Metabolic consequences of sleep restriction in rats

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General introduction and scope of the thesis

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Insufficient sleep may have serious consequences for health and well-being. It has been suggested that short sleep may in the long run contribute to metabolic diseases such as obesity and type 2 diabetes. To address this issue, we experimentally restricted or disrupted sleep in rats under controlled laboratory conditions, which allowed for a detailed assessment of the metabolic consequences of sleep loss and its underlying mechanisms.

Sleep restriction and health

Over the past 50 years, average sleep duration in Western societies has decreased by almost 2 hours per night (Figure 1). This may not be without consequences. A decrease in the length and quality of sleep has been identified as a risk factor for the development of various diseases, such as mood disorders (Breslau et al., 1996;Ford and Kamerow, 1989;Neckelmann et al., 2007), immune system dysfunction (Imeri and Opp, 2009;Irwin, 2002;Opp and Toth, 2003) and cardiovascular disorders (Gangwisch, 2009;Knutson, 2010;Mullington et al., 2009).

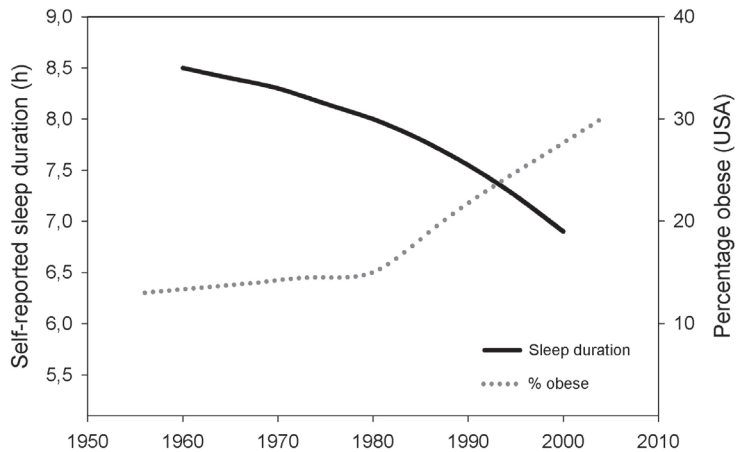

Figure 1: Self-reported sleep duration (adapted from (Van Cauter et al., 2005)) and the percentage of obese people (adapted from (Ogden & Carroll., 2010)) over the past 50 years in the USA.

Along these same lines, many epidemiological studies in the last decade have shown correlations between short sleep and increased prevalence of metabolic disorders such as obesity and type 2 diabetes, in both adults and children (Chaput et al., 2006;Horne, 2011;Van Cauter and Knutson, 2008). In addition, decreased sleep quality has been linked to metabolic disorders. One typical example of reduced sleep quality in our modern society is shift work. Shift workers are expected to sleep at times when their biological clock is competing to maintain wakefulness. Hence, shift work is often associated with disturbed sleep (Akerstedt, 2003). Several studies have shown that shift work constitutes a risk factor for

obesity and type 2 diabetes (Akerstedt, 2003;Antunes et al., 2010;Atkinson et al., 2008;Knutsson, 2003;van Amelsvoort et al., 1999), providing support for the suggested link between reduced sleep quality and metabolic disorders.

Another example for a relation between decreased sleep quality and metabolic disorders comes from people who suffer from obstructive sleep apnea (OSA), which is characterized by airway collapses during sleep, causing brief but frequent awakenings to resume breathing (Bandla and Gozal, 2000;Svanborg and Guilleminault, 1996). These frequent awakenings result in a serious reduction in sleep quality, even though the overall amount of sleep is not dramatically decreased. Vgontzas and colleagues reported a positive correlation between OSA and the development of obesity, type 2 diabetes and cardiovascular disease (Vgontzas et al., 2003). In fact, the relationship between OSA and obesity seems to be bidirectional: sleep apnea leads to obesity and weight gain also negatively impacts breathing and may lead to poor sleep quality (Peppard et al., 2000;Pillar and Shehadeh, 2008).

Although epidemiological and clinical studies report a clear association between restricted or disrupted sleep and metabolic disorders, it is unclear whether the relationship is causal. A causal relationship can only be established by experimental studies. Understandably, no study in humans has ever applied chronic sleep disturbance to the point that it resulted in diabetes type 2 or obesity. However, there are several experimental studies that assessed more immediate effects of sleep deprivation or restriction on metabolic processes that contribute to our understanding of this relationship.

Sleep restriction and metabolism in humans

Different approaches and terms have been used in literature to study sleep loss. Many studies in both humans and rodents have focused on the negative consequences of total sleep deprivation, which indicates that the subjects were not allowed to sleep at all for a certain period of time. However, chronic sleep restriction or disruption is a more common problem. Sleep restriction is characterized by shortening the amount of sleep per night, whereas sleep disturbance is characterized by arousals occurring throughout the night, without severely reducing the total amount of time spent asleep.

One of the first experimental studies that provided evidence for a direct effect of sleep restriction on glucose homeostasis was performed by Spiegel and colleagues in 1999 (Spiegel et al., 1999). Healthy subjects were exposed to 5 nights of restricted sleep, with 4h of sleep per night, and then subjected to an intravenous glucose tolerance test (IVGTT). An IVGTT is used to evaluate insulin response and glucose clearance after glucose infusion. It has been shown previously that an IVGTT is a successful tool to study glucose homeostasis (Strubbe and Bouman, 1978). Sleep restricted subjects displayed a decreased insulin response and an attenuated clearance of glucose from the blood in response to an IVGTT. The authors interpreted these results as a decrease in glucose tolerance (i.e., glucose intolerance) and concluded that sleep restriction has a harmful impact

on metabolism. It was suggested that the sleep restriction-induced decrease in glucose tolerance might reflect a first step towards the development of type 2 diabetes. A follow-up study showed that several consecutive nights of reduced deep sleep without changes in total sleep time resulted in reduced insulin sensitivity and glucose intolerance (Tasali et al., 2008). A more recent study demonstrated that only one night of sleep restriction (4h of sleep allowance) is sufficient to cause moderate insulin resistance as measured by a hyperinsulineamic euglycemic clamp (Donga et al. 2010).

Regulatory hormones such as the orexigenic hormone ghrelin and the adiposity modulators leptin and insulin have a clear influence on food intake, fat mass, and body weight (review: Spiegelman and Flier, 2001). For that reason, several studies have assessed the effects of sleep restriction on feelings of hunger as well as on these regulatory hormones. A few days of sleep restriction lead to increased feelings of hunger accompanied by a decrease in leptin and an increase in ghrelin (Spiegel et al., 2004a;Spiegel et al., 2004b;Taheri et al., 2004). In contrast, another study with a similar degree of sleep restriction failed to reproduce these results, showing increased concentrations of leptin with no changes in feelings of hunger (van Leeuwen et al., 2010). The reason for this inconsistency is unclear, but it might be explained by differences in the length of the sleep restriction period, the amount of activity allowed and/or differences in energy intake.

The finding that sleep restriction has direct effects on glucose tolerance, insulin sensitivity and feelings of hunger is in agreement with epidemiological data suggesting that sleep restriction may serve as a risk factor for developing obesity and type 2 diabetes. However, it is not known how persistent the metabolic changes in these experimental studies are. It is important to investigate whether these metabolic changes would really lead to metabolic dysfunction in the long run, in case restricted sleep would truly become a chronic condition. Experimental studies in animals are of importance here. Animal models allow us to investigate the underlying mechanisms of sleep restriction induced changes in metabolic regulation both during acute and prolonged sleep restriction.

Sleep restriction and metabolism in rats

Experimental studies in rats have shown that sleep restriction leads to changes in food intake, body weight and regulating hormones such as insulin and leptin. In almost all studies, sleep deprivation led to an attenuation of weight gain, often associated with increased food intake (Everson and Crowley, 2004;Hipolide et al., 2006;Koban and Stewart, 2006;Koban et al., 2008). In rats, the adiposity modulators leptin (Everson and Crowley, 2004;Koban and Swinson, 2005) and insulin (Hipolide et al., 2006) generally decrease during sleep deprivation, which may be secondary to the attenuation of weight gain. Acute sleep deprivation does not affect body weight and leptin concentrations, but it does increase the concentration of the hunger hormone ghrelin (Bodosi et al., 2004).

While results from experimental rat studies are relatively consistent, the interpretation of these data remains difficult. For example, the reported reductions

in basal concentrations of insulin provide little information regarding insulin responses and glucose regulation under challenging conditions, i.e., in response to food intake. Additionally, none of these animal studies assessed glucose homeostasis by means of an IVGTT or a hyperinsulineamic euglycemic clamp. Also, while changes in metabolic hormones in animal studies appear to be consistent with data from human studies, the reduction in body weight found in rats has not been reported in humans. In fact, this finding is in contrast to the proposed role of short sleep in obesity as based on epidemiological studies. One should realize that the data on sleep restriction and metabolism in laboratory animals are derived from a wide variety of sleep restriction protocols, from short and prolonged total sleep deprivation to selective REM sleep deprivation. Unfortunately, few studies so far assessed the metabolic consequences of chronic partial sleep deprivation or sleep disturbance as it often occurs in our society, which is therefore the specific aim of the current project.

Sleep restriction methods for rat studies

As mentioned before, there is a wide variety of methods available for sleep deprivation in animals, each of which may have its own specific effects and confounding factors (Rechtschaffen et al., 1999). Not all of these methods are suitable for studies on chronic sleep restriction and metabolism. A commonly used method for acute sleep deprivation is the gentle handling method. When used in our lab, animals are kept awake by a protocol consisting of tapping on the cage, gently shaking the cage and, if necessary, disturbing the nest (Hagewoud et al., 2010;Van der Borght et al., 2006). While this method is relatively mild and appears to produce little stress in the animals, it is only practical for sleep deprivation of short duration because it requires a direct and continuous involvement of the experimenter. For chronic purposes, the following methods are available.

The flower pot method or platform method is known for its ability to selectively deprive rats of rapid-eye-movement (REM) sleep for prolonged periods of time (Cohen and Dement, 1965;Mendelson et al., 1974). The method is based on the fact that REM sleep is characterized by a complete loss of muscle tone. Rats are placed on a small platform or upside-down flower pot, surrounded by water. As soon as the rat enters the REM sleep stage and loses muscle tone, it will touch or fall into the water and wake up. The movement restriction and risk of falling into the water makes this method rather stressful and may have immediate and non-specific effects on metabolism that could confound the result of our experiments.

Another method is the disk-over-water method, which is described by Rechtschaffen and colleagues (Rechtschaffen and Bergmann, 1995;Rechtschaffen and Bergmann, 2002). This method is based on continuous recording and online analysis of brain activity (electroencephalogram, EEG) and neck muscle activity (electromyogram, EMG). Rats are housed on a horizontal disk above water. As soon as the EEG and EMG signals indicate the onset of sleep, the disk starts to rotate at a low speed which awakens the rat and forces it to walk to avoid being carried into the water. This method has proven to be highly effective for prolonged

sleep deprivation studies but requires a computer-driven sleep deprivation set up for each individual rat. It also has the disadvantage that all rats in each experiment need to be equipped with electrodes for recording EEG and EMG. Moreover, the risk of rats eventually falling in the water is again not preferable in the context of studies on sleep restriction and metabolism.

A slightly different but simpler method is the rotating drum method, which is also based on forced locomotion. This method was originally described by Borbely and colleagues (Borbely and Neuhauss, 1979;Borbely et al., 1984). With this method, rodents are kept awake by placing them in a slowly and continuously rotating drum. In our lab, the rotating drum method has been used extensively for studies in rats that were aimed at mimicking restricted sleep as it often occurs in our society. In these studies, rats are generally allowed 4h of sleep per day. During the remaining 20h, rats are placed in the rotating drums and are forced to maintain wakefulness. To control for the mild forced locomotion in this sleep restriction protocol, another group of rats is subjected to forced locomotion for 10 h in the drum rotating at double the speed of the experimental group. With this protocol, rats walk the same distance as sleep restricted rats, but have sufficient time to sleep (14h). This approach has previously been validated in the context of mood disorders. In a series of studies, it was shown that chronically restricted sleep leads to neurobiological and neuroendocrine changes that are similar to what has been reported for depressed patients, e.g., reduced serotonin 1A receptor sensitivity (Novati et al., 2008;Roman et al., 2005), altered hypothalamic-pituitary-adrenal axis regulation (Novati et al., 2008), and reduced hippocampal volume (Novati et al., 2011). Some of these changes did not occur after acute, short sleep deprivation but only developed gradually in the course of a prolonged period of sleep restriction (Novati et al., 2008;Roman et al., 2005). Moreover, some of the changes, such as the decrease in serotonin (5-HT) 1A sensitivity, proved to be rather long-lasting. Together these findings indicate that chronically restricted sleep may induce gradually developing and long-lasting consequences that have suggested implications for disease sensitivity. Therefore, sleep restriction induced by the rotating drum may serve as an appropriate approach for studies on the metabolic consequences of sleep loss as well.

Aim and scope of the thesis

Taken together, the data from experimental studies in humans and rodents do not yet provide a complete picture on the consequences of chronic sleep restriction for metabolic regulation. Moreover, most rat studies are based on acute or prolonged total sleep deprivation or on selective REM sleep deprivation. Few studies assessed the consequences of chronic partial sleep deprivation as it often occurs in our modern society. Therefore, the aim of this thesis is to provide a detailed assessment of metabolic regulation in rats under conditions of both acute and chronic sleep restriction.

In **Chapter 2** we investigated the effects of chronic sleep restriction on sleep-wake patterns, body weight, food intake, and regulatory hormones such as leptin

and insulin. Since sleep deprived or sleep restricted rats commonly show weight loss or attenuated weight gain despite an increase in food intake, we measured whether this attenuated weight gain might be the consequence of increased energy expenditure. Energy expenditure was measured during sleep restriction by means of the doubly labeled water method.

Although it is known that sleep deprivation may cause a decrease in basal concentrations of insulin, the interpretation of this result in terms of glucose regulation is met with difficulty. Changes in basal concentrations of insulin do not provide a clear picture of insulin responses and glucoses homeostasis under challenging conditions such as food intake. In **chapter 3** we investigated if chronic sleep restriction affects insulin responses and glucose homeostasis by means of a controlled IVGTT.

Since our rotating drum method for sleep deprivation involves forced locomotion, we performed an experiment to study whether metabolic changes following chronic sleep restriction might be mediated by increased activity or by sleep loss per se. We therefore investigated the effect of forced and voluntary running activity on insulin regulation and glucose homeostasis by means of an IVGTT, without disturbing the sleep-wake pattern or total sleep time (**chapter 4**).

We then asked the question whether metabolic changes following chronic sleep restriction are due to a critical reduction in sleep duration or due to disruption of the sleep-wake rhythm, i.e., a circadian disruption irrespective of sleep time. For that reason we assessed the effects of a shift work protocol by forcing rats to be active during their normal resting phase without restricting their sleep time and measured the effects on insulin regulation and glucose homeostasis (**chapter 5**).

The attenuation of weight gain in sleep deprived or sleep restricted rats is in conflict with the hypothesis that sleep loss may lead to obesity according to epidemiological correlations. This apparent difference with the human situation may be caused by the alternation between periods of sleep restriction and sleep allowance that often occur in real life. Therefore, in **chapter 6** we studied the metabolic consequences of a chronic sleep restriction protocol that modeled working weeks with restricted sleep time alternated with weekends of sleep allowance. We hypothesized that these weekends might not only allow the rats to recover from a weight deficit but that it could even lead to an overall weight gain.

Another explanation for this apparent difference in weight gain between rats and the human situation may be a difference in diet. Therefore we assessed in **chapter 7** the effects of a medium fat diet versus a standard chow diet during chronic sleep restriction on body weight, food intake and regulatory hormones such as leptin and insulin. In addition we studied the effects of both diets during sleep restriction on the serotonergic system in order to evaluate a potential connection with previous experiments performed in our laboratory, which demonstrated that chronic sleep restriction leads to reduced serotonin 1A receptor sensitivity. We hypothesized that metabolic consequences of sleep restriction underlie these neurobiological changes. Thus, a medium fat diet may, at least partially, prevent the attenuation of weight gain seen during sleep restriction and protect against the

desensitization of the serotonin 1A receptor seen previously after chronic sleep restriction.

Chapter 8 summarizes and discusses the main outcomes of our sleep restriction on glucose homeostasis data and how it relates to the literature, whereas **chapter 9** discusses all the data presented in this thesis.

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Metabolic consequences of chronic sleep restriction in rats: changes in body weight regulation and energy expenditure

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Abstract

Epidemiological studies have shown an association between short or disrupted sleep and an increased risk to develop obesity. In animal studies, however, sleep restriction leads to an attenuation of weight gain that cannot be explained by changes in energy intake. In the present study, we assessed whether the attenuated weight gain under conditions of restricted sleep is a consequence of an overall increase in energy expenditure. Adult male rats were subjected to a schedule of chronic sleep restriction (SR) for 8 days with a 4h window of unrestricted rest per day. Electroencephalogram and electromyogram recordings were performed to quantify the effect of the sleep restriction schedule on sleep-wake patterns. In a separate experiment, we measured sleep restriction-induced changes in body weight, food intake, and regulatory hormones such as glucose, insulin, leptin and corticosterone. To investigate whether a change in energy expenditure underlies the attenuation of weight gain, energy expenditure was measured by the doubly labeled water method from day 5 until day 8 of the SR protocol. Results show a clear attenuation of weight gain during sleep restriction but no change in food intake. Basal plasma glucose, insulin and leptin levels are decreased after sleep restriction which presumably reflects the nutritional status of the rats. The daily energy expenditure during SR was significantly increased compared to control rats. Together, we conclude that the attenuation of body weight gain in sleep restricted rats is explained by an overall increase in energy expenditure together with an unaltered energy intake.

Introduction

A substantial number of studies have demonstrated a correlation between short sleep and increased prevalence of obesity ((Bjorvatn et al., 2007;Chaput et al., 2006;Chaput et al., 2009;Gangwisch et al., 2005;Hasler et al., 2004), for an overview see (Cappuccio et al., 2008)). While these studies form an important basis for the hypothesis that restricted sleep may contribute to metabolic diseases, they do not provide information on the cause and consequence in this relationship (Cizza et al., 2005;Marshall et al., 2008). To determine the relationship between insufficient sleep and altered metabolic regulation, controlled studies with experimental sleep restriction are required.

The present study aimed to assess the effects of chronic sleep restriction on energy metabolism in rats. For this purpose, we used a well-established rotating drum system to keep rats awake. Previously, we reported that chronic sleep restriction induced by this rotating drum method leads to gradual and, in some cases, persistent changes in a variety of neurobiological systems (e.g., serotonergic signaling:(Roman et al., 2005;Roman et al., 2006)), neuroendocrine regulation (e.g., hypothalamus-pituitary-adrenal (HPA-) axis regulation: (Meerlo et al., 2002;Novati et al., 2008)), and physiological processes (e.g., glucose homeostasis: (Barf et al., 2010)). We now investigated the effect of sleep restriction on specific metabolic parameters, including body weight, food intake and circulating regulatory hormones, in particular insulin, leptin and corticosterone. Leptin and insulin are known adiposity signals that regulate both food intake and body weight (for reviews see: Woods and Seeley, 2000;Woods and D'Alessio, 2008). Corticosterone levels reflect HPA-axis activity, which may alter metabolic function and regulation of metabolic hormones, as seen during stress (for reviews see: Pecoraro et al., 2006;Sapolsky et al., 2000).

We particularly focused on changes in energy expenditure during sleep restriction. It has been shown that experimentally disturbed sleep in rats leads to an attenuation of weight gain, despite normal (Barf et al., 2010) or increased food intake (Everson and Crowley, 2004;Hipolide et al., 2006;Koban and Stewart, 2006;Koban et al., 2008;Rechtschaffen and Bergmann, 1995;Rechtschaffen and Bergmann, 2002). An increase in energy expenditure might explain this, since being awake and active costs more energy than being asleep (Brebbia and Altshuler, 1965;Ryan et al., 1989). Spending a larger part of the day awake may therefore increase overall energy expenditure.

To assess the effects of sleep restriction on different aspects of energy balance, we exposed male rats to sleep restriction for 8 days. Body weight and food intake were measured daily and at the end of the sleep restriction protocol and after a recovery period of 5 days blood samples were taken to determine blood glucose and plasma insulin, leptin and corticosterone levels. Energy expenditure during sleep restriction was studied by the doubly labeled water method. To quantify the effect of sleep restriction on sleep-wake patterns, measurements of sleep electroencephalograms (EEG) and electromyograms (EMG) were performed.

Methods

Animals and housing

All experiments were performed in adult male Wistar rats (Harlan Netherlands BV, Horst, The Netherlands) weighing approximately 320 g at the start of the experiment. The rats were individually housed in Plexiglas cages in a climate-controlled room (21 °C ± 1) under a 12:12 h light-dark cycle (lights on at 10:00 am). Rats had unrestricted access to water and were maintained *ad lib* on medium fat food (45 % fat diet; Arie Blok Diervoeding B.V., Woerden, The Netherlands). Food intake and body weights were measured daily. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Chronic sleep restriction

Rats were subjected to chronic sleep restriction (SR) according to a previously published method (Meerlo et al., 2002). The rats were allowed to sleep in their home cage for 4 hours per day at the beginning of the light phase, i.e., their normal resting phase. During the remaining 20 hours, they were kept awake by placing them in drums rotating at a constant speed of 0.4 m/min (Barf et al., 2010;Novati et al., 2008;Roman et al., 2005). Rats were subjected to this schedule of sleep restriction for 8 days during which they had unlimited access to food and water inside the drums. All rats were habituated to the experimental conditions by placing them in the drums for 1-2 hours on 3 consecutive days before the onset of the sleep restriction protocol. Control rats (Control) were housed in the same room but were left undisturbed in their home cage throughout the experiment. During the recovery period afterwards, all rats were left undisturbed in their home cage.

Experiment 1: Sleep-wake patterns and sleep EEG

In experiment 1, we assessed the actual sleep loss during the chronic sleep restriction protocol by measuring sleep-wake patterns and sleep EEG. To be able to record EEG and EMG inside the rotating drums, we used a wireless datalogger system mounted on the head of the animals (NeuroLogger mobile system, TSE, Homburg, Germany).

The NeuroLogger head plug with electrodes for recordings of EEG and EMG was fixed to the skull under general isoflurane anesthesia (2%). Holes were drilled in the skull and 3 brass screws served as electrodes for epidural EEG (one 2.0 mm lateral of *sutura sagittalis*, 1.5 mm rostral of lambda and one 2.0 mm rostral of the measurement electrode on the right side) and a reference electrode (2.0 mm mediocaudal of lambda). For placement of EMG electrodes, the neck muscle was pierced twice with a 21-gauge needle, approximately 2mm apart. Electrodes were then guided through these perforations and fixed into place using non-absorbable wire. Afterwards electrodes and head plug were covered with a layer of dental cement. A “dummy”, in size and weight comparable to the NeuroLogger, was attached to the head holder. After recovery from anesthesia the rat was placed back in its home cage. For postoperative care, rats received a single subcutaneous injection of finadyne (1.0 mg/kg). Rats were allowed to recover for at least 10 days before the start of the experiments.

Specialized software (CommSW, Newbehavior, Zurich, Switserland) was used to configure and start the NeuroLogger. EEG and EMG signals were sampled at 200 Hz and directly stored on a built-in 512mb data storage. The data was first saved in a hexadecimal format. These files were then transformed to text files using a MatLab routine and were further analyzed using SleepSign® for animals (KISSEI COMTEC, Nagano, Japan). At first, an automatic scoring took place using the wave form recognition and logic setup algorithm of the screening module of SleepSign®. Each file and epoch was then checked visually, and if necessary, corrected by an experienced observer. On the basis of this scoring, time spent in each vigilance state was calculated. In addition, the signals were subjected to spectral analysis by Fast Fourier Transformation (SleepSign®). For all NREM sleep epochs, the EEG power in the 1-4 Hz delta range was calculated as an indicator of sleep intensity. To correct for inter-individual differences in strength of the EEG signal, the delta power values were normalized by expressing them as a percentage of each rats' own average 24 hour baseline delta power. The normalized EEG delta power is referred to as slow wave activity (SWA).

In this study, EEG and EMG measurements were done for baseline and day 1 of sleep restriction as well as day 8 of sleep restriction and the first day of recovery. In this experiment, sleep restricted rats (SR: n=4) served as their own controls.

Experiment 2: Plasma hormone levels

Experiment 2 established the effects of chronic sleep restriction on body weight, food intake and baseline circulating levels of blood glucose and plasma insulin, leptin and corticosterone. All rats in this experiment were equipped with a chronic jugular vein catheter allowing repeated and stress free blood sampling according to a previously described method (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 where it was fixed with dental cement and could be used to connect the rats to sampling tubes. Rats were allowed to recover for at least 10 days before the start of the experiment. Rats were then divided over two groups: a sleep restricted group (SR: n=11) and a home cage control group (Control: n=7). SR rats spent the first 4h of the light phase in their regular home cages, where after they were transferred to the rotating drums. Blood samples were taken after 8 days of SR/Control (8d experiment) and after 5 days of recovery (5d recovery) during the fourth hour of the light period (ZT 4), at the end of the daily 4h sleep window. In case of blood sampling, food was removed at ZT0.

Blood samples (500 µL) were collected in tubes with EDTA (20 µL/ml blood) on ice. About 50 µL of fresh blood was immediately stored at □ 20 °C for later determination of blood glucose levels by Hoffman's ferrocyanide method. The remaining blood was centrifuged at 2600 g for 10 min and the plasma was then stored at □ 20 °C until further analysis. Plasma levels of insulin were measured by Millipore Rat Insulin Radioimmunoassay (Linco Research, St Charles, MO, USA), plasma levels of leptin were measured by Linco Research Rat

leptin Radioimmunoassay (Linco Research), and plasma levels of corticosterone were measured by ImmuChem 125I Corticosterone Radioimmunoassay (MP Biomedicals, Orangeburg, NY, USA).

Experiment 3: Energy balance

Experiment 3 aimed to assess energy expenditure during chronic sleep restriction. Rats were divided over 2 groups: sleep restriction (SR: n=8) and home cage controls (Control: n=8). Body weight and food intake (45% fat diet: 1g = 4.8 kCal) were measured daily. Measurement of energy expenditure during sleep restriction was achieved by the doubly labeled water method as described previously (Speakman, 1998). Energy expenditure was measured over a 3-day period, from day 5 until day 8 of the sleep restriction protocol. In brief, at day 5 of the sleep restriction protocol, an intraperitoneal injection of a mixture of $^{2}H_{2}{}^{16}O$ (mixture enrichment of ^{2}H = 33.32% atom%) and $^{1}H_{2}{}^{18}O$ (mixture enrichment of ^{18}O = 65.62 atom%) was administered. The syringes containing the mixture were weighed to 0.1 mg before and after injection to obtain a dose mass. Following isotope injection rats returned to their home cage to allow isotope equilibration with the rat's water pool. Two-and-a-half hour after injection an initial blood sample was drawn from the tail (Fluttert et al., 2000;Meerlo et al., 2002). At day 8 of the sleep restriction protocol, a second blood sample was taken at the same circadian time as the first blood sample. All samples were collected in 50 µl Vitrex pre-calibrated capillaries and were immediately flame-sealed and stored until analysis. Analysis of the blood samples was achieved by previously described methods (Speakman, 1998).

Data analysis

In experiment 1, we measured time spent in NREM sleep, REM sleep or Wake and NREM sleep EEG SWA during a baseline day, day 1 of SR, day 8 of SR and the first day of recovery. All parameters were compared to baseline by a Paired t-test. In experiment 2, body weight and food intake was measured daily during the sleep restriction protocol and recovery period afterwards. At day 8 of SR, regulatory metabolic hormones were measured. To test the effect of 8 days of sleep restriction and the effect of 5 days of recovery thereafter on body weight, data were subjected to analysis of variance (ANOVA) with repeated measures. To test for effects of sleep restriction on food intake and glucose, insulin, leptin and corticosterone levels, data were subjected to One Way ANOVA. In experiment 3, body weight and food intake were measured daily. Energy expenditure was measured by doubly labeled water during day 5 until day 8 of the experimental protocol. Energy balance was calculated by subtracting energy expenditure from energy intake. To test for effects of sleep restriction on delta body weight, data were subjected to repeated measures ANOVA. To test for the effects of sleep restriction on food intake, energy expenditure and energy balance, data were subjected to One Way ANOVA. For all three experiments, data in text and figures are expressed as averages ± SEM and $P<0.05$ was considered statistically significant.

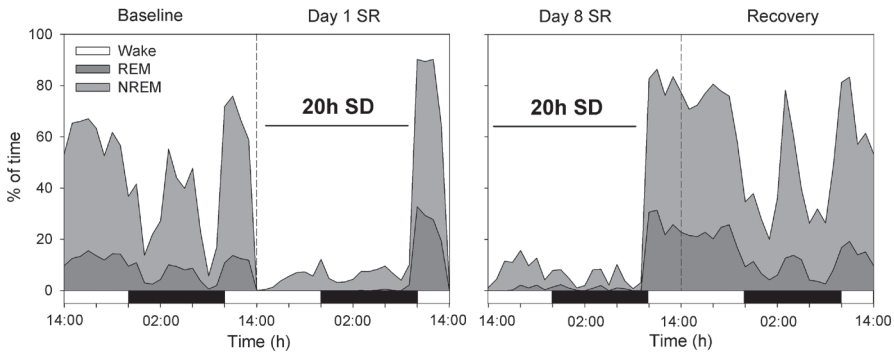

Figure 1: Average sleep-wake patterns under baseline conditions, during day 1 and day 8 of the sleep restriction protocol and on the first recovery day. Rats (n=4) were subjected to sleep deprivation (SD) for 20 h per day. The horizontal black-and-white bar at the bottom of the panels represent the light-dark cycle.

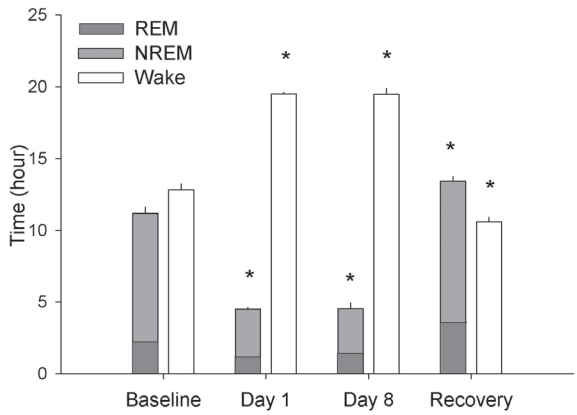

Figure 2: The total time spent asleep or awake during the baseline day, during day 1 and day 8 of SR protocol, and on the first recovery day. Time spent asleep is divided into NREM and REM sleep. Data are average values ± SEM (n=4). Statistics is done on total sleep time and total wake time. Asterisks indicate a significant difference in comparison to the baseline day (* $P<0.05$).

Table 1: Time (minutes) spent in NREM, REM or Wake.

	Baseline	Day 1	Day 8	Recovery
NREM (min)				
20h SR	411.8 ± 4.7	65.8 ± 9.9*	65.1 ± 13.6*	473.0 ± 9.2*
4h recovery	132.8 ± 12.1	142.1 ± 4.1	131.5 ± 3.7	130.8 ± 11.3
24h total	544.7 ± 16.7	207.9 ± 9.0*	196.6 ±17.3*	603.8 ±11.3
REM (min)				
20h SR	99.5 ± 6.9	2.1 ± 1.0*	9.2 ± 1.6*#	162.3 ± 12.0*
4h recovery	26.7 ± 3.8	60.2 ± 3.1*	65.7 ± 6.8*	39.1 ± 2.2
24h total	126.2 ± 10.2	62.4 ± 3.4*	74.9 ± 8.2*	201.4 ± 12.0*
Wake (min)				
20h SR	688.6 ± 10.3	1132.1 ± 9.9*	1125.7 ± 15.1*	564.7 ± 18.6*
4h recovery	80.5 ± 15.5	37.6 ± 6.7*	42.8 ± 10.4	70.1 ± 1.1
24h total	769.1 ± 25.3	1169.8 ± 7.4*	1168.8 ± 19.3*	634.8 ± 19.3*

Time spent in various sleep stages during the total 24h, 20h of sleep restriction and 4h window of sleep allowance (recovery) during day 1 and 8 of SR. The same time points were used for the baseline and the first recovery day. Data are average ± SEM. * P<0.05 compared to baseline, # P<0.01 compared to day1.

Results

Experiment 1: Sleep-wake patterns and sleep EEG

The average sleep-wake pattern is shown in Figure 1 for baseline, day 1 and day 8 of sleep restriction and the first day of recovery. The sleep restriction protocol reduced sleep time and increased overall waking time compared to baseline, both on the first and eighth day of the experiment (paired t-test: total sleep time day 1 SR: t=16.27, P<0.01; total sleep time day 8 SR: t=18.11, P<0.01; total wake time day 1 SR: t= -26.97, P<0.001; total wake time day 8 SR: t= -72.59, P<0.001; see Figure 2 and Table 1). NREM and REM sleep time were significantly decreased during first and eighth day of sleep restriction (total NREM sleep day 1 SR: t= 25.69, P<0.001; total NREM sleep day 8 SR: t= 34.42, P<0.01; total REM sleep day 1 SR: t= 15.94, p<0.01; total REM sleep day 8 SR: t= 11.85, P<0.01; see Table 1).

Although the sleep restriction procedure reduced NREM and REM sleep time, rats did have occasional micro sleeps in the rotating drum, which added up to approximately 1 hour of sleep during the daily 20h sleep deprivation phase, both on the first and eighth day of the experimental protocol (Table 1). Most of the sleep in the rotating drum consisted of NREM sleep, although sporadic REM sleep epochs occurred as well. In fact, the amount of REM sleep in the rotating drum significantly increased from day 1 to day 8 of the sleep restriction protocol (t= -7.65, P<0.05).

During the daily 4 hour sleep window, REM sleep time was significantly increased as compared to the same period under baseline conditions (day 1 SR: t= -20.96, P<0.001; day 8 SR: t= -10.73, P<0.01). NREM sleep time during this 4 hour window was not significantly changed compared to baseline conditions. However, the average NREM sleep EEG SWA during this 4h window was higher on sleep restriction days than during baseline. Due to the small sample size and variation, this did not reach statistical significance (SWA Baseline: 116.7 ± 1.1%, SWA Day 1 SR: 132.5 ± 5.1%, SWA Day 8 SR: 129.0 ± 10.2%: Paired t-test: Baseline vs. Day 1 SR: t= -3.06, P=0.055, Baseline vs. Day 8 SR: t= -1.17, P=0.36).

During the first day of recovery total sleep time was significantly increased (t=-4.99, P<0.05) and total wake time significantly decreased when compared to baseline (t= 5.00, P<0.05). Total REM sleep time was significantly increased (t=-29.13, P<0.01), but NREM sleep time was only increased during 20 h (t=-12.32, P<0.05) and not during total 24 h of recovery.

Experiment 2: Plasma hormone levels

Sleep restriction by forced locomotion significantly suppressed the weight gain that was seen in home cage control rats (Repeated Measures ANOVA: time x treatment interaction: F(18,306)=17.38, P<0.001; see Figure 3A). Upon termination of the sleep restriction protocol, weight gain seemed to normalize, but the overall increase in body weight remained significantly lower compared to controls even after 5 days of recovery. There were no significant differences in total food intake (Figure 3B).

The changes in blood glucose and plasma insulin, leptin and corticosterone levels after 8 days of sleep restriction and after a subsequent 5 day recovery period are shown in Figure 4. Blood glucose levels were significantly decreased after 8 days of SR (One way ANOVA: F(1,14)=8.16, P<0.05) but levels had returned to control levels after 5 days of recovery (One way ANOVA F(1,10)=0.88, P>0.5).

Insulin levels were decreased after 8 days of SR compared to controls (One way ANOVA: F(1,14)=17.42, P<0.01) and after 5 days of recovery they were no longer significant different compared to controls (One way ANOVA: F(1,9)=1.30, P>0.1). Plasma levels of leptin showed the same pattern. After 8 days of SR, leptin levels were significantly decreased compared to control rats (One way ANOVA: F(1,12)=11.63, P<0.01) and after 5 days of recovery they were no longer significant different compared to controls (One way ANOVA: F(1,12)=0.71, P>0.1). Corticosterone levels after 8 days of SR and 5 days of recovery did not differ between groups.

Experiment 3: Energy balance

Energy expenditure in SR rats, assessed by the doubly labeled water method from day 5 until day 8 of the sleep restriction protocol, was significantly higher compared to energy expenditure in home cage control rats (F(1,14)= 118.0, P<0.0001; see Figure 5). During the same 3-day period, food intake was not different between the groups, leading to a significant difference in energy balance (energy intake

minus energy expenditure: Control: 18.8 ± 2.5 kCal/day; SR: 0.1 ± 2.2 kCal/day: One Way ANOVA: $F(1,14)= 30.7$, $P<0.001$). In agreement with this, weight gain during this 3-day period was significantly lower in sleep restricted rats than control animals ($F(1,14)= 58.1$, $P<0.0001$; see Figure 5). Body weight over the 3-day period was significantly increased over time for control rats (Repeated Measures ANOVA: $F(1,7)= 63.57$, $P<0.001$) and significantly decreased over time for SR rats ($F(1,7)= 13.32$, $P<0.01$).

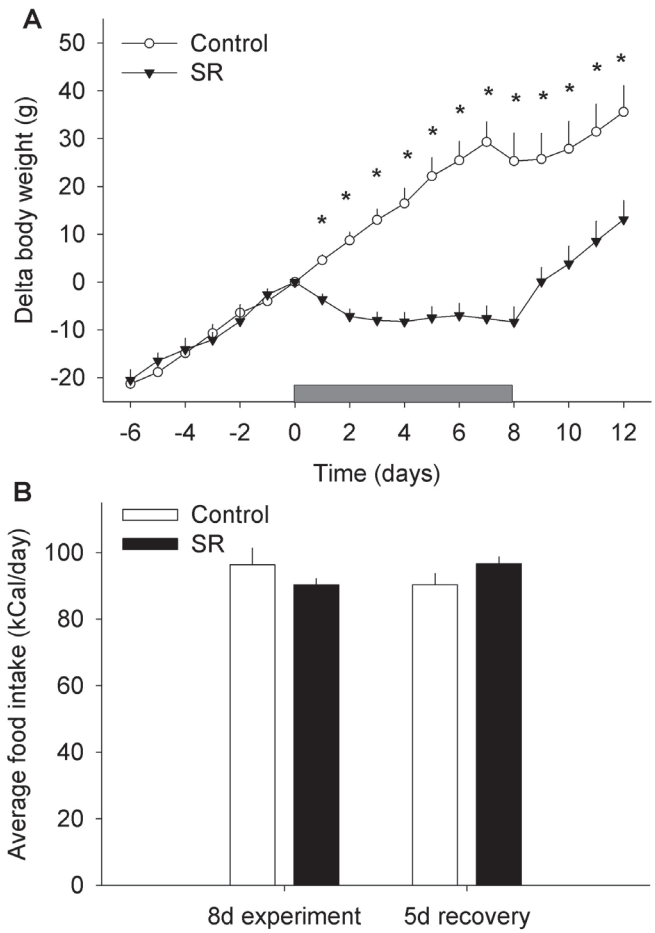

Figure 3: Daily body weight (A) and food intake (B) during the baseline, experimental and recovery phase of the experiment for sleep restricted rats (SR, n=11) and control rats (Control, n=7). The horizontal grey bar at the bottom of panel A represents the 8 day sleep restriction period. Data are average values ± SEM. Asterisks indicate a significant difference between SR and control rats (* $P<0.01$).

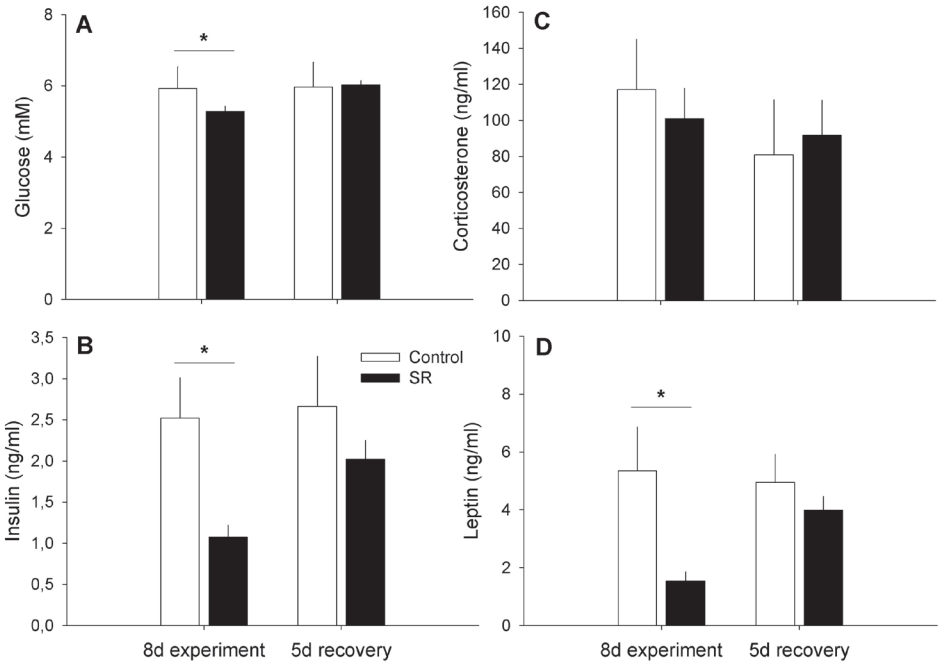

Figure 4: Plasma levels of glucose, insulin, corticosterone and leptin at the end of the 8d experiment and after 5d of recovery in sleep restricted rats (SR, n=11) and control rats (Control, n=7). Data are average values ± SEM. Asterisks indicate a significant difference (* P<0.05).

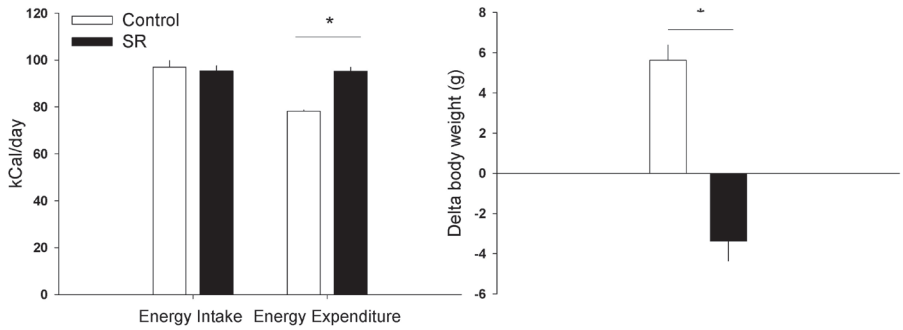

Figure 5: Daily energy expenditure and energy intake measured during the last 3 days of the 8-day sleep restriction protocol (left panel) and the change in body weight during the same 3d-period (right panel). Measurement of energy expenditure was performed by the doubly labeled water method and energy intake was calculated on the basis of food intake and the caloric value of the diet. n=8 for both groups. Data are average values ± SEM. Asterisks indicate a significant difference (* P<0.05).

Discussion

The slowly rotating drum method effectively restricted sleep time in rats. The reduction of total sleep time led to an attenuation of weight gain. During the sleep restriction period, energy intake in terms of food consumption was not affected while daily energy expenditure was significantly increased. This implies that the attenuation in weight gain during sleep restriction is caused by an increase in energy expenditure. Plasma levels of glucose, insulin and leptin were reduced, reflecting the nutritional status and attenuation of weight gain.

In the present study, chronic sleep restriction, achieved by the slowly rotating drum method, significantly reduced total sleep time. Total sleep decreased from 11 h on the baseline day to about 4.5 h on sleep restriction days. Most of this sleep occurred in the daily 4 h sleep window, but during the daily 20 h forced wakefulness periods, inside the rotating drums, rats had occasional micro sleeps as well. The latter only added up to approximately one hour of sleep. It cannot be excluded that sleep restricted rats experienced some additional sleep like processes, perhaps even locally in specific brain regions but this was not accounted for in our global analysis of vigilance states. A recent study in rats showed that under conditions of sleep deprivation, local clusters of cortical neurons may go offline while the rest of the brain stays awake (Vyazovskiy et al., 2011). However, it is unlikely that such local processes are sufficient to compensate for the substantial deficit of sleep that our rats experienced.

Weight gain was attenuated as a consequence of chronic sleep restriction. This attenuation appears to be a direct result of increased energy expenditure. In control rats, energy intake was higher than energy expenditure, resulting in weight gain. In sleep restricted rats, energy expenditure was increased compared to controls. One has to keep in mind that energy expenditure measurement by means of the doubly labeled water method is a relative underestimation (Kaiyala and Ramsay, 2011). This method does not take heat production and anaerobic phosphorylation into account. This may explain why SR rats lost some weight during the 3-day period when energy expenditure was measured, even though energy intake and energy expenditure seem to be in balance.

There may be multiple explanations for the increase in energy expenditure in sleep restricted rats. First, being awake costs more energy than being asleep (Brebbia and Altshuler, 1965;Ryan et al., 1989;Brebbia and Altshuler, 1965;Jung et al., 2011). During wakefulness and sleep deprivation, the activity of the sympathetic autonomic nervous system is higher than during sleep (for review, see Meerlo et al., 2008). As a consequence, body temperature (Bergmann et al., 1989;Bodosi et al., 2004;Everson et al., 1994;Sgoifo et al., 2006) and heart rate are increased (Everson et al., 1989;Spiegel et al., 2004). As a result, it is not surprising to see an increase in energy expenditure during prolonged wakefulness.

A second explanation for the increase in energy expenditure might be that our sleep deprivation procedure involves a certain degree of (forced) locomotion. However, our lab previously found that rats on both a forced or voluntary exercise protocol did not show an attenuation of body weight gain, even though

both groups of rats walked approximately 5500 m/day (Boersma et al, submitted). The latter is ten times as much as the distance walked by SR rats in the present study. Therefore, it is unlikely that the daily forced locomotion during sleep restriction is the only factor involved in the increase in energy expenditure and in turn the attenuation of weight gain.

A third explanation for the increase in energy expenditure during sleep restriction may be that the procedure causes stress in rats. Various studies have shown that uncontrollable stress leads to increased energy expenditure (Fuchs and Kleinknecht, 1986) together with an attenuation of weight gain (Meerlo et al., 1996). However, in our experiment, plasma levels of the stress hormone corticosterone were not changed after 8 days of SR, suggesting that forced locomotion as a sleep restriction method is not a major stressor.

It is intriguing that rats in our sleep restriction model do not increase their food intake, despite an increase in energy expenditure. Studies by other investigators have shown hyperphagia in sleep deprived rats, which may indicate an attempt to compensate for the increased energy use (Everson and Crowley, 2004;Galvao et al., 2009;Koban et al., 2008;Rechtschaffen and Bergmann, 1995). However, even in those studies, sleep deprived rats lost weight compared to controls. We showed that plasma insulin and leptin levels were decreased after 8 days of SR. These hormonal changes are likely a reflection of the nutritional state of the rats, as seen in literature (Benoit et al., 2004;Levin and Keesey, 1998). At the end of the 5 day recovery phase, body weight of SR rats remained significantly lower compared to controls, which still seemed to be reflected in leptin and insulin levels, even though these levels were no longer significantly different from controls. Interestingly, since leptin is a satiety signal (Woods and Seeley, 2000;Woods and D'Alessio, 2008), one might expect that a decrease in the levels of this hormone would lead to an increase in food intake. However, in the present study SR rats refrained from increasing their food intake, despite a decrease in leptin and insulin levels.

Since the decreased levels of leptin and insulin did not lead to increased food intake, it may be that these neuroendocrine signals are processed differently at the central level. Some sleep deprivation studies in literature show hyperphagia together with increased neuropeptide Y mRNA levels and orexin/hypocretins levels in the cerebrospinal fluid (Koban et al., 2006;Koban et al., 2008;Martins et al., 2010). Thus it may be that the increases in these neuropeptides, together with decreased levels of leptin and insulin, are necessary to induce hyperphagia. One can speculate that we did not see hyperphagia in our experiment due to the fact that these neuropeptides are not changed with this particular sleep restriction protocol. Future experiments should be performed to verify this.

In conclusion, eight days of sleep restriction leads to an attenuation of weight gain which is largely explained by an increase in energy expenditure. During sleep restriction, food intake is not changed despite a decrease in the

regulatory hormonal factors insulin and leptin. An explanation may be that sleep restriction disturbs the regulation of food intake at a more central level such that the decrease in plasma leptin and insulin is not sufficient to induce hyperphagia.

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Chronic sleep disturbance impairs glucose homeostasis in rats

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Abstract

Epidemiological studies have shown an association between short or disrupted sleep and an increased risk for metabolic disorders. To assess a possible causal relationship, we examined the effects of experimental sleep disturbance on glucose regulation in Wistar rats under controlled laboratory conditions. Three groups of animals were used: a sleep restriction group (RS), a group subjected to moderate sleep disturbance without restriction of sleep time (DS), and a home cage control group. To establish changes in glucose regulation, animals were subjected to intravenous glucose tolerance tests (IVGTT) before and after 1 or 8 days of sleep restriction or disturbance. Data show that both RS and DS reduce body weight without affecting food intake and also leads to hyperglycemia and decreased insulin levels during an IVGTT. Acute sleep disturbance also caused hyperglycemia during an IVGTT, yet, without affecting the insulin response. In conclusion, both moderate and severe disturbances of sleep markedly affects glucose homeostasis and body weight control.

Introduction

Sleep and metabolism seem to be related. Epidemiological studies have established a link between disturbed sleep and increased risk for the development of obesity and type 2 diabetes (Cappuccio et al., 2008;Chaput et al., 2006;Chaput et al., 2007;Gottlieb et al., 2005). These studies revealed that habitual short sleep is a risk factor, independent of classical risk factors such as BMI, food intake and reduced exercise [for reviews, see (Knutson et al., 2007;Penev, 2007;Spiegel et al., 2009)]. Whether or not these relationships are causal is still a matter of debate (Horne, 2008).

Experimental studies in both humans and animals have shown clear effects of sleep deprivation on body temperature, food intake, body weight gain and energy expenditure (Banks and Dinges, 2007;Nedeltcheva et al., 2009;Rechtschaffen and Bergmann, 1995;Vaara et al., 2009). Sleep deprivation also leads to changes in the activation of the sympathetic nervous system, to reduced levels of leptin and to increased levels of ghrelin in the general circulation (Taheri et al., 2004). Finally, a number of recent experimental studies suggest that even mild sleep disturbance leads to glucose intolerance, the first step in the development of type 2 Diabetes (Spiegel et al., 1999;Tasali et al., 2008).

While epidemiological studies mainly focused on mild but chronic sleep disturbances, laboratory studies mostly focus on the consequences of acute and short-lasting sleep deprivation. Frequent or chronic sleep disruption may gradually lead to changes in brain and body that are not noticeable after acute sleep deprivation (Meerlo et al., 2002;Novati et al., 2008;Roman et al., 2005). Yet, studies on metabolism and glucose regulation under conditions of mild but chronic sleep disturbance in a controlled experimental setting are scarce (Everson and Szabo, 2009;Spiegel et al., 1999). Therefore, in the current study we applied an animal model to investigate the effect of chronically disturbed sleep on glucose homeostasis. To this end, rats were subjected to a series of intravenous glucose tolerance tests (IVGTT) before and after a period of either moderate sleep disturbance or severe sleep restriction. To compare the effects of acute and chronic sleep disturbance, the experiment was performed after a period of 1 or 8 days of sleep disturbance.

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 °C ± 1) under a 12h: 12h light-dark cycle (lights on at 10:00 am). Animals were maintained ad lib on medium fat food (45% fat; Arie Blok Diervoeding B.V., Woerden, The Netherlands). Water was available ad lib throughout the study. Food intake and body weights were measured daily. Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Surgery

All animals were instrumented with chronic heart catheters bilaterally in the jugular vein (Steffens, 1969) allowing stress free blood sampling during an intravenous glucose tolerance test (IVGTT). Surgeries were carried out under general isoflurane (2%) anesthesia. Animals had at least 10 days to recover before the start of the experiments. Cannulas were checked every week for patency.

Sleep restriction and sleep disturbance

The animals were divided over three groups (see Figure 1): a sleep restricted group (restricted sleep: RS), a moderately sleep disturbed group (disturbed sleep, DS) and a home cage control group (controls). The sleep restricted animals (RS) were allowed to sleep in their home cage for only 4 hours per day at the beginning of the light phase. During the remaining 20 hours, the rats were kept awake by placing them in slowly rotating drums (diameter 40cm), rotating at a constant speed of 0.4 m/min (Novati et al., 2008;Roman et al., 2005). The animals of the sleep disturbed group (DS) were forced to walk in the rotating drums for a total of 10 hours/day with the aim to disturb their normal sleep-wake cycle without restricting their sleep time. The 10h of forced activity in this group was divided in 4 blocks of 2 or 3h with 3 or 4h of rest in between (Figure 1). The animals of the DS group walked at double speed (0.8 m/min) and therefore covered the same distance as the RS animals (0.48 km/day). For comparison, rats run approximately 2-3 km/day when allowed to run voluntarily (Scheurink et al., 1999). Both RS and DS animals spent the first 4h of the light phase in their regular home cages for IVGTT's and blood sampling. All animals were habituated to the experimental conditions by placing them in the drums for 1-2h for 3 consecutive days before the onset of the experiments. Control animals were left undisturbed in their home cage.

Intravenous glucose tolerance test and chemical analyses

To assess the effects of sleep restriction and/or sleep disturbance on glucose regulation, rats were subjected to a series of intravenous glucose tolerance tests (IVGTT). The IVGTT's were performed during the third and fourth hour of the light phase. Food was removed at lights on and rats were connected to the blood sampling and infusion tubes at least one hour before the IVGTT. During the IVGTT, a 15% glucose solution was infused for 30 minutes at a rate of 0.1 ml/min. The start of the infusion was designated time point t = 0 min. Blood samples (0.2 ml) for determination of blood glucose and plasma insulin levels were taken before, during and after the infusion of glucose at time points t = -10, -1, 5, 10, 15, 20, 25, 30, 35, 40 and 50 min. Note that the glucose infusion prevented any hypovolemic effect of the blood sampling. Blood samples were collected in EDTA (20µL/ml blood) containing tubes on ice. Blood was centrifuged at 2600g for 10 min and plasma was stored at □ 20 °C until analysis. Blood glucose levels were measured by Hoffman's ferrocyanide method and plasma levels of insulin were measured by Millepore Rat Insulin Radioimmunoassay (Linco Research, St Charles, MO, USA).

Experimental design

Two experiments were performed. Experiment 1 was designed to study glucose homeostasis under conditions where sleep was disrupted or restricted chronically. In this experiment, the animals were subjected to an IVGTT before (pre-experimental baseline) and after an 8-day period of sleep disturbance (RS or DS). Rats that remained in their home cage without any sleep disturbance served as controls. Experiment 2 served as a control experiment for the chronic sleep disturbance study and assessed the effects of acute sleep disturbance. In this second experiment a single IVGTT was performed after 1 day of sleep disturbance. In both experiments, blood samples were collected for measurement of glucose and insulin levels. In the second experiment an additional 0.1 ml blood sample was taken at t = -10 minutes for determination of plasma corticosterone levels (ImmuChem 125I Corticosterone Radioimmunoassay, MP Biomedicals, Orangeburg, NY, USA).

Statistical analysis

Data are expressed as averages ± SEM. Body weight is expressed as the change in weight relative to day 0 (the onset of sleep disturbance). The effects of RS and DS on food intake and body weight, as well as glucose and insulin responses to IVGTT were tested by comparing the experimental and control groups with each other and, in Experiment 1, with the pre-experimental baseline using repeated measures analysis of variance (ANOVA). When appropriate, a posthoc Tukey test was applied to establish differences between the three groups (controls, RS and DS). $P < 0.05$ was considered statistically significant.

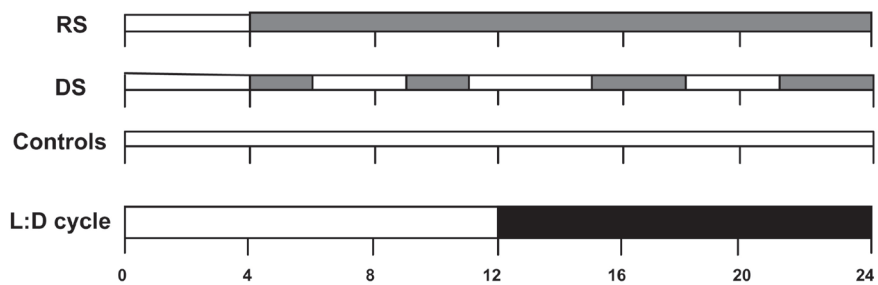

Figure 1: Schematic overview of the sleep disturbance protocols for the restricted sleep group (RS), disturbed sleep group (DS), and control group. For each treatment group, periods of wakefulness induced by forced locomotion are shown in light grey. The RS group was subjected daily to one consolidated block of 20h forced activity while the DS group was subjected to blocks of 2-3h forced activity interspersed by 3-4h blocks of rest. Control animals were left undisturbed.

Results

The average 24 hour food intake before, during and after the treatment for the different groups is shown in figure 2A. There were neither differences in food intake between the 3 groups, nor changes over time within the groups. Body weights are shown in figure 2B. The RS and DS animals were significantly lower in body weight than the home cage controls already after 2 days of sleep disturbance (Repeated Measures ANOVA: $F(36,486)=11.02$, $P<0.001$; posthoc Tukey Test: controls vs. RS $P<0.01$ and controls vs. DS $P<0.01$). There were no body weight differences between the RS and the DS animals.

Figure 3 depicts the glucose and insulin levels before, during and after the 30-min intravenous infusion of glucose, both under baseline (pre-experimental) conditions and after 8 days of sleep disturbance (RS and DS versus controls). In all groups, intravenous infusion of glucose led to an increase in both blood glucose and plasma insulin levels. After termination of the infusion, both glucose and insulin returned to pre-infusion levels. Eight days of sleep disturbance markedly changed the glucose and insulin responses to an IVGTT. Blood glucose levels were higher and plasma insulin levels were lower in the RS and DS animals compared to the pre-experimental IVGTT levels (Glucose RS: $F(10,140)=10.05$, $P<0.0001$; Glucose DS: $F(10,160)=9.64$, $P<0.0001$; Insulin RS: $F(10,150)=10.53$, $P<0.0001$; Insulin DS: $F(10,160)=8.97$, $P<0.0001$). Also in comparison to the home cage controls, glucose levels were higher and insulin levels were lower in both experimental groups (Glucose: $F(20,200)=3.37$, $P<0.0001$; posthoc Tukey Test: RS vs. controls $P<0.05$ and DS vs. controls $P<0.05$; Insulin: $F(20,190)=3.70$, $P<0.0001$; posthoc Tukey Test: RS vs. controls $P<0.01$ and DS vs. controls $P<0.05$). No differences were found between the RS and DS rats.

Figure 4 shows the glucose and insulin levels before, during and after the glucose infusion after a single day of sleep disturbance. In both the RS and DS animals, glucose levels were significantly higher than the levels in undisturbed home cage controls ($F(21,210)=12.49$, $P<0.0001$; posthoc Tukey Test: RS vs. controls $P<0.001$ and DS vs. controls $P<0.001$) There were no differences between the groups with regard to the plasma insulin response to an IVGTT after one day of sleep disturbance.

In Experiment 2, after 1 day of RS or DS, at time point t = -10 min immediately preceding the IVGTT, plasma levels of corticosterone were low and not different between the groups (RS: 1.4 ± 0.2 µg/dl, DS: 1.3 ± 0.1 µg/dl, controls: 2.4 ± 0.2 µg/dl).

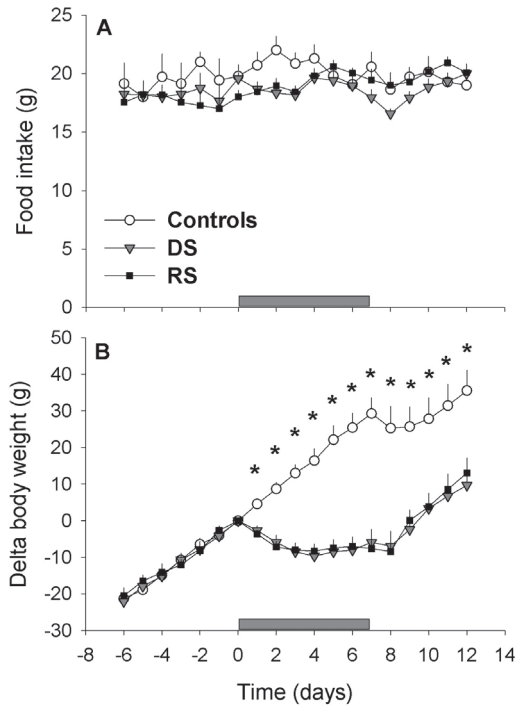

Figure 2: Average daily food intake (a) and body weight (b) in the baseline, experimental and recovery phase of the experiment for RS (n=11), DS (n=12) and control (n=7) animals. The horizontal grey bars at the bottom of the graphs represent the 8 day period of RS or DS. Data are average values ± SEM. Asterisks indicate a significant difference between sleep disturbed (DS and RS) and control animals (* P<0.01).

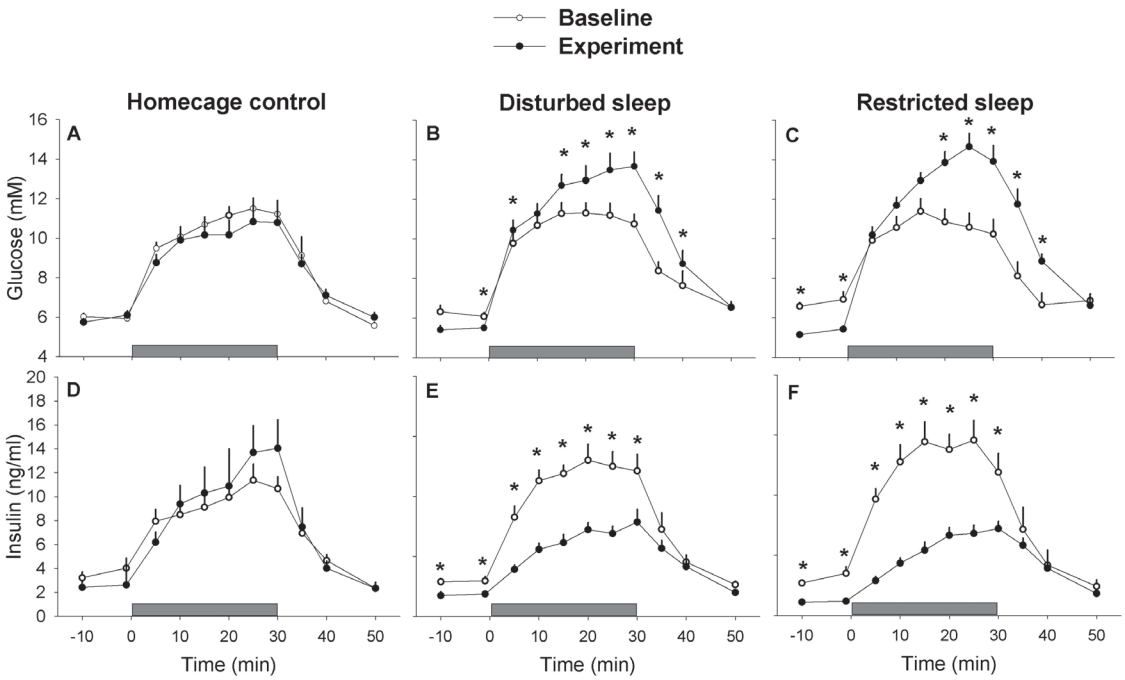

Figure 3: Blood glucose and plasma insulin levels in response to a 30-min intravenous glucose infusion after 8 days of sleep restriction (graphs C and F, n=11), sleep disturbance (graphs B and E, n=12) or control (graphs A and D, n=7). Each graph presents the glucose or insulin profiles under pre-experimental baseline conditions (Baseline: open circles) and after 8 days of sleep disturbance (Experiment: closed circles). The horizontal grey bars at the bottom of each graph represent the 30 min of 15% glucose infusion. Data are average values ± SEM. Asterisks indicate a significant difference between baseline and experimental conditions (* $P<0.05$).

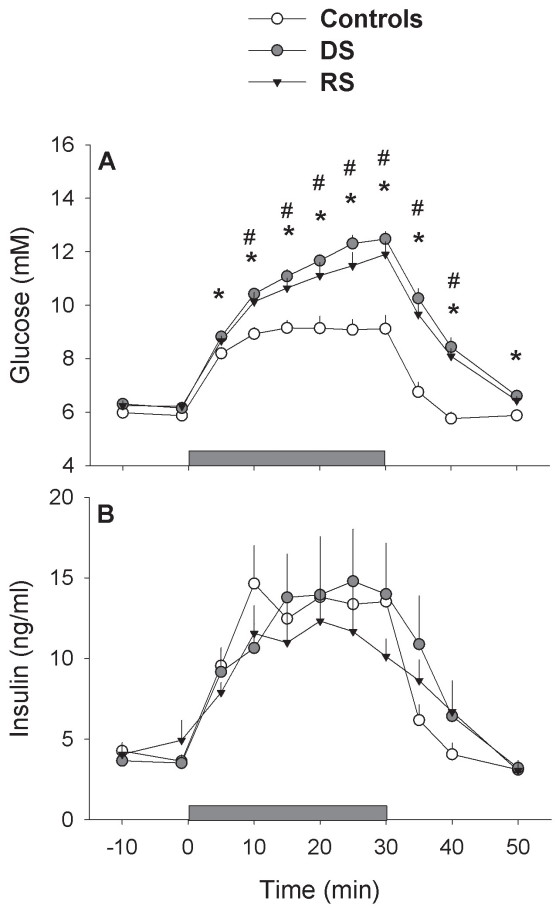

Figure 4: Blood glucose (a) and plasma insulin levels (b) in response to a 30-min intravenous glucose infusion after 1 day of sleep restriction (closed triangles, n=8), sleep disturbance (closed circles, n=8) or control (open circles, n=8). The horizontal grey bars at the bottom of the graphs represent the 30 min of 15% glucose infusion. Data are average values ± SEM. Asterisks indicate a significant difference between sleep restricted rats and controls (* $P<0.05$) and # indicates a significant difference between sleep disturbed rats and controls (# $P<0.05$).

Discussion

This study shows that eight days of sleep disturbance markedly interferes with body weight maintenance and glucose metabolism in rats. The main findings were: 1) chronic sleep disturbance reduces body weight without changes in food intake; 2) chronic sleep disturbance leads to hyperglycemia and a concomitant reduction in the insulin response to an IVGTT; 3) acute sleep disturbance also leads to hyperglycemia without changes in the insulin response to an IVGTT; 4) the metabolic effects of moderate sleep disturbance and more severe sleep restriction are remarkably similar.

The elevated glucose levels that occurred after both short and long term sleep disturbance confirm data from previous studies in humans in which was found that moderate sleep restriction or even suppression of sleep intensity without affecting sleep time may lead to glucose intolerance (Spiegel et al., 1999;Tasali et al., 2008). In our study, the increase in blood glucose during the IVGTT already occurred after one day of sleep disturbance. The data from the chronic sleep disturbance experiment might suggest that the elevated glucose levels are caused by a reduced insulin response. However, the finding of hyperglycemia without changes in plasma insulin response after acute sleep disturbance makes this explanation less likely. An alternative explanation might be that the hyperglycemia is caused by increased HPA-axis activity reflecting the stress of sleep disturbance. To test this possibility we measured plasma corticosterone levels in the sleep disturbed animals just prior to the infusion of glucose. Since corticosterone levels were not different between the groups, elevated HPA-axis activity can also not explain the hyperglycemia after sleep disturbance. Therefore, the reason for the hyperglycemia following both short and long term sleep disturbance remains unclear. Our current studies focus on the hypothesis that this hyperglycemia may be secondary to changes in hypothalamic orexin, a neuropeptide known to be involved in both the sleep/wake cycle and glucose metabolism (Sakurai, 2007;Tsujino and Sakurai, 2009;Yi et al., 2009). A number of recent studies suggest that REM sleep deprivation increases orexin immunoreactivity in the lateral hypothalamic area and orexin levels in the CSF, which may underlie some of the metabolic changes described after restricted or disrupted sleep (Galvao et al., 2009;Pedrazzoli et al., 2004).

Eight days of sleep disturbance caused a reduction in body weight together with a decrease in basal levels of glucose and insulin and a decrease in IVGTT levels of insulin. The literature suggests that the lower levels of glucose and insulin and the attenuated insulin response to the glucose tolerance test are most likely a direct consequence of the drop in body weight (Redman and Ravussin, 2009).

Surprisingly, the weight loss in our rats was not accompanied by a change in food intake, which may suggest that sleep disturbance leads to increased daily energy expenditure. The latter indeed is supported by data in the literature (Everson, 1995;Rechtschaffen and Bergmann, 1995). One cause of an increased energy expenditure in our protocol of sleep disturbance might be the forced locomotion in the rotating drums. However, one should note that in both the RS and DS condition the rats walked only 480 m/day, which is less than 20% of the

distance they would voluntarily run in a running wheel (Scheurink et al., 1999). Furthermore, although long term exercise may lead to improved insulin sensitivity and therefore reduced plasma insulin levels (Borghouts and Keizer, 2000), in rats, it does not lead to extensive weight loss and/or hyperglycemia (Donovan and Sumida, 1990). Therefore, the decrease in body weight and hyperglycemia in our study are not likely a result of the mild increase in activity involved in our sleep disturbance protocols.

The metabolic changes after sleep disturbance were similar in the RS and DS animals. This was unexpected because the degree of sleep restriction was markedly different between the two groups. The DS rats were subjected to a disruption of the normal sleep-wake cycle without restriction of their sleep time, whereas the RS rats were genuinely sleep restricted. Based on this observation, we speculate that the metabolic consequences of sleep curtailment are mainly related to the occurrence of frequent sleep interruptions and a disturbed sleep-wake cycle rather than sleep loss per se. In other words, it is the quality rather than the quantity of sleep that is important. Indeed, a recent study in humans found that suppression of sleep intensity without changes in total sleep time was sufficient to cause glucose intolerance and a decreased acute insulin response (Tasali et al., 2008). Patients suffering from obstructive sleep apnea (OSA) provide comparable evidence (Bandla and Gozal, 2000;Svanborg and Guilleminault, 1996). Total sleep time in OSA patients is not dramatically altered, still there are direct correlations between OSA and obesity, type 2 diabetes and cardiovascular diseases (Vgontzas et al., 2003). The opposite is true as well: modest weight gain or weight loss lead to a significant worsening or improvement, respectively, of sleep apnea in middle-aged individuals (Peppard et al., 2000;Pillar and Shehadeh, 2008). Thus, several lines of evidence together suggest that disturbed sleep by itself is sufficient to affect glucose homeostasis.

In conclusion, our data reveal that disturbance of the regular sleep-wake rhythm has a marked effect on glucose homeostasis and body weight control. Sleep disturbance directly leads to glucose intolerance and hyperglycemia and, on the long term, to weight loss accompanied with reduced insulin responses. The data further suggest that a disturbance of the normal sleep pattern, even without restriction of total sleep time, is sufficient to affect glucose metabolism and body weight maintenance.

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Forced and voluntary exercise counteracts insulin resistance in rats: role of coping style

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Submitted

Abstract

There are large individual differences in the success rate of exercise intervention programs aimed at the prevention and treatment of obesity related disorders. In the present study we tested the hypothesis that differences in coping style may have repercussion for the success rate of these intervention programs. To this end we tested the insulin responses before and after voluntary wheel running, via intravenous glucose tolerance tests (IVGTT), in both passive (insulin resistant) Roman Low Avoidance (RLA) and proactive (insulin sensitive) Roman High Avoidance (RHA) rats. To control for the potential difference between voluntary and forced exercise, we also included RLA and RHA rats that were subjected to forced-running. We found that 1) when given the opportunity to run voluntarily in a running wheel passive RLAs run more than proactive RHAs, 2) voluntary exercise leads to a normalization of the insulin response during an IVGTT in the RLA rats, and 3) there were no behavioral and physiological differences in efficacy between voluntary and forced running. We thus conclude that exercise, both forced and voluntary, is a successful lifestyle intervention for the treatment of hyperinsulinemia, especially in individuals with a passive coping style.

Introduction

Successful life style intervention may halt the ever increasing prevalence of metabolic disorders such as obesity, the metabolic syndrome and type 2 diabetes (Dunstan et al., 1997; Lindstrom et al., 2006). Exercise-based intervention programs are particularly successful (Vanninen et al., 1992; Torjesen et al., 1997), for a recent review see (McCall and Raj, 2009). Exercise reduces body adiposity, improves glucose tolerance and increases insulin sensitivity (DeFronzo et al., 1987; Ebeling et al., 1993; Hughes et al., 1993; Mayer-Davis et al., 1998; Afonso and Eikelboom, 2003; Alessio et al., 2005). There are, however, large individual differences in the success rate of exercise intervention programs (Teixeira et al., 2004), partly due to the large individual differences in the susceptibility for these metabolic disorders (recently reviewed in (Andreassi, 2009)). Our working hypothesis is that differences in coping style may explain the large individual variation in the success rate of exercise interventions programs in metabolic disorders such as type 2 Diabetes and the metabolic syndrome.

Differences in coping style are a wide-spread phenomenon in the animal kingdom (Careau et al. 2010; Wolf et al., 2008). This is, however, largely ignored in animal studies modeling the development of type 2 Diabetes, insulin resistance or the metabolic syndrome. In our most recent studies, we have filled in this gap using the Roman High and Low Avoidance rat selection lines (Boersma et al. 2009; Boersma et al. 2010; Boersma et al., 2011b). Rats from these selection lines are different at the level of several neuroendocrine, cardiovascular and metabolic parameters (Corda et al., 1997; Giorgi et al., 2003; Boersma et al., 2009), but do also differ in emotional reactivity and coping style. Roman Low Avoidance (RLA) rats are highly emotional individuals with a passive coping style, Roman High Avoidance (RHA) rats are (pro)active rats with low emotional reactivity. We recently demonstrated that the passive animals display, already at normal weight, several characteristics of the metabolic syndrome, such as insulin resistance, visceral adiposity and hypertension (Boersma et al. 2009; Boersma et al. 2010; Boersma et al. 2011a; Boersma et al., 2011b). We have extended and confirmed these findings in passive and proactive individuals in outbred wild-type Groningen rats, in which coping style appeared to predict changes in metabolic profiles analogous to those observed in the RHA/RLA rats (Boersma et al. 2010; Boersma et al. 2011b).

In the present study we particularly focused on the importance of differences in coping style on the potential beneficial effect of exercise on insulin resistance and visceral adiposity. To this end, we performed a series of experiments in which the insulin response during an intravenous glucose tolerance test (IVGTT) was measured in both passive (insulin resistant) RLA and proactive (insulin sensitive) RHA rats, under sedentary conditions and after 18 days of exercise in a running wheel.

In most animal studies, exercise consists of voluntary running in a wheel. The voluntary running in rats is used to mimic exercise programs in humans. However, these programs are, at least by a part of the participants perceived as unpleasant,

stressful and/or aversive. This means that there is a discrepancy between voluntary exercise in the rat model and forced exercise in the human. Therefore, to control for the potential difference between stressful and stress-free exercise, we also included two groups of RHA and RLA rats that were subjected to 18 days of forced running.

Materials and methods

Animals and housing

The experiments were approved by the local animal experimental welfare and care committee (DEC, Groningen, the Netherlands). Roman High (RHA) and Low (RLA) Avoidance rats, obtained from a breeding colony at the Clinical Psychopharmacology Unit (APSI) of the University of Geneva, were housed in a room controlled for temperature and humidity (20 ± 2 °C; 60%). The room was kept at a 12-12 hours light-dark cycle (lights on = CT0 at 01:00 hrs., lights off = CT12 at 13:00 hrs.). The rats were fed a standard lab chow diet (Hope Farms, Arie Blok Diervoeding, Woerden, NL; 3.7 kcal/g, 14 % fat). Food and water was available ad libitum.

Experimental design

The following experiments were performed: 1) Experiment 1 in which both RHA and RLA rats were submitted to an intravenous glucose tolerance test (IVGTT) at baseline and after 18 days of voluntary wheel running and 2) Experiment 2 in which both RHA and RLA rats were submitted to IVGTTs at baseline and after 18 days of forced wheel running. For both studies, the rats underwent surgery to place two indwelling jugular vein catheters for infusion and blood sampling (Steffens, 1967). Rats were accustomed to the infusion and blood sample procedures before the onset of the experiments (Steffens, 1969). The experiments started two weeks after surgery. Body weights and food take was measured daily throughout the experiment. The experimental design is summarized in figure 1.

Experiment 1

Twelve rats (6 RHA and 6 RLA) were housed in standard cages (24x24x32 cm). Two weeks after surgery, at day -14, a baseline IVGTT was performed. At day -10, the rats were transferred to standard running wheel cages (Nalgene polycarbonate running wheel cages [50-27-36 cm]) with free access to a running wheel (diameter 27cm, Mini Mitter, Oregon, USA). The rats were allowed to habituate to the wheel running for 10 days. During this habituation period running activity typically increases after which it stabilizes. After habituation, the rats were allowed to run voluntary for 18 days (intervention period: day 0 until day 18). A second IVGTT was performed on day 18. Four days later, the rats were sacrificed for carcass analysis.

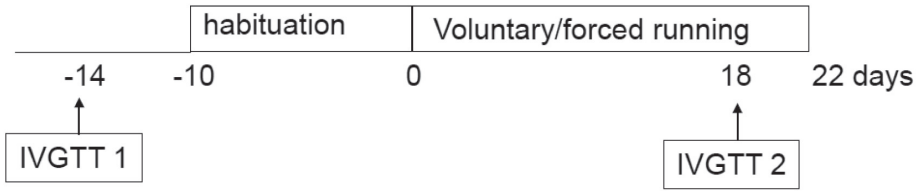

Figure 1: The experimental design for experiment 1 and experiment 2.

Experiment 2

Sixteen rats (8 RLA and 8 RHA) were housed in standard cages (24x24x32 cm). A baseline IVGTT was performed at day -14. At day -10, the rats were transferred to forced activity cages (TSE, Bad Homburg, Germany). These cages contain running wheels with a diameter of 25 centimeter that force the rat to run. Both running speed and time spent on running are controlled. All animals were forced to run in a schedule that mimicked the voluntary running activity patterns of the pro-active RHA rats that participated in Experiment 1 (see Figure 3B). Since we observed that rats are running in bouts of circa 5 minutes, we decided to force the animals to run in a schedule of 5 minutes running and 5 minutes rest. The speed (max 20 m/min) was adjusted so that the total distance per hour was similar to that of the RHA rats. The rats were accustomed to the forced running paradigm for 10 days (day -10 until day 0). Intensity and duration was slowly built up, in parallel to the increased running in the habituation period in the voluntary running animals in Experiment 1. Running only took place in the dark phase, and the running pattern mimicked the average hourly running activity patterns of the voluntary running RHA rats. Hereby the forced running rats, similar to voluntary running rats, had ample time to eat, sleep and drink, and effects of alterations in circadian rhythms were minimized. During the intervention the rats were forced to run 5000 m/day. The forced activity intervention period lasted from day 0 until day 18. A second IVGTT was performed on day 18. Four days later, the rats were sacrificed for carcass analysis.

Intravenous glucose tolerance test

At the day of an IVGTT, food was removed at the beginning of the light phase at CT 0. The IVGTTs were performed in the light phase, between CT 4 and CT 6. An IVGTT consisted of 30 minutes infusion of 15 mg glucose in 0.1 ml saline per minute (total 450 mg in 3 ml). Before the onset of the infusion, two baseline samples (0.2 ml) were taken at time points t = -11 and 1 minutes. The infusion of glucose was started at time point t = 0 minutes. Additional blood samples were taken at time points t = 5, 10, 15, 20, 25, 30, 35, 40, and 50 minutes. A total blood volume of 2.2 ml was taken. Blood samples were kept on ice and stored in files with 10 µl EDTA (0.09g/ml). For glucose determination 50 µl of full blood with

450 µl Heparin solution (2%) was stored at -20°C until analysis. Blood glucose levels were determined using the ferry-cyanide method (18) in a Technicon auto analyzer. The remaining blood was centrifuged for 15 minutes and plasma was stored for insulin and corticosterone determination. Plasma levels of insulin and corticosterone were measured with commercial radioimmunoassay (RIA) kits (Linco Research and.M P Biomedicals).

Carcass analysis

An extensive carcass analysis was performed 4 days after the last IVGTT. 3 hours before lights off, rats were sacrificed using an overdose of pentobarbital. Epididymal and retroperitoneal fat pads and the liver were taken out and weighed. The skin with the subcutaneous fat was removed from the carcass. The liver, skin, and carcasses were dried at 80 °C for 5 days. The fat content was determined by extracting the fat from the tissue using a petroleum based Soxlet fat extractor. After fat extraction the tissue was dried for 5 days again. The difference between dry tissue weight before and after fat extraction provides information on the fat content of the tissue.

Data analysis

Food intake and body weight data are presented as daily averages with standard error of the mean (SEM). Average running wheel activity was calculated as averages from day 0 until day 18 for each individual animal. Glucose and insulin levels are presented in group averages with standard error of mean. Statistical differences between groups were determined with repeated measures ANOVA followed by Tukey post-hoc test using coping style and type of intervention as between subjects factors and time of measurement as within subjects factor. The area under the curves (AUC) of the insulin responses were calculated and averaged. Percentage fat mass was calculated by dividing total dry fat mass by total dry lean body mass and multiplying this with 100%. Fat mass and weight of the different fat pads are presented as group averages with standard error of the mean. Differences in the area under the insulin response curve, and the body composition were statistically tested with one-way ANOVA followed by Tukey post hoc analysis using coping style and type of intervention as the between subject factors. All statistical analyses used a 5% confidence interval.

Results

Figure 2 displays body weight gain (A) and food intake (B) of the different groups during the intervention period from day 0-18. There were no differences in food intake or body weight gain among any of the groups. In all groups, food intake was higher during the intervention period when compared to the intake during the baseline period (baseline: 101 ± 4.8 kcal/day; intervention: 120 ± 5.6 kcal/day; $F(1,27)= 4.562$, $p<0.05$). Figure 3 displays the running activity of all groups. In Experiment 1, RLA rats ran significantly more than RHA rats ($F(1,15)= 9.332$, $p< 0.01$).

Blood glucose and plasma insulin levels are presented in Figure 4. There were no significant differences in blood glucose levels among the groups. Insulin responses were significantly different ($F(5,39)= 6.294$, $p<0.01$): 1) at baseline, when RLAs have much higher levels than RHAs ($p<0.01$), 2) in the RLAs, in which the baseline levels were much higher than those after 18 days of both voluntary and forced exercise (voluntary running $p<0.01$; forced running $p<0.01$) and 3) in the RHAs when baseline levels were higher than those after 18 days of voluntary but not forced exercise (voluntary running $p<0.05$; forced running $p=0.103$). There were no differences in plasma insulin responses between the voluntary runners and the forced runners, both under baseline conditions and after 18 days of exercise.

Corticosterone levels at the end of the light phase were not different between the forced and voluntary running rats under any circumstances (RLA baseline: 250 ± 35.3 ng/ml; RHA baseline: 225 ± 29.3 ng/ml; RLA voluntary running: 242 ± 29.7 ng/ml; RHA voluntary running: 226 ± 25.3 ng/ml; RLA forced running: 263 ± 29.7 ng/ml; RHA forced running: 233 ± 25.26 ng/ml). Baseline levels of corticosterone at circadian peak level tended to be higher in the passive RLAs when compared to the proactive RHAs, but this difference did not reach statistical significance.

Carcass analysis showed there were no differences in the percentage of body fat at the end of the study (RLA voluntary running: 35.9 ± 0.47 %; RHA voluntary running: 34.9 ± 0.54 %; RLA forced running: 33.3 ± 0.56 %; RHA forced running: 33.1 ± 0.62 %). The distribution of body fat was however different between the groups: passive RLAs have relatively more fat in the epididymal depot in comparison to proactive RHAs (RLA voluntary running: 4.42± 0.24 g; RHA voluntary running: 3.91 ± 0.21 g; RLA forced running: 5.8 ± 0.49 g; RHA forced running: 3.6 ± 0.26 g; ($F(3,25) = 6.426$ $p<0.05$)). There were no differences between RLAs and RHAs in the amount of fat distributed in the retroperitoneal fat depot (RLA voluntary running: 7.7 ± 0.77 g; RHA voluntary running: 7.1 ± 0.69 g; RLA forced running: 8.1 ± 0.76 g; RHA forced running: 7.6 ± 0.84g).

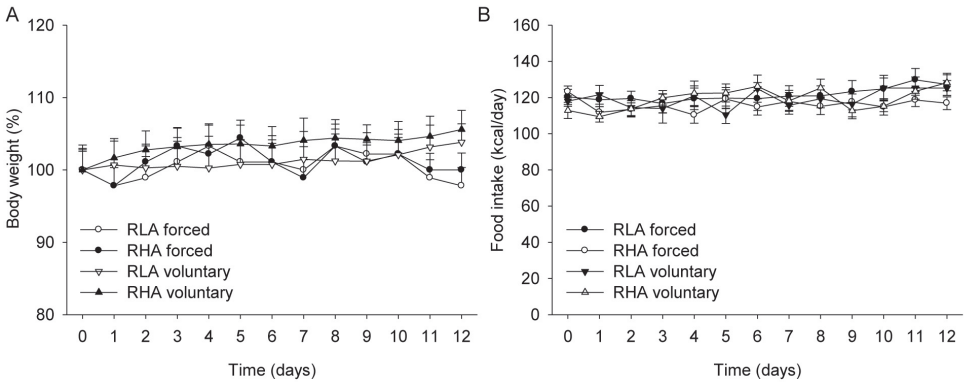

Figure 2: Body weight gain and food intake of passive and proactive rats that ran voluntarily or forced. Black circles = proactive forced runners (n=8), white circles = passive forced runners (n=8), black triangles = proactive voluntary runners (n=6), white triangles = passive voluntary runners (n=6).

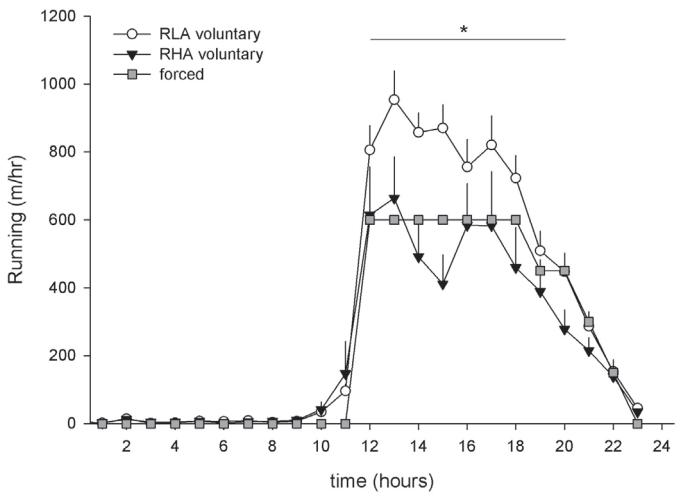

Figure 3: Running activity in experiments 1 and 2. White symbols = voluntary running RLA rats, Black symbols = voluntary running RHA rats, Grey symbols = forced running rats of both strains * indicates a significant difference between voluntary running RLAs and RHAs.

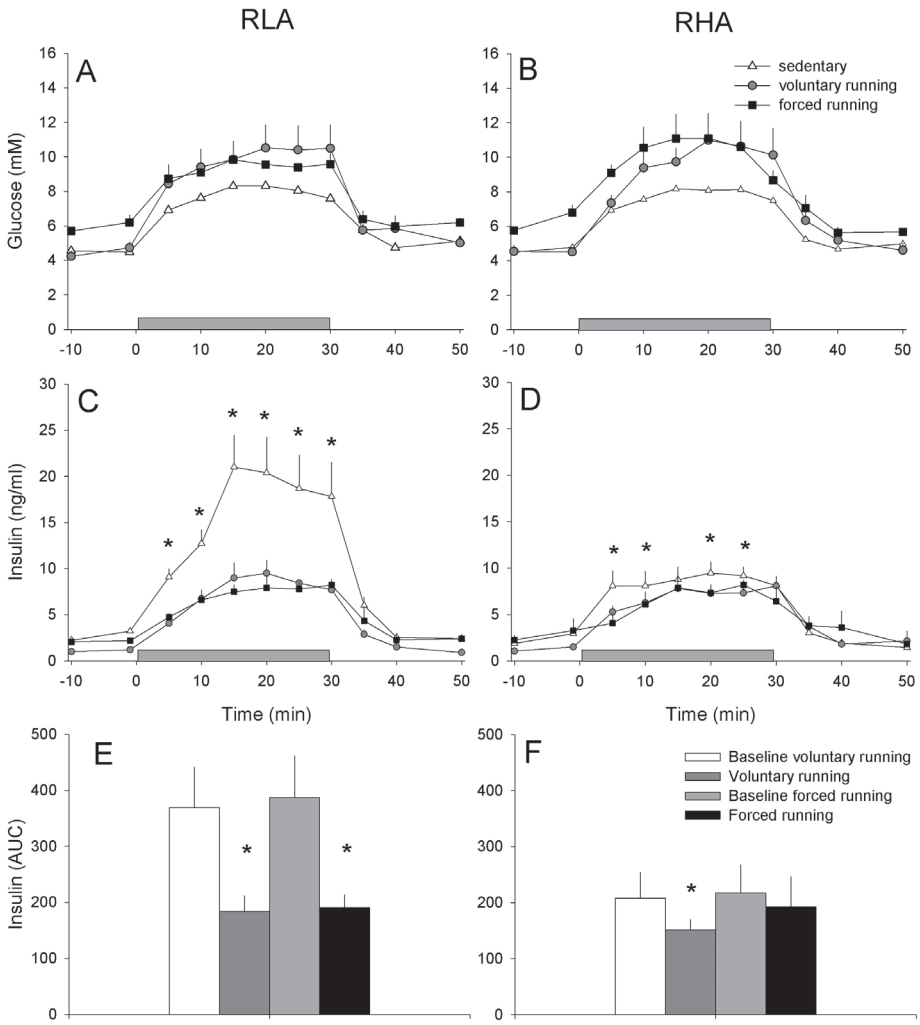

Figure 4: Blood glucose (A and B) and plasma insulin (C and D) levels before, during and after an IVGTT at baseline and after 18 days voluntary or forced running in both RLAs (A and C) and RHAs (B and D). Baseline values in experiment 1 and 2 are combined in one graph. Black triangles = baseline, light grey circles = voluntary runners, dark grey squares = forced runners. Area under the insulin curve at baseline and after 18 days of voluntary and forced running in RLA (E) and RHA (F) rats. Black bars = baseline voluntary runners, medium grey bars = 18 days voluntary running, dark grey bars = baseline forced runners, light grey bars = 18 days forced running. vr = voluntary running, fr = forced running. * indicates a significant difference between baseline conditions and both voluntary running and forced running conditions.

Discussion

The aim of the current study was to investigate the interaction between coping style and exercise in relation to the treatment of hyperinsulinemia. The major findings of this study were: 1) when given the opportunity to run voluntarily in a running wheel, passive RLAs run more than proactive RHAs, 2) voluntary exercise leads to normalization of the insulin response during an IVGTT in the RLA rats, and 3) there are no behavioral and physiological differences between voluntary and forced running.

Consistent with our previous studies, passive RLA rats displayed a much higher insulin response to an intravenous glucose tolerance test under baseline conditions when compared to the RHA rats (Boersma et al., 2009). Exercise completely normalized this elevated insulin response to control levels indicating that exercise is a successful life style intervention for the treatment of hyperinsulinemia, in particular in rats with a passive coping style.

The most interesting finding is the increased running activity in the passive RLA rats when they were allowed to run voluntarily. This is remarkable since these so-called passive rats are generally characterized as having lower locomotor activity. This 'passive' behavior was observed in several different experimental conditions such as the open field test, the Porsolt forced swim procedure and the elevated plus maze test (Ferre et al., 1995; Steimer and Driscoll, 2005; Smith and MacKenzie, 2006). However, these tests are all short term responses to unfamiliar conditions, whereas in our study we monitored the internal motivation to be active in a familiar environment.

Increased running in the RLAs resulted in the normalization of the insulin response during an IVGTT, which is a strong indication of improvement of insulin sensitivity. Such a phenomenon, i.e. increased spontaneous wheel running activity in metabolically deranged rodents has been reported before, among others in overweight animal models such as the OLEFT rat (Bi et al., 2005) and the MC4 knockout mouse (Haskell-Luevano et al., 2009). Both the OLEFT and the MC4 knockouts have an obese and insulin resistant phenotype under sedentary conditions, but compensate for this by increased activity when allowed to run spontaneously in wheels, leading to normalization of their body weight. In the present study, we observe that presumably insulin resistant rats show increased running to normalize their insulin sensitivity. Therefore it is tempting to speculate that the increased running may be considered as a behavioral strategy to compensate for the reduced insulin sensitivity in the sedentary state. Like insulin, muscular contractile activity causes glucose transporter type 4 (GLUT4) translocation and increases glucose uptake (Ploug and Ralston, 1998), hence exercise would benefit RLA rats more than RHA rats. Along these lines, it may be speculated that exercise has a larger impact on glucose availability to neuronal circuitry (Bequet et al., 2000) in RLA rats than in RHA rats, which might be a mechanisms by which RLA rats sustain a higher level of running wheel activity than RHA rats. Another implication of these results is that the sedentary state, at least in rodents, should not be considered as a proper control condition because

physical activity and health are inevitably linked (Booth et al., 2006). A point that is illustrated by the healthy insulin profiles in the voluntary running RLA rats.

We argued in the introduction section that the translational value of voluntary exercising animals might be limited, because humans subjected to exercise-based interventions may perceive it as a stressful workload. Our second study therefore investigated difference in the efficacy of forced and voluntary exercises in the RLA rats. We showed that both forced and voluntary running resulted in “normalized” insulin responses to an IVGTT in the RLA rats. This suggests that the exercise itself rather than the voluntary or forced nature of the running determines the beneficial effects of the wheel running on insulin sensitivity. In the current study, the amount of forced running was based on the average voluntary running activity of proactive RHA rats. Proactive rats were shown to run less voluntarily than passive rats. Since this forced running improved insulin signaling in the RLA rats, one may argue that the amount of running might not be crucial for the attenuation of hyperinsulinemia in the RLAs.

The current set-up was chosen to minimize the stress of the forced running paradigm, especially since it might be perceived differently in RLA and RHA rats. A difference in perception of the workload imposed on them might, however, prove important when studying exercise based lifestyle interventions. In humans it is argued that individuals with proactive personality traits have a lower perception of exertion and endure higher amounts of exercise than individuals with passive personality traits (Hassmen et al., 1993). Nevertheless, the observation that there are no behavioral and physiological differences between voluntary and forced running animals, strengthens the face validity of the voluntary rat model for translation to human studies. Finally, we may conclude that exercise, either forced or voluntary, may serve as a successful lifestyle intervention for the treatment of hyperinsulinemia, especially in rats with a passive coping style.

Acknowledgements

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Shift work in rats affects body weight and food intake, without changes in glucose homeostasis

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Abstract

Epidemiological and clinical studies suggest that working in shifts may lead to health problems and contribute to the development of obesity and type 2 diabetes. In support of this idea, an experimental study in rats showed that a simulated shift work protocol leads to loss of glucose rhythmicity and increased body weight. Based on these findings, we hypothesized that simulated shift work in rats will lead to glucose intolerance. Two groups of rats were included in this experiment. One group was subjected to a shift work protocol for 14 days. Rats were forced to be active for 8h per day during the light phase, i.e., their normal resting phase, which was achieved by placing them in slowly rotating drums. The second group was forced to be active for 8h during the dark phase, i.e., their normal active phase. All rats were subjected to an intravenous glucose tolerance test (IVGTT) before and after 14 days of (shift) work. Rats subjected to the shift work protocol showed a gradual increase in food intake during the forced waking period but immediately returned to their normal baseline pattern of food intake during the recovery period following shift work. Despite this shift in food intake, total food intake was not changed, body weight was slightly attenuated and no effect was found on glucose homeostasis. In conclusion, our current data do not support the hypothesis that shift work leads to disturbed metabolic regulation and glucose intolerance.

Introduction

Shiftwork is a common phenomenon in our modern industrialized societies. Several epidemiological and clinical studies suggest that working during the normal sleep phase on a regular basis is a risk factor for the development of various diseases including metabolic disorders such as obesity and type 2 diabetes (de Assis et al., 2003;Knutsson, 2003;Nagaya et al., 2002;van Amelsvoort et al., 1999). The negative metabolic consequences of shift work may be mediated by a variety of different factors, e.g., circadian disturbance, sleep disturbance, altered food intake and increased snacking behavior (for review see: (Akerstedt, 2003; Antunes et al., 2010;Atkinson et al., 2008;Boggild and Knutsson, 1999;Knutsson, 2004). Unfortunately, few experimental and well-controlled studies have been devoted to this topic.

A study in rats demonstrated that 8h of forced activity during the sleep phase shifted food intake from the normal activity phase to the forced activity phase (Salgado-Delgado et al., 2008). This was accompanied by an increase in body weight and loss of glucose rhythmicity. Since there were no changes in the expression profiles of the circadian clock proteins PER1 and PER2 in the suprachiasmatic nucleus (SCN), these observations indicate that in rats shift work leads to an uncoupling of metabolic function from the biological clock in the SCN.

Loss of glucose rhythmicity per se does not provide information on the effect of shift work on glucose homeostasis. An intravenous glucose tolerance test (IVGTT) is more informative in this respect, since an IVGTT is used to evaluate insulin response and glucose clearance after glucose infusion. It has been shown previously that an IVGTT is a successful tool to study glucose homeostasis (Strubbe and Bouman, 1978).

In the present study, we assessed the effect of shift work on food intake patterns, body weight and glucose homeostasis. The experiment included two groups of rats that were subjected to forced activity during different phases of the circadian cycle. The first group was forced to be active during the light (inactive) phase (shift work group). A second group was forced to be active during the dark (active) phase and was therefore not disturbed in their normal circadian organization (control work group). The rats were subjected to IVGTTs before and after a 2-week period of control work or shift work. We first assessed if shift work leads to a change in food intake pattern, similar to data of Salgado-Delgado and colleagues (Salgado-Delgado et al., 2008). Second, we hypothesized that shift work rats would have an impaired glucose tolerance.

Methods

Animals and housing

Adult male Wistar rats (Harlan Netherlands BV, Horst, The Netherlands) weighing 322.3 ± 1.6 g at start of the experiment were individually housed in Plexiglas cages in a climate-controlled room (21 °C ± 1) under a 12h:12h light-dark cycle. Rats were maintained ad lib on medium fat food (45% fat diet: Arie Blok Diervoeding B.V., Woerden, The Netherlands). Water was available *ad lib*

throughout the study. Food intake and body weights were measured daily. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Surgery

All rats underwent surgery to place two bilateral jugular vein catheters (Steffens, 1969). This allowed stress free glucose infusion and frequent blood sampling. Surgery was carried out under general isoflurane (2%) anesthesia. Rats were allowed to recover for at least 10 days prior the start of the experiments. Cannulas were checked and flushed at least once a week.

Shift work and Control work protocol

The rats were divided over two groups (Figure 1): a shift work group (n=7) and a control work group (n=8). Shift work rats were forced to be active for 8 hours during the light phase, i.e., their normal circadian resting phase. Control rats were forced to be active for 8h during the dark phase, i.e. the normal activity phase. Forced activity was accomplished by placing the rats in slowly rotating drums (diameter 40 cm), rotating at a constant speed of 0.4 m/min. Both experimental groups were subjected to this protocol for 14 consecutive days. 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 experiments.

Body weight and food intake

During the experimental period, body weight and food intake were measured daily. Food intake was measured both in the rotating drums (8h) and in the regular home cage (16h). In addition, on the baseline day and on day 13 of the experiment, food intake was measured during the 12h light and 12h dark phase.

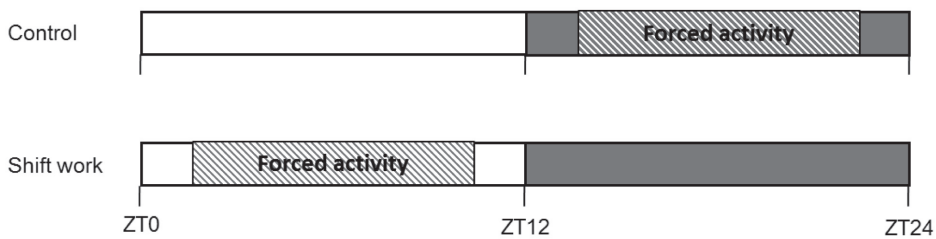

Figure 1: The experimental protocol for the control work and shift work rats. Dark grey bars represent the 12h dark phase of the 24h light-dark cycle. The hatched light grey boxes with indicate the 8 hours when rats are placed into the slowly rotating drums and forced to be active.

Intravenous glucose tolerance test and chemical analyses

To assess the effects of shift work on glucose regulation, rats were subjected to an intravenous glucose tolerance test (IVGTT) before (pre-experiment) and immediately after the experimental 14 day period (experiment). We decided to standardize the timing of the IVGTT at the end of the experiment relative to the work schedule and performed the test 17h after the last session of forced activity in the rotating drum for both the control work and shift work group. This means that the IVGTTs in the two treatment groups were performed at different circadian times. For shift work rats, IVGTTs were performed during the third and fourth hour of the light phase, whereas for control work rats IVGTTs were performed during the third and fourth hour of the dark phase. These circadian times were used for both the pre-experimental and experimental IVGTT. Food was removed 3 hours before the start of the IVGTT and 1 hour before the test rats were connected to the tubings for blood sampling and glucose infusion. During the IVGTT, a 15% glucose solution was infused for 30 minutes at a rate of 0.1 ml/min. Blood samples for determination of blood glucose and plasma insulin levels were taken before, during and after the infusion of glucose at time points t = -10, -1, 5, 10, 15, 20, 25, 30, 35, 40 and 50 min (t=0 was the start of the glucose infusion). All blood samples had a volume of 200 µl and were collected in EDTA (20 µL/ml blood) containing tubes on ice. The samples were centrifuged at 2600 g for 10 min and the plasma was stored at □ 20 °C until analysis. Blood glucose levels were measured by Hoffman's ferrocyanide method. Plasma levels of insulin were measured by Millipore Rat Insulin Radioimmunoassay and plasma levels of leptin were measured by Millipore Rat Leptin Radioimmunoassay (Linco Research, St. Charles, MO, USA). Plasma levels of corticosterone were measured by ImmuChem 125I Corticosterone Radioimmoassay (MP Biomedicals, Orangeburg, NY, USA).

Data analysis

All data in results section and figures are expressed as averages ± SEM. The effect of shift work on food intake and body weight was assessed by repeated measures analyses of variance (ANOVA). An effect of treatment on the percentage of food intake during the light and dark phase at baseline day (day 0) and experimental day 13 was analyzed with one way ANOVA. To test for an effect of shift work on glucose homeostasis, we compared the glucose and insulin profiles during the pre-experimental IVGTT with the profiles after the 2-week experiment by repeated measures ANOVA. Since circadian time per se might affect insulin regulation and glucose clearance, glucose and insulin responses to an IVGTT were also tested by comparing shift work with control rats. For all tests, $P<0.05$ was considered statistically significant.

Figure 2: Body weight gain in rats subjected to shift work (n=7) or control work (n=8). Data are shown for a baseline day (day 0), 14 experimental days and at the end of the recovery phase (day 21). Data are average values ± SEM. Asterisks indicate a significant difference between both groups (* P<0.05).

Results

Changes in body weight in the course of the experiment are shown in Figure 2. On average, during the 2-week work protocol from day 0 to 14, both the control work and shift work rats continued to gain weight (Repeated measures ANOVA; time effect: $F(14,182)= 14.67$, $P<0.001$). Although the shift work rats on average gained less weight than control work rats, this difference did not reach statistical significance (time * group interaction: $F(14,182)= 1.52$, $P=0.11$; group effect: $F(1,13)= 1.62$, $P=0.23$). However, at the end of the recovery week (day 21), the shift work rats had gained significantly less weight than control animals (One Way ANOVA: $F(1,13)= 9.20$, $P<0.05$).

Figure 3A represents total 24h food intake at baseline (day 0), during the experimental period (day 13) and after recovery (day 21). We specifically tested for group differences in overall food intake on day 13 and 21 to be at the end of the experimental protocol and recovery phase, when all rats presumably were adapted to the protocol and food intake was not confounded by the IVGTT on day 14. Overall, shift work rats ate slightly less than control work rats (Repeated measures ANOVA: group effect ($F(1.13)= 6.29$, $P<0.05$). However, there was no significant time effect or time x group interaction, suggesting these effects were independent of the shift work protocol per se.

Figure 3B shows the intake of food during the 8h sessions in the rotating drum for the same 3 days (day 0, 13 and 21). On all 3 days, shift work rats ate significant less compared to control rats (Repeated measures ANOVA: group effect ($F(1,13)= 67.36$,$P<0.001$), but there was no time x group interaction. ANOVA also revealed an overall time effect ($F(2,26)= 42.10$, $P<0.001$) and posthoc tests showed a significant increase in food intake at experimental day 13 compared to baseline. At recovery day 21, food intake had returned to baseline.

Figure 4 shows depicts food intake during the 8h in the rotating drums in more detail. In control rats, food intake during the daily 8h forced activity period did not change during the experimental days (nonlinear regression: R=0.16, One Way ANOVA: F(1,11)= 0.30, P>0.5). However, in shift work rats, food intake during the 8 hours in the drums gradually increased in the course of the 14 experimental days (nonlinear regression: R=0.90, One WayANOVA: F(1,11)= 47.40, P<0.0001).

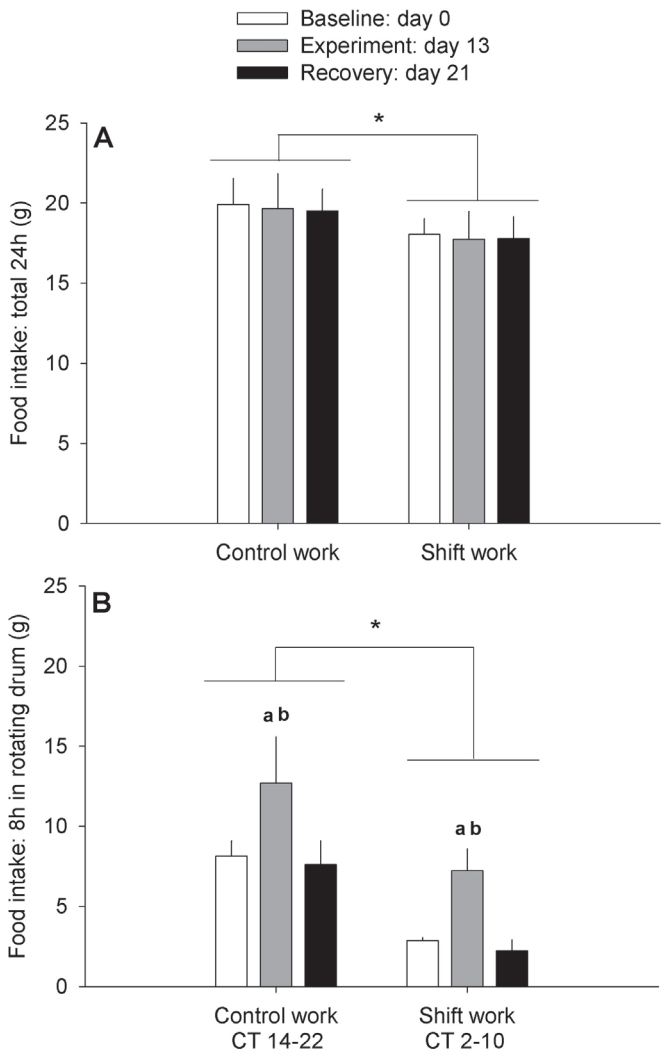

Figure 3: Effects of shift work on food intake. (A) Total 24h food intake for control work and shift work rats on baseline day 0, experimental day 13, and recovery day 21. (B) Food intake during the forced activity phase in the rotating drum (8h) at the same days. Data are averages ± SEM. Asterisks indicate a significant different between both groups, a: significant difference in comparison to baseline; b: significant difference in comparison to recovery day 21 (* P<0.05).

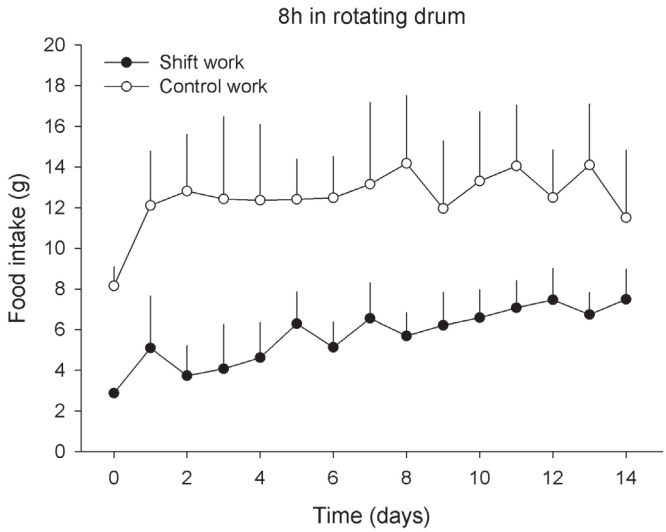

Figure 4: Food intake during the daily 8h of forced activity in the rotating drum for the shift work group (during light phase) and the control work group (during dark phase). Data are shown for a baseline day (day 0) and 14 experimental days. Data are average ± SEM. See text for details on statistics.

Percentage of food consumed during the total 12h light and 12h dark phase was calculated for baseline day 0 and experimental day 13 (Figure 5). During the baseline day, there was no difference between both groups. However, during the experimental day shift work rats had significantly higher food intake during the light phase (which included their 8h work phase), compared to control rats (One Way ANOVA: $F(1,13)= 32.93$, $P<0.001$) and compared to their own baseline ($F(1,12)= 15.80$, $P<0.01$). Control work rats on average showed a small increase in food intake during the dark phase of the experimental day (which included their 8h work phase) compared to the dark phase of their own baseline day, but this did not reach statistical significance ($F(1,14)= 3.99$, $P=0.07$).

Figure 6 depicts the glucose and insulin levels in response to an intravenous infusion of glucose, under pre-experimental baseline conditions and after 14 days of shift work or control work conditions. Intravenous infusion of glucose led to an increase in blood glucose and plasma insulin levels. After termination of the 30-min glucose infusion, both glucose and insulin returned to pre-infusion levels. Fourteen days of shift work or control work conditions did not change the glucose and insulin responses to an IVGTT within each group. Shift work seems to lead to a small decrease in insulin response, but this effect did not reach statistical significance (Area under the curve: One Way ANOVA: $F(1,12)= 2.20$, $P=0.16$).

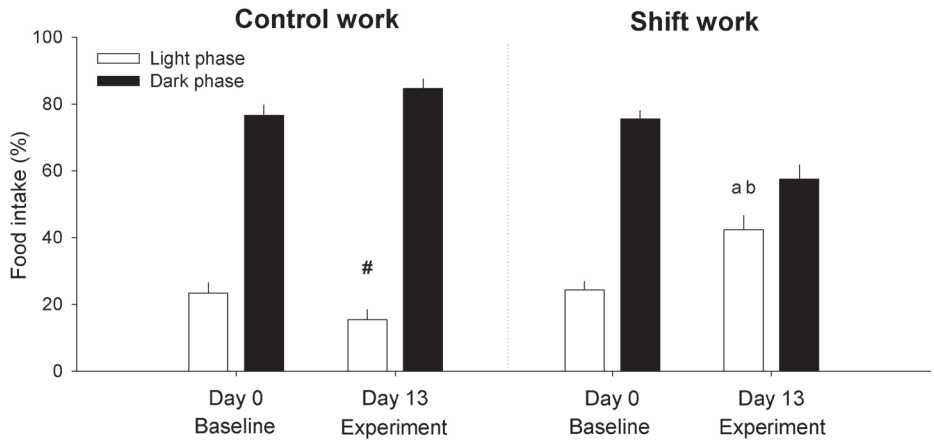

Figure 5: Percentage of food consumed during the light and dark phase on baseline day 0 and experimental day 13. For shift work rats, the 8h of activity was in the light phase, and for control work rats, the 8h of activity was in the dark phase. Data are averages ± SEM. a: significant difference in comparison to control rats, b: significant difference in comparison to baseline ($P<0.05$); # indicates a trend in comparison to baseline ($P=0.07$).

As explained in the methods section, the time of day when the IVGTT was performed was different for shift work and control work rats. This difference in time of day is associated with different glucose and insulin profiles. Even under baseline conditions, the two experimental groups differed in their glucose and insulin responses (Repeated measures ANOVA: time x group interaction effect for the pre-experimental glucose profile: $F(10,120)= 10.37$, $P<0.001$; pre-experimental insulin profile: $F(10,120)= 6.73$, $P<0.001$). This difference persisted during the experimental phase of the protocol (time x group interaction effect for the experimental glucose profile: $F(10,120)= 5.49$, $P<0.01$; experimental insulin profile: $F(10,120)= 3.79$, $P<0.01$).

Figure 7 depicts the area under the curve (AUC) of blood glucose and plasma insulin levels in response to the IVGTT. The AUC of blood glucose levels were significantly lower in shift work rats compared to control work rats during both the pre-experimental (One Way ANOVA: $F(1,12)=22.43$, $P<0.001$) and the experimental IVGTT ($F(1,12)= 10.26$, $P<0.01$). The AUC of plasma insulin levels were significantly higher in shift work rats compared to control work rats during the pre-experimental IVGTT ($F(1,12)= 15.85$, $P<0.01$) but this did not reach significance during the experimental IVGTT ($F(1,12)= 3.54$, $P=0.08$).

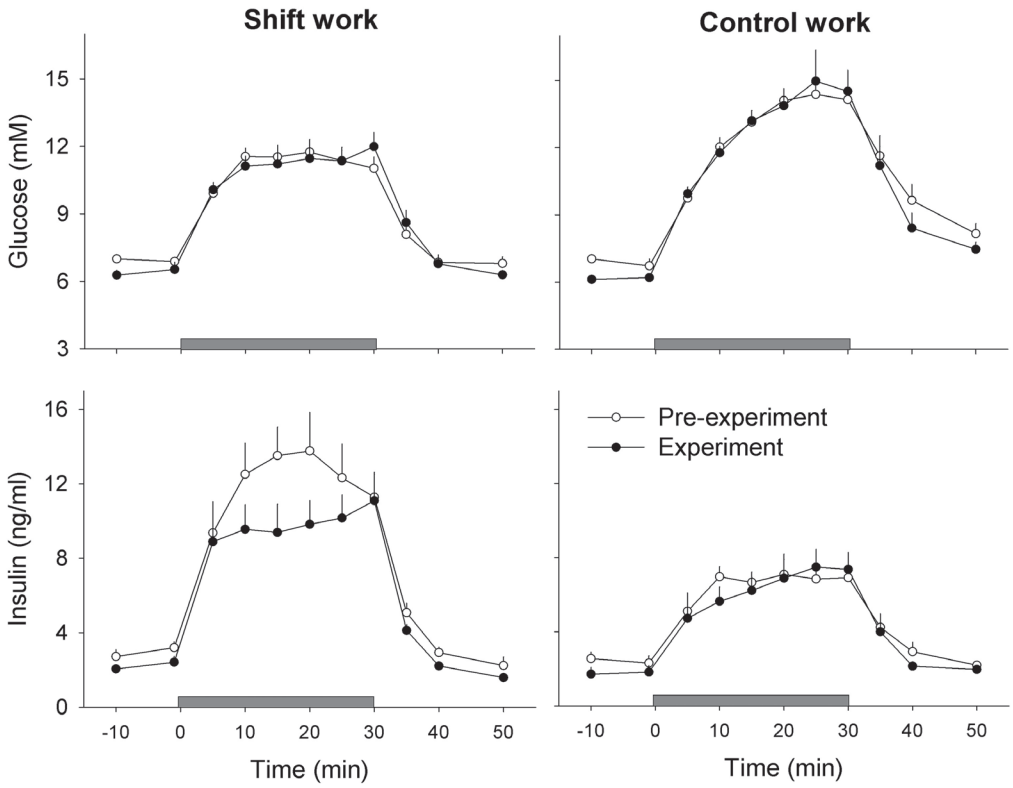

Figure 6: Blood glucose and plasma insulin levels in response to a 30-min intravenous glucose infusion under baseline conditions or after 14 days of shift work (graphs A and C, n=7) or control work (graphs B and D, n=8). Each graph presents the glucose or insulin profiles under pre-experimental baseline conditions (open circles) and after 14 days of shift work or control work (closed circles). The horizontal grey bars at the bottom of each graph represent the 30 min of 15% glucose infusion. Data are average values ± SEM.

Discussion

In this study we assessed the effects of a shift work protocol on food intake, body weight and glucose homeostasis in rats. Rats subjected to a 14-day shift work protocol with 8h of forced activity during their circadian resting phase displayed a gradual shift in food intake from their normal activity phase to their forced activity phase. Overall, total daily food intake did not differ between shift work rats and control work rats that were subjected to 8h of forced locomotion during their activity phase. We also investigated whether circadian disorganization affected glucose homeostasis during an IVGTT test, but no effect of shift work was found. In contrast to an earlier study on shift work (Salgado-Delgado et al., 2008), we did not find increased weight gain in shift work rats compared to control work rats. One explanation may be a difference in the protocol used. Although the amount and the timing of forced locomotion was similar in both studies, we used a continuous shift work protocol for 2 weeks, whereas Salgado-Delgado and colleagues alternated 5 days of shift work with undisturbed weekends and continued this protocol for 5 weeks. Thus, it might be that the length

of the protocol and the alternation between shift work and periods of rest are important factors to induce the body weight changes the authors found. Indeed, we recently found that the alternation between weeks of sleep restriction and weekends of sleep allowance prevents the attenuation of weight gain as seen during a continuous sleep restriction protocol (Barf et al., 2012).

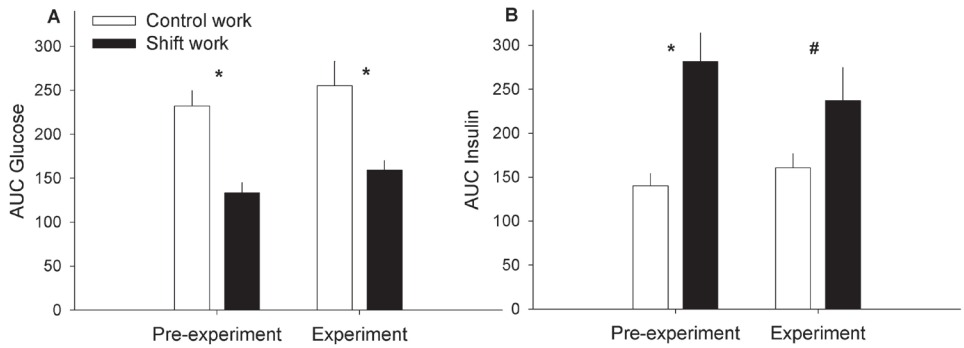

Figure 7: Area under the curve (AUC) of blood glucose (A) and plasma insulin (B) levels in response to a 30-min intravenous glucose infusion under pre-experimental conditions or after 14 experimental days. Data are average values ± SEM. Asterisks indicate a significant difference between both groups (* P<0.01); # indicates a trend towards a difference between the groups (P=0.08).

Also, our finding of unaltered glucose tolerance in rats subjected to shift work may seem at odds with the loss of glucose rhythmicity reported in the study by Salgado-Delgado and colleagues (Salgado-Delgado et al., 2008). However, one should keep in mind that glucose rhythmicity and glucose homeostasis are not necessarily linked.

Importantly, while glucose tolerance was not affected by two weeks of shift work, in an earlier study we reported glucose intolerance after 8 days of restricted or disrupted sleep (Barf et al., 2010). It thus appears that the consequences of sleep disruption reported earlier are not mediated by circadian disruption but, rather, by changes in sleep per se (i.e., the amount of sleep or architecture of sleep).While rats on a shift work protocol may have an altered sleep-wake rhythm, total sleep time and sleep quality are not necessarily affected. Indeed, it has been shown in a human laboratory experiment that one week of shift work did not affect total sleep time or sleep quality afterwards (Lamond et al., 2003). Therefore it may be that changes in food intake patterns and changes in glucose homeostasis have independent underlying mechanisms, which are differently affected by shift work or sleep disruption.

Insulin and glucose responses to the IVGTT in this experiment were quite different for shift work and control work rats. This difference was already present under baseline conditions before the start of the experimental protocol and is a consequence of the fact that the IVGTTs were performed at different times of day.

The IVGTT was performed 17h after the last forced activity period in the rotating drum for both groups. For shift rats, the IVGTT took place in the 4th hour of the light phase, whereas for control rats the IVGTT took place in the 4th hour of the dark phase. Literature has reported that glucose homeostasis varies across the 24h cycle (Kalsbeek et al., 2010;La Fleur et al., 2001). During the inactive phase, the body is less glucose tolerant and less insulin sensitive, resulting in increased insulin levels in response to an IVGTT. During the active phase, more glucose is needed for daily activities, leading to increased glucose levels and decreased insulin levels. This is indeed also visible in our data.

In conclusion, our current data do not support the hypothesis that shift work leads to disturbed metabolic regulation and glucose intolerance. Since our previous data demonstrated clear effects of sleep restriction on glucose homeostasis (Barf et al., 2010), it might be that the circadian organization is not the crucial factor, but disturbed sleep is.

Acknowledgements

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Increased food intake and changes in metabolic hormones in response to chronic sleep restriction alternated with short periods of sleep allowance

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Abstract

Rodent models for sleep restriction have good face validity when examining food intake and related regulatory metabolic hormones. However, in contrast to epidemiological studies where sleep restriction is associated with body weight gain, sleep restricted rats show a decrease in body weight. This difference with the human situation might be caused by the alternation between periods of sleep restriction and sleep allowance that often occurs in real life. Therefore we assessed the metabolic consequences of a chronic sleep restriction protocol that modeled working weeks with restricted sleep time alternated by weekends with sleep allowance. We hypothesized that this protocol could lead to body weight gain. Male Wistar rats were divided over three groups: sleep restriction (SR), forced activity control (FA) and home cage control (HC). SR rats were subjected to chronic sleep restriction by keeping them awake for 20h per day in slowly rotating drums. To model the human condition, rats were subjected to a 4-week protocol with each week consisting of a 5-day period of sleep restriction followed by a 2-day period of sleep allowance. During the first experimental week, SR caused a clear attenuation of growth. In subsequent weeks, two important processes occurred: 1) a remarkable increase in food intake during SR days, 2) an increase in weight gain during the weekends of sleep allowance, even though food intake during those days was comparable to controls. In conclusion, our data revealed that the alternation between periods of sleep restriction and sleep allowance lead to complex changes in food intake and body weight, that prevented the weight loss normally seen in continuous sleep restricted rats. Therefore this “week-weekend” protocol may be a better model to study the metabolic consequences of restricted sleep.

Introduction

Sleep loss is a common problem in our modern society. Both epidemiological and clinical data suggest that disturbed sleep may contribute to the development of various diseases, e.g. obesity and type 2 diabetes (Cappuccio et al., 2008;Chaput et al., 2006;Chaput et al., 2007;Gottlieb et al., 2005;Spiegel, 2008;Spiegel et al., 2009). Restricted sleep also leads to alterations in food intake and its regulatory hormones, particularly an increased appetite and preference for fat, together with increased levels of ghrelin and decreased levels of leptin (Spiegel et al., 2004;Taheri et al., 2004).

There are several rodent models for sleep deprivation; for example, the disk-over-water method (Everson and Crowley, 2004;Rechtschaffen and Bergmann, 1995), the inverted flowerpot (or platform) paradigm (Hipolide et al., 2006;Mendelson et al., 1974), and the slowly rotating drum paradigm (Barf et al., 2010;Novati et al., 2008;Roman et al., 2005). In general, the changes in blood hormone levels and food intake in these models are similar to the findings in humans. Sleep deprivation decreased plasma insulin (Hipolide et al., 2006) and leptin levels (Everson and Crowley, 2004) and increased food intake was observed in some (Everson and Crowley, 2004;Hipolide et al., 2006;Koban and Stewart, 2006;Koban et al., 2008;Rechtschaffen and Bergmann, 2002;Rechtschaffen and Bergmann, 1995) but not all studies (Barf et al., 2010). However, the most consistent finding among these studies is that sleep deprived rats lose weight (Barf et al., 2010;Everson and Crowley, 2004;Hipolide et al., 2006;Koban and Stewart, 2006;Koban et al., 2008;Rechtschaffen and Bergmann, 2002;Rechtschaffen and Bergmann, 1995), which is in contrast to the human finding where a lack of sleep generally is associated with weight gain (Chaput et al., 2006;Knutson and Van Cauter, 2008).

The reason for this difference between rats and humans is unknown. There is some indirect evidence that the differences in weight gain vs. weight loss might be related to the nature of the sleep restriction protocol. Sleep loss in humans is in general an alternation between sleep restriction during the week and recuperation from sleep loss in the weekend (Valdez et al., 1996). In contrast, experimental studies in rats often consist of a continuous period of sleep restriction without periods of recovery. Indirect evidence comes from a shiftwork study by Salgado-Delgado and colleagues who subjected rats to a protocol consisting of alternating 5-day periods of shift work and 2-day period of undisturbed sleep-wake rhythms, and indeed they found a clear increase in body weight (Salgado-Delgado et al., 2008;Salgado-Delgado et al., 2010).

Based on these studies, we hypothesized that an alternation between periods of sleep restriction and periods of sleep allowance is critical for the induction of body weight gain in a sleep restriction paradigm. Therefore we evaluated the behavioral and metabolic consequences of a sleep restriction protocol consisting of 5 days of sleep restriction alternated by 2 days in which the rats were allowed to recover. This protocol was continued for four weeks. We hypothesize that the 2 days of sleep allowance per week will prevent the weight loss normally seen in chronically sleep restricted rats.

Methods

Animals and housing

Male Wistar rats (weight 302.1 ± 1.2 g at start of the experiment, derived from Harlan Netherlands BV, Horst, The Netherlands) were individually housed in Plexiglas cages in a climate-controlled room (21 °C ± 1) under a 12h: 12h light-dark cycle (lights on at 1:00 PM). Animals were maintained ad lib on medium fat food (45 % fat: Arie Blok Diervoeding B.V., Woerden, The Netherlands), which mimics the human diet and is the standard diet in our previous studies on metabolism (Barf et al., 2010). Water was available ad lib throughout the study. Food intake and body weights were measured daily. Experiments were approved by the Ethical Committee of Animal Experiments of the University of Groningen.

Chronic sleep restriction and forced activity

The rats were assigned to one of three groups. The first group was subjected to chronic sleep restriction (SR, n-12). SR was achieved by placing the rats in slowly rotating drums, according to previously described methods (Meerlo et al., 2002). Briefly, rats were allowed to sleep in their home cage for only 4 hours per day at the beginning of the light phase. During the remaining 20 hours, the rats were kept awake by placing them in slowly rotating drums (diameter 40cm), rotating at a constant speed of 0.4 m/min (Barf et al., 2010;Novati et al., 2008;Roman et al., 2005). To model the human condition with alternating working weeks and weekends of rest, rats were subjected to a 4-week schedule with each week consisting of a 5-day “working week” (5 consecutive days with SR or FA) followed by a 2-day “weekend” (2 days of uninterrupted sleep allowance in the home cage). The second group was a forced activity control group (FA: n=7). The FA rats served as controls for the amount of exercise and walked the same distance as the SR rats in 2 hours at the end of the dark phase (wheels by TSE, Bad Homburg, Germany). Thus, the FA group walked the same distance but was not sleep restricted. Both the SR and FA groups were forced to walk 480m/day during the “working week”, which is approximately 10-20% of the distance rats cover voluntarily (Scheurink et al., 1999). The rats of the SR and FA groups had unlimited access to food and water inside the drums. The third group of rats consisted of home cage controls (HC, n=5) which remained in their home cage throughout the experiment.

Blood samples and chemical analysis

To assess the effects of sleep restriction on metabolic hormones, blood samples were taken in week 1 and week 4 of the experiment for analysis of glucose, insulin, leptin and corticosterone. Samples were taken immediately after the 5-day working week (working week: day 5 and day 26) at the beginning of the lights phase (ZT0) and after four hours of rest (ZT4). Another blood sample was taken after two days of rest (weekend: day 7 and day 28) during the 4th hour of the light phase (ZT4). Blood samples of approximately 0.5 ml were drawn from the tail (Fluttert et al., 2000;Meerlo et al., 2002) and collected in pre-cooled cups

containing EDTA. Afterwards, the samples were centrifuged at 4 °C for 10 min at 2600 g, and the plasma was stored at □20 °C until further analysis. Blood glucose was measured by Hoffman's ferrocyanide method and plasma levels of insulin, leptin and corticosterone were measured by Millipore Rat Radioimmunoassays (Linco Research, St Charles, MO, USA).

Indirect calorimetry

At the first day of the final weekend of sleep allowance (day 26), immediately after the last SR/FA period, rats were transferred to respirometric chambers (45×25×30 cm) to determine oxygen consumption (V·O2, l/h) and carbon dioxide production (V·CO2, l/h). Oxygen and carbon dioxide concentration of dried inlet and outlet air (drier: molecular sieve 3 Å, Merck) from each chamber was measured with a paramagnetic oxygen analyzer (Servomex Xentra 4100) and carbon dioxide by an infrared gas analyzer (Servomex 1440). The system recorded the differentials in oxygen and carbon dioxide between dried reference air and dried air from the metabolic chambers. Flow rate of inlet air (60 l/h) was measured with a mass-flow controller (Type 5850 Brooks). Samples were collected every 10 min (allowing optimal air mixing) for each animal and automatically stored on a computer. Behavioural activity of the animals was recorded with calibrated passive infrared detectors (PIR: Optex Wonderex FX-35; OPTEX (Europe) LTD., Berkshire, UK). Animals were measured at an ambient temperature of 21 °C and food and water were provided ad libitum over the whole period. Energy expenditure (kJ) was calculated using the following equation of Ferrannini (Ferrannini, 1988):

$$EE=(16.18\times VO2\times 0.001) + (5.02\times VCO2\times 0.001).$$

During the 24 hours in the indirect calorimetry, fecal pellets were collected. The rotating drum system to sleep restrict rats could not be combined with the respirometry system and therefore it was not possible to measure oxygen consumption during the working weeks of SR/FA.

Statistical analysis

The data in the figures and text are expressed as averages ± SEM. The effects of the sleep restriction and forced activity protocols on food intake, body weight and blood hormone levels were tested by repeated measures analysis of variance (ANOVA) with between-subjects factor 'treatment' (SR, FA, or HC) and within-subjects factor 'time' (day of the experimental protocol). Indirect calorimetry data, body weight gain, average daily food intake and feces weight was tested by One Way ANOVA with factor 'treatment' (SR, FA, or HC). When appropriate, a posthoc Tukey test was applied to establish differences between the three groups (controls, SR and FA). $P<0.05$ was considered statistically significant.

Results

Daily body weight and food intake are shown in Figure 1. Both body weight and food intake differed significantly over time (Repeated Measures ANOVA effect of time for body weight: $F(32,672)= 411.73$, $P<0.001$; food intake: $F(30,600)= 7.57$, $P<0.001$). The increase in body weight was significantly attenuated in SR rats compared to home cage controls (Repeated Measures ANOVA treatment x time interaction: $F(64,672)= 3.26$, $P< 0.001$; posthoc Tukey test: SR vs. HC: $P<0.05$). Food intake of SR rats increased after the first recovery weekend compared to both FA and HC rats (Repeated Measures ANOVA treatment x time interaction: $F(60,600)= 6.48$, $P<0.001$; posthoc Tukey test: experimental week 2-4: SR vs. FA and SR vs. HC: $P<0.05$).

In Figure 2, data are averaged for week 2-4, when the effects of sleep restriction and patterns of body weight and daily food intake had stabilized (average body weight gain or food intake divided by the amount of days (7, 5 or 2 days)). SR rats had a slight reduction in weight gain during the 5-day working weeks ($F(2,21)=3.48$, $P<0.05$, posthoc Tukey test: SR vs. HC: $P<0.05$). Both SR and FA rats showed an increase in their body weight gain during the weekends of rest ($F(2,21)= 8.38$, $P<0.01$, posthoc Tukey test: SR vs. HC and FA vs. HC: $P<0.05$). The total body weight gain was not different between groups.

During week 2-4, average daily food intake was significantly increased during the 5-day sleep restriction periods (One Way ANOVA: $F(2,21)= 43.26$, $P<0.001$, posthoc Tukey test: SR vs. FA and SR vs. HC: $P<0.001$) but returned to control levels during the 2-day weekends of rest ($F(2,21)= 1.65$, $P>0.1$). For the week totals, including both the working week and recovery weekend, average daily food intake remained significantly increased for SR rats ($F(2,21)= 23.38$, $P<0.001$, posthoc Tukey test: SR vs. HC and SR vs. FA: $P<0.001$).

Glucose, insulin, leptin and corticosterone levels (Table 1) were measured at the end of the first and fourth working week, immediately after the rats had returned to their home cage at the beginning of the light phase (ZT0), after 4 hours of rest (ZT4) and after 2 days rest (weekend: ZT4). No changes over time or between groups were found in glucose levels. For insulin levels, Repeated Measures ANOVA revealed a significant time effect ($F(5,105)= 2.60$, $P<0.05$) and a significant treatment x time interaction ($F(10,105)= 2.26$, $P<0.05$). Posthoc Tukey revealed decreased insulin levels in SR and FA rats as compared to HC rats at the end of working week 1 and 4. Insulin levels returned to control values after 2 days of rest.

Leptin levels showed a similar pattern. Repeated Measures ANOVA revealed a significant time effect ($F(5,105)= 6.48$, $P<0.001$) and a nearly significant treatment x time interaction ($F(10,105)= 1.74$, $P=0.08$). Posthoc Tukey revealed decreased leptin levels after both 1 and 4 weeks of SR but not FA, which are back to control levels after 2 days of rest.

Corticosterone levels showed a significant time effect ($F(5,105)= 26.00$, $P<0.001$) and a significant treatment x time interaction ($F(10,105)= 10.77$, $P<0.001$). At the end of the first 5-day working week, immediately after the last 2h forced locomotion

session, FA rats had significantly increased corticosterone levels (posthoc Tukey test, FA vs. HC and FA vs. SR: $P<0.05$), which had returned to control levels after 4 hours of rest. The same pattern was visible after 4 weeks of FA (Posthoc Tukey test, FA vs. HC and FA vs. SR: $P<0.05$), although the increase in corticosterone levels after FA was less pronounced compared to week 1 (Paired t-test: $t=4.11$, $P<0.01$).

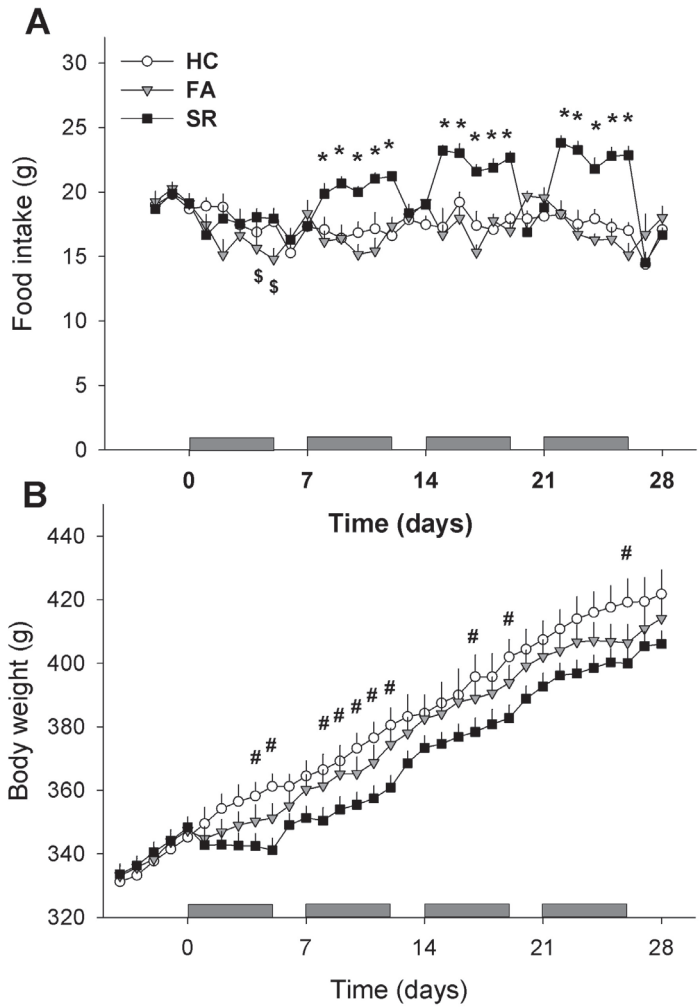

Figure 1: Daily food intake (A) and body weight (B) during baseline, 4 experimental weeks and 4 weekends of sleep allowance for SR (n=12), FA (n=7) and HC (n=5) rats. The grey bars represent the 5-day weeks of SR or FA. Data are average values ± SEM. Significant differences ($P<0.05$): * SR vs. both control groups; # SR vs. HC; † FA vs. HC.

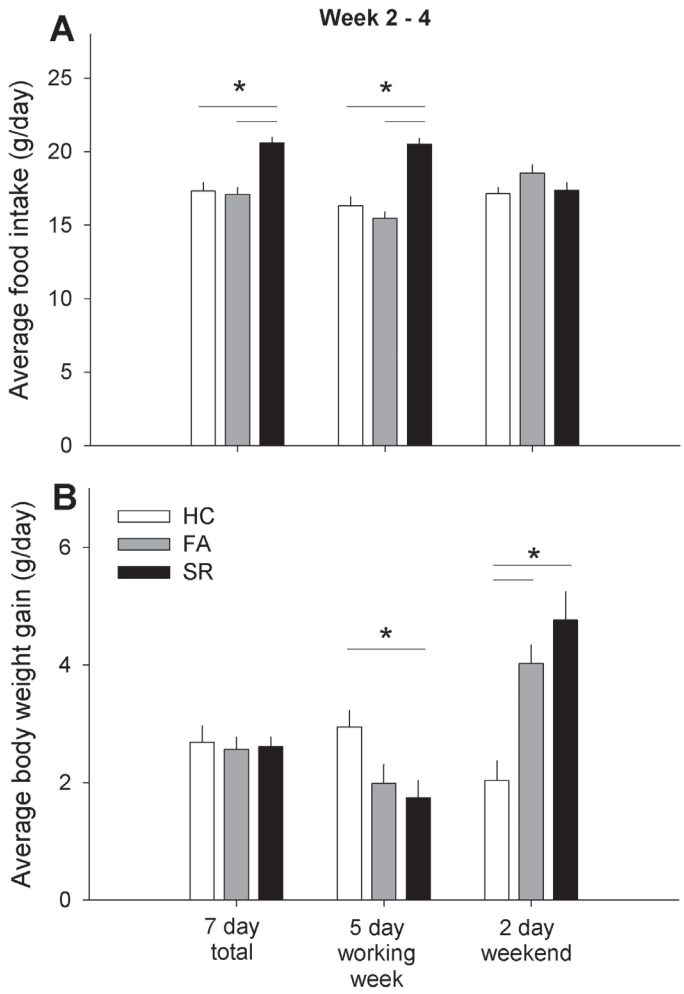

Figure 2: Average food intake and body weight gain per day during experimental week 2-4 for SR (n=12), FA (n=7) and HC (n=5) rats. Averages are shown per total 7 days, per experimental week (5 days) and per weekend of sleep allowance (2 days). Data are average values ± SEM. * P<0.05.

Table 1: Effect of chronic sleep restriction on plasma levels of glucose, insulin, leptin and corticosterone.

	Working week 1		Weekend 1	Working week 4		Weekend 4
	ZT0	ZT4	ZT4	ZT0	ZT4	ZT4
Glucose (mM)						
HC	5.9 ± 0.2	6.1 ± 0.1	6.2 ± 0.2	5.9 ± 0.1	6.1 ± 0.1	6.2 ± 0.3
FA	6.0 ± 0.1	5.8 ± 0.1	6.1 ± 0.1	6.0 ± 0.2	5.8 ± 0.2	6.0 ± 0.1
SR	5.9 ± 0.1	5.7 ± 0.1	6.0 ± 0.1	6.1 ± 0.2	5.9 ± 0.1	6.1 ± 0.1
Insulin (ng/ml)						
HC	4.8 ± 1.1	4.5 ± 0.5	4.4 ± 0.9	4.9 ± 0.9	5.2 ± 0.7	3.7 ± 0.5
FA	2.3 ± 0.2*	3.4 ± 0.5	3.8 ± 0.5	2.6 ± 0.6*	3.1 ± 0.4*	3.4 ± 0.4
SR	2.7 ± 0.3*	2.6 ± 0.3*	3.7 ± 0.4	2.9 ± 0.2*	3.9 ± 0.4	3.3 ± 0.3
Leptin (ng/ml)						
HC	16.3 ± 3.7	11.9 ± 1.1	11.6 ± 1.7	17.6 ± 2.7	15.3 ± 2.8	13.5 ± 2.4
FA	10.7 ± 3.1	8.3 ± 2.0	10.8 ± 2.3	10.7 ± 2.7	10.0 ± 3.2	12.3 ± 4.5
SR	8.3 ± 0.9*	6.3 ± 0.6*	8.7 ± 0.8	10.2 ± 0.7*	9.3 ± 0.9	10.3 ± 1.3
CORT (ng/ml)						
HC	19.2 ± 2.1	16.2 ± 5.0	31.7 ± 9.7	11.3 ± 2.5	7.7 ± 2.3	26.8 ± 14.1
FA	376.6 ± 71.9*	11.7 ± 1.3	18.0 ± 2.2	158.9 ± 27.5*	4.9 ± 1.3	14.4 ± 1.7
SR	132.4 ± 38.7†	11.2 ± 0.7	21.3 ± 2.3	77.8 ± 20.5†	10.3 ± 1.4	27.8 ± 5.3

Blood samples were taken during the 1st and 4th week of the experiment. For both weeks, samples were taken immediately after 5 experimental days (working week) at the beginning of the light phase (ZT0) and after 4 hours of rest (ZT4); another sample was taken after 2 days of rest (weekend) during the 4th hour of the light phase (ZT4). Data are average values ± SEM (SR: n=12, FA n=7 and HC n=5). Values are means ± SEM. Significant differences ($P<0.05$): * compared to HC, † compared to FA.

Data derived from indirect calorimetry on the first recovery day after the fourth working week is shown in Figure 3. Daily energy expenditure did not differ between groups during the light phase, dark phase and total 24 hours. The respiratory quotient (RQ: CO2 production/O2 consumption) during the 24h respirometry measurement was significantly lower in SR rats as compared to control rats (SR: 0.92 ± 0.01; FA: 0.95 ± 0.01; HC: 0.95 ± 0.01; $F(2,21)= 3.85$, $P<0.05$; Posthoc Tukey test: SR vs. FA and SR vs. HC: $P<0.05$). Levels of activity were significantly decreased for SR rats during the light phase ($F(2,15)= 7.47$, $P<0.01$, posthoc Tukey test, SR vs. FA: $P<0.05$). Total 24h activity levels tended to be lower but this did not reach statistical significance ($F(2,15)= 3.11$, $P=0.07$). Furthermore, feces were weighed for all rats. SR rats had significantly less feces during the first day of sleep allowance in the indirect calorimetry (SR: 3.8 ± 0.2g; FA: 4.8 ± 0.3g; HC: 4.7 ± 0.3g; One Way ANOVA: $F(2,20)= 4.79$, $P<0.05$, posthoc Tukey test, SR vs. FA: $P<0.05$).

Discussion

The most striking result of the present study is the significant increase in food intake, which appeared after the first weekend of sleep allowance. The first period of sleep restriction was similar to our previously published data, in which 8 days of sleep restriction led to unchanged food intake, but a clear weight loss (Barf et al., 2010). After the first weekend of sleep allowance, rats become hyperphagic, preventing further weight loss. A second interesting finding is that the rats have normal food intake during the weekends of sleep allowance, but significant weight gain in this period. Together, these data support the hypothesis that an alternation between periods of sleep restriction and sleep allowance can prevent the weight loss normally seen in sleep restricted rats. Therefore this "week-weekend" protocol has increased face validity in comparison to our earlier sleep restriction protocol that did not include the intervening periods of sleep allowance.

It is important to note that our experimental rats had increase body weight gain only during the weekends of sleep allowance as compared to the home cage controls, but not during the working weeks. Overall, even at the end of the 4-week protocol, the SR rats were still slightly lighter than controls. Even though our current protocol of sleep restriction alternated with weekends of sleep allowance attenuates the weight loss seen with continuous sleep restriction in literature, rats still do not become overweight or obese. This suggests that sleep restriction per se is not sufficient to produce obesity in rats.

The present experiment was indirectly based on a study by Salgado-Delgado and colleagues, who showed that subjecting rats to a shift work protocol with forced locomotion during the normal rest phase led to a significant increase in body weight as compared to controls (Salgado-Delgado et al., 2008;Salgado-Delgado et al., 2010). In their study, shift working weeks were alternated with weekends of undisturbed rest, which may have been a crucial factor in the reported body weight increase. The reason why their shift work protocol resulted in a net increase in body weight above control levels while our current protocol only attenuated the body weight loss seen in previous studies may lie in the methodological differences. While both models interfered with sleep by subjecting rats to forced activity, the Salgado-Delgado shift work protocol specifically disrupted circadian organization whereas our model only aimed to shorten sleep. It may thus be that disrupting circadian organization has additional effects beyond sleep disruption that contribute to the body weight increase.

In our present study, SR rats lost only weight during the first week of the protocol, similar to what was reported before (Barf et al., 2010). This decrease in weight may be a result of increased energy expenditure associated with prolonged wakefulness and increased activity. Indeed several studies have shown increased energy expenditure during sleep deprivation (Bergmann et al., 1989;Caron and Stephenson, 2010;Hipolide et al., 2006;Koban and Swinson, 2005) and one explanation for this change in energy expenditure, and in turn the attenuation of body weight gain, could be an increase in the gene expression of uncoupling protein-1 (UCP-1) in the brown adipose tissue (BAT). BAT is known for its

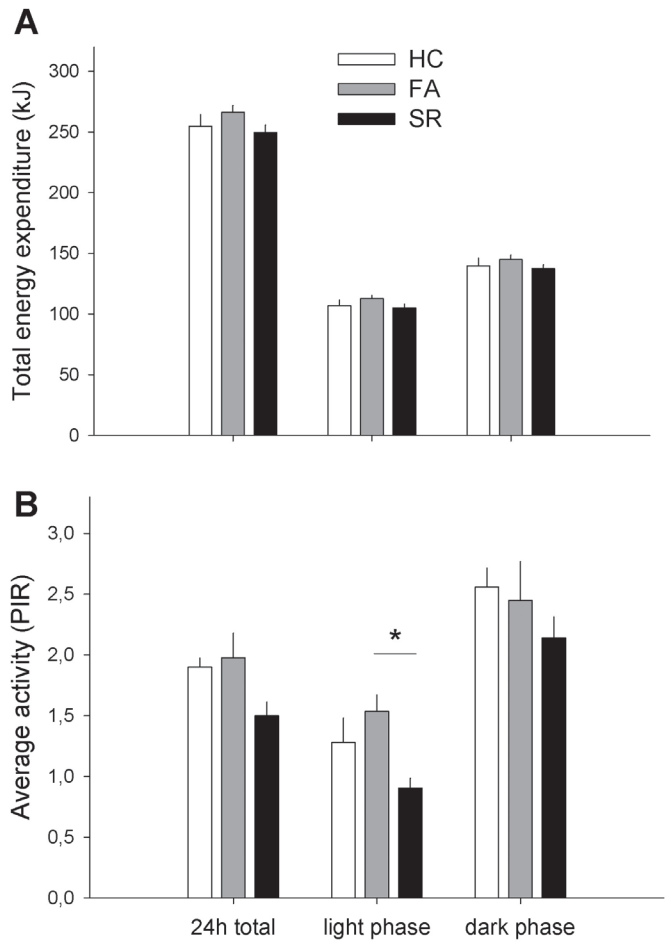

Figure 3: Total energy expenditure and average cage activity (PIR) measured with indirect calorimetry during the first day of sleep allowance after week 4 for SR (n=12), FA (n=7) and HC (n=5) rats. Data are average values ± SEM. * P<0.05.

regulatory non-shivering thermogenesis in rodents and heat production is mediated by UCP-1 (Cannon and Nedergaard, 2004). Indeed, Koban and Swinson have demonstrated that during sleep deprivation UCP-1 is increased over time, together with an increase in O2 consumption (Koban and Swinson, 2005). Thus, in our study it might be that the 5 days of sleep restriction leads to increased energy expenditure and increased UCP-1, whereas during the weekends of sleep allowance both return to baseline.

During the second week of the protocol, rats started compensating for the presumed increased energy expenditure associated with sleep restriction by increasing their food intake. These changes in food intake may be related to changes in hypothalamic neuropeptides such as orexin and neuropeptide Y.

Orexin is involved in the regulation of both the sleep/wake cycle and food intake regulation (Sakurai, 1999;Sakurai, 2002). Some studies have demonstrated that REM sleep deprivation increases orexin levels in the CSF and orexin immunoreactivity in the lateral hypothalamic area (Galvao et al., 2009;Pedrazzoli et al., 2004). Furthermore, orexin neurons project densely to the arcuate nucleus, which is known for its involvement in food intake regulation (Nambu et al., 1999). Indeed, REM sleep deprivation leads to significant increases in neuropeptide Y mRNA levels (Koban et al., 2006;Koban et al., 2008). Recently, it has also been demonstrated that sleep deprivation increases orexin mRNA levels which in turn activate the arcuate neuropeptide Y neurons that could lead to hyperphagia (Martins et al., 2010). Thus, it might be that in our experiment, sleep restriction after the first weekend of sleep allowance leads to increased orexin and neuropeptide Y levels in the brain, causing the rats to increase their food intake only during periods of sleep restriction. During the weekends rats are allowed to sleep, which might lead to a decrease in central orexin levels, and in turn cause food intake to return to baseline values. Future experiments should be performed to verify this.

The data in figure 2 reveal that the sleep restricted rats are only hyperphagic during the periods of sleep restriction, whereas the weight gain only occurs during the periods of sleep allowance when the rats are not hyperphagic. Why the rats do not gain weight when they are hyperphagic during the periods of sleep restriction may be due to a number of factors. One may argue that the exercise protocol of 480 m/day may have increased the energy expenditure of the rats. However, the FA control rats walked the same distance per day, did not increase their food intake during these periods of forced locomotion and did not lose body weight. Nevertheless, it might be that the sleep restriction protocol itself has effects on the energy expenditure beyond the increase in locomotor activity. Due to methodological limitations we were not able to measure energy expenditure during the 5-day periods of SR, therefore we can only speculate that energy expenditure most probably is increased during SR, similar to what has been published before (Bergmann et al., 1989;Caron and Stephenson, 2010;Hipolide et al., 2006;Koban and Swinson, 2005).

The finding that SR rats gain weight when they are not hyperphagic during the periods of sleep allowance can be interpreted in relationship to the effects of sleep restriction on sleep time and intensity. It is tenable to assume that recovery from sleep restriction is associated with increased sleep time and sleep intensity, as others have demonstrated that rats do sleep longer and deeper after sleep acute sleep deprivation and chronic sleep restriction (Leemburg et al., 2010;Machado et al., 2005). However, the data in figure 4 demonstrate that energy expenditure is not different between groups during these periods of sleep allowance, even though total activity is decreased. The question of how SR rats grow faster during the fourth weekend of sleep allowance, despite similar food intake and overall energy expenditure in the different groups, remains. One explanation might be increased food efficiency. One may argue that increased energy absorption in the intestinal tract can be used for recovery, storage and

thus weight gain. This is indirectly supported by the fact that the feces weight of SR rats is decreased during weekends of sleep allowance compared to the control groups. This decrease indicates that, even though energy intake and energy expenditure are similar compared to controls, the food efficiency might be higher in SR rats during a period of sleep allowance, leading to increased body weight gain.

Although SR rats increase their food intake from week 2 onwards, leptin and insulin levels were still decreased at the end of the fourth working week, which is in agreement with the fact that these rats still had a slightly lower weight than control rats. These hormones reflect the nutritional status of the rat. However, both leptin and insulin are also satiety hormones, which could also be another explanation for the increase in food intake during the periods of sleep restriction. The corticosterone levels of FA rats, immediately after the experimental period, were strongly increased. For SR rats this did not reach significance. This increase in corticosterone for FA rats is in agreement with the notion that corticosterone may in part reflect and support behavioral activity (Koolhaas et al., 2011). For instance, Koolhaas and colleagues have demonstrated that stressful events but also pleasurable events, such as a sexual experience, can lead to similar increases in corticosterone. Therefore, an increase in corticosterone is associated with behavior and in our case, forced locomotion. The fact that corticosterone levels rapidly returned to baseline during 4 hours of sleep allowance suggests that our sleep restriction protocol is not a chronic stressor.

Perspectives and Significance

Our data revealed that the alternation between periods of sleep restriction and periods of sleep allowance lead to complex changes in food intake and body weight that prevented the negative energy balance normally seen during continuous sleep restriction in rat studies. Although the discrepancy between epidemiological studies and rat studies remains, the alternation between periods of sleep loss and periods of sleep allowance seems to be a crucial factor and an important addition to the sleep deprivation literature.

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CH. 7

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

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Abstract

The serotonergic system plays an important role in the regulation of mood. Experimental studies in rats show that chronic sleep restriction leads to altered serotonergic function, including a desensitization of the serotonin 1A receptor system. Such changes may in part be an indirect consequence of changes in body weight. To determine whether a medium fat (MF) diet could protect against these effects of sleep restriction by preventing the decrease in body weight, we subjected rats to a schedule of chronic sleep restriction during which half of the sleep restricted rats were maintained on a MF diet. 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. We found that a MF diet strongly attenuated the drop in body weight normally seen in sleep restricted rats on Chow. However, this did not prevent desensitization of the serotonin 1A system. Sleep restricted rats on both diets showed a similarly blunted body temperature response and pituitary ACTH response upon serotonin 1A stimulation. Insulin and leptin levels were equally decreased in both sleep restricted groups despite differences in body weight. These results indicate that changes in serotonin 1A receptor sensitivity following sleep restriction are not dependent on diet and body weight changes. The desensitization might be the result of altered regulation of physiological and endocrine factors 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 (Breslau et al., 1996;Chang et al., 1997;Ford and Kamerow, 1989). 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 rats showing that chronic sleep restriction causes a gradually developing desensitization of the serotonin 1A receptor system and an attenuation of 1A-mediated functions (Novati et al., 2008;Roman et al., 2005;Roman et al., 2006). 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 (Donga et al., 2010;Spiegel et al., 2002;Spiegel et al., 2004). Also, numerous studies on prolonged sleep deprivation in rodents have reported increased food intake and reduced body weight (e.g. Everson, 1995;Hipolide et al., 2006;Rechtschaffen and Bergmann, 1995). In recent studies, we focused on the metabolic consequences of sleep restriction in our rat model. Sleep restriction 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, literature shows that weight loss affects serotonin levels (Bailer et al., 2005;Brewerton, 1995;Haleem, 2009;Kaye et al., 2005) and a decrease in body weight due to food restriction is associated with a desensitization of the serotonin 1A receptor in rats (Li and France, 2008;Li et al., 2009). Furthermore, partially 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).

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. A study was performed in 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 rats 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) (Novati et al., 2008;Roman et al., 2005).

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 °C ± 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, 14% vs. 45.0% fat, 63% vs. 30% carbohydrates and 23% vs. 25% proteins; Arie Blok Diervoeding B.V., Woerden, The Netherlands). Ten days prior to the onset of the experiments animals were introduced to the MF diet. 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

Rats were subjected to chronic sleep restriction according to a previously published method (Barf et al., 2010;Novati et al., 2008;Roman et al., 2005). Sleep restriction groups were allowed to sleep in their home cage for 4 hours per day at the beginning of the light phase, i.e., their normal resting 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. Rats 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 rats 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 (Roman et al., 2005;Roman et al., 2006). All rats 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, rats 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 fat food 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., 2005;Roman et al., 2006).

Experiment 2: Blood sampling and serotonin 1A mediated pituitary ACTH response

In the second experiment we studied the effects of MF food and sleep restriction on the sensitivity of the serotonin 1A system by measuring the pituitary ACTH response to an 8-OHDPAT challenge (Novati et al., 2008). Serotonin 1A receptors at the level of both the hypothalamic paraventricular nucleus and the pituitary play an important role in regulating ACTH release (Dinan, 1996;Fuller, 1992). All rats were equipped with a chronic heart catheter in the jugular vein allowing repeated and stress free blood sampling according to a previously described method (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 rats to sampling tubes. After 10 days of recovery from surgery, rats were divided over four groups (n=8 in each group): home cage control on standard Chow food (Control-Chow) or medium fat food (Control-MF), and sleep restriction on standard Chow food (SR-Chow) or medium fat food (SR-MF). After 8 days of sleep restriction, rats 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 stress free 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 °C until 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 was 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

During the 8-day experiment, sleep restricted rats on standard Chow food significantly decreased in body weight compared to control rats on Chow (Repeated Measures ANOVA, sleep restriction x time: $F(11,154)=33.58$, $P<0.001$) (Figure 1A). Sleep restricted rats on Chow also lost significantly more weight than sleep restricted rats on MF food (Repeated Measures ANOVA, diet x time: $F(11,154)=7.19$, $P<0.001$). 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).

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

Experiment 2: serotonin 1A induced endocrine responses

Similar to experiment 1, both sleep restriction and diet affected body weight (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 rats 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 activation of the hypothalamic-pituitary-adrenal axis in all treatment groups (ACTH: Figure 2B, corticosterone: Figure 2C). The ACTH response was not affected by diet but ANOVA revealed a

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

Eight days of sleep restriction decreased both leptin and insulin levels compared to control rats (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 rats 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 (Figure 3).

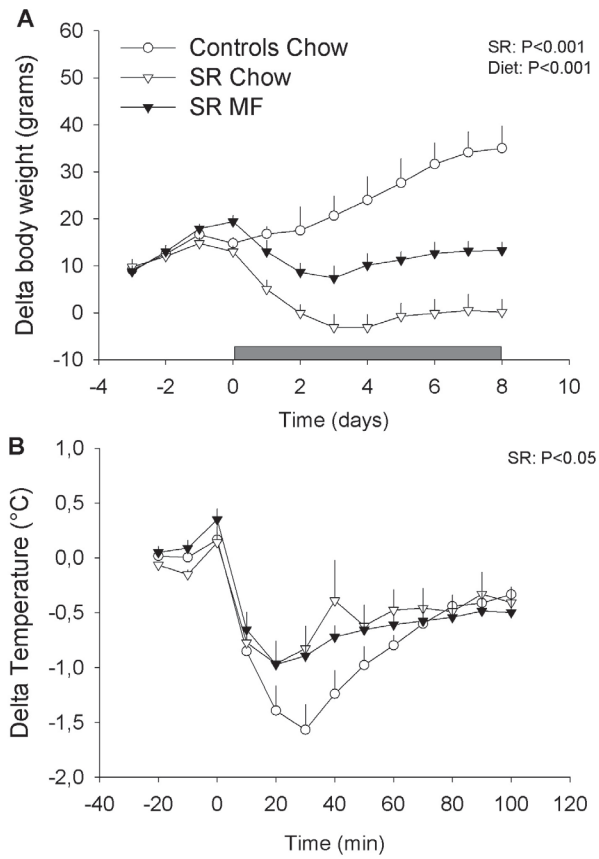

Figure 1: The effects of 8 days of sleep restriction on body weight and serotonin 1A sensitivity in rats receiving a standard Chow diet or a MF diet: (A) body weight changes in the course of the experiment; (B) body temperature responses to an injection with the serotonin 1A agonist 8-OHDPAT (0.25 mg/kg) 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.

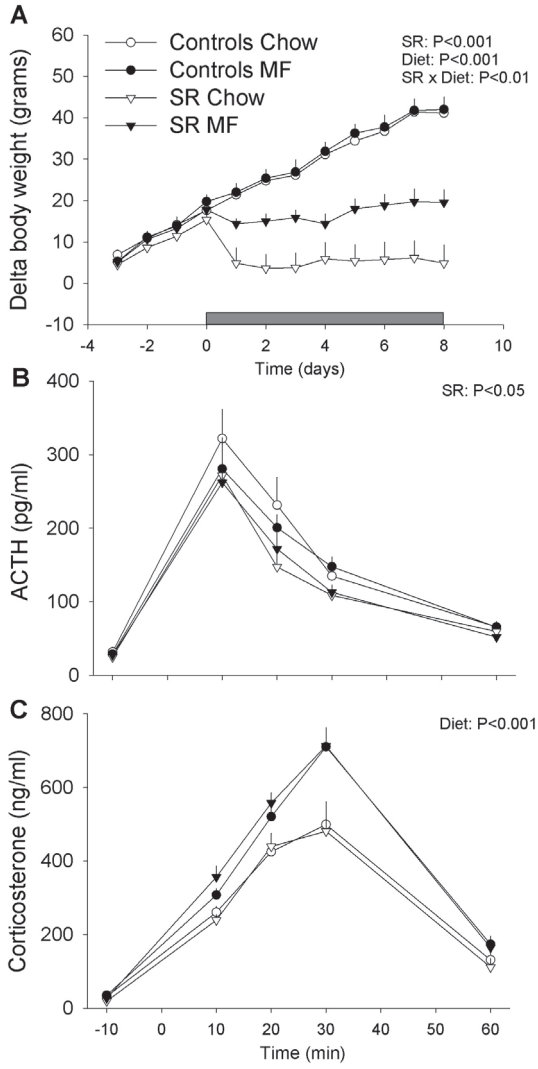

Figure 2: The effects of 8 days of sleep restriction on body weight and serotonin 1A sensitivity in rats receiving a standard Chow diet or a MF diet: (A) body weight changes in the course of the experiment; (B) ACTH and (C) corticosterone responses to an injection with the serotonin 1A agonist 8-OHDPAT (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.

Discussion

In the present study we aimed to assess whether SR induced changes on serotonin 1A sensitivity 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 rats on a standard Chow diet. However, this did not prevent the desensitization of the serotonin 1A system. Sleep restricted rats on both MF and standard Chow showed a similarly blunted body temperature response and pituitary ACTH response upon stimulation of the serotonin 1A receptor system by an injection of 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, rats 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, rats on the MF diet did not lose as much weight as rats on regular Chow did. Nevertheless, the MF diet did not prevent the changes in serotonin 1A sensitivity seen after sleep restriction. Since the MF diet did not prevent the decrease in body weight completely, an effect of body weight loss during sleep restriction on the 5-HT1a desensitization cannot be fully excluded. One might argue that even the smaller drop in weight in the MF rats could be sufficient to cause a maximal 1A desensitization that could not worsen by the additional weight loss in the Chow rats. However, this possibility does not seem likely in light of other published studies. Particularly, a study on the consequences of social stress showed that a high fat diet was able to partially prevent the stress-induced drop in body weight and at the same time completely prevented the stress-induced desensitization of the serotonin 1A receptor (Buwalda et al., 2001). Thus, whereas fat food ameliorates the effects of stress on the serotonergic system, it does not seem to protect against the effects of sleep restriction. Apparently, the mechanisms through which stress and sleep disturbance affect the serotonin 1A sensitivity are different.

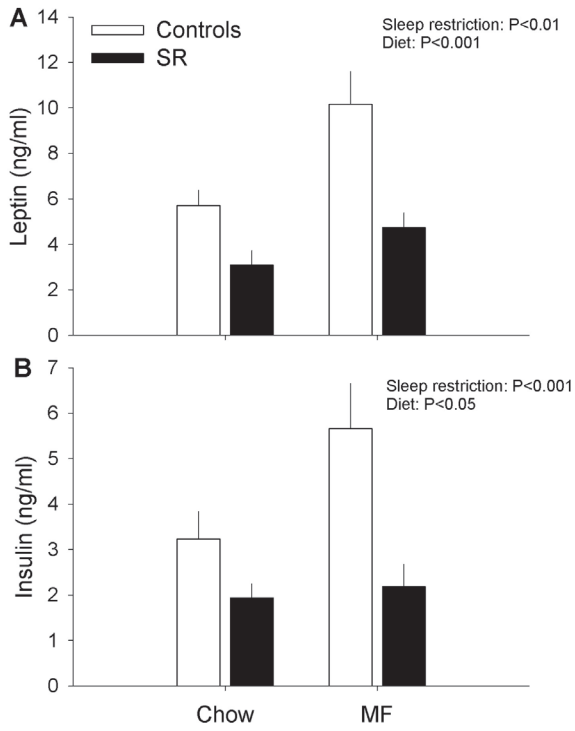

Figure 3: The effects of 8 days of sleep restriction on plasma levels of leptin (A) and insulin (B) in rats receiving a standard Chow diet or a MF diet. N=8 in each group. Data are presented as average values ± SEM. See text for details on statistics.

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 rats 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;Koolman et al., 2010;Morens et al., 2006). 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 rats 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 compared to the sleep restricted rats 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 □-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;Friedman, 1978;Friedman and Ramirez, 1994). Li and colleagues (Li et al., 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 both a standard Chow or MF diet showed a similarly 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.

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Sleep disturbances and glucose homeostasis

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Abstract

Sleep disturbances, induced by either life style, shift work or sleeping disorders, have become more prevalent in our Western 24/7 society. Sleep disturbances are associated with impaired health including metabolic diseases such as obesity and type 2 Diabetes. The question remains whether there is a direct effect of disturbed sleep on glucose homeostasis. Experimental studies under controlled laboratory conditions in both humans and experimental animals revealed that there are differences between the effects of acute or chronic sleep disturbance. Acute sleep restriction clearly leads to glucose intolerance often combined with insulin resistance. Glucose intolerance does also occur after chronic sleep disturbance but the changes in insulin may vary, dependent on the body weight changes in the various studies. The underlying mechanism that may cause the changes in glucose homeostasis after sleep disturbance remain unclear but both the biological clock located in the nucleus suprachiasmaticus as well as orexinergic mechanisms in brain and periphery seem to be involved.

Introduction

Over the past 50 years, average sleep duration in Western societies has decreased by 2 hours per night (Van Cauter et al., 2005). Initially, this decrease is sleep has been mainly observed in adulthood, but recent epidemiological studies have shown a similar decrease in children and adolescents (for reviews see: Horne, 2008;Van and Knutson, 2008). A decrease in sleep includes not only the duration but also the quality of sleep. Quality of sleep is generally defined in terms of changes in sleep architecture, the content of dreaming and the amount of awakenings (for a review see: Vandekerckhove and Cluydts, 2010).

A decrease in sleep length or quality of sleep has important consequences on an individual's well-being. Sleeping problems are linked to many health and lifestyle problems such as increased errors, loss of productivity, sleepiness during the day, impaired social activities and an elevated risk for accidents (for a review see: Banks and Dinges, 2007;Horne and Reyner, 1999). Disturbed sleep has also been identified as risk factors for various diseases including psychiatric disorders such as depression (Breslau et al., 1996;Ford and Kamerow, 1989;Neckelmann et al., 2007) and immune system dysfunctions (Imeri and Opp, 2009;Irwin, 2002;Opp and Toth, 2003). These health problems are increasing since sleep loss and decreased sleep quality are becoming more and more prevalent in our current 24/7 Western society,

Metabolic consequences of sleep disturbances

Sleep disturbances are also linked to metabolic dysfunctioning. Studies in shift workers provided the first (indirect) evidence for a relation between sleep disturbances and impaired metabolism. Shift work has become more prevalent in the last decades and shows clear negative effects on sleep timing, length and quality (Dumont et al., 1997). Shift workers are active during the night and they sleep and eat at abnormal hours. They fall asleep in the morning, but are awakened ahead of time due to their circadian rhythm, causing them to exhibit sleepiness and reduced performance (Akerstedt, 1990). The shift work - induced changes, in particular sleep loss and disturbed circadian rhythmicity in eating and energy expenditure, are associated with an increased susceptibility to develop obesity, type 2 diabetes and cardiovascular disorders (Akerstedt et al., 2004;Knutsson, 2003).

There is also a direct correlation between disturbed sleep and obesity, the main risk factor for developing cardiovascular diseases and type 2 diabetes. Evidence for this comes from several epidemiological studies, among others described by Caput and Van Cauter (Chaput et al., 2007;Van Cauter et al., 2007). In addition, the group of Gottlieb and co-workers (Gottlieb et al., 2005) provided evidence for a direct correlation between disturbed sleep and the increased prevalence of type 2 diabetes, independent of changes in body weight.

Another striking example for the relation between disturbed sleep and increased risk on metabolic disorders derives from studies on patients with obstructive sleep apnea (OSA). OSA is characterized by the recurrent collapse of the airway during

sleep, which usually leads to arousals to resume breathing. The patients suffer from sleep fragmentation and hypoxemia, causing disturbed sleep architecture and increased amount of awakenings (Bandla and Gozal, 2000;Svanborg and Guilleminault, 1996). OSA primarily changes the quality of sleep while total sleep time is not dramatically altered. Vgontzas and colleagues (Vgontzas et al., 2003) were the first to show that there is a relation between OSA and obesity, type 2 diabetes and cardiovascular disease. In fact, there is bidirectional relationship: OSA leads to obesity but obesity does also directly affect OSA: weight gain or weight loss leads to a significant worsening or improvement, respectively, of sleep apnea in adults (Peppard et al., 2000;Pillar and Shehadeh, 2008). OSA is also directly associated with insulin resistance and glucose intolerance, independent of changes in weight (Punjabi et al., 2002).

Human studies on sleep and glucose homeostasis

The main point of the above is that disturbed sleep is clearly associated with an increased risk on type 2 diabetes, even when corrected for BMI or fat content of the body (Chaput et al., 2009;Gottlieb et al., 2005). This leads to the question: is there a direct effect of (disturbed) sleep on glucose and insulin regulation? To answer this question, experimental studies under controlled laboratory conditions are required. These studies, both in humans and experimental animals, are recently performed in different labs.

In experimental studies in humans, most experimenters standardize the experimental protocol by providing standard meals at fixed time points of the day and by requiring the subjects to stay in bed during the sleep restriction hours to minimize activity. Unfortunately, there is still a large variation in the experimental set up of the sleep restriction protocols, in particular in the length of the sleep restriction period which may vary from acute one night total sleep deprivation up to periods of 6 nights of partial sleep deprivation. These differences in length of the sleep restriction protocols markedly influenced the outcomes of the different studies.

The first experimental study in humans was published in 1993, when VanHelder and colleagues showed that sixty hours of sleep deprivation led to an increased plasma insulin response without changes in blood glucose during an oral glucose tolerance test (OGTT) (VanHelder et al., 1993). Similar data after one night of sleep restriction have been found in a recent study by Donga et al (Donga et al., 2010) in which insulin sensitivity was measured with the gold standard method for measuring insulin sensitivity: a hyperinsulineamic euglycemic clamp. In this study, one night of sleep restriction (four hours in bed) was sufficient to develop moderate insulin resistance, reflected by a decreased glucose infusion rate, a reduced glucose disposal rate and increased endogenous glucose production. In this study, the baseline glucose and insulin levels were not different from controls.

In 1999, Spiegel and colleagues showed that six days of partial sleep restriction (subjects were allowed to sleep only four hours per night) led to glucose intolerance

reflected by a significant elevation of blood glucose levels during an intravenous glucose tolerance test (IVGTT) (Spiegel et al., 1999). Surprisingly, there was no reduction in insulin sensitivity. Instead, it was observed that plasma insulin levels were decreased after six days of sleep disturbance which is, of course, in sharp contrast with the data from VanHelder and Donga (Donga et al., 2010;VanHelder et al., 1993). However, in a follow up study from the same lab (Tasali et al., 2008), they found that sleep disturbance could indeed lead to reduced insulin sensitivity and glucose intolerance. In this particular study, the subjects were submitted to an IVGTT and sleep disturbance was defined as three nights of experimentally suppressed slow wave sleep without changes in total sleep time. This also allows the conclusion that it is not only the quantity of sleep but also the quality of sleep that is important.

Although many of these human data are seemingly in conflict with each other, one may still conclude that sleep disturbance has a marked impact on glucose and insulin homeostasis. When searching for a common denominator, it seems that acute sleep disturbances may to lead to insulin resistance and in some cases glucose intolerance, whereas longer periods of disturbed sleep has no direct effect on insulin sensitivity but the glucose intolerance remains. One may speculate that the insulin resistance after acute total sleep deprivation is secondary to the (stress-induced) activation of the HPA-axis elevation reflected by the elevated cortisol levels in the evening after a night without sleep (Leproult et al., 1997). This acute stress response may be less pronounced in the longer sleep restriction studies where the subjects are still allowed to sleep for a few hours per night. However, Spiegel and colleagues (Spiegel et al., 1999) still found increased cortisol levels after the six days of sleep restriction although this increase was less pronounced when compared to the acute response after one night without sleep.

Animal studies on sleep and glucose homeostasis - rat studies

As mentioned before, human studies may vary in length between acute sleep deprivation to a maximum of up to six days of sleep restriction. Animal studies are required for studies on chronic sleep disturbance for investigation of the underlying mechanisms. Most animal studies in (chronic) sleep research are performed in rodents and experimental methods to prevent the animals from sleeping may vary from gentle handling to small balance platforms and forced activity. The different methods are discussed below.

The gentle handling method (Van Der Borght et al., 2006) is comparable to the sleep restriction protocol in human experiments. The animals, mostly rats, are kept awake by a protocol that includes tapping on the cage, shaking the cage gently or, if required, disturbing the nest. This method is mainly used in acute or short time sleep deprivation experiments, since the experimenter is required to be present continuously during the experiments. In our laboratory, 12h of sleep deprivation by means of gentle handling had no effect at all on baseline levels of blood glucose, plasma insulin and plasma corticosterone.

The balance platform method is more commonly used in chronic sleep restriction

experiments. Two methods are described in literature the flower pot and the single platform method. Both methods are used to deprive rats of REM sleep only and the principle is as follows: to induce REM sleep deprivation, rats are placed inside a water chamber onto a flower pot or a platform of about 7.0cm in diameter. The platform is in the water up to 1.0cm of the platform upper surface. The method is based on the loss of muscle tonus that occurs during REM sleep. This means that if the rat enters the REM sleep stage, it loses muscle tonus and will touch the water after which the rat will wake up (Cohen and Dement, 1965). When this method is used to deprive rats of REM sleep for 4 days, it leads to a decrease in plasma insulin levels which is secondary to a significant reduction in body weight (Hipolide et al., 2006).

Another commonly used method in animal sleep restriction research is the disk over water method, extensively described by Rechtschaffen and colleagues (Rechtschaffen and Bergmann, 1995;Rechtschaffen and Bergmann, 2002) In short, the experimental animal is housed on a horizontal disk above water. The electromyograph (EMG) and the electroencephalograph (EEG) are continuously recorded to detect sleep states. When the experimental rat starts to sleep or enters a certain sleep stage, the disk starts to rotate at a low speed. This causes the awakening of the rat and forces it to walk to avoid being carried into the water. There is only one study in which this method was used to investigate glucose homeostasis after a longer period of sleep deprivation. In this study by Everson and colleagues, they measured glucose disappearance after a dextrose injection. The data revealed that there was a tendency towards somewhat lowered glucose levels after sleep deprivation (Everson et al., 1989).

In our laboratory, we use a forced activity paradigm known as the slowly rotating drum to sleep deprive the rats. This method was originally described in 1984 by Borbely and colleagues (Borbely et al., 1984). In our studies, the experimental animals are placed for twenty hours per day in a rotating drum (Figure 1) that is rotating at a constant speed of 0.4cm/min (Barf et al., 2010;Novati et al., 2008;Roman et al., 2005). In this way the animals are allowed to sleep only for (the remaining) four hours per days. We generally include a control group that walks twice the speed (0.8cm/min) but half the time to control for the forced activity in this protocol. Our latest studies focused on the difference between acute and chronic sleep deprivation on glucose homeostasis and insulin levels. To this end, we performed an intravenous glucose tolerance test (IVGTT) after 1 day and 8 days of sleep restriction. The data revealed that a reduction in sleep markedly interfered with glucose metabolism (Barf et al., 2010). Acute sleep restriction was accompanied with elevated blood glucose profiles without any changes in plasma insulin levels during an IVGTT. The effects of chronic sleep reduction are presented in Figure 2. The observed glucose intolerance is still present after 8 days of sleep restriction which is similar to the data obtained in most human studies. Baseline insulin levels and glucose-stimulated insulin responses were lower after chronic sleep reduction. This effect on insulin was, similar to the data in humans obtained by Spiegel et al in 1999, secondary to the weight loss after chronic sleep

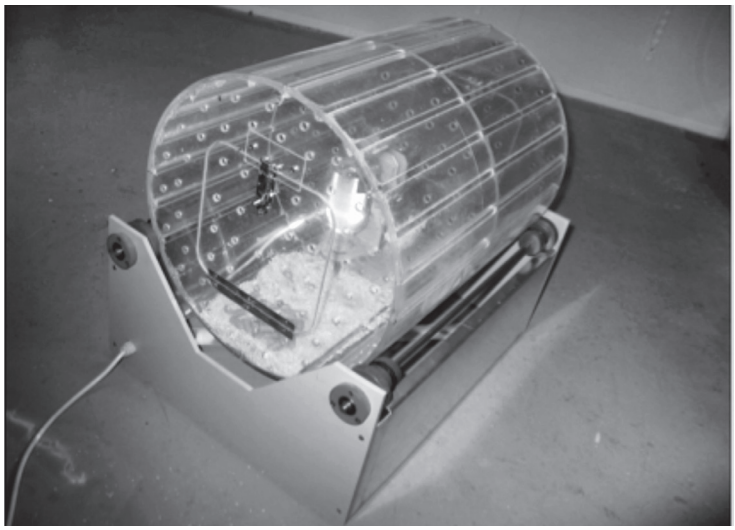

Figure 1: Slowly rotating drum

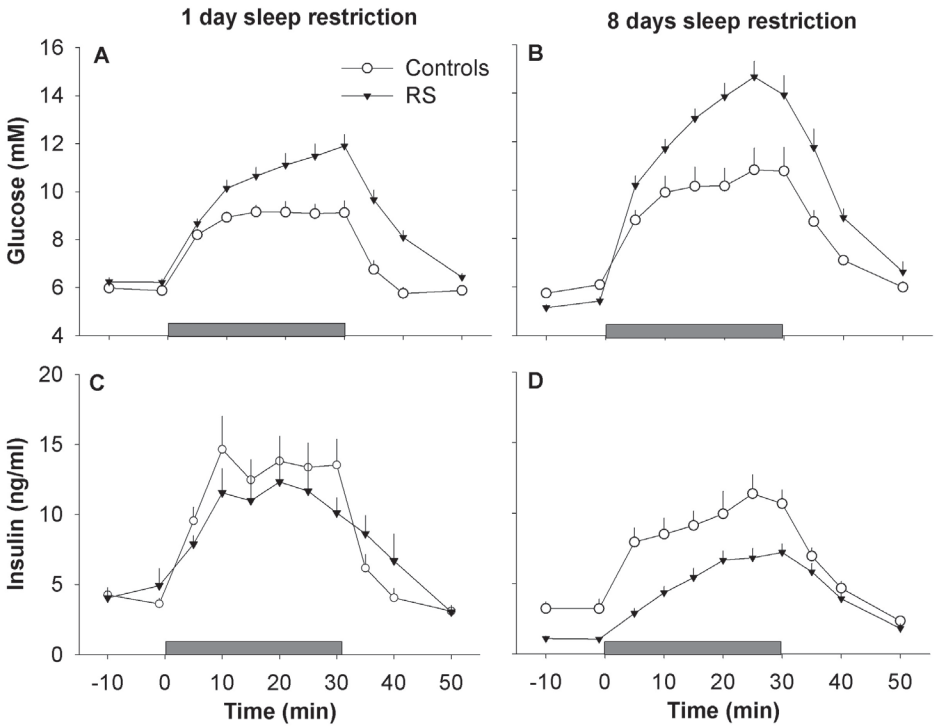

Figure 2: Blood glucose and plasma insulin levels in response to a 30-min intravenous glucose infusion after one (graphs A and C) and 8 (graphs B and D) days of restricted sleep (RS) or controls. The horizontal grey bars at the bottom of each graph represent the 30 min of 15% glucose infusion. Data are average values ± SEM (Barf et al., 2010).

reduction. Based on the data above, we may conclude that the effects of sleep restriction on glucose homeostasis in experimental animals are remarkably similar in rats and humans. Acute sleep restriction leads to glucose intolerance in combination with unchanged insulin levels (all rat models and some human studies) or insulin resistance (other human studies). The glucose intolerance remains prevalent after chronic sleep restriction, both in humans and rats. Chronic sleep restriction may also lead to a reduction in plasma insulin levels but this effect seems secondary to a reduction in body weight. The reduction in body weight is typical for experimental studies under controlled conditions and in sharp contrast with epidemiological data that suggest that long term sleep disturbances are associated with weight gain and the development of obesity and, consequently, the development of insulin resistance and type 2 Diabetes. The insulin resistance that occurs after one night of total sleep deprivation in the (human) experimental studies seems primarily caused by a (stress-induced) activation of the HPA-axis, reflected by the elevated cortisol levels under these circumstances.

Possible mechanisms underlying glucose intolerance

The data above raises the question: what is the cause of the glucose intolerance that occurs after both acute and chronic sleep deprivation? Is it mainly a behavioral effect, related to changes in food intake, physical activity and energy expenditure? Or is it caused by (circadian) disturbances in hormonal outflow and/or the activation of the autonomic nervous system? Or is hyperglycemia mainly secondary to changes at the level of the central nervous system, in particular at orexinergic neurons in the lateral hypothalamus? Some of these options will be discussed below.

The biological clock, located in the suprachiasmatic nucleus (SCN), has a marked effect on glucose homeostasis. There are clear differences in the glucose and insulin responses to an IVGTT in both rats and humans at different times of the circadian clock (for review see: Kalsbeek et al., 2010). This circadian rhythmicity in glucose regulation disappears when the SCN is lesioned (La Fleur et al., 2001). One may state that the biological clock does not only influence the sleep-wake cycle, but also prepares the glucose regulatory mechanisms for the changes in energy uptake and expenditure at different time points of the day/night cycle. These circadian systems are tightly integrated and problems may occur when our daily activities are not in synchrony with our sleep-wake cycle, for example during shift work and periods of disturbed sleep (Knutsson, 2003). For example, when rats are forced to be active in their non-active phase, they will eat at the wrong time of the day and consequently increase in body weight compared to controls (Salgado-Delgado et al., 2008). This weight gain can be prevented when the animals are only allowed to eat in the (normal) active phase and not in the shift work period (Salgado-Delgado et al., 2010).

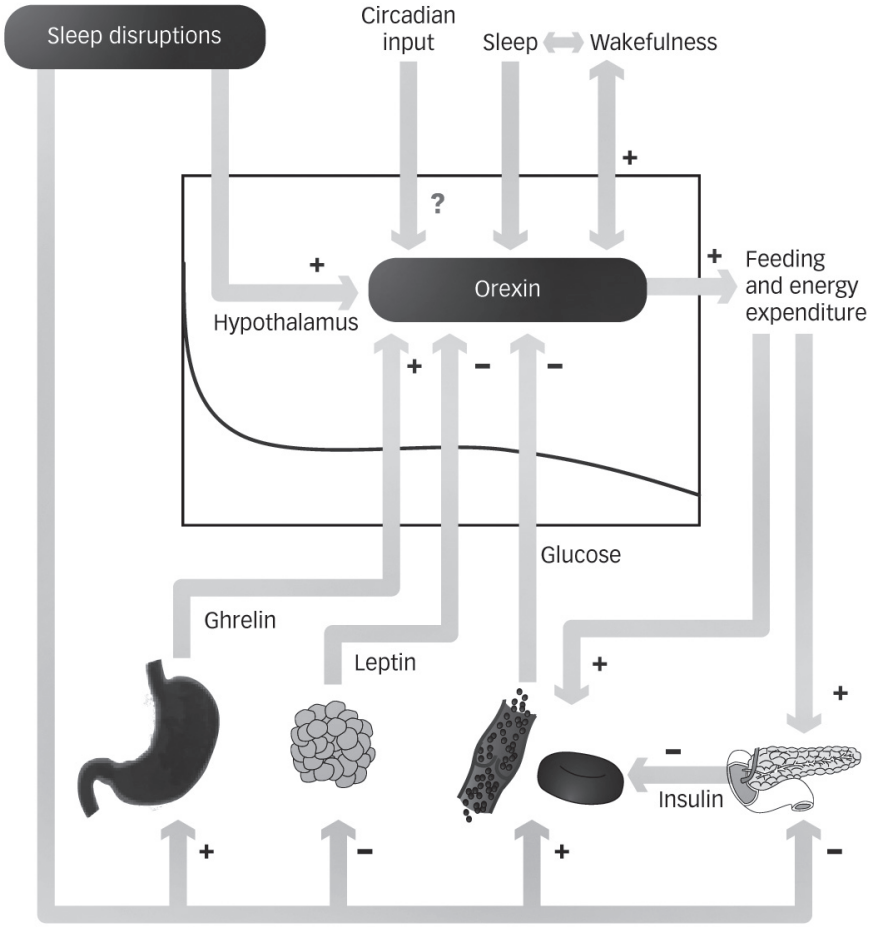

Figure 3: Overview of the connections between sleep, sleep loss, orexin and metabolic hormones.

Hormonal influences also seem to be involved in the effects of disturbed and restricted sleep in glucose metabolism. In humans, sleep deprivation has been shown to decrease plasma leptin and to increase plasma ghrelin levels, leading to increased hunger and appetite (Spiegel et al., 2004). At a central level, orexinergic system in the brain seems to play a role as well. Orexin is involved in both food intake, energy expenditure and the sleep-wake cycle and could therefore be an interesting link between sleep disturbances and the increased prevalence of obesity and type 2 diabetes (Sakurai, 2002;Sakurai, 2007). For example, narcolepsia is a disease that is characterized by reduced orexin levels in the central nervous system causing less consolidated wake periods, leading to a sudden appearance of sleep periods. Narcolepsia is associated with an increased frequency of type 2 diabetes (Honda et al., 1986). Peripheral orexin seems to be involved as well since orexin receptors have been found on the pancreas and other peripheral organs (Adeghate et al., 2010). It is also known that orexin plays a direct role in the regulation of glucose homeostasis. Tsuneki and colleagues showed that

orexin neurons directly respond to the nutritional status of an individual and is modulated by metabolic signals such as glucose, leptin and ghrelin (Tsuneki et al., 2010;Yi et al., 2009). Studies in orexin knockout mice point to a contribution of orexin in the age-related development of impaired glucose tolerance, independent of obesity (Tsuneki et al., 2008). Finally, it is known that orexin is directly involved in the regulation of energy expenditure. Increased orexin levels correspond with increased physical activity and increased non-exercise thermogenesis (NEAT) and, consequently, glucose utilization. Taken together, these data show that orexin may serve as a crucial factor in the relation between sleep loss, circadian rhythms, physical activity and the effects on glucose metabolism (Figure 3).

In summary, sleep disturbances are directly associated changes in glucose homeostasis. Experimental studies under controlled laboratory conditions in both humans and experimental animals revealed that there are differences between the effects of acute or chronic sleep disturbance. Acute sleep restriction clearly leads to glucose intolerance often combined with insulin resistance. Glucose intolerance does also occur after chronic sleep disturbance but the changes in insulin may vary, dependent on the body weight changes in the various studies. The underlying mechanisms that may cause the changes in glucose homeostasis after sleep disturbance remain unclear but both the biological clock located in the nucleus suprachiasmaticus as well as orexinergic mechanisms in brain and periphery seem to be involved.

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General discussion

9

Summary

This thesis aimed to study restricted or disrupted sleep in rats in a controlled laboratory setting to allow a detailed assessment of the metabolic consequences of sleep loss and its underlying mechanisms. Based on the human epidemiology, we expected that chronic sleep restriction would lead to increased food intake, weight gain and insulin resistance. However, we found that chronic sleep restriction attenuates weight gain with no change in food intake or insulin resistance. In addition, we observed that sleep restriction leads to hyperglycemia, both acutely and after 8 days of sleep restriction (chapter 3). Furthermore, chronic sleep restriction led to increased energy expenditure, which may explain the attenuated weight gain (chapter 2). Changing the sleep restriction model by including 2 days of undisturbed sleep per week did lead to increased food intake and weight gain in comparison to continuously sleep restricted rats although the weight gain was still lower than in home cage controls (chapter 6). The hyperglycemia and attenuated weight gain appears to be a direct consequence of disturbed or insufficient sleep and not a non-specific byproduct of our sleep deprivation model, since hyperglycemia was not observed in control studies in which rats were exposed to forced running (chapter 4) or a circadian disruption protocol in which rats were forced to be active for 8 hours during the light (inactive) phase (chapter 5).

Sleep quantity vs. sleep quality

An important question is whether the metabolic consequences of sleep restriction are due to a change in the sleep quantity or sleep quality. In chapter 3 we demonstrated that chronically disturbed sleep led to an attenuation of weight gain, despite the fact that rats were allowed to sleep for 14h per day. In addition, intravenous glucose tolerance test (IVGTT) data demonstrated a hyperglycemia together with a decrease in insulin response, similar to sleep restricted rats. To compare total sleep time between both groups we measured sleep EEG also in our sleep disturbed rats (Figure 1). During a baseline day, rats slept approximately 11h. In chapter 2 we demonstrated that our sleep restriction protocol led to a 60% decrease of total sleep time. Although sleep disturbed rats had 14h of sleep allowance per day, the protocol still led to a 20-30% decrease in total sleep time. Thus, sleep disturbed rats slept more than sleep restricted rats, but considerably less compared to their own baseline day. We cannot exclude that this reduction in total sleep time was responsible for the changes in glucose tolerance and weight gain. However, since the consequences were not more pronounced in the sleep restricted rats, we can conclude that a more severe sleep restriction protocol does not necessarily lead to more extreme consequences.

Interestingly, when we only disturbed the circadian aspect of sleep, by means of a shift work protocol that forced rats to be active during their normal sleep phase, a small attenuation of weight gain was found but no effect on glucose tolerance (chapter 6). We did not perform EEG measurements in these shift work rats, but it may be that this protocol only affected the timing of sleep and not sleep quantity and sleep quality per se. Clearly, while shift work under real life conditions is

often associated with disturbed sleep (Akerstedt, 2003), such disturbances may not occur under optimal laboratory conditions. Indeed, it has been shown in at least one human laboratory experiment that, under optimal conditions, one week of shift work did not affect total sleep time or sleep architecture (Lamond et al., 2003). Similarly, our shift work rats may have had shifted, but normal amounts of consolidated sleep, thereby preventing the metabolic consequences seen in our sleep restricted and sleep disturbed rats.

To conclude, our data suggest that shifting sleep allowance to the subjective day per se does not affect glucose homeostasis, whereas disturbed and/or reduced sleep induces glucose intolerance. Therefore, our rat data regarding sleep quantity vs. sleep quality are comparable to the human literature, since both restricted sleep (Spiegel et al., 1999) as well as decreased sleep quality (Tasali et al., 2008), by means of suppressed deep sleep without changes in total sleep time, lead to reduced glucose tolerance.

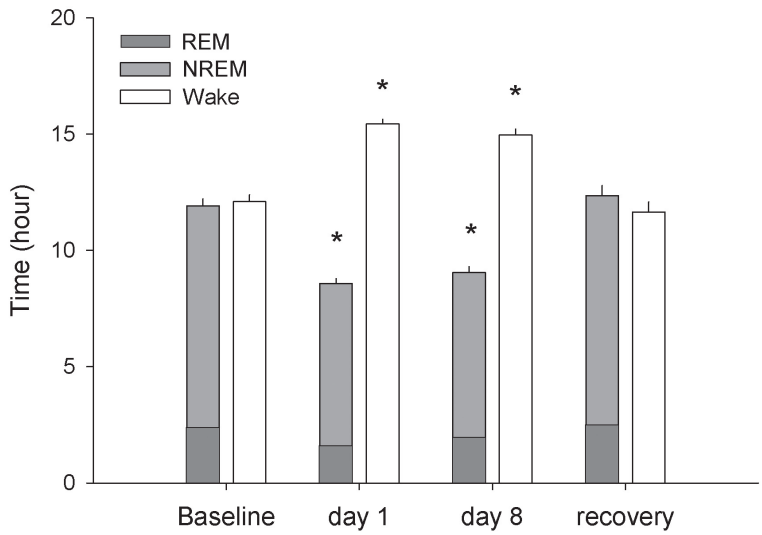

Figure 1: Time spent asleep or wakefulness during baseline, day 1 and day 8 of sleep disturbance and a recovery day (n=5). Time spent asleep is divided into NREM and REM sleep. Data are average values ± SEM. Statistics are done on total sleep time and total wake time. Asterisks indicate a significant difference in comparison to baseline (* P<0.05).

Sleep restriction and the orexinergic system

The neuropeptides orexin A and orexin B (also known as hypocretin 1 and hypocretin 2) may play an important role in the metabolic consequences of chronic sleep restriction. Orexin neurons were independently discovered by two different groups (de Lecea et al., 1998;Sakurai et al., 1998;Peyron et al., 1998). Both found that orexin neurons are localized to the lateral hypothalamus (LH). Since the

LH area is known as the feeding center, it was thought that orexin was involved in food intake. Indeed, ICV injections of orexins during the light period induced feeding behavior in both rats and mice (Haynes et al., 2000;Sakurai et al., 1998). In addition to the effects of orexin on feeding behavior, orexin knockout mice also show a remarkably similar phenotype to human narcolepsy patients. Narcolepsy is a disease that is characterized by severe sleep disturbance, less consolidated wake periods and sudden appearances of sleep bouts. Studies in patients indeed confirmed that narcolepsy is associated with a pronounced orexin deficiency (Peyron et al., 2000;Thannickal et al., 2000). Importantly, narcolepsy patients often suffer from metabolic disorders such as obesity and type 2 diabetes (Honda et al., 1986). Likewise, studies in orexin knockout mice point to a contribution of orexin in the age related development of impaired glucose tolerance independent of obesity (Tsuneki et al., 2008). In addition, it has been found that orexin neurons directly respond to metabolic signals such as glucose, insulin, leptin and ghrelin that reflect the nutritional status of the body (Tsuneki et al., 2010;Yi et al., 2009). Finally, it is known that orexin is directly involved in the regulation of energy expenditure. An increase in orexin levels increases physical activity, non-exercise thermogenesis and, consequently, glucose utilization (for review: Ganjavi and Shapiro, 2007). Taken together, the literature suggests that orexin may be a crucial factor in the relationship between sleep loss, physical activity, food intake and glucose metabolism.

Other research has focused on the effects of sleep deprivation on orexins and its receptors. However, there are still many inconsistencies. REM sleep deprivation using the flower pot method leads to increased orexin A levels in the CSF (Pedrazzoli et al., 2004) and an increased number of immune-reactive orexin A neurons in the LH (Galvao et al., 2009), but has no effect on the mRNA levels of prepro-orexin, the precursor of both orexin A and B (D'Almeida et al., 2005). After 24h of recovery sleep, orexin A levels in the CSF were reduced (Pedrazzoli et al., 2004), whereas another study showed a significant increase in prepro-orexin mRNA expression in the LH as well as a pronounced increase in orexin receptor 1 mRNA expression in multiple brain areas after sleep rebound (D'Almeida et al., 2005). Total sleep deprivation for 24 hours increases the concentration of orexin A in CSF of dogs (Wu et al., 2002), and 6 hours of sleep deprivation increases orexin A in the LH as measured by micro-dialysis in rats (Yoshida et al., 2001). In contrast, the same amount of sleep deprivation did not affect orexin A mRNA expression in rats (Terao et al., 2000). Thus, it seems that variations in sleep deprivation differentially affect orexin at the mRNA and protein level. Nonetheless, sleep deprivation leads to increased orexin A expression independent of method or length of the protocol.

If, along the same lines, orexin A expression is increased in our sleep restriction protocol as well, one might expect that sleep restriction leads to changes at the receptor level in certain brain areas. Abundant amounts of orexin may lead to desensitization and, in turn, to down-regulation of the receptors (Freedman and Lefkowitz, 1996;Jackson, 1991). To test this hypothesis, we performed western

blot analyses for measurements of orexin receptor 1 in both the prefrontal cortex and the thalamus (including hypothalamus) after 8 days of sleep restriction. The results in Figure 2 show no differences between sleep restricted rats and home cage controls, thus we can conclude that the orexin receptor 1 is not down regulated. These data do not support the idea that the metabolic consequences of chronic sleep restriction are mediated by changes in the orexinergic system. However, to completely understand the effect of sleep restriction on orexins and its receptors, future studies should focus on all aspects of the neuropeptides as opposed to focusing only on mRNA levels, proteins, or its receptors.

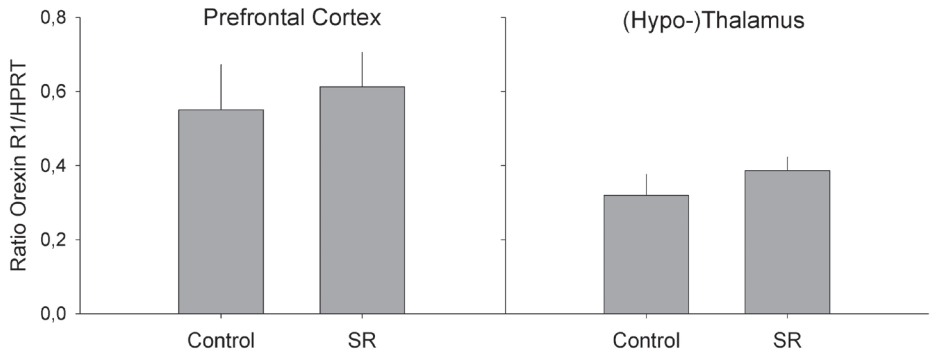

Figure 2: Orexin receptor 1 expression measured by western blotting in both the prefrontal cortex and the thalamus-hypothalamus divided by HPRT data, which is intended for use as a positive control (n=8). Data are average values ± SEM.

Sleep restriction and obesity

In our studies, chronic sleep restriction led to an attenuation of weight gain in rats, whereas the literature reports a clear association between short sleep and the development of obesity in humans (Chaput et al., 2006;Chaput et al., 2008;Knutson and Van Cauter, 2008;Snell et al., 2007). The question remains what the reason for this discrepancy between human epidemiology and animal experiments is. One explanation is that exposure to chronic sleep restriction differently affects energy expenditure in rats and humans. In rats, we demonstrated that the attenuation of weight gain in sleep restricted rats is a consequence of increased energy expenditure (chapter 2). In humans, however, energy expenditure is not affected during sleep restriction (Buxton et al., 2010;Penev, 2007). In fact, it might be that chronic sleep restriction in humans, by causing fatigue, is associated with reduced day-time activity and thereby a decrease in overall daily energy expenditure. In contrast, sleep restricted rats in our forced activity model are not able to reduce their activity and thereby maintain an increased energy expenditure. This difference in energy expenditure may explain the assumed difference in weight changes between human and rat studies.

Alternatively, differences in energy intake may explain the discrepancy between

weight changes in sleep restricted laboratory rats and humans as well. In chapters 2 and 3 we demonstrated that rats did not increase their food intake despite the attenuation of weight gain during sleep restriction. Research has shown that total sleep deprivation as well as REM sleep deprivation leads to hyperphagia, yet rats lose weight (Everson and Crowley, 2004;Hipolide et al., 2006;Koban and Stewart, 2006;Koban et al., 2008). In humans, several days of sleep restriction did not affect normal food intake but did significantly increase snacking behavior in the evening (Nedeltcheva et al., 2009). Although body weights were unchanged during the experimental period, authors concluded that the increase in snacking behavior, and thus an increase in caloric intake, could be obesity-promoting. In our experiments, rats do not have the availability of high caloric snacks or choice between different types of food, which may be another explanation for the assumed difference in weight changes between human and rat studies.

Interestingly, alternating sleep restriction with periods of rest is more common in real life. Sleep loss in humans is generally an alternation between sleep restriction during the week and recuperation from sleep loss on the weekend (Valdez et al., 1996). In contrast, experimental studies in rats often consist of a continuous period of sleep restriction without periods of recovery. Therefore, we assessed the metabolic consequences of a chronic sleep restriction protocol that modeled working weeks with restricted sleep time alternated by weekends with unrestricted sleep allowance (chapter 6). We showed that the alternation between periods of sleep restriction and sleep allowance led to complex changes in food intake and body weight thus preventing weight loss that is normally seen during continuous sleep restriction. Yet, even though this protocol of sleep restriction alternated with weekends of sleep allowance prevented the attenuation of weight gain, rats did not become overweight or obese.

Perhaps, the duration of sleep restriction is important as well. Experimental studies in rats are rarely longer than a few days or weeks. Even in our own lab, rats were never sleep restricted for more than a month. It may be that insufficient sleep only contributes to obesity when sleep restriction truly becomes a chronic condition.

To summarize, epidemiological studies in humans demonstrate clear associations between short sleep and obesity, which has led to the hypothesis that insufficient sleep may be a causal factor in the development of obesity. Unfortunately, the finding that, in rats, sleep restriction attenuates weight gain does not support this hypothesis. There may be several complicating factors that could result in different effects of sleep restriction in laboratory rats and humans in real life, particularly differences in the way sleep restriction affects overall energy expenditure and energy intake. Furthermore, it may be that sleep restriction is a risk factor for developing obesity only for certain personality types or people with a certain lifestyle. Personality differences may therefore be an important factor in the metabolic consequences of sleep restriction as well, which we will further address in the next section.

Sleep restriction and personality differences

The negative consequences of sleep restriction can differ between individuals (Van Dongen et al., 2005). Personality differences may play a major role in sensitivity to the development of metabolic disorders. To investigate this, we performed a pilot study in which we subjected two selection lines of rats with different coping styles to our chronic sleep restriction protocol. For this purpose we studied the Roman high avoidance (RHA) and Roman low avoidance (RLA) rats. These selection lines were founded by Bignami in 1965 (Bignami, 1965). The rats originate from the Wistar strain and were selectively bred on the basis of their performance in a two-way active avoidance test. RHA rats were bred to rapidly learn shock avoidance, whereas RLA rats were bred for non-acquisition of avoidance. Subsequent studies showed that these rats not only differ in their shock-avoidance performance but, in fact, differ in a wide range of behaviors, such as emotional reactivity and coping style. RLA rats are highly emotional individuals with a passive coping style, whereas RHA rats are (pro)active rats with low emotional reactivity (Steimer and Driscoll, 2005).

Research demonstrated that sleep architecture is different between both lines as well (Steimer et al., 1999). Total sleep time was not different, but RHA rats did have an overall increase in REM sleep time, with a concomitant decrease in NREM sleep time. However, it is not known whether they respond differently to sleep restriction. In our pilot experiment rats of both lines underwent 8 days of sleep restriction (n=4 per group). Food intake and body weight was measured daily. We hypothesized that, since RLA rats are more emotional and therefore sensitive to changes in the environment, they will lose more weight in comparison to RHA rats. Interestingly, both RLA and RHA rats had an attenuated weight gain, but there were no differences between the lines. Furthermore, we did not detect any differences when compared to Wistar rats in our previous experiments (chapters 2 and 3).

In chapter 4, we demonstrated that both forced and voluntary running decreases the insulin response to an IVGTT in RLA and RHA rats, but this effect is most prominent in the RLA rats, since these rats are already insulin insensitive under sedentary conditions. The same decrease in insulin response to an IVGTT is seen in our Wistar rats during sleep restriction (chapter 3). The question that remains is whether RLA and RHA rats differ in their glucose response to an IVGTT after sleep restriction, since we found a clear hyperglycemia in Wistar rats after 8 days of sleep restriction. It might be that, although both lines of rats have attenuated weight gain, they do have a difference in glucose clearance during an IVGTT. This would support the notion that personality differences are an important aspect in the metabolic consequences of sleep restriction and perhaps the sensitivity to metabolic disorders.

Concluding remarks

Our data demonstrated that chronic, as well as acute, sleep restriction in rats has profound effects on glucose homeostasis and energy balance. In addition, we

found that alternating sleep restriction with periods of unrestricted sleep prevented the attenuated weight gain as seen during continuous sleep restriction. For that reason, this week-weekend protocol is interesting, both in the context of glucose homeostasis and weight gain. The attenuation of weight gain during continuous sleep restriction has always been an important, but unwanted, confounding factor when looking at the development of insulin resistance and glucose intolerance, since decreased body weight has effects on insulin sensitivity in itself. Therefore, this week-weekend protocol is a significant addition to existing research. Future experiments should emphasize the effects of chronic sleep restriction, alternated with periods of sleep allowance, on glucose homeostasis and insulin sensitivity.

Based on our data, future research should also focus on the underlying mechanisms of the sleep restriction induced glucose intolerance, especially at the central level. The neuropeptides orexin A and orexin B are assumed to be involved in the effects of sleep restriction on glucose homeostasis and energy balance, but it is not clear to what extent. Once the involvement of these peptides is elucidated it might be that the negative consequences of sleep restriction can be controlled by therapies targeting the orexinergic system.

Nevertheless, it is a well-known notion that preventing is better than curing. In addition to personality differences in sleep architecture (Steimer et al., 1999) it is known that there are individual differences in the amount of total sleep one requires. Sleeping 8 hours per night may be right for one, but not enough for another. In any case, data obtained from the current studies suggest that chronic sleep restriction in the rat is a useful model for determining mechanisms by which insufficient sleep may lead to metabolic diseases such as type 2 diabetes and obesity.

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Nederlandse samenvatting

De metabole gevolgen van slaapverstoringen in ratten

Metabole ziekten als overgewicht en type 2 diabetes komen steeds meer voor in de Westerse wereld. Deze toename kan niet alleen verklaard worden door een verhoogde voedselinname en/of verminderde fysieke activiteit. De hoeveelheid slaap is de laatste jaren ook sterk afgenomen. Recent onderzoek, ondermeer door de groep van Van Cauter in Chicago, heeft aangetoond dat ook een tekort aan slaap of een verstoring van de slaap kan leiden tot de ontwikkeling van deze metabole ziekten. Het is echter nog niet duidelijk of er daadwerkelijk sprake is van een oorzakelijk verband. Experimentele studies zijn nodig om een oorzaak-gevolg relatie tussen slaaptekort en gezondheidsproblemen te kunnen bepalen. Om deze reden hebben wij in dit proefschrift in detail onderzocht wat het effect is van chronisch slaaptekort op de metabole regulatie in ratten.

Om ratten op een gecontroleerde manier bloot te stellen aan chronische slaaprestrictie werden ze 8 dagen gehuisvest in langzaam draaiende wielen voor 20 uur per dag. De dieren mochten de eerste 4 uur van hun normale rustfase slapen, hetgeen niet voldoende was om te herstellen van de opgedane slaapschuld. Wij hebben hetzelfde schema aangehouden om chronisch slaaptekort te induceren en de metabole gevolgen te bestuderen. Om het slaaptekort te quantificeren werden de dieren in **hoofdstuk 2** voorzien van electrodes voor het meten van hersenaktiviteit en het vaststellen van de slaap-waak patronen. De methode bleek zeer efficient in het verminderen van slaap tijdens de 20 uur per dag, wat leidde tot een toename in slaaptijd tijdens de 4 uur slaapmogelijkheid per dag en tijdens de 24 uur herstelperiode na 8 dagen met verminderde slaap. Tevens werd geconstateerd dat 8 dagen met verminderde slaap leidde tot een afname in lichaamsgewicht in vergelijking met controle dieren die onbeperkt konden slapen. De verklaring voor deze afname in lichaamsgewicht bleek een duidelijke toename in energie uitgave tijdens de periode van slaaprestrictie, vastgesteld met behulp van de dubbel gelabeld water methode.

In een volgende studie werd op dag 1 en dag 8 van het slaaprestrictie protocol een intraveneuze glucosetolerantietest (IVGTT) uitgevoerd om de invloed van acuut en chronisch slaaptekort op de glucoseregulatie te bepalen (**hoofdstuk 3**). Tijdens deze test kregen de ratten een glucose-infuus en werden veranderingen in glucosewaarden en de insulinerespons gemeten. Ondanks dat de basale bloedglucose en plasma-insulinewaarden verlaagd waren was een toename in glucose zichtbaar tijdens de IVGTT, zowel op dag 1 als op dag 8 van het slaaprestrictie-protocol. Een tweede groep ratten onderging slaapverstoring in plaats van slaaptekort. Slaapverstoring werd geinduceerd door middel van meerdere kortdurende periodes geforceerde activiteit afgewisseld met periodes waarin de ratten vrij mochten slapen. Ondanks dit protocol hadden de ratten genoeg tijd per dag om te slapen (14 uur). Deze slaapverstoorde ratten lieten ook een hyperglycemie zien. Daarentegen was de insulinerespons tijdens de IVGTT juist verlaagd voor zowel ratten die blootgesteld waren aan slaaptekort als aan slaapverstoring. Hieruit concludeerden wij dat de glucosetolerantie van het lichaam verlaagd is na zowel acute als chronische slaapverstoring.

Om te bepalen of de resultaten die we zien echt een gevolg waren van slaaptekort werden 2 controle experimenten uitgevoerd. In onze experimenten maakten wij gebruik van langzaam draaiende wielen om slaaprestrictie te induceren, wat inhoudt dat de ratten geforceerd werden om actief te zijn. In **hoofdstuk 4** hebben we laten zien dat als ratten gedwongen werden om te lopen in een loopwiel, zonder slaaptekort te induceren, dit geen invloed had op de glucose homeostase. De veranderingen in glucoseregulatie in de dieren die blootgesteld werden aan slaaprestrictie lijken dus niet het gevolg te zijn van gedwongen activiteit.

Het slaaprestrictie model kan mogelijk ook een verstoring van de slaap-waak ritmiek teweeg brengen. Verstoring van de ritmiek kan een alternatieve verklaring zijn voor de veranderingen die wij zien op het metabolisme. Het is mogelijk dat het moment van de dag waarop de geforceerde activiteit plaatsvindt van belang is in het induceren van de metabole verandering. Om deze reden werd in **hoofdstuk 5** een model voor ploegendienst opgezet om te meten wat de effecten van een veranderd dag-nacht ritme zijn op het metabolisme in vergelijking tot het slaaprestrictie model. De dieren in dit ploegendienst model hadden genoeg tijd om te slapen, maar de slaapfase en waakfase werden omgedraaid. Deze ratten werden geforceerd tot activiteit met behulp van de langzaam draaiende wielen voor 8 uur per dag tijdens de lichtfase, wat normaal hun slaapfase is. Een controle groep ratten werd geforceerd tot 8 uur activiteit tijdens de donkerfase, wat normaal ook de actieve fase is. Een duidelijk effect van ploegendienst op voedselinname was zichtbaar. De voedselinname verschoof geleidelijk naar de lichtfase waarin ze nu actief moesten zijn. Echter, de totale voedselinname per dag was niet veranderd. Aan het einde van het protocol, na 14 dagen ploegendienst, hebben we een IVGTT uitgevoerd om het effect van ploegendienst op de glucoseregulatie te bepalen. Er waren geen veranderingen zichtbaar in de respons op een IVGTT in vergelijking met controle ratten. We kunnen dus concluderen dat een verstoring in dag-nacht ritme niet leidt tot glucose intolerantie zoals zichtbaar was tijdens slaaprestrictie en slaapverstoring (hoofdstuk 3).

In al onze experimenten laten we zien dat ratten afvallen tijdens slaaptekort. Dit lijkt niet in overeenstemming te zijn met epidemiologische studies die suggereren dat slaaptekort kan leiden tot overgewicht. Een belangrijk verschil is dat slaaprestrictie in rattenexperimenten vaak ononderbroken zijn terwijl in de humane situatie het vaak een afwisseling is van periodes met slaaptekort (doordeweeks) en periodes van bijslapen (weekends). Het kan zijn dat deze continue afwisseling van slaaptekort en periodes van rust juist cruciaal zijn in de ontwikkeling van overgewicht. Om die reden hebben wij ratten op hetzelfde “week-weekend” protocol gezet. Dit houdt in dat ratten blootgesteld werden aan verminderde slaap voor 5 dagen per week, en vrij mochten slapen in de overige 2 dagen van de week. Dit protocol hebben we 4 weken aangehouden (**hoofdstuk 6**). Onze hypothese was dat dit “week-weekend” protocol de afname in lichaamsgewicht zou voorkomen, aangezien ratten nu tussendoor tijd hadden om te herstellen. Tijdens de eerste week van het protocol was een verlaging in lichaamsgewicht en geen verandering in voedselopname zichtbaar, vergelijkbaar

met de data in hoofdstuk 2 en 3. Echter, na het eerste weekend, waarin de ratten vrij mochten slapen, was er een extreme toename in voedselinname zichtbaar tijdens de slaaprestrictie dagen. Deze verhoging in voedselinname zorgde ervoor dat er geen verlaging in lichaamsgewicht meer plaats vond. Om precies te zijn, slaaprestrictie ratten bleven parallel lopen aan de controle dieren. Schijnbaar zorgde het weekend rust ervoor dat de dieren zich beter konden aanpassen aan het slaaprestrictie protocol, waardoor de voedselinname verhoogd werd en de ratten op deze manier konden voorkomen dat ze nog meer zouden afvallen.

Een ander verschil tussen ratten experimenten en de humane situatie is het verschil in dieet. In het dagelijkse leven hebben mensen de keuze uit vele types eten met verschillende hoeveelheden vet. In tegenstelling tot vet voedsel krijgen ratten vaak automatisch een standaard type voer (chow) met een laag vetgehalte. Wij wilden onderzoeken of de effecten van slaaptekort op lichaamsgewicht, voedselinname en regulerende hormonen leptine en insuline beinvloed zou worden door een medium vet dieet in vergelijking met standaard chow dieet (**hoofdstuk 7**). Voedselinname was niet verschillend tussen de ratten op de verschillende diëten, maar tijdens slaaptekort was het lichaamsgewicht van de ratten op een medium vet dieet minder extreem gedaald vergeleken met ratten op een chow dieet. Deze minder extreme daling in lichaamsgewicht kan ook een beschermende invloed hebben op de neurobiologische gevolgen van slaaptekort die eerder in ons lab zijn geconstateerd. Om die reden hebben wij onderzocht of een verschil in dieet tijdens slaaptekort invloed heeft op de gevoeligheid van de serotonine 1A receptor. De afname in gevoeligheid van de serotonine 1A receptor tijdens slaaptekort, zoals die in eerder onderzoek was vastgesteld, bleef bestaan, ook bij dieren op een medium vet dieet. De minder extreme afname in lichaamsgewicht van ratten op een medium vet dieet tijdens slaaptekort had dus geen effect op de serotonine 1A receptor gevoeligheid. Hieruit kunnen we concluderen dat de veranderingen in serotonine 1A receptor gevoeligheid tijdens slaaptekort niet het gevolg zijn van veranderingen in dieet of lichaamsgewicht, maar waarschijnlijk het gevolg zijn van een direct effect van slaaptekort op de hersenen.

Conclusie

In dit proefschrift hebben we onderzoek gedaan naar de invloed van slaaptekort op metabole processen in de rat. Onze data laten zien dat chronische slaapverstoring in de rat leidt tot veranderingen in de energiebalans (energie-inname en energie-uitgave) en glucoseregulatie wat op de lange termijn zou kunnen leiden tot de ontwikkeling van type 2 diabetes en obesitas. Het is belangrijk om te onderzoeken wat de onderliggende mechanismen zijn van de metabole gevolgen van slaapverstoringen, in zowel de hersenen als in de periferie. De neuropeptide orexine zou hierin een belangrijke rol kunnen spelen. Het is bekend dat orexine betrokken is bij een aantal metabole processen zoals glucoseregulatie en de energie balans. Het is echter nog niet geheel duidelijk wat de precieze functie van orexine is in deze processen. Als dit eenmaal opgehelderd is, zou orexine een belangrijk doel kunnen zijn voor medicatie om de metabole gevolgen van slaaptekort te verbeteren.

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Wetenschap is iets wat je samen doet en waarbij sociale interacties echt belangrijk zijn. Ik wil dan ook al mijn kamergenootjes door de tijd heen bedanken. Marcelo, although we only shared an office for a few months at the BC, we have always been close and good friends. You were always there for me; this meant a lot to me and it still does. Gretha, mijn grote voorbeeld! Vanaf het moment dat ik begon aan dit project ben jij er geweest om zowel praktisch als theoretisch te helpen. Jij hebt me veel technieken geleerd en zonder jou was het een stuk zwaarder geweest. Ik zal onze congresmomenten ook niet snel vergeten, vooral onze nachtelijke acties blijven spectaculair. Als jij sliep, begon ik te praten en te wandelen en als ik eindelijk rustig was en even mijn ogen open deed, keek ik recht in jouw (geheel wakkere en heldere) ogen en dan wist ik dat het alweer 6 uur was! Heel erg bedankt voor alles! Caroline (CC), jouw extreem nuchtere en zakelijke manier van denken was vaak zeer verhelderend. Super leuk om te zien hoe duidelijk je kunt zijn naar anderen; of dit nou gaat om mede AIOs of onze bazen, het maakt jou niet uit. Ondanks dat we vaak even moed moesten verzamelen om te gaan sporten, hebben we toch altijd doorgezet en was het achteraf een super gezellig sportuurtje (of kletsuurtje?). Heel erg bedankt hiervoor! (Klaar nu! Doeg!) Dear Stefano and Federica, it was a pleasure to share an office with you in the new building. Thanks for the ‘gezelligheid’! Grazie! Jan en Fiona, ik ken jullie beide al erg lang. Eerst als studenten en erna als mede AIOs. Ik kan me geen betere nieuwe AIO’s voorstellen in onze groep, zowel qua werk, maar ook qua gekheid. Iemand moet mijn rol toch een beetje gaan overnemen en ik weet zeker dat ik dit aan jullie kan overlaten! Bedankt voor alles, vooral jullie steun in de laatste maanden.

Dear Arianna, although we technically shared an office in the new building, I probably spent more time in your office at the BC. If I needed advice, talk about data, or just personal stuff, you were there. I am really happy that we worked together on some of our projects and will not forget the moments in the basement during the IVGTTs/challenges. Lots of fun! Grazie! Lieve Henriette, het is ongelooflijk hoeveel tijd wij samen doorgebracht hebben. In onze kantoren, samen werken in de kelder, basketbal, Buckshot, de bioscoop of gewoon hangend op de fiets bij het kruispunt waar we beide een andere kant op moesten. Het laatste jaar zagen we elkaar al minder, maar we hebben altijd mailcontact gehouden en het is mijn doel om dit ook zeker voort te zetten. We moeten elkaar toch een beetje op de hoogte blijven houden! En anders moet je maar zo denken; we hebben altijd Marco Borsato nog!

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(en geordend) was. Ik ben erg blij dat ik nog gebruik heb kunnen maken van jouw kennis in de afgelopen jaren! Ten tweede wil ik Gertjan bedanken. Ik ben erg blij dat we in mijn laatste jaar nog echt hebben kunnen samenwerken en gelukkig zijn de data die daaruit voort kwamen ook nog eens super mooi. Buiten dat om wil ik je ook bedanken voor het feit dat jij zag als er mij iets dwars zat en dat ik dan altijd mijn verhaal bij jou kwijt kon als ik die behoefte had.

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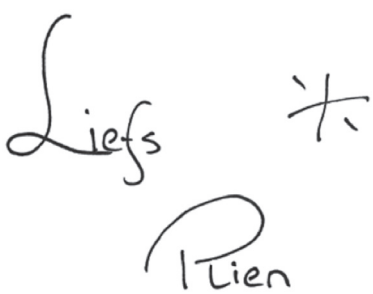

Curriculum Vitae

Reina Paulien Barf was born on February the 12th, 1984, in Hollum on the island of Ameland. She studied Biology at the University of Groningen from 2002 to 2007. She specialized in behavioral neurosciences and concluded her research master at the Graduate School for Behavioral and Cognitive Neuroscience (BCN). Her first master research project focused on the role of clock genes in time-place learning in mice under supervision of Prof. Dr. Menno Gerkema and Prof. Dr. Eddy van der Zee. Her second research project focused on the effects of social stress on sleep-wake behavior and sleep EEG in rats under supervision of Dr. Peter Meerlo. She graduated cum laude.

In August 2007, Paulien started her PhD project “the metabolic consequences of sleep restriction in rats” under supervision of Prof. Dr. Anton Scheurink and Dr. Peter Meerlo, which resulted in this thesis. She continued her scientific carrier in December 2011 at the University of Washington in Seattle, WA, USA, in the group of Prof. Dr. Mark Opp.

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