short-term total sleep deprivation in the rat increases antioxidant responses in multiple brain regions without impairing spontaneous alternation behavior. Behav Brain Res, 207: 305-309

Ramos A (2008). Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci, 29: 493-498

Rao MS, Shetty AK (2004). Efficacy of doublecortin as a marker to analyze the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. Eur J Neurosci, 19: 234-246

Rasmussen T, Gulati DR (1962). Corticosterone in treatment of post operative cerebral edema. J Neurosurgery, 19: 535-&

Rechtschaffen A, Bergmann BM (1995). Sleep deprivation in the rat by the disk-overwater method. Behav Brain Res, 69: 55-63

Redman LM, Ravussin E (2009). Endocrine alterations in response to calorie restriction in humans. Mol Cell Endocrinol, 299: 129-136

Rerup CC (1970). Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol Rev, 22: 485-518

Riemann D, Berger M, Voderholzer U (2001). Sleep and depression-results from psychobiological studies: an overview. Biol Psychol, 57: 67-103

Riemann D, Voderholzer U (2003). Primary insomnia: a risk factor to develop depression? J Aff Disord, 76: 255-259

Riemann D, Voderholzer U, Spiegelhalder K, Hornyak M, Buysse DJ, Nissen C, Hennig J, Perlis ML, van Elst LT, Feige B (2007). Chronic insomnia and MRI-measured hippocampal volume: a pilot study. Sleep, 30: 955-958

Riemann D, Workshop Participants (2009). Does effective management of sleep disorders reduce depressive symptoms and the risk of depression? Drugs, 69: Suppl 2: 43-64

Roane BM, Taylor DJ (2008). Adolescent insomnia as a early risk factor for depression and substance abuse. Sleep, 31: 1351-1356

Robertson DAF, Beattie JE, Reid IC, Balfour DJK (2005). Regulation of corticosteroid receptors in the rat brain: the role of serotonin and stress. Eu J Neurosci, 21: 1511-1520

Roenneberg T, Kuehnle T, Juda M, Kantermann T, Allebrandt K, Gordijn M, Merrow M (2007). Epidemiology of the human circadian clock. Sleep Med Rev, 11: 429-438

Roman V, Van der Borght K, Leemburg S, Van der Zee EA, Meerlo P (2005a). Sleep restriction by forced activity reduces hippocampal cell proliferation. Brain, 1065: 53-59

Roman V, Walstra I, Luiten PGM, Meerlo P (2005b). Too little sleep gradually desensitizes the 5-HT1A receptor system in rats. Sleep, 28: 1505-1510

Roman V, Hagewoud R, Luiten PG, Meerlo P (2006). Differential effects of chronic partial sleep deprivation and stress on serotonin-1A and muscarinic acetylcholine receptor sensitivity. J Sleep Res, 15: 386-394

Roth T, Jaeger S, Jin R, Kalsekar A, Stang PE, Kessler RC (2006). Sleep problems, comorbid mental disorders, and role functioning in the national comorbidity survey replication. Biol Psychiatry, 60: 1364-1371

Roy M, Sapolsky RM (2003). The exacerbation of hippocampal excitotoxicity by glucocorticoids is not mediated by apoptosis. Neuroendocrinology, 77: 24-31

Ruskin DN, Liu C, Dunn KE, Bazan NG, LaHoste GJ (2004). Sleep deprivation impairs hippocampus-mediated contextual learning but not amygdala-mediated cued learning in rats. Eur J Neurosci, 19: 3121-3124

Sahay A, Hen R (2007). Adult hippocampal neurogenesis in depression. Nat Neurosci, 10: 1110-1115

Sakai K, Crochet S (2001). Differentiation of presumed serotonergic dorsal raphe neurons in relation to behavior and wake-sleep states. Neuroscience, 104: 1141-1155

Sagaspe P, Sanchez-Ortuno M, Charles A, Taillard J, Valtat C, Bioulac B, Philip P (2006). Effects of sleep deprivation on color-word, emotional, and specific stroop interference and on self-reported anxiety. Brain Cogn, 60: 76-87

Samuels BA, Hen R (2011). Neurogenesis and affective disorders. Eu J Neurosci, 33: 1152-1159

Sapolsky RM, Krey LC, McEwen BS (1984). Stress down-regulates corticosterone receptors in a site specific manner in the brain. Endocrinology, 114: 287-292

Sapolsky, RM, Krey LC, McEwen BS (1985). Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. J Neurosci, 5: 1222-1227

Sapolsky RM, Pulsinelli WA (1985). Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science, 229: 1397-1400

Sapolsky RM, 1986. Glucocorticoid toxicity in the hippocampus. Temporal aspects of synergy with kainic acid. Neuroendocrinology, 43: 440-444

Sapolsky RM, 2000. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry, 57: 925-935

Sargent PA, Kjaer KH, Bench CJ, Rabiner EA, Messa C, Meyer J, Gunn RN; Grasby PM, Cowen PJ (2000). Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635. Arch Gen Psychiatry, 57: 174-180

Sattler R, Tymiansky JM (2000). Molecular mechanisms of calcium-dependent excitotoxicity. J Mol Med, 78: 3-13

Sattler R, Tymianski JM (2001). Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol, 24: 107-129

Scharf MT, Naidoo N, Zimmerman JE, Alan IP (2008). The energy hypothesis of sleep revisited. Prog in Neurobiol, 86: 264-280

Schmidt-Kastner R, Wetmore C, Olson L (1996). Comparative study of brain-derived neurotrophic factor messenger RNA and protein at the cellular level suggests multiple roles in hippocampus, striatum and cortex. Neuroscience, 74: 161-183

Schmitt KR, Kern C, Berger F, Ullrich O, Hendrix S, Abdul-Khaliq H (2006). Methylprednisolone attenuates hypothermia- and rewarming-induced cytotoxicity and IL-6 release in isolated primary astrocytes, neurons and BV-2 microglia cells. Neurosci Lett, 404: 309-314

Schrader GD (1997). Does anhedonia correlate with depression severity in chronic depression? Compr Psychiatry, 38: 260-263

Schüle C, Baghai T, Zwanger P, Minov C, Padberg F, Rupprecht R (2001). Sleep deprivation and hypothalamic-pititary-adrenal (HPA) axis activity in depressed patients. J Psychiat Res, 35: 239-247

Sei H, Saitoh D, Yamamoto K, Morita K, Morita Y (2000). Differential effect of short-term REM sleep deprivation on NGF and BDNF protein levels in the rat brain. Brain Res, 877: 387-390

Semba K, Reiner PB, McGeer EG, Fibiger HC (1988). Non-cholinergic basal forebrain neurons project to the contralateral basal forebrain in the rat. Neurosci Lett, 84: 23-28

Semkova I, Wolz P, Krieglstein J (1998). Neuroprotective effect of 5-HT1A receptor agonist, Bay X 3702, demonstrated in vitro and in vivo. Eur J Pharmacol, 359: 251-260

Senthilvelan M, Ravindran R, Samson J, Devi RS (2006). Serotonin turnover in different duration of sleep recovery in discrete regions of young rat brain after 24 h REM sleep deprivation. Brain Dev, 28: 526-528

Sgoifo A, Buwalda B, Roos M, Costoli T, Merati G, Meerlo P (2006). Effects of sleep deprivation on cardiac autonomic and pituitary-adrenocortical stress reactivity in rats. Psychoneuroendocrinology, 31:197-208

Shapira B, Newman ME, Gelfin Y, Lerer B (2000). Blunted temperature and cortisol responses to ipsapirone in major depression: lack of enhancement by electroconvulsive therapy. Psychoneuroendocrinology, 25: 421-438

Sharma V (2003). Role of sleep in the causation of puerperal psychosis. Med hypotheses, 61: 477-481

Shibley HL, Malcom RJ, Veatch LM (2008). Adolescents with insomnia and substance abuse: consequences and comorbidities. J Psichiatr Pract, 14: 146-153

Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, Nakazato M, Watanabe H, Shinoda N, Okada,S, Iyo M (2003). Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol Psychiatry, 54: 70-75

Silva RH, Kameda SR, Carvalho RC, Takatsu-Coleman AL, Niigaki ST, Abilio VC, Tuffik S, Frussa-Filho R (2004). Anxiogenic effect of sleep deprivation in the elevated plus-maze test in mice. Psychopharmacology, 176: 115-122

Simpson N, Dinges DF (2007). Sleep and inflammation. Nutr Rev, 65: S244-6252

Smeraldi E, ZAnardi R, Benedetti F, Di Bella D, Perez J, Catalano M (1998). Polymorphism within the promoter of the serotonin transporter gene and antidepressant efficacy of fluvoxamine. Mol Psychiatry, 3: 508-511

Sobczak S, Honig A, van Duinen MA, Riedel WJ (2002). Serotonergic dysregulation in bipolar disorders: a literature review of serotonergic challenge studies. Bipolar Disord, 4: 347-56

Sousa N, Paula-Barbosa MM, Almeida OF (1999). Ligand and subfield specificity of corticoid induced neuronal cell loss in the rat hippocampal formation. Neuroscience, 89: 1079-1087

Southmayd SE, David MM (1990). Sleep deprivation in depression – pattern of relapse and characteristics of preceding sleep. Biol Psychiatry, 11: 979-988

Sowell ER, Trauner DA , Gamst A , Jernigan TL (2002). Development of cortical and subcortical brain structures in childhood and adolescence: a structural MRI study. Dev Med Child Neurol, 44: 4-16

Spiegel K, Leproult R, Van Cauter E (1999). Impact of sleep debt on metabolic and endocrine function. Lancet, 354: 1435-1439

Spiegel K, Tasali E, Penev P, Van Cauter E (2004). Brief communication: sleep curtailment in healthy young men is associated with decreased leptin levels, elevated ghrelin levels, increased hunger and appetite. Ann Intern Med, 141: 846-850

Sportiche N, Suntsova N, Methippara M, Bashir T, Mitrani B, Szymusiak R, Mc Ginty D (2010). Sustained sleep fragmentation results in delyed changes in hippocampal-dependent cognitive function associated with reduced dentate gyrus neurogenesis. Neurosci, 170: 245-258

Steffens A.B (1969). A method for frequent sampling of blood and continuous infusions of fluids in the rat without disturbing the animal. Physiol Behav, 4: 833-836

Stewart GR, Price M, Hartman BK, Cozzari C (1986). N-Methylaspartate: an effective tool for lesioning basal forebrain cholinergic neurons of the rat. Brain Res, 369: 377-382

Stockmeier CA (2003). Involvement of serotonin in depression: evidence from postmortem and imaging studies of serotonin receptors and the serotonin transporter. J Psychiatr Res, 37: 357-373

Suchanek B, Struppeck H, Fahrig T (1998). The 5-HT1A receptor agonist BAY x 3702 prevents staurosporine-induced apoptosis. Eur J Pharmacol, 355: 95-101

Suchecki D, Lobo LL, hipolide DC, Tufik S (1998). Increased ACTH and corticosterone secretion induced by different methods of paradoxical sleep deprivation. J Sleep Res, 7: 276-281

Suchecki D, Tiba PA, Tufik S (2002). Hormonal and behavioural responses of paradoxical sleep-deprived rats to the elevated plus-maze. J Neuroendocrinol, 14: 549-554

Supko DE, Johnston MV (1994). Dexamethasone potentiates NMDA receptor-mediated neuronal injury in the postnatal rat. Eur J Pharmacol, 270: 105-113

Tannenbaum B, Brindley D, Tannenbaum G, Dallman J, McArthur D, Meaney M, (1997). High fat feeding alters both basal and stress-induced hypothalamic–pituitary– adrenal activity in the rat. Am J Physiol, 273: E1168-1177

Tartar JL, Ward CP, Cordeira JW, Legare SL, Blanchette AJ, McCarley RW, Strecker RE (2009). Experimental sleep fragmentation and sleep deprivation in rats increases exploration in an open field test of anxiety while increasing plasma corticosterone levels. Behav Brain Res 197: 450-453.

Taylor DJ (2008). Insomnia and depression. Sleep, 31: 447-448

Taylor DJ, Lichstein KL, Durrence HH, Reidel BW, Bush AJ (2005). Epidemiology of insomnia, depression, and anxiety. Sleep, 28: 1457-1464

Thakkar MM, Winston S, McCarley RW (2003). A1 receptor and adenosinergic homeostatic regulation of sleep-wakefulness: effects of antissense to the A1 receptor in the cholinergic basal forebrain. J Neurosci, 23: 4278-4287

Tiba PA, Oliveira MG, Rossi VC, Tufic S, Succhecki D (2008). Glucocorticoids are not responsible for paradoxical sleep deprivation-induced memory impairments. Sleep, 31: 505-515

Toga AW, Thompson PM, Sowell ER (2006). Mapping brain maturation. TRENDS Neurosci, 29: 148-159

Tononi G, Cirelli C (2006). Sleep function and synaptic homeostasis. Sleep Med Rev, 10: 49-62

Törk I (1990). Anatomy of the serotonergic system. Ann N Y Acad Sci, 600: 9-34

Torelli F, Moscufo N, Girolamo G, Placidi F, Romigi A, Zannino S, Bozzali M, Fasano F, Giulietti G, Djonlagic I, Malhotra A, Marciani MG, Guttmann CRG (2011). Cognitive profile and brain morphological changes in obstructive sleep apnea. NeuroImage, 54: 787-793

Tsigos C, Chrousos GP (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res, 53: 865-871

Tsuno N, Besset A, Ritchie K (2005). Sleep and depression. J Clin Psychiatry, 66:1254-1269

Tung A, Takase L, Fornal C, Jacobs B (2005). Effects of sleep deprivation and recovery sleep upon cell proliferation in adult rat dentate gyrus. Neuroscience, 134: 721-723

Twiggs DG, Popolow HB, Gerall AA (1978). Medial preoptic lesions and male sexual behavior: age and environmental interactions. Science, 200: 1414-1415

Ulrich-Lay YM, Herman JP (2009). Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci, 10: 397-409
Valera E, Sanchez-Martın FJ, Ferrer-Montiel AV, Messeguer A, Merino JM (2008). NMDA-induced neuroprotection in hippocampal neurons is mediated through the protein kinase A and CREB (cAMP-response element-binding protein) pathway. Neurochem Int, 53: 148-154

Van den Burg W, Beersma DGM, Bouhuys, Van den Hoofdakker RH (1992). Self-reported arousal concurrent with the antidepressant response to total sleep-deprivation of patients with a major depressive disorder-a disinhibition hypothesis. J Sleep Res, 1: 211-222

Van den Bulck J (2004). Television viewing, computer game playing, and Internet use and self-reported time to bed and time out of bed in secondary-school children. Sleep, 27: 101-104

Van Dongen HP, Maislin G, Mullington JM, Dinges DF (2003). The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation. Sleep, 26: 117-126

Van der Borght K, Ferrari F, Klauke K, Roman V, Havekes R, Sgoifo A, van der Zee EA, Meerlo P (2006). Hippocampal cell proliferation across the day: increase by running wheel activity but no effect of sleep and wakefulness. Behav Brain Res, 167: 36-41

Van Der Werf YD, Altena E, Schoonheim MM, Sanz-Arigita EJ, Vis JC, De Rijke W, Van Someren EJ (2009). Sleep benefits subsequent hippocampal functioning. Nature Neurosci, 12: 122-123

Van Praag H, Kempermann G, Gage FH (2000). Neural consequences of environmental enrichment. Nature Rev Neurosci, 1: 191-198

Vgontzas AN, Bixler EO, Lin HM, Prolo P, Mastorakos G, Vela-Bueno A, Kales A, Chrousos GP (2001). Chronic insomnia is associated with nyctohemeral activation of the hypothalamic-pituitary-adrenal axis: clinical implications. J Clin Endocrinol Metab, 86: 3787-3794

Von Treuer K, Norman TR, Armstrong SM (1996). Overnight human plasma melatonin, Cortisol, prolactin, TSH, under conditions of normal sleep, sleep deprivation, and sleep recovery. J Pineal Res, 20: 7-14

Vyazovskiy VV, Cirelli C, Pfister-Genskow M, Faraguna U, Tononi G (2008). Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. Nat Neurosci, 11: 200-208

Vyazovskiy VV, Olcese U, Hanlon EC, Nir Y, Cirelli C, Tononi G (2011). Local sleep in awake rats. Nature, 472: 443-447

Walker JM, Berger RJ (1980). Sleep as an adaptation for energy conservation functionally related to hibernation and shallow torpor. Prog Brain Res, 53: 255-252

Walker MA, Highley JR, Esiri MM, McDonald B, Roberts HC, Evans SP, Crow TJ (2002). Estimated neuronal populations and volumes of the hippocampus and its subfields in schizophrenia. Am J Psychiatry, 159: 821-828

Wang GP, Huang LQ, Wu H-J, Zhang L, You Z-D, Zhao Z-X (2009). Calcineurin contributes to spatial memory impairment induced by rapid eye movement sleep deprivation. Neuroreport, 20: 1172-1176

Watanabe Y, Gould E, McEwen BS (1992). Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res, 588: 341-345

Wehr TA (1991). Sleep loss as a possible mediator of disease causes of mania. Br J Psychiatry, 159: 576-578

Weitzman ED, Zimmerman JC, Czeisler CA, Ronda J (1983). Cortisol secretion is inhibited during sleep in normal man. J Clin Endocrinol Metab, 56: 352-358

Wenk H, Bigl V, Meyer U (1980). Cholinergic projections from magnocellular nuclei of the basal forebrain to cortical areas in the rats. Brain Res, 2: 295-316

Willner P, Muscat R, Papp M (1992). Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev, 16: 525-534

Wolfson AR, Carskadon MA (2003). Understanding adolescents'sleep patterns and school performance: a critical appraisal. Sleep Med Rev, 7: 491-506

Wu D, Hersh LB (1994). Choline acetyltransferase: celebrating its fiftieth year. J Neurochem, 62: 1653-1663

Xifrò X, Garcia-Martinez JM, del Toro D, Alberch J, Perèz-Navarro E (2008). Calcineurin is involved in the early activation of NMDA-mediated cell death in mutant huntingtin knock-in striatal cells. J Neurochem, 105: 1596-1612

Yanik G, Radulovacki M (1987). REM sleep deprivation up-regulates adenosine A1 receptors. Brain Res, 402: 362-364

Yau JL, Noble J, Seckl JR (2001). Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus. Neurosci Lett, 309: 141-144

Yudt MR, Cidlowski JA (2002). The glucocorticoid receptor: Coding a diversity of proteins and responses through a single gene. Mol Endocrinol, 16: 1719-1726

Nederlandse samenvatting

De neurobiologische gevolgen van chronisch slaaptekort: implicaties voor depressie

Slaaptekort komt in toenemende mate voor in onze Westerse samenleving. Veel mensen kampen met regelmatige slaapproblemen of lijken gewoonweg niet voldoende tijd te hebben voor slaap. De redenen hiervoor zijn divers en kunnen variëren van stress, hoge werkdruk, en ploegendienst tot ouderschap, maar ook recreatieve bezigheden in de late avonduren krijgen regelmatig voorrang boven slaap. Frequent slaaptekort is niet een specifiek probleem van hardwerkende volwassenen maar lijkt ook steeds meer voor te komen bij adolescenten en jonge kinderen. De redenen hiervoor zijn voorspelbaar: de jongeren kijken 's avonds nog laat naar de televisie of surfen op het internet maar moeten wel de volgende ochtend vroeg weer op vanwege schoolverplichtingen.

Er wordt algemeen aangenomen dat slaap belangrijk is voor herstel en plasticiteit van zenuwcellen en daardoor uiteindelijk voor een goed functioneren van de hersenen. Wat slaap precies doet op moleculair en cellulair niveau is nog grotendeels onbekend maar het is duidelijk dat te weinig slapen directe effecten heeft op concentratievermogen, cognitieve prestaties en stemming. Deze acute effecten van slaaptekort lijken vrij snel te verdwijnen wanneer slaap wordt ingehaald. Echter, wanneer slaapverstoring of slaapgebrek een chronisch probleem wordt zouden er wel eens veranderingen in hersenen en lichaam kunnen optreden die ernstige gevolgen kunnen hebben voor de psychische en fysieke gezondheid.

Er zijn het afgelopen decennia talloze studies verschenen die een relatie aantonen tussen slaapduur en het risico op allerlei aandoeningen. Weinig slaap is geassocieerd met een verhoogde kans op infectieziektes inclusief griep en verkoudheid. Verder is een levensstijl met weinig slaap geassocieerd met een verhoogde kans op hart- en vaat ziektes, metabole stoornissen als diabetes, en stemmingsstoornissen als depressie, manie, en post-traumatisch stress syndroom. Vooral de relatie tussen verstoorde slaap en depressie lijkt erg sterk en is in talloze studies vastgesteld. Meestal is het onduidelijk wat oorzaak en gevolg is maar sommige studies laten zien dat insomnia vaak vooraf gaat aan veranderingen in stemming. Echter, ook in dat geval is het niet bewezen dat de verstoorde slaap daadwerkelijk een oorzaak is van de verstoorde stemming. Om dergelijke causale verbanden aan te tonen zijn goed gecontroleerde experimentele studies nodig waarbij men chronisch slaapverstoring toepast en vastlegt of dit leidt tot bepaalde kenmerken of symptomen van depressie.

In dit proefschrift zijn een aantal studies beschreven waarin werd gekeken naar de neurobiologische en neuroendocriene gevolgen van chronische slaaprestrictie in ratten. De experimenten waren in het bijzonder gericht op hersensystemen en processen die mogelijk betrokken zijn bij depressie. Het model van chronische slaaprestrictie dat werd gebruikt was gericht op het nabootsen van regelmatig slaaptekort in onze maatschappij. De dieren konden slechts 4 uur per dag slapen, veel minder dan de ruim 10 uur die ratten in het laboratorium normaliter slapen, en vermoedelijk niet voldoende voor een volledig herstel en verwerking van de dagelijkse wakker activiteiten. De ratten werden wakker gehouden door ze in langzaam draaiende

trommels te plaatsen. De rotatiesnelheid van de trommels was net voldoende om de dieren wakker te houden zonder een zware lichamelijke inspanning te vereisen. De dieren werden blootgesteld aan slaaprestrictie voor een periode van een week tot een maand en in de meeste experimenten vergeleken met twee controle groepen. De ratten in de eerste controle groep werden eveneens in trommels geplaatst. Deze trommels draaiden echter op dubbele snelheid voor de helft van de tijd waardoor deze dieren evenveel fysieke activiteit moesten uitvoeren maar toch voldoende tijd hadden om te slapen. Hierdoor konden effecten van slaaptekort en geforceerde activiteit worden onderscheiden. De tweede controle groep bestond uit dieren die gedurende het hele experiment ongestoord in hun thuiskooi verbleven.

Het experiment in **hoofdstuk 2** is gebaseerd op het algemene idee dat slaaptekort op langere termijn het brein kwetsbaarder maakt voor neurodegeneratieve processen. Hoewel er in de literatuur weinig aanwijzingen zijn dat slaaptekort direct leidt tot celdood zou het wel de gevoeligheid kunnen verhogen voor een daarop volgende neurodegeneratief insult. Om dit te testen werd bij ratten na een maand van slaaprestrictie een gecontroleerde locale hersenschade geïnduceerd door injectie van een neurotoxische dosis N-methyl-d-aspartate (NMDA). De dieren kregen een unilaterale NMDA injectie in de nucleus basalis magnocellularis (NBM) welke resulteerde in een duidelijk verlies van cholinerge neuronen in de NBM zelf en vermindering van cholinerge vezelprojecties in de cortex. Tegen de verwachting in was de schade in ratten blootgesteld aan slaaprestrictie significant minder dan in controle dieren. Een mogelijke verklaring voor deze bevinding is dat chronische slaaprestrictie een milde stress is voor de hersenen die weliswaar niet direct tot schade leidt maar wel beschermende mechanismen activeert en daardoor de schade van een later optredend insult vermindert. Deze resultaten zijn duidelijk in strijd met de hypothese dat slaaptekort de gevoeligheid voor neurodegeneratie verhoogt.

Hoewel slaaptekort niet direct leidt tot de dood van hersencellen kan het de morfologie en plasticiteit van het brein wel op andere, meer subtiele manieren aantasten die uiteindelijk grote gevolgen zouden kunnen hebben voor het functioneren van het brein. In **hoofdstuk 3** werd onderzocht wat de invloed is van chronische slaaprestrictie op het serotonerge systeem, dat een belangrijke rol speelt bij de regulatie van emoties en stress. Tevens werd gekeken naar de effecten van slaaptekort op regulering en reactiviteit van de hypothalamus-hypofyse-bijnier (HPA) as, een van de belangrijkste neuroendocriene stress systemen van het lichaam. Ratten werden na slaaprestrictie blootgesteld aan verschillende stressoren door ze te onderwerpen aan een zogenaamd ‘fear conditioning paradigm’. Deze bestond allereerst uit een korte sessie met enkele milde elektrische schokken (een acute fysieke stressor), een dag later gevolg door hernieuwde blootstelling aan de schokkamer maar nu zonder schokken (een emotionele stressor). De HPA as respons werd bepaald door de plasmaspiegels van het hypofyse hormoon ACTH en het bijnierhormoon corticosteron te meten. Na beide stressoren vertoonden de dieren met chronisch slaaptekort een significant verminderde ACTH response. Deze verandering in hypofyse respons was alleen zichtbaar na 8 dagen slaaprestrictie maar niet na 1 dag. Dit laatste is een belangrijke

ondersteuning van het idee dat slaaptekort effecten kan hebben die niet onmiddellijk zichtbaar zijn maar zich geleidelijk aan opstapelen wanneer slaaptekort chronisch wordt.
De activiteit van de hypofyse en de afgifte van ACTH in reactie op stress staat onder controle van diverse centraal-nerveuze signalen. In de eerste plaats is dat CRH vanuit de hypothalamus maar ook het serotonerge systeem speelt hierbij een belangrijke rol, vooral via de serotonine 1A receptor die aanwezig is in zowel de hypothalamus als de hypofyse. Teneinde het mechanisme dat ten grondslag ligt aan de veranderingen in hypofyse ACTH response na slaaprestrictie te onderzoeken, werd de HPA as direct gestimuleerd door infusie van een standaard dosis CRH of door infusie van een serotonine-1A receptor agonist (8-OH-DPAT). In beide gevallen vertoonden de dieren na chronisch slaaptekort een verminderde ACTH respons. Dit bevestigt eerdere bevindingen dat chronische slaaprestrictie leidt tot een verminderde gevoeligheid van het serotonine-1A receptorsysteem en suggereert ook een verminderde gevoeligheid van CRH receptoren. Beide kunnen bijdragen aan de verminderde hypofyse respons wanneer dieren met slaaptekort worden blootgesteld aan stress. Ondanks de verandering in hypofyse ACTH respons vertoonden ratten na chronisch slaaptekort een vrijwel normale bijnier corticosteron afgifte. Dit zou kunnen betekenen dat de bijnieren onder condities van slaaprestrictie juist gevoeliger worden voor ACTH zodat zelfs de verminderde ACTH afgifte leidt tot een even hoge corticosteron respons.

Het is bekend dat slaapdeprivatie ook directe effecten heeft op het metabolisme en bij ratten vaak leidt tot een vermindering van groei of zelfs een daling van het lichaamsgewicht. Dit laatste kan op zijn beurt weer effecten hebben op het serotonerge systeem. Daarom werd in **hoofdstuk 4** onderzocht of de slaaprestrictie-geinduceerde veranderingen in serotonine-1A gevoeligheid mede afhankelijk zijn van gewichtsverlies. Om gewichtsverlies door slaaprestrictie te voorkomen kreeg een deel van de ratten gedurende het experiment vet-verrijkt voedsel. Ondanks het feit dat dit dieet de vermindering in groei van dieren met slaaptekort deels voorkwam was de verminderde gevoeligheid van serotonine-1A receptor systeem nog steeds aanwezig. De slaaprestrictiedieren vertoonden nog steeds een verminderde hormonale respons en temperatuur respons in reactie op de seroninin-1A agonist 8-OH-DPAT. Deze resultaten ondersteunen dus niet het idee de verminderde gevoeligheid van het serotonin-1A systeem na slaaprestrictie een consequente is van veranderingen in metabolisme en lichaamsgewicht.

In **hoofdstuk 5** werd verdere studie verricht naar de effecten van slaaprestrictie op de gevoeligheid voor stress en met name op de expressie van corticoïde receptoren die een belangrijke rol spelen bij de effecten van het bijnier-stresshormoon corticosteron op hersenfuncties. De expressie van deze receptoren in de hersenen wordt gereguleerd door verschillende systemen en processen die op zich weer beïnvloed kunnen worden door slaaptekort. Er werd gekeken naar de expressie van mineralocorticoïd receptoren (MR) en glucocorticoïd receptoren (MR) in verschillende hersengebieden na chronische slaaprestrictie alleen en na slaaprestrictie in combinatie met herhaalde blootstelling aan immobilisatie stress. In situ hybridisatie liet echter in geen van de onderzochte gebieden veranderingen zien in de

expressie van MR/GR mRNA. Verder onderzoek is nodig om na te gaan of slaaprestrictie effect heeft op de concentratie van het functionele MR/GR receptor eiwit of op de gevoeligheid en werking van deze receptoren.

Omdat slaaptekort in onze maatschappij een probleem is dat ook op jonge leeftijd steeds groter lijkt te worden, en een rol zou kunnen spelen in de toename van stemmingsstoornissen op deze leeftijd, werd in **hoofdstuk 6** gekeken naar de gevolgen van chronische slaaprestrictie in adolescente ratten van postnatale dag 30 tot 61. De experimenten waren deels gericht op de morfologie en plasticiteit van de hippocampus, een hersenstructuur die een belangrijke rol speelt bij cognitieve processen en emoties. De belangrijkste bevinding van dit experiment was een significante 10% reductie in het volume van de hippocampus, zoals die ook vaak is gerapporteerd bij depressieve patiënten. De oorzaak van deze vermindering in hippocampaal volume is vooralsnog onduidelijk maar lijkt niet veroorzaakt te worden door een vermindering van neurogenese. Hoewel eerdere studies in volwassen dieren hadden aangetoond dat chronische slaapverstoring kan leiden tot een sterke reductie in de aanmaak van nieuwe neuronen in de hippocampus, werd dat in de huidige studie in jongere dieren niet bevestigd. Er werden ook diverse testen uitgevoerd om angst- en depressieachtig gedrag bij de jonge dieren te meten. In overeenstemming met de literatuur bleek slaaprestrictie geen duidelijk effect te hebben op het gedrag in klassieke angsttesten (open field, elevated plus maze). Wel veroorzaakte slaaptekort een tijdelijke onderdrukking in de normale voorkeur voor suikerwater boven normaal drink water, hetgeen kan wijzen op een tijdelijke anhedonie of depressieachtig gedrag. We konden in dit experiment echter niet uitsluiten dat deze anhedonie veroorzaakt werd door de geforceerde activiteit van de slaaprestrictie procedure in plaats van slaapgebrek per se.

Conclusie. Veel van de waargenomen neurobiologische en neuroendocriene veranderingen na chronische slaaprestrictie in ratten komen sterk overeen met afwijkingen die zijn vastgesteld bij depressieve patiënten, met name de verminderde gevoeligheid van het serotonine-1A systeem, de veranderingen in HPA-as regulatie, en de reductie in hippocampaal volume. De resultaten van deze studies leveren dan ook een sterke ondersteuning van de hypothese dat verstoorde slaap niet enkel een symptoom is van depressie maar, bij een deel van de patiënten, mogelijk een causale rol speelt bij het ontstaan van de stemmingsstoornis. Tevens leggen deze resultaten potentiele mechanismes bloot waarlangs dat kan gebeuren.

Summary in Italian

Sintesi in italiano

Carenza di sonno, vulnerabilità cerebrale e psicopatologia

Una varietà di fenomeni nel nostro stile di vita, quali stress, ritmi di lavoro elevati ed attività ricreative notturne, causano la perdita di ore di sonno, un fenomeno sempre più diffuso non soltanto nella popolazione adulta, ma anche tra i bambini e gli adolescenti.

Il sonno è fondamentale per la plasticità ed il recupero neuronali e di conseguenza anche per il normale funzionamento del cervello. La sua azione a livello molecolare è in gran parte sconosciuta, ma è ben noto che dormire troppo poco influisce negativamente sulle capacità cognitive, sull'abilità di concentrarsi ed anche sull'umore. Mentre questi effetti indotti dalla privazione acuta del sonno scompaiono abbastanza presto semplicemente recuperando il sonno perso, la carenza cronica di sonno potrebbe causare serie alterazioni fisiche e cerebrali rappresentando una minaccia per la salute.

Diversi studi dimostrano una relazione tra la durata del sonno e il rischio per determinate patologie. Chi dorme poco contrae più facilmente infezioni respiratorie come l'influenza ed il raffreddore ed ha un'aumentata probabilità di sviluppare malattie cardiocircolatorie, diabete e disturbi dell'umore tra cui depressione, mania e disturbo post traumatico da stress. L'associazione tra disturbi del sonno e depressione è particolarmente forte ed è stata presa in considerazione in diversi studi, ma nel complesso rimane ignoto quale ne sia la relazione causa effetto. Alcune ricerche hanno rivelato che spesso, periodi di insonnia precedono l'insorgenza di stati depressivi, ma questo non vuol dire necessariamente che sia la perdita del sonno a causare la depressione. Per stabilire l'esistenza di una relazione causale, sono necessari degli studi sperimentali al fine di testare se la restrizione cronica del sonno porta ad alterazioni e sintomi tipici della depressione.

Questa tesi consiste di una serie di studi sulle conseguenze neurobiologiche e neuroendocrine della restrizione del sonno nel ratto, con particolare attenzione a quei fenomeni e processi che sono alterati nella depressione. Allo scopo di riprodurre il fenomeno della perdita del sonno in modo simile a come avviene nella nostra società, viene usato un modello di studio in cui i ratti possono dormire soltanto quattro ore al giorno. Essendo considerevolmente inferiore alla normale durata del sonno nei ratti da laboratorio (~10 ore), tale quantità è insufficiente a garantire un normale recupero. In questo modello, i ratti vengono mantenuti svegli forzandoli a camminare in ruote rotanti a bassa velocità. Il lento movimento di rotazione è sufficiente ad impedire agli animali di addormentarsi senza indurre un forte stress fisico. Negli esperimenti qui riportati, i ratti vengono sottoposti alla restrizione cronica del sonno per periodi variabili da una settimana ad un mese e nella maggior parte dei casi sono messi a confronto con due gruppi di controllo. Come i ratti sottoposti a restrizione del sonno, quelli del primo gruppo di controllo vengono fatti camminare nelle ruote che però in questo caso, girano a velocità doppia e per la metà del tempo. In tal modo, i livelli di attività fisica sono simili per i due gruppi, ma i ratti nel gruppo di controllo non sono soggetti a restrizione del sonno. Dal paragone tra questi due gruppi sperimentali è possibile distinguere gli effetti causati dalla restrizione del sonno per sé da quelli indotti dal

metodo usato in questo modello. Gli animali appartenenti al secondo gruppo di controllo rimangono indisturbati nelle loro gabbie per tutto il corso degli esperimenti.

Gli esperimenti nel **secondo capitolo** di questa tesi si basano sull'idea che la privazione cronica del sonno rende il cervello più sensibile a processi neurodegenerativi. Anche se nella letteratura le evidenze in favore di una perdita neuronale come diretta conseguenza della privazione del sonno sono poco convincenti, la perdita del sonno potrebbe comunque aumentare la vulnerabilità del cervello ad insulti di tipo neurodegenerativo. Al fine di testare questa ipotesi, in ratti sottoposti a trenta giorni di restrizione del sonno, è stato indotto un evento neurodegenerativo mediante iniezione unilaterale di una dose neurotossica di N-metil-D-aspartato (NMDA) nel nucleo basale magnocellulare (NBM). L'iniezione di NMDA è risultata in una diminuzione del numero di cellule nel NBM e delle rispettive proiezioni alla corteccia nell'emisfero cerebrale iniettato. Contrariamente alle ipotesi, il danno era meno accentuato nei ratti sottoposti a restrizione del sonno rispetto ai controlli. Una possibile ragione per questo effetto è che la restrizione del sonno, agendo da stress moderato, sia insufficiente ad indurre un danno neurodegenerativo, ma attivi dei meccanismi di difesa che successivamente attenueranno il danno causato da un insulto più forte.

Pur non inducendo neurodegenerazione, la restrizione del sonno potrebbe alterare plasticità e morfologia cerebrali in modo più subdolo, compromettendo il normale funzionamento del cervello. Gli esperimenti descritti nel **capitolo tre** hanno lo scopo di investigare l'effetto della restrizione del sonno sia su regolazione ed attività dell'asse ipotalamo-ipofisi-surrene (HPA) che è uno dei maggiori sistemi neuroendocrini del nostro organismo, sia sul sistema serotoninergico che ha una funzione molto importante nella regolazione dello stress e delle emozioni. In seguito al periodo di restrizione del sonno, i ratti sono stati sottoposti ad un paradigma noto come 'condizionamento alla paura'. In questo paradigma gli animali vengono prima esposti ad una breve sequenza di scosse elettriche a basso voltaggio (stress fisico) all'interno di un box e il giorno successivo, vengono reintrodotti nello stesso box senza scosse (stress emotivo). La risposta dell'HPA viene determinata in base ai livelli plasmatici dell'ormone adrenocorticotropo (ACTH) e del corticosterone. Gli animali sottoposti a restrizione del sonno hanno dimostrato un'attenuazione nei livelli di ACTH in risposta ad entrambe le situazioni di stress (fisico ed emotivo). Tale attenuazione nei livelli di ACTH non era evidente dopo un giorno di privazione del sonno, ma soltanto dopo otto giorni di trattamento. Ciò suggerisce la possibilità che gli effetti della perdita del sonno non siano immediatamente evidenti, ma si accumulino nel tempo.

L'attività dell'ipofisi e la risposta dell'ACTH allo stress vengono controllate da diversi segnali nervosi centrali. Tra questi, sono molto importanti l'ormone di liberazione della corticotropina (CRH) e il sistema serotoninergico che agisce specialmente attraverso il recettore 1A per la serotonina presente a livello dell'ipotalamo e dell'ipofisi.
Allo scopo di investigare i meccanismi alla base dei cambiamenti nella risposta dell'ACTH in seguito a restrizione del sonno, l'asse ipotalamo-ipofisi-surrene è stato stimolato direttamente, attraverso l'infusione di una dose standard di CRH o di un agonista del recettore 1A della

serotonina (8-OH-DPAT). In entrambi i casi, gli animali con restrizione del sonno hanno dimostrato una risposta ridotta dell'ACTH. Questi risultati confermano una ridotta sensibilità del recettore 1A della serotonina, riportata in studi precedenti e suggeriscono inoltre un'attenuazione della sensibilità dei recettori per il CRH in seguito a restrizione cronica del sonno. Entrambi questi effetti possono contribuire alla ridotta risposta pituitaria quando gli animali privati del sonno vengono esposti a stress. Nonostante il cambiamento della risposta dell'ACTH, ratti assoggettati a restrizione di sonno cronica hanno mostrato un rilascio quasi normale di corticosterone dalle ghiandole surrenali. Ciò potrebbe indicare che le ghiandole surrenali in condizioni di restrizione del sonno sono più sensibili all'ACTH e di conseguenza anche un rilascio ridotto di ACTH indurrebbe un rilascio normale di corticosterone.

È noto che la privazione del sonno ha effetti diretti sul metabolismo e nei ratti spesso porta ad una diminuzione del peso corporeo che a sua volta può influenzare il sistema serotoninergico. Nel **capitolo quattro** si esamina se le alterazioni indotte dalla restrizione del sonno a livello del sistema serotoninergico, sono dipendenti dalla perdita di peso. Per prevenire il calo del peso corporeo causato dalla restrizione del sonno, parte dei ratti nell'esperimento sono stati alimentati con una dieta arricchita di grassi. Nonostante questa dieta abbia prevenuto parzialmente la perdita di peso corporeo causata dal trattamento di privazione del sonno, la ridotta sensibilità del sistema recettoriale 1A della serotonina era ancora presente nei ratti con restrizione del sonno. Questi animali hanno dimostrato ancora un'attenuazione delle risposte ormonali e della temperatura in seguito a somministrazione di 8-OH-DPAT. Questi risultati non supportano quindi l'idea che la desensitizzazione del sistema serotoninergico negli animali con restrizione del sonno sia l'effetto di cambiamenti di peso.

Nel **quinto capitolo** si studiano gli effetti della restrizione del sonno sui recettori corticoidi che sono fondamentali per la regolazione delle funzioni cerebrali da parte del corticosterone. L'espressione di questi recettori nel cervello viene regolata da diversi sistemi e processi che a loro volta sono sensibili alla privazione del sonno. Negli esperimenti di questo capitolo l'espressione dei recettori mineralcorticoidi (MR) e glucocorticoidi (GR) è stata misurata in diverse aree cerebrali in seguito a restrizione del sonno da solo o in combinazione con l'esposizione ripetuta a stress da immobilizzazione. L'ibridazione in situ non ha dimostrato cambiamenti nell'espressione dell'mRNA di questi recettori in nessuna delle aree considerate. Sono necessarie ulteriori ricerche per determinare se la restrizione del sonno influisce sull'espressione recettoriale a livello proteico o sulla funzionalità di questi recettori.

Siccome dormire poco è un'abitudine che interessa sempre di più la popolazione più giovane e potrebbe avere una funzione nell'insorgenza di disturbi dell'umore in individui di giovane età, nel **capitolo sei** si esaminano gli effetti della restrizione del sonno cronica nel ratto adolescente (periodo post-natale 30 – 61). Parte degli esperimenti sono stati centrati sulla morfologia e la plasticità dell'ippocampo, una struttura cerebrale che svolge un ruolo importante per i processi cognitivi e le emozioni. Ratti sottoposti a restrizione del sonno hanno dimostrato una riduzione del volume ippocampale di circa 10%, come osservato anche in soggetti affetti da

depressione. La causa di questa diminuzione del volume dell'ippocampo non è chiara, ma sembra essere indipendente da cambiamenti nei livelli di neurogenesi. Mentre studi in animali adulti hanno dimostrato che la privazione del sonno può ridurre la produzione di nuovi neuroni nell'ippocampo, ciò non è stato confermato dai risultati degli esperimenti qui riportati, con gli animali giovani. Un'altra parte degli esperimenti in questo capitolo riguarda gli effetti della privazione del sonno in ratti adolescenti sulla tendenza a sviluppare comportamnenti di tipo ansioso e depressivo. In accordo con altri studi nella letteratura, la restrizione del sonno non ha influenzato il comportamento di questi ratti in test classici per misurare l'ansia (open field, elevated plus maze). Negli stessi animali si poteva osservare una soppressione temporanea della preferenza per la bevanda dolce verso l'acqua non zuccherata, indicando la possibile presenza di anedonia. In ogni caso in questo esperimento non è stato possibile determinare se tale effetto era il risultato della privazione del sonno o era invece dovuto alla locomozione forzata usata per mantenere gli animali svegli.

Conclusione. Molte tra le alterazioni neurobiologiche e neuroendocrine osservate nei ratti in seguito a restrizione cronica del sonno, sono simili a quelle riportate in pazienti che soffrono di depressione. I risultati di questi studi forniscono quindi supporto all'idea che nei soggetti depressi, i disturbi del sonno non siano soltanto un sintomo, ma contribuiscano all'insorgenza della depressione e suggeriscono alcuni dei meccanismi attraverso cui ciò potrebbe avvenire.

Acknowledgments

It is a pleasure to thank all the people who helped and accompanied me in the course of my PhD and in the realization of this thesis.

My first acknowledgment goes to Dr Peter Meerlo, my supervisor. Peter, thank you for the opportunity to work on this project and for guiding me through it. I am very grateful to you for your help, for the discussions about experiments and results and for giving me many directions. I am also grateful to you for making sure that I had a very good welcome in Groningen and for arranging many practical aspects upon my arrival.

I wish to express my gratitude to Prof Jaap Koolhaas, the promoter of this thesis for his important feedbacks, especially during the writing phase.

I would like to thank Prof Paul Luiten for his kind interest and his comments on chapter three. I am grateful to Prof Paul Lucassen for his valuable comments on chapter six. I am thankful to Prof Onno Meijer for his contribution to chapter five and for welcoming me in his lab to do the GR/MR analysis. A special word of thanks to Servane Lachize for her patient explanations about in situ hybridization and for the work done together with Tim De Jager.

I would like to thank the members of the reading committee, Prof Paul Lucassen, Prof Anton Scheurink and Prof Tarja Stenberg for the time spent reading this thesis.

Thanks to all the colleagues of the Animal Physiology department for creating a pleasant working atmosphere. I am very thankful to the people that gave technical support during my PhD. So thanks to: Jan B for analysing many plasma samples, for the skilled surgeries on the rats and the handy waterproof sleeves; Jan K for plenty of help in the lab, especially setting up all the macros, and finding a valuable solution to any type of problem; Folkert who gave me many advises on how to improve a staining; Bert, Wanda and Jan G for their support in the lab. I am very thankful to Auke, Linda and Jaap for their support in the animal facility. Auke, thank you for being the first aid when facing eight-legged danger. I am very grateful to Henk, Joke and Pleunie for taking care of many administrative and bureaucratic matters. Henk, dank je voor al je hulp, je vriendelijkheid en om mij te doen lachen met je leuke humor. A big thanks to the PhD students that I met at the department: Alinde, Amalia, Angelique, Anghel, Anniek, Caroline, Deepa, Doretta, Erin, Federica, Fiona, Girste, Giulia, Gretha, Henriëtte, Ivi, Izabella, Jan, Josè, Kees, Marcelo, Martijn, Nikoletta, Paulien, Pieter, Robbert, Roelina, Simon, Stefano, Timur, Viktor and to the senior academic staff: Bauke Buwalda, Sietse de Boer, Uli Eisel, Paul Luiten, Ad Nelemans, Ingrid Nijholt, Csaba Nyakas, Anne de Ruiter, Jan Strubbe, Anton Scheurink, Gertjan van Dijk and Eddy van der Zee. For the valuable help and support with the oxytocin experiment, I am grateful to Dr Sietse de Boer, Federica, Christa and Dr Ad Nelemans. I am thankful to Prof Csaba Nyakas and Ivi for the useful conversations during the experiments of chapter two. Ivi, thanks also for the help with small things that made some practical work much easier. Paulien, I enjoyed your presence in the lab as well as your company beyond working hours. Thank you for your clear and helpful attitude: working with you goes just by itself. Girste, with your everlasting happy mode, it was nice sharing an office with you. Thank you a lot Josè for your cheerful support. Izabella, I enjoyed our conversations and your wonderful yoga classes. Deepa I have a

nice memory of the many absorbing talks and those very yummy dishes. Alinde, thank you for keeping the “magnet” alive on your returns to Groningen.

I am very thankful to Dick Visser for his detailed and patient work making the thesis cover and to the people from Van Denderen for finalizing this book.

A very special thank to my paranimfen, Francesca and Henriëtte. Francesca, thank you for your precious friendship. Grazie per tutto il bel tempo trascorso assieme, per essere tanto premurosa e mettermi così spesso di buon umore con le tue trovate spontanee. Ripenso con il sorriso alle vacanze di Erik en Nicky, i lunch all’indimenticabile ‘cantina di Peppuzzo’, il lopende-lopende-lopende, la frittata della buurvrouw, e molte altre. Grazie anche per la tua generosissima ospitalità e per gli eventi in compagnia di Marco e Willem. Henriëtte, more than a great colleague, you are also a very dear friend. Thank you for being so caring, encouraging and willing to help me with everything. I enjoyed all the work we did together and the very interesting discussions. I appreciated your effort in looking for some ‘no neutral music’ and the new Dutch words you taught me, especially the curios-sounding ones. It was always fun to spend some time in town between a cup of tea and one of your unique jokes, when the clock was apparently ticking often way too fast.

Linda, thanks a lot for your spontaneity, your honest comments and the positive influence of your hyper-energetic waves. I appreciated that despite your unbeatable working rhythm, you always found the time for some fun hour together or for the exercise in the early morning on Sundays. Thanks to Ivan, Lucile, Nina, Ronald, Saleta, Fran & Xulia and Alejo for being such a nice and happy company with whom to spend fun time and many dinners. Huub, thank you for offering me your help so many times and for your daily company with thee or coffee. Elisa, grazie per la tua gentilezza, per le numerose chat e per l’entusiasmo in tutte le tue visite a Groningen. Grazie Roberta, Lara, Martina e Sara per la vostra accoglienza ad ogni mio ritorno.

Mickey, your support and care through these years of PhD have meant so much to me. Thanks for being so generous in giving me your help and patience and thank you for all the happy things we share.

Il ringraziamento più caro va a tutta la mia famiglia e specialmente a mamma e papà. Grazie di cuore per il vostro amorevole sostegno in questo lungo periodo lontano da casa.