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PALATABLE FOOD, CLOCK GENES AND THE REWARD CIRCUITRY

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Academic thesis

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TABLE OF CONTENTS

CHAPTER 1 General introduction **9**

CHAPTER 2 Diet-induced obesity and circadian disruption of feeding behavior: role of homeostatic and reward systems..... **25**

CHAPTER 3 Effects of a free-choice hgh-fat high-sugar diet on brain PER2 and BMAL1 protein expression in mice **49**

CHAPTER 4 A free-choice high-fat high-sugar diet alters day-night *per2* gene expression in reward-related brain areas in rats..... **71**

CHAPTER 5 Glutamatergic inhibition of the lateral habenula in rats affects food intake in a time-dependent manner **87**

CHAPTER 6 Eating behaviour in npas2 clock gene mitant mice under a free-choice high-fat high-sugar condition **105**

CHAPTER 7 General discussion **127**

CHAPTER 8..... **163**

Thesis Summary **164**

Résumé de la thèse **169**

Samenvatting **175**

APPENDICES..... **183**

Phd Portfolio..... **184**

Publications **186**

Acknowledgments **187**

Chapter 1

General Introduction

Biological Rhythms

The cyclic environment caused by the rotation of the earth around its own axis, generates the day-night cycle with a period length of exactly 24 hours. In living organisms, also a temporal circadian organization has also been developed, this internal temporality is important to adapt to the cyclic environmental changes, for instance, by keeping the organisms awake and aroused at the time when the chances of food availability are higher and predator menace are lower, increasing the odds for survival (DeCoursey, Walker, & Smith, 2000). These internal cyclic changes with a ~24h period duration are known as "circadian", a word with Latin etymologies: circa (about) and dies (day). Food intake is among many biological processes that are expressed with a circadian pattern (Aschoff, 1981), in mammals such as humans and rodents, feeding behavior is higher during the normal active phase and minimal during the resting phase. Within the body, virtually all processes in cells show circadian variation, whether it is electrical activity, peptide/hormonal production or gene expression (Dibner, Schibler, & Albrecht, 2010; Kalsbeek, Perreau-Lenz, & Buijs, 2006). However, the rhythm of each cell however is not acting in isolation but there is a cell synchronization within tissue/organ cells to coordinate physiology and behavior(Welsh, Logothetis, Meister, & Reppert, 1995). Central in the hierarchy of the circadian system, coordinating all body oscillators is the suprachiasmatic nucleus (SCN), located in the hypothalamus, also referred to as the central clock. This coordination of body oscillators (the peripheral clocks) produces an internal synchronization where all cycles are coupled among each other and to the SCN (Figure 1).

When the internal rhythmicity is coupled to the external cyclic environment, it is called external synchronization (Husse, Eichele, & Oster, 2015). The SCN exhibits circadian physiology observed for instance, on gene expression or electrical activity (Ono, Honma, & Honma, 2015; Yamazaki, Kerbeshian, Hocker, Block, & Menaker, 1998). The rhythmic activity of the SCN is synchronized to the outside world by environmental stimuli of which light is the strongest stimulus (Dibner et

al., 2010). These stimuli that are able to entrain the circadian rhythms are referred to as Zeitgeber, a German word that means: time-giver. In addition to light, other Zeitgebers such as temperature, fasting/feeding cycles, arousal and social cues can synchronize circadian rhythms (Hirao et al., 2010; Landry & Mistlberger, 2007; Rensing & Ruoff, 2002; Tahara et al., 2015). Proper synchronization between internal and external rhythms is important for a healthy state, whereas internal desynchronization between different organs or external desynchronization (such as shift work, or frequent jet-lag) has been related to development of disease (Scheer, Hilton, Mantzoros, & Shea, 2009; Voigt et al., 2014; Wyse, Selman, Page, Coogan, & Hazlerigg, 2011).

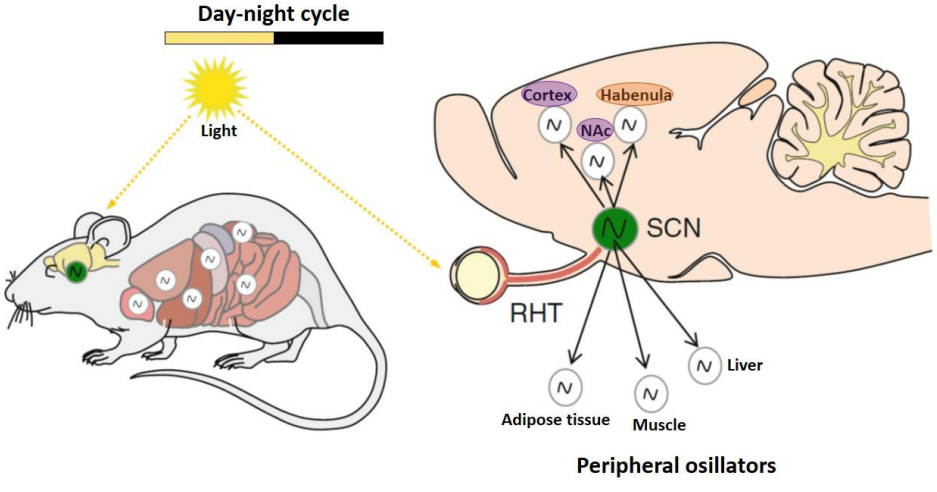

Figure1. The circadian system is hierarchically organized. Light provides the main input to the SCN through the RHT. The SCN (green clock) is the central clock, whereas other brain areas are semi-autonomous oscillators such as the habenula (in orange) and not self-sustained oscillators like accumbens and cortex (in purple). In the periphery, all organs have rhythmic functions (white clocks) and are synchronized by the central clock.. Modified from Horst-Werner and von Gall, 2016. (suprachiasmatic nucleus: SCN; retinohypothalamic tract: RHT; nucleus accumbens: NAc)

The “western” lifestyle, where food is available every moment of the day and artificial light enables us to be active at all times, makes it easy to disrupt rhythmic synchrony among body oscillators. For example, the SCN is sensitive to light, whereas peripheral oscillators are more sensitive to feeding, thus when light exposure and feeding times are providing conflicting stimuli to different oscillators this can result in disruption of energy balance (Damiola et al., 2000; Jorge Mendoza, 2007; Wyse et al., 2011). In addition, food composition can also affect rhythmicity. For example, animals rendered obese with high energy diet also exhibit altered feeding patterns, consuming abnormally higher amounts of food during the inactive period, pointing to effects of highly caloric diets on the circadian system (Kohsaka et al., 2007; la Fleur, Luijendijk, van der Zwaal, Brans, & Adan, 2014). Given the growing obesity epidemic, it is important to investigate how the circadian system affects feeding behavior and metabolism and which factors are involved in disruption of the circadian system.

The biological clock(s)

The endogenous circadian system in mammals, including humans and rodents, is hierarchically organized, where the SCN is the main clock that coordinates rhythms in physiology and behavior (Dibner et al., 2010; Stephan & Zucker, 1972). The SCN consists of two nuclei that are located bilaterally over the optic chiasm, in the hypothalamus, an area controlling basic survival functions like food intake, body temperature regulation and mating (L. R. Squire et al., 2008; Weaver, 1998). In the 70s, the SCN was identified as the main biological clock, as it was shown that rats with a lesioned SCN displayed arrhythmic general locomotion and water intake which, instead of remaining nocturnal, was spread equally throughout the 24h of the day (Stephan & Zucker, 1972). Further evidence supporting the idea of the SCN being the main body clock came from brain tissue transplantation experiments with tau mutant hamsters. When lacking the *tau* gene, the hamsters displayed a period of locomotor activity shorter than 24h, and when their SCN was lesioned, they became arrhythmic. When these animals are subsequently being

transplanted with an SCN from wild-type hamsters (with normal 24h rhythmicity), the mutant SCN-lesioned hamsters recovered their locomotor activity rhythms but displaying the 24h period rhythms from the wild-type hamsters (LeSauter, Lehman, & Silver, 1996; Ralph, Foster, Davis, & Menaker, 1990). Thus, the SCN has its own endogenous rhythm. Further studies revealed that within the SCN, different rhythmic parameters can be measured *in vitro* as well as *in vivo* such as electrical activity (Albus et al., 2002; Shibata & Moore, 1988) and gene expression within its cells (Ono et al., 2015; Yoo et al., 2004). The SCN receives the information from the cyclic external environment through photic cues that are first received in the retina after light stimulates the intrinsically photosensitive retinal ganglion cells (ipRGCs), which contain a photopigment called melanopsin, that project directly to the SCN, using glutamatergic signaling at the synapses (Albrecht, 2012; Evans & Silver, 2016; Schmidt et al., 2011). The SCN then transmits the rhythmic information by sending direct projections to several brain nuclei including hypothalamic areas such as the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), paraventricular hypothalamic nucleus (PVH), the lateral hypothalamus (LH) and arcuate nucleus (ARC) (Abrahamson & Moore, 2001; Buijs et al., 2017). Moreover, several nuclei involved in reward processing also receive projections from the SCN such as the lateral septum and the lateral habenula (LHb) (Abrahamson & Moore, 2001). Within the hierarchical organization of the circadian system, the LHb has been recognized as a semi-autonomous oscillator together with the pineal gland, it has strong electrical oscillations and circadian variation in gene expression even when it is studied ex-vivo (C Guilding, Hughes, & Piggins, 2010). The LHb is an epithalamic area that has been involved in predicting a negative outcome and it gets activated when an expected outcome is not present (Matsumoto & Hikosaka, 2007). The LHb activity has a modulatory effect on the dopaminergic tone from the mesolimbic system that is importantly involved in reward processing. The LHb projects to the GABAergic tail of the ventral tegmental area/Rostromedial tegmentum (tVTA/RMTg), an area projecting to the dopaminergic ventral tegmental area (VTA) which has efferent

projections to forebrain areas like the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Matsumoto & Hikosaka, 2007; Meye, Lecca, Valentinova, & Mameli, 2013). This mesolimbic circuit show daily activity in some functional parameters but most of its areas have been labeled as "slave" oscillators because their rhythmicity is not self-sustained and depend on a functioning SCN (Abrahamson & Moore, 2001). When the SCN is lesioned and its rhythmic control disappears, the normal day-night variation of gene expression is also blunted in both core and shell subregions of the NAc (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2010). Both SCN and LHb thus seem to participate in the control of the rhythmic function of the mesolimbic dopaminergic system. Within the SCN, LHb and several nuclei controlling the homeostatic and reward processing of food intake, a group of genes forming a feedback loop of expression/repression with a circadian duration are known as clock-genes (Takahashi, 2015). These genes are not only expressed in a circadian fashion but they can also control the overall rhythmicity of the organism, modifying rhythmic patterns. However, at the same time, some environmental factors are able to regulate its intrinsic expression.

The molecular clock-genes

The molecular clock-mechanism in the cell is formed by a group of different genes and proteins that form a feedback loop with an induction and repression rhythmic activity of ~24h. In a simplified clock-gene model, the core components of this system are represented by a positive and a negative limb that signal each other. The positive limb of the loop comprises the genes *clock* and *Bmal1* which protein products form a dimer in the cytoplasm which is translocated to the nucleus and binds the promoter region of the *Per* and *Cry* genes, part of the negative limb. Subsequently, these genes are translated into proteins in the cytoplasm that form a dimer and enter back into the nucleus where they act as a repression signal to the different components of the positive limb (Takahashi, 2015). When the PER/CRY dimers degrade, the expression of *clock* and *Bmal1* starts again (Figure 2)

(Takahashi, 2015). This genetic clock system is redundant since other homologue genes can take over the functions of another canonical clock-genes. This is the case for the *npas2* gene which is able to substitute *clock* in the SCN when it is genetically knocked down (DeBruyne, Weaver, & Reppert, 2007). There is evidence of clock-gene function hierarchy dependent on the brain area. For instance, in the SCN, the main gene that forms a dimer with *bmal1* is *clock*, although *npas2* can substitute it. Nevertheless, in forebrain areas, *npas2* has a more important role to *bmal1* dimer formation (Reick, Garcia, Dudley, & McKnight, 2001).

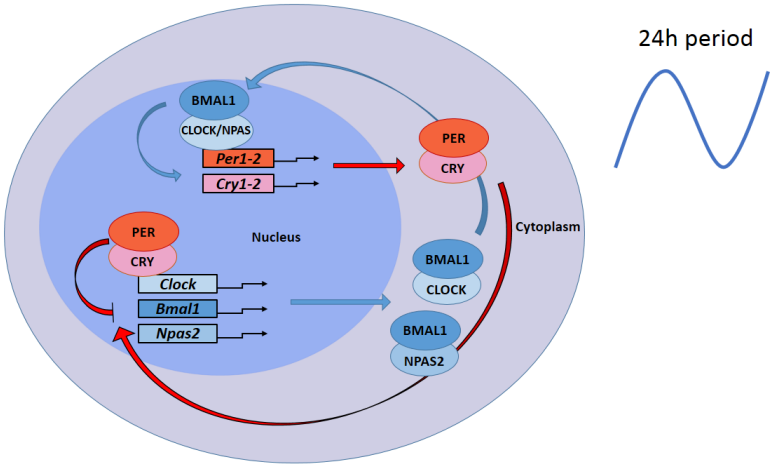

Figure 2. Simplified model of clock-gene expression.Clock, Bmal1 and Npas2 genes (blue tone rectangles)form the positive limb of the feedback loop, its proteins (blue tone ovals) form dimers in the cytoplasm and are traslocated into the nucleus (signaled by the long blue arrow). In the nucleus, the BMAL1/CLOCK or BMAL/NPAS2 dimer bind to the negative limb (signaled by the short blue arrow) elements of the clock-gene loop, Per and Cry genes (red tone rectangles). The proteins PER and Cry will dimerize in the cytoplasm and translocate to the nucleus (signaled by the long red arrow) and inhibit the expression of the clock-gene positive limb elements (signaled with the short blue blind-ended arrow).

Systemic deletion of *Npas2* in mice does not produce behavioral arrhythmicity, but it does produce impairments in the adaptation to a timed cue such as food access. In a protocol of time-restricted feeding (TRF) where the food availability is narrowed to a time period of 2 to 4 h, the wild-type mice develop an anticipatory locomotor activity to food access, however, the *Npas2* mutant mice take significantly longer to develop this anticipatory behavior to food access (C. A. Dudley et al., 2003). Moreover, *Npas2* mutant mice ate less food during the TRF protocol and also they were more prone to weight loss compared to the wild-type mice (C. A. Dudley et al., 2003). Another study showed that during TRF protocol, the *Npas2* mutant mice ate the same amount of food but lost more weight than the wild-type group (Wu, Wiater, & Ritter, 2010). When the Npas2 gene is ablated in mice, the adaptation to a photoperiod shift, like the one a human would be exposed during a travel across multiple time zones, is affected. Npas2 mutant mice need significantly less time to adapt to the new light schedule compared to the wild-type mice (C. A. Dudley et al., 2003; Wu et al., 2010). This suggests that whereas the *Npas2* mice have difficulties to adapt their behavior to a timed food-cue, they are more sensitive to photic signals, as observed by the increased adaptability to photoperiodic changes. Moreover, the relation of this clock-gene to drugs of abuse such as cocaine has been studied. Mutant mice lacking the *Npas2* gene show a decreased conditioned placed-preference (CPP) to cocaine and furthermore, the specific ablation of this gene in the NAc caused the same behavioral effects in the CPP protocol as in the systemic *Npas2* mutant mice. Furthermore, the lack of Npas2 had also repercussions on the NAc dopaminergic system by blunting the normal day-night expression of the dopaminergic receptors (Ozburn et al., 2015). Conversely, cocaine administration has effects on *Npas2* expression, producing arrhythmicity in the NAc after chronic exposure and interestingly, this effect was restricted to *Npas2* but not its analog *clock* gene. Similar to *Npas2*, the *Per2* gene, part of the negative limb of the clock-gene mechanism also resulted in a blunted rhythmic expression in the NAc following cocaine exposure (Falcon, Ozburn, Mukherjee, Roybal, & McClung, 2013). Moreover, ablation of the *Per2* gene also

affected the behavioral approach to other drugs. Mice with *Per2* gene ablation show higher cocaine sensitization after long term withdrawal from cocaine administration (Abarca, Albrecht, & Spanagel, 2002). Additionally, *Per2* mutant mice have a disrupted brain glutamatergic system and show a higher intake and motivation to ingest alcohol (Spanagel et al., 2005). These results point towards a modulatory role of *Per2* in the reward system. In fact, mice lacking *Per2* gene exhibit a disrupted mRNA expression rhythm of monoamine oxidase (Mao), an important enzyme for dopamine degradation, in the VTA. In line, dopamine levels are elevated in the NAc of Per2 mutant mice compared to the WT mice (Hampp et al., 2008). It is evident that clock-genes have a role as a time-keeper of cellular functions but it might be important to also acknowledge their role in non-circadian functions such as metabolic regulation and reward processing.

Setting the time to the clock

Circadian rhythms in mammals, from the clock-gene function to the behavioral level can be temporarily arranged and modified by different environmental signals. For humans and rodents, the main Zeitgeber is light, which is detected in the eye's retina by the intrinsically photosensitive retinal ganglion cells (ipRCG's), a specialized cell group which project to the SCN via the retinohypothalamic tract (Schmidt et al., 2011). Light is able to entrain the circadian rhythm of the SCN and, subsequently, the SCN entrains the rest of the body through its rhythmic output signals to other hypothalamic nuclei (Dibner et al., 2010). General locomotor activity and daily sleep/arousal rhythmic patterns are some of the major outputs of SCN entrainment, but also food and water ingestion as well as some metabolic processes result from the communication of the SCN with the autonomic nervous system (Kalsbeek et al., 2006). However, rhythms can be altered and modified by different stimuli such as, for instance, drugs of abuse and food (Escobar, Salgado, Rodriguez, Blancas Vazquez, et al., 2011; Hsu, Patton, Mistlberger, & Steele, 2010; Kosobud et al., 2007; McClung, 2007), which can act as a Zeitgeber. When normocaloric food is provided *ad libitum*, the SCN paces the rhythmic overall

behavior and the main Zeitgeber is light, thus, mice and rats eat mainly during their active phase, during the dark period. However, when food availability is limited in time of access, food becomes a strong zeitgeber due to its vital nature. In experiments where normal chow food availability is restricted to a few hours a day in a restricted feeding schedule (RFS), different animal species quickly develop an anticipatory locomotor activity prior to food access (Mistlberger, 1994). Physiological changes are developed to prepare the organism to the expected food access (Díaz-Muñoz, Vázquez-Martínez, Aguilar-Roblero, & Escobar, 2000) and it is important to note that this anticipatory mechanism is reflecting the biological clock timing system which is entrained to the time of food access. During the RFS protocol, both metabolic and rewarding properties of food are signals to the circadian system. In order to separate the homeostatic from the hedonic properties of food, a palatable food item is provided daily at the same time, but keeping the animals with regular chow food *ad-libitum*. This protocol results in anticipation to the hedonic food but the behavioral anticipation is significantly less than during the RFS protocol (Escobar, Salgado, Rodriguez, Blancas Velazquez, et al., 2011; Hsu et al., 2010; J Mendoza, Angeles-Castellanos, & Escobar, 2005), highlighting the additive components from the reward and metabolic system and the ability of a natural rewarding stimulus to modify rhythmic behavior.

As mentioned above, physiology can be affected by the type of nutrients in the diet. Food with high amounts of sugar and/or fat are highly palatable. The long term ingestion of these type of nutrients produce body weight gain due to fat accumulation and concomitantly, results in metabolic disturbances such as hyperglycemia and leptin resistance (Hans-Rudolf Berthoud & Morrison, 2008). Furthermore, high caloric diets, besides producing obesity, can also alter the rhythmic physiology of the organism. Some, but not all studies have shown that hypercaloric diets affect rhythmic behavioral outputs such as eating patterns and general locomotor activity. Because the information has been controversial, we reviewed and discussed the studies that have investigated rhythmic behavioral

outcomes after chronic high caloric consumption which is described and discussed in Chapter 2.

The free choice High Fat High Sugar diet

Different high caloric diets have been used to study the development and consequences of obesity using animal models. A common paradigm is the use of homogeneous custom made pellets that contain all diet nutrients, have a consistent quality and can be bought from a commercial company. These types of diets are successful to generate increased caloric intake during the first days of exposure. On the other hand, these high caloric pellet diets lead to larger meals but also compensation in meal interval resulting in a progressive decrease of total calorie intake. Moreover, these kind of diets contain all nutrients within a single pellet which, as a model for human obesity, fails to mimic the human situation where different food items can be chosen, a process that includes the processing of reward and motivation. In order to better model this situation where the individual is able to choose among healthy and palatable/high caloric diet components, the free choice High-Fat High-Sugar diet (fcHFHS) has been developed and studied as a model of high caloric consumption and obesity development (la Fleur et al., 2007). Rats fed the fcHFHS diet increase their body weight gain and display hyperphagia throughout the 4-week experimental period differently from the animals which had all food components in a single pellet (Non-free choice High-Fat High-Sugar diet, ncHFHS) which showed decreased caloric consumption overtime (la Fleur et al., 2014). It has also been observed that when the animals have the 4 different components available, the ingestion patterns for each diet component differs. The daily pattern of chow and fat remain rhythmic with higher ingestion during the night, whereas the sugar is ingested as much during the day as during the night (la Fleur et al., 2014). Interestingly, the sugar consumption is not the main factor for the development of the day-night disruption observed in animals on the fcHFHS diet, as animals kept their nocturnal ingestion patterns if the sugar is presented alone with the chow food (Bainier, Mateo, Felder-Schmittbuhl, & Mendoza, 2017;

la Fleur et al., 2014). Because food ingestion is able to alter the brain oscillators differentially as described previously with the TRF and palatable entrainment protocols, and because the SCN is mainly driven by light, the disrupted ingestion patterns of sugar intake and its specificity to this rewarding diet component raises the question whether some nuclei in the brain that direct hedonic food intake might be implicated and engaged in directing this "abnormal" behavior. Taking into account that the day/night ingestion of chow food remains unaltered, and that the SCN is also implicated in directing food intake rhythmicity, we hypothesize that the SCN is still exerting its timing coordination for homeostatic feeding and thus, a possible desynchronization between nuclei from the reward system and those involved in homeostatic eating, including the SCN might be occurring in this situation.

Synchronization of body clocks in health and disease

Entrainment of the body's physiology to light indicates that the SCN is synchronized to the environmental photoperiod and thus, a coordinated rhythmicity for other brain nuclei is signaled by the SCN, resulting in rhythmic behavioral outputs as locomotor activity and food intake. In this situation, the central and peripheral oscillators work in rhythmic synchrony which means in a controlled and constant phase relationship, with their acrophases or maximum expression peaks at the same time, or in antiphase with their acrophases in the opposite phase of the 24h rhythm. This coordination is important to preserve a healthy state as shown with experiments aiming to alter the normal rhythmicity of a determined variable. Evidence in humans has pointed out the relationship of internal desynchronization to metabolic syndrome in people who shifted their normal schedules to work during the night. Circadian disruption has been linked to cancer development probably mediated by clock-genes involved in the cell cycle process (Soták, Sumová, & Pácha, 2014). Moreover, shift workers such as nurses that alter their normal rhythmic patterns, are prone to develop insulin resistance, high glucose levels, increased blood pressure and obesity (Peplonska, Bukowska, & Sobala,

2015). These metabolic alterations come together with alterations in normal rhythmic physiology including clock-gene expression. To study the effects of this rhythmic disruption, animal models of shift-work have been developed where the animals are forced to remain awake during the light phase and the food and water is present *ad libitum.* These studies have shown that the animals ingest more food during the light phase than controls. Interestingly, humans prefer high palatable foods during their shift working periods, highlighting the influence of the rewarding system in driving food choices at night in humans. This activation during the normal resting period and the increased food intake is related to development of metabolic syndrome (Garaulet & Madrid, 2010). Eating at the wrong time of day can produce internal desynchrony of different brain oscillators as shown in rats fed a normocaloric chow diet during the day which resulted in blunted rhythmic oscillation of orexinergic function in the LH and arrhythmic *Per1* and *Per2* clock-gene expression in the LH and ARC (Opperhuizen et al., 2016; Wang et al., 2017). Feeding during the light period also has consequences on the rhythmic expression of genes involved in hepatic function, by shifting the normal rhythm to the feeding time in mice and rats (De Vries et al., 2017; Hatori et al., 2012; Landgraf et al., 2015). There is clear evidence that the time of food intake has an impact on rhythmic function of the brain and peripheral organs, however, little is known about the effects of obesogenic high caloric diets on rhythmic behavior and different body oscillators. The understanding of these processes would shed light on the interaction processes between the circadian and the feeding system which could serve to generate prevention actions and/or interventions against obesity.

Scope of the thesis

Rhythmicity of food intake relies on the circadian system of which the main clock is the SCN. The SCN signals to other brain oscillators to generate this feeding rhythm, allowing the individual to eat during its active phase. The SCN also generates rhythmic output to metabolic tissues through its influence on the autonomic nervous system and hormonal release, coupling the metabolic processes to be in sync with feeding patterns. Nevertheless, in humans, the modern environment seems to be altering the normal physiological processes. The exposure to artificial light has promoted the lengthening of our active phases, and the production of fast and ultra-processed food which is easily accessible at any time, are factors promoting obesity and rhythmic disruptions. Several studies in humans and in animal models have found that these life style changes could have a detrimental effect on health as there is a relation between high caloric feeding during the normal resting phase and the development of obesity.

Overeating of highly palatable foods can alter brain function in specific regions of the reward system (e.g. the mesolimbic dopamine pathway) .The neuronal and molecular mechanisms involved in overeating of palatable food are unclear but, interestingly, recent studies reported that the circadian clock in SCN and clock genes, have been implicated in the regulation of the consumption of high caloric diets. Nevertheless, the role of different clock genes and circadian clocks (SCN and other brain clocks) in the development of palatable eating is still poorly explored.

The aim of the thesis was therefore to understand how nutrients affect circadian activity the brain and how these changes mediate the overeating of high palatable diets. We first characterized, in mice and rats, how a high caloric diet with the possibility to consume saturated fat and a sugar solution in addition to regular nutritionally balanced chow (the fcHFHS diet), alter circadian rhythms in feeding behaviour and the expression of clock genes in reward related brain structures, and how day-night changes in specific brain areas are involved in daily feeding

rhythms. Secondly, we investigated whether deletion of a clock gene mainly expressed in forebrain, is involved in the response to a highly palatable diet.

Many studies have suggested a disruption of behavioural features such as locomotor activity and eating patterns with obesity. Therefore, in **Chapter 2** we reviewed the available information about the effects of hypercaloric diets on rhythmic locomotor activity and eating patterns as well as its effects on rhythmic parameters in feeding-related brain areas. Our main findings highlighted the disruption of rhythmic eating patterns and, to a lesser extent, general locomotor activity. However, the information about the effects of high caloric diet on rhythmic features of brain areas controlling food intake is still unclear. We know that the fcHFHS diet is effective in generating hyperphagia and obesity in rats but this model had not been tested in mice before. The use of genetically modified mice strains would be an approach to study the interaction between the fcHFHS diet and specific genes, including clock-genes. Therefore, in **Chapter 3** we characterized the food intake patterns of mice fed the fcHFHS diet and, we described the effects of this diet on clock-protein expression in the LHb, ARC and SCN. Although the effect of the fcHFHS diet in the brain's rat has been studied, its effects on brain clock-gene expression had not been investigated. In **Chapter 4** we addressed that question by measuring clock-genes mRNA in the feeding related areas of the rat's brain. The results from Chapters 3 and 4 pointed to a possible role for the LHb in rhythmic food intake. We focused on the role of the LHb in normal and high caloric food intake. In **Chapter 5** we investigated the effect of pharmacologically inhibiting the LHb, either during the night or during the day time, of rats fed normal chow food or the fcHFHS diet. Finally, we studied the influence of the *Npas2* clock-gene on feeding patterns using genetically modified mice with an ablation of the *Npas2* gene. The behaviour of *Npas2* mutant mice exposed to either chow or fcHFHS diet and their *ex-vivo* PER2::Luc expression of brain oscillators are shown in **Chapter 6**.

Chapter 2

Diet-induced obesity and circadian disruption of feeding behavior: role of homeostatic and reward systems.

Aurea Blancas-Velazquez, Jorge Mendoza, Alexandra N. Garcia and Susanne E. la Fleur

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Diet-induced obesity and circadian disruption of feeding behavior: role of homeostatic and reward systems.

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Abstract

Feeding behaviour shows a rhythmic daily pattern, which in nocturnal rodents is observed mainly during the dark period. This rhythmicity has been shown to be under the influence of the hypothalamic suprachiasmatic nucleus (SCN), the main biological clock. Various studies have shown that animal models, using high-energy diet to render animals obese, disrupt general locomotor activity and feeding rhythms. Feeding behaviour is regulated by a neural circuitry that responds to changes in energy balance. The ingestion of hypercaloric diets alters the normal physiology of the neural circuits of feeding. These alterations disrupt the feedback to properly adjust energy balance. The neural circuitry that regulates feeding includes the hypothalamus, which has several structures that have reciprocal interactions with the SCN, and reward related areas involved in the motivational and hedonic aspects of feeding. This review focuses on the effects of diet-induced obesity on feeding patterns, related physiological and behavioral parameters, as well as brain sites within the neural circuitry involved in feeding behaviour. We further discuss the effects of disrupted feeding patterns on overall energy balance.

Keywords: Circadian, hypothalamus, reward, feeding, obesity, clock-genes, palatable, dopamine.

Introduction

Biological rhythms are the cyclic variations of any biological process of a living organism. Rhythms with a ~24 h duration are called circadian, a word with Latin etymologies that means *circa* (around) and *dies* (day). Circadian rhythms are adaptive to the cyclic environment caused by the rotation of the earth on its own axis, where the most evident variation is the light-dark (LD) cycle causing the day and night. General locomotor activity and food intake are two of the behavioral outputs of the endogenous circadian system which, in normal feeding and LD conditions, are coupled and synchronized to the activity period of the organism; predominantly during the day in humans and during the night in most rodents (Aschoff, 1981; Silver & LeSauter, 2008). Biological rhythms are not displayed only as a response to the environmental changes but they are inherently paced by a timekeeping system comprised of several organs, tissues and brain nuclei called oscillators. The rhythmic properties of these oscillators can be observed for instance, in the electrical activity of the cells, neurotransmitter and molecule synthesis and release, or gene expression, among other variables. In natural conditions, these oscillators can be entrained by several external or environmental factors (such as the alternance of day-night, food availability and/or temperature) that set the timing of their functions (Challet, 2010; Rensing & Ruoff, 2002). The main synchronizer or *zeitgeber* (ZT; a German noun adopted to define a time-giver) is the solar time, which is able to pace the activity/inactivity cycles (in chronobiology ZT0 is used to indicate the start of the light period). Thus, activity cycles are entrained by photic signals that are received by the ganglion cells in the retina and transmitted via the optic tract to the hypothalamic suprachiasmatic nuclei (SCN) (Albrecht, 2012). These nuclei, located bilaterally adjacent to the third ventricle and dorsal to the optical chiasm, are considered the main biological clock since its physical or genetic function ablation causes disorganized locomotor activity as well as disrupted eating and drinking rhythmic patterns (Albus et al., 2002; Stephan & Zucker, 1972).

Food ingestion, an essential part of energy balance, is controlled by two main processes in the brain: one that evaluates the quantity of the required energy intake and another that regulates the quality of the food including its hedonic properties (Hans-Rudolf Berthoud & Morrison, 2008). The first system, in charge of the energy balance, resides in the hypothalamus, a central area that receives and sends information from and to the peripheral organs via neuronal and hormonal signals (Lenard & Berthoud, 2009; Schwartz, Woods, Porte, Seeley, & Baskin, 2000). Whereas, the reward-limbic system processes the characteristics and quality of the food, reinforcing the preference for palatable/rewarding items, which in general contain high levels of sugar and/or fat (Avena, Bocarsly, & Hoebel, 2012). Nowadays, the relatively easy access to ultra-processed food high in fat and sugar, together with social variables like education and socioeconomic status, influence the food choices made by an individual (Specter, 2004). These situations that facilitates the consumption of hypercaloric food have a correlation to the increasing obesity epidemic and other concomitant metabolic diseases (Juul & Hemmingsson, 2015; Louzada et al., 2015). During obesity, one of the main characteristics is the caloric consumption beyond homeostatic need, which has led scientist to question why energy homeostasis is malfunctioning when faced with the food-choice enriched modern world. One hypothesis is that the exposure to a rich environment stimulates our visual, olfactory and gustatory senses, overriding the energy-balance system by means of over excitation of the reward system (H. Zheng, N. Lenard, A. Shin, 2009). In obese humans, behavioral changes such as an increase in depressive symptoms and disruption of sleep patterns indicates a close relationship between the reward and circadian systems (Kudlow, Cha, Lam, & McIntyre, 2013; Ulrich-lai, Fulton, Wilson, Petrovich, & Rinaman, 2016)

This review focuses on the effects of hyper caloric diets on the daily rhythms of locomotor activity and feeding behavior. Furthermore we describe the current evidence how hyper caloric diets affect circadian properties of the homeostatic and reward systems.

CYCLES OF THE HOMEOSTATIC HYPOTHALAMIC CLOCK(S)

The hypothalamus is a brain center that integrates internal and external signals to produce vital behaviors such as eating. Within this region, several nuclei important for homeostatic regulation have been identified, including the SCN, the hypothalamic arcuate nuclei (ARC), ventromedial and dorsomedial hypothalamic nuclei (VMH and DMH), paraventricular nuclei (PVN) and lateral hypothalamic area (LH) (Gonnissen, Hulshof, & Westerterp-Plantenga, 2013; Schwartz et al., 2000). These nuclei contain different cell populations that synthesize and release neuropeptides and neurotransmitters that are important for regulating food intake. Given the vital function of food ingestion, it is not surprising that redundant systems exist within the hypothalamus to ensure this important behavior continues. The presence of several orexigenic molecules in different areas such as neuropeptide Y (NPY) and agouti related peptide (AgRP) in the ARC and the melanin-concentrating hormone (MCH) and the hypocretines/orexin in the LH are evidence of the complex regulation of feeding. In the ARC two anorexigenic peptides pro-opiomelanocortins (POMC) and cocaine and amphetamine regulated transcript (CART) produce signals of satiety (Schwartz et al., 2000). The decrease of circulating glucose and nutrients as well as other hunger signals such as an empty stomach are sensed and processed by the brain to trigger feeding behavior (Hans-Rudolf Berthoud & Morrison, 2008). In conditions where the organism ingests a nutritionally balanced diet (i.e. a laboratory chow diet) feeding behavior is arranged in a temporal manner, which is coupled with the activity period. Although the SCN is considered to be the main biological clock, there are also several organs and brain areas containing oscillatory properties and thus, they have been called peripheral circadian oscillators. In normal healthy conditions, these peripheral oscillators show different rhythmic features such as a variation in electrical activity, neurotransmitter release and/or gene expression.

Neuronal activity

The SCN cells show clear rhythmic electrical activity. In brain slices of hamsters, rats and mice, electrophysiological experiments have revealed a rhythmic firing rate with higher levels during the light period (Albus et al., 2002; Gillette & Reppert, 1987; Shibata & Moore, 1988). Similarly, the ARC shows clear circadian firing rate even in the absence of the SCN input as evidenced with ex-vivo slice preparations(Clare Guilding, Hughes, Brown, Namvar, & Piggins, 2009). In the DMH, although circadian rhythms are present, the amplitude of the oscillations is decreased and dampens faster than those observed in the ARC, whereas the VMH has no clear oscillation after tissue culture (Clare Guilding et al., 2009). Nevertheless, another study recorded the electrical activity of the VMH of rats *in-vivo,* finding a rhythmic activity with an acrophase (highest activity around the 24h) in the dark period. Moreover, when the SCN was lesioned, the *in-vivo* rhythmicity of the VMH was blunted (Inouye, 1983). The fact that some nuclei show self-sustained rhythms (although in a lesser extent compared to the SCN), and some others are not able to oscillate in the absence of SCN signals is evidence of the hierarchic nature of the circadian system.

Diurnal variation of neurotransmitter levels

Neuropeptides and neurotransmitters within the hypothalamus also show circadian rhythmicity. These oscillations can be observed in neuropeptides such as NPY, which has receptors located within hypothalamic nuclei, forebrain and cortex (Kash et al., 2015; Keen-rhinehart, Dailey, & Bartness, 2010). In rats, the expression of both NPY and its receptor Y1R in the ARC and PVN are higher during the active period (Cohen et al., 2015). Interestingly, the NPY and its Y1R receptor were detected in the hippocampus and the basolateral amygdala but no clear day and night difference was observed (Cohen et al., 2015). In mice, gene expression of the AgRP, NPY (Stütz, Staszkiewicz, Ptitsyn, & Argyropoulos, 2007) and orexin (Opperhuizen et al., 2016; Stütz et al., 2007), molecules that

increase appetite and decrease metabolism and energy expenditure, exhibit diurnal variation. Moreover, a clear time difference was also seen for MCH and the leptin receptor but not in the levels of POMC or CART (Stütz et al., 2007). A study using immunohistochemistry reported no day-night variation of orexin peptide in the hypothalamus of mice, however, the c-fos co-localization with ORX cells was higher during the active period (Marston et al., 2008). Taken together, the evidence suggests that not all hypothalamic molecules implicated in the regulation of feeding and energy balance have the ability to oscillate. To gain a better understanding of how a single variable contributes to the circadian system, scientist have conducted studies where they either suppress or over-express a single molecule. In a study made by Waiter et al., 2011, they silenced NPY signaling, which interfered with the functioning of the NPY receptors in the rat mediobasal hypothalamus. The ablation of NPY signaling produced disturbed sleep and food intake patterns; rats increased feeding during the light period compared to the control rats with an intact NPY-ergic system (Wiater et al., 2011). A separate study manipulated the NPYergic system by producing an overexpression of NPY using a viral gene transfer either in the PVN or the LH. The constant overexpression produced a reduced amplitude of locomotor activity and disrupted the eating pattern rhythm (Tiesjema, Adan, Luijendijk, Kalsbeek, & la Fleur, 2007). Thus, when the NPYergic system is taken out of the rhythmic system, either by suppression or constant over-expression, alterations in rhythmic behavior can be observed. These studies demonstrate that a single molecule can be important in building the circadian rhythms. Further investigation is needed to understand why some molecules have a circadian variation and what implications these oscillations have on physiology.

Rhythmic clock-gene expression

The molecular gene machinery can be observed in cells throughout the body and is comprised of several molecules that generate rhythms of transduction/translation with a ~24 h duration. In this oscillating mechanism *Bmal1* and *Clock* genes form

part of the positive loop, which promotes the transcription of the Period (Per 1-3) and Cryptochrome (Cry 1-2) genes. The latter genes form the negative loop components, which in turn suppresses the activity of *Bmal1* and *Clock* dimer (Takahashi, 2015). These genes and their products are relevant molecules for building-up the circadian variations in physiology and behavior. *Per1* and *Per2* clock-proteins have been evaluated with immunohistochemistry in both the rat and mouse hypothalamus at different time points of the day. Results showed that there is daily rhythmic expression in the ARC, DMH, and VMH with higher levels at night (Céline A Feillet, Mendoza, Albrecht, Pévet, & Challet, 2008; M Verwey, Khoja, Stewart, & Amir, 2007). Studies have also examined PER2 using the *ex-vivo* bioluminescence technique, where the PER2 protein is coupled to a luciferase reporter, which allows the measurement of the photon emission produced when PER2 protein is expressed. Using this method, PER2 in the ARC and DMH of animals kept in light/dark (LD) and dark/dark (DD) conditions has been shown to oscillate even when these nuclei are isolated from the rest of the brain (Clare Guilding et al., 2009; Hughes, Guilding, & Piggins, 2011). Similar results have been found for PER1 bioluminescence in the PVN and LH with the acrophase during the night time (Abe et al., 2002b). This technique has made it possible to describe rhythmic properties of clock-genes and proteins in different brain areas. Moreover, results indicate that the rhythm amplitude in the SCN is stronger and longer-lasting than the peripheral oscillators in which, with ex-vivo bioluminescence, the oscillations dampen with a faster rate compared to the SCN (Abe et al., 2002b). As for the electrical activity of peripheral oscillators, the rhythmic expression of clock genes varies in intensity depending on the brain nuclei, but its functionality is not fully understood (Clare Guilding et al., 2009). Several experiments have demonstrated that the complete knockdown of clock-genes in the body alters physiology and behavior. For instance, the mutation of the *clock* gene includes loss of normal locomotor activity patterns as well as metabolic alterations and obesity (Rudic et al., 2004; Turek et al., 2005). The knockout of systemic *Per2* produces a phenotype that displays disrupted rhythmicity of

locomotor activity in constant darkness (DD) conditions, as well as lower body weight and disrupted lipid metabolism (Grimaldi et al., 2010; Zheng et al., 1999). Although there is a large body of evidence that links the clock gene function to metabolic physiology, the implications of the rhythmic clock-gene expression within the different areas of the hypothalamus are still unclear. An attempt to determine the effect of a single clock-gene in a specific area was made within the hypothalamic tuberomammilar nucleus (TMN), an area that integrates inputs from the circadian and the sensory stimuli to modulate locomotion and arousal (Torrealba, Riveros, Contreras, & Valdes, 2012). When *Bmal1* is knocked down from the histaminergic cells in the TMN of mice their normal rhythmic levels of histamine are lost and they display fragmented sleep but there are no changes in overall locomotor activity (Yu et al., 2014). This highlights the important role a single clock-gene can play in specific brain areas. Also, it is interesting to note that the disruption of the molecular clock can alter only certain rhythmic parameters. These findings lead to questions about which functions are controlled by each clock-gene in different brain areas.

THE CYCLES OF THE REWARD SYSTEM

The reward system comprises several nuclei as well as different neurotransmitters, with the mesolimbic dopaminergic pathway playing a central role. This circuit includes the ventral tegmental area (VTA), a nucleus containing dopaminergic cells that projects to the nucleus accumbens (NAc) and the cortex (Koob & Volkow, 2010). Other nuclei that send projections to the NAc are the amygdala and hippocampus, which are involved in the regulation of emotion and memory consolidation (Sesack & Grace, 2010). The bed nucleus of the stria terminalis (BNST) is known to densely project to the amygdala and is considered part of the extended amygdala, where the corticotropin-releasing factor, a neuropeptide involved in stress response, is a key signal (Daniel & Rainnie, 2015; Kash et al., 2015). The septum forms part of the limbic system, where it receives dopaminergic projections from the VTA and regulates affective behaviors (Mogenson, Jones, &

Yim, 1980; Sokolowski & Corbin, 2012). Beyond the classical mesolimbic dopaminergic circuitry, other structures integrate the wiring of the reward process such as the lateral hypothalamus (LH) and the lateral habenula (LHb). The LH is part of the feeding behavior neurocircuitry and is anatomical connected to the dopaminergic system with which it regulates motivation and arousal (Hans-Rudi Berthoud & Münzberg, 2011; Harris, Wimmer, & Aston-Jones, 2005; Stuber & Wise, 2016). The Habenula, an epithalamic area, can be divided into different sub regions with the major distinction being between the medial and lateral part. The medial habenula (MHb) receives inhibitory and excitatory projections from the septum (Qin & Luo, 2009). The LHb has more anatomical and functional communication to the VTA, sending excitatory glutamatergic projections to the tail of the VTA (Bianco & Wilson, 2009; Matsumoto & Hikosaka, 2007). The LHb is able to modulate dopamine release and has been shown to be involved in the processing of the reward-prediction error and negative motivational states (Proulx, Hikosaka, & Malinow, 2014; Salaberry & Mendoza, 2016). The reward system is known to regulate the appetitive state of the organism by influencing the approach and intake of food. Recently, the function of the LH and the LHb have been shown to communicate and to modulate the control of motivational feeding (Stamatakis et al., 2016). Areas of the reward system have different cyclic properties that can be observed in neuronal activity, neurotransmitter release and gene expression.

Neuronal activity

By using the neuronal activity marker c-fos, an immediate early gene, Baltazar and colleagues (2013) found that there is a day-night rhythm of neuronal activity in the rat PFC, NAc, and VTA, with higher levels during the night (Baltazar, Coolen, & Webb, 2013). A diurnal variation of c-Fos has also been observed in the LH and LHb, with the LH having higher levels during the night in mice (27) and the LHb during the daytime around ZT6 in rats (56,57). However, other studies have found in hamsters and also in mice higher levels of c-fos during the night time (2 h after lights off), compared to daytime (2h before lights off; 55,56). The different results

found for c-fos could be due to species differences and/or the time point of when the c-fos was evaluated. Despite these discrepancies, the findings point to the existence of rhythmic c-fos expression in the LHb. Another piece of evidence of direct neuronal activity comes from experiments using cellular multi-unit recordings, where a cyclic variation of electrical activity in the NAc and medial septum of hamsters has been measured *in-vivo* (Yamazaki et al., 1998). This observation has also been reported in *in-vitro* for the LHb cells of mice, where the firing rate was found to peak during the latter part of the light period (Sakhi et al., 2014). In rats this activity has been evaluated in *in-vivo* and *in-vitro* with both showing the highest activity around ZT6 (H. Zhao & Rusak, 2005).

Diurnal variation of neurotransmitter levels

As stated previously, the neuronal activity can vary throughout the day, and this oscillation might be reflected in the cyclic functions of other variables like the neurotransmitter production and/or release. Dopamine (DA), adrenaline, and noradrenaline (NA) are part of the catecholamine family of neurotransmitters in which tyrosine hydroxylase (TH) is the precursor of all (L. R. Squire et al., 2008). The dopaminergic system is highly oscillating, in mice higher levels of TH mRNA production in the VTA have been shown during the early resting period (Chung et al., 2014). Similar results have been found in rats, with higher protein expression of TH at ZT6, during the light period (Webb et al., 2009). Furthermore, not only the production, but also the release of DA is highly rhythmic during the LD cycle, within the dorsal striatum (DS) of rats (Ferris et al., 2014; Paulson & Robinson, 1994; Smith, Olson, & Justice, 1992) as well as ventral striatum of rats and mice (Castañeda, de Prado, Prieto, & Mora, 2004; Frustaci et al., 2012; Hampp et al., 2008; Hood et al., 2010). The endogenous rhythmicity of DA release in the NAc has also been observed in DD conditions where higher levels are observed during the active period of rats (Castañeda et al., 2004). The rhythmic dopaminergic activity in the mesolimbic system has been reviewed previously by Webb et al (Webb, Lehman, & Coolen, 2015). The rhythmicity of adrenaline and NA,

implicated in the activation of the fight or flight behavior in response to a threatening situation (L. R. Squire et al., 2008) has also been studied. Measured from the blood of human adults, a clear rhythmic pattern is present, showing the highest levels during the active period (Scheer et al., 2009).

Serotonin (5HT) is another important neurotransmitter within the reward system, which is produced in the raphe nuclei and projects throughout the brain. It is involved in the regulation of mood, food intake and circadian rhythms (Versteeg, Serlie, Kalsbeek, & la Fleur, 2015). The levels of 5HT and its main metabolite, 5-HIAA, have been measured during different time points. Interestingly, when measuring 5-HIAA with microdialysis in the DS and NAc of rats there is a rhythm, which is synchronized to the LD cycle where the highest levels are observed during the night. Nevertheless, when the light condition is changed to constant light (LL), the rhythms of 5HT and 5-HIAA in both DS and NAc are ablated whereas in the DD condition the rhythm is still present in the NAc but not in the DS (Castañeda et al., 2004). A different report on the diurnal levels of 5HT assessed the variations in rats at 6 different time points but found no diurnal variation of this neurotransmitter in the anterior hypothalamus or the cortex (Cagampang, Yamazaki, Otori, & Inouye, 1993). Nevertheless, studies using microdialysis to measure 5HT levels in the SCN of rats (Cagampang et al., 1993) and hamsters (T. E. Dudley, DiNardo, & Glass, 1998) have shown that there is clear rhythmicity though there are some differences between species. Rats show higher levels during the light period whereas levels are higher during the dark period in hamsters. Despite species differences, the results point towards a rhythmic function of 5HT that varies across brain regions.

Rhythmic clock-gene expression

The rhythmic variation of the function and activation of the reward system can also be extended to clock-genes, which are widely expressed in these areas. In the mesolimbic system including the NAc, the PFC, DS, BNST and amygdala the

genes *Per 1-3*, *Clock*, and *Npas2* oscillate in a circadian manner (Harbour, Weigl, Robinson, & Amir, 2013; Webb et al., 2015). The habenula, which regulates mesolimbic dopaminergic release, also shows daily variations of the *Per2* gene and protein (Z. Zhao et al., 2015). However, when the BNST, NAc and VTA are isolated and cultured for *ex-vivo* bioluminescence recordings no rhythmicity of PER1 is observed (Abe et al., 2002b). Recently, a study assessing *ex-vivo* bioluminescence showed rhythmicity of PER2 in NAc cells (Logan et al., 2015), which differs from the findings on PER1. This may indicate that different molecules from the molecular clock-machinery might persist more than others, and thus be a stronger molecular timekeeper. Using the same technique, it has been shown that in the habenula PER2 also displays robust oscillations (C Guilding et al., 2010). Taken together these results indicate that the reward system has several parameters that are able to oscillate, but further research is needed to understand the physiological repercussions of these diurnal and circadian variations. The circadian function of the reward system might be altered by external factors that stimulate and modify its function. One of these possible factors is food intake, especially the ingestion of highly rewarding food that facilitates the development of obesity.

INFLUENCE OF HIGHLY CALORIC INTAKE ON THE CIRCADIAN SYSTEM OUTPUT

Locomotor activity

Feeding is generally coupled with the period of arousal and (locomotor) activity. In mice and rats, the highest locomotor activity is performed at night, although some bouts of activity are also present during the day-time. This activity pattern is observed when the animals are under normo-caloric feeding conditions. However, when rats and mice are offered a high caloric diet, the normal rhythm of locomotor activity is altered showing an overall decrease in the amount of activity during the night (Jorge Mendoza, Pévet, & Challet, 2008; Pendergast et al., 2013; Sherman et al., 2012; Sun et al., 2015). Moreover, some studies in mice have found that the

activity during the daytime is also disrupted, generating spare activity throughout the light period resulting in a-rhythmic activity (Branecky, Niswender, & Pendergast, 2015; Pendergast et al., 2013). Kosaka et al, didn't find differences in the amount of activity during the day on high-fat fed vs. normal chow-fed mice under LD conditions, but the behavioral recordings (actograms) resembles the disrupted behavior reported by Pendergast et al., 2014 (Kohsaka et al., 2007; Pendergast, Branecky, Huang, Niswender, & Yamazaki, 2014). In DD conditions mice fed with a hyper caloric diet increased the length of their activity period compared to the normo-caloric fed mice during the first week of diet exposure (Kohsaka et al., 2007; Jorge Mendoza et al., 2008). In addition to the locomotor activity changes, similar disturbances have been observed in sleep-wake physiology recorded in mice and rats. The electroencephalogram of the animals fed with a high-fat diet showed decreased awake time as well as wake fragmentation and more rapid eye movement (REM) and non-REM sleep episodes (Guan, Vgontzas, Bixler, & Fang, 2008; Jenkins et al., 2006; Luppi et al., 2014). This highlights the effects of the diet content on general activity, which might evolve together with changes in sleep patterns. Data presented in this section reflects the influence of the ingestion of a high-fat diet in a pellet over the disruption of the locomotor activity. However, other types of highly caloric diets that are not only high in fat but also offer free access to sugar did not clearly find an effect on general locomotion (la Fleur et al., 2007; Oosterman et al., 2015) suggesting that not only the highly caloric content but the quality of food influences the changes observed in behavior.

Eating patterns

When a highly-caloric diet is presented, the locomotor patterns can be affected in a small extent, but the alterations are mainly observed in the food intake patterns. The first alteration in eating behavior is the over ingestion of calories and secondly, there is a change in the diurnal rhythmic patterns of intake. Mice and rats fed with a high-fat pellet amplitude of the rhythm of feeding is lowered, which implies that

the animals eat less during the night period and increase their intake during the day time, compared to the control group (Branecky et al., 2015; Kohsaka et al., 2007; Mifune et al., 2015; Pendergast et al., 2013). The small but frequent bouts of intake, outside the main meals, throughout the 24 h cycle resemble human snacking behavior. This snacking observed even during the resting period might reflect an alteration in circadian rhythmicity. This pattern is also observed when the hyper caloric foods (fat and sucrose) are offered separately from the normal laboratory chow food. In this situation, an overall caloric consumption is produced with snacking behavior mainly observed in the consumption of sugar (la Fleur et al., 2014; Oosterman et al., 2015). In a similar situation where the normal chow was offered to rats, together with canola oil (low in saturated fatty acids) or butter (high saturated fatty acids), the normal rhythm of the hyper caloric components was altered. This alteration was reflected in a snacking pattern that extended to the daytime, especially when they were exposed to butter (Hariri & Thibault, 2011). Over ingestion of food and obesity does not develop in all individuals, even when the general environment might be more or less homogenous. This difference is also found with experimental animals and it has been shown that some neural substrates such as neurotransmitter levels in the brain underlie the preference for rewarding food (Chang, Karatayev, Barson, Chang, & Leibowitz, 2010; Holtz, Zlebnik, & Carroll, 2012; Tõnissaar, Herm, Rinken, & Harro, 2006).

Effects of hyper caloric diets on rhythmic activity of the homeostatic neural system.

When the system is challenged with a hyper caloric diet, several changes occur such as an increase in fat storage and dysregulation of circulating hormones (Buettner, Schölmerich, & Bollheimer, 2007). At the level of the brain different alterations ranging from a dysregulation of the normal levels of NPY, 5-HT and DA among others have been shown to occur (Gumbs, van den Heuvel, & la Fleur, 2015; Karin Eva Koopman, Booij, Fliers, Serlie, & la Fleur, 2013; Pritchet & Hajnal, 2011). The effects of a hyper caloric diet on the rhythmicity and the

expression of clock genes has been reported in peripheral organs such as the liver and adipose tissue with varying effects depending on the energy content of the diet, the age and species of the animals used in the studies as well as the duration of the diet (Branecky et al., 2015; Cunningham et al., 2016; Pendergast et al., 2013; Wong et al., 2015). Few studies have focused on the effects of a diet-induced obesity (DIO) on the rhythmic expression of neuropeptides and clock-genes in the brain. In mice, *Bmal1*, *Per2* and *Clock* gene expression has been assessed using qPCR in animals fed a normo-caloric and a hypercaloric diet, no differences were found within the hypothalamic area (Jang et al., 2012; Kohsaka et al., 2007). Moreover, measured with *ex-vivo* bioluminescence PER2 expression was not changed in the arcuate complex of mice fed with a hypocaloric diet vs. chow diet (Pendergast et al., 2013). When the hypothalamic structures were separately evaluated for *Per2* gene expression using qPCR, the high-fat diet did not exert an effect on the ARC or the DMH in DD conditions. A separate study examined the expression of *Bmal1* in the SCN of DIO mice utilizing *in situ* hybridization where a decreased amplitude was found in DD conditions (Cunningham et al., 2016). Nevertheless, in other peripheral tissues such as the liver, remarkable changes caused by a DIO can be observed in the clock gene expression. These changes include a PER2 phase advance in the liver of mice DIO mice, evidenced by bioluminescence (Branecky et al., 2015). Other changes include the blunted rhythmicity of Per2, Bmal1 and *clock* mRNA in the liver (Kohsaka et al., 2007; Sun et al., 2015) and white adipose tissue (Kohsaka et al., 2007). Taken together, the main findings are that there are little to no effects of hyper caloric diets on the clock-genes expression in the hypothalamus but a large impact of hypercaloric diet on the rhythmicity of some peripheral organs. The DIO state seems to be generating an uncoupling of hypothalamic and peripheral organ oscillators.

Table 1. Effects of *ad libitum* highly caloric diets on rhythmic behavioral outputs and clock gene expression. NR: not reported.

Ref	Locomotor activity	Eating patterns	Clock genes	Diet	Species
(Branecky et al., 2015)	No differences	<amplitud	*Ex-vivo* bioluminiscence Liver PER2 phase advanced	Pellet 45% kcal from fat	Male Mice C57BL/6J::LUC
(Sherman et al., 2012)	Overall decrease	NR		Pellet 42% kcal from fat	Male Mice C57BL/6J
(Sun et al., 2015)	Overall decrease	NR	qPCR Liver *Clock*, *Bmal1* and *Per2* lost rhythm	Pellet 45% kcal from fat	Male Mice C57BL/6J
(Pendergast et al., 2013)	not a clear effect	<Feeding during day	PER2::Luc in the ARC complex. No change	45% kcal from fat	Male Mice C57BL/6J::LUC
(Jorge Mendoza et al., 2008)	>Wheel running at night	NR	NR	53% kcal from fat	Male Mice C57BL/6J
(Kohsaka et al., 2007)	No differences	< Feeding at day >Feeding at night	*Clock*, *Bmal1* and *Per2* qPCR Hypothalamus No change. Fat tissue and liver > amplitude	45% kcal from fat	Male Mice C57BL/6J
(Guan et al., 2008)	>Wake <Non REM sleep	NR	NR	59.3% kcal from fat	Male Mice C57BL/6J
(Jenkins et al., 2006)	>wakefulness and <NREMS	NR	NR	59.3% kcal from fat	Male Mice C57BL/6J
(Luppi et al., 2014)	<REM and nREM sleep	NR	NR	35% fat	Male Rat Sprague-Dawley
(Oosterman et al., 2015)	No differences	NR	NR	Fat and sugar choice. Average: 30.1% from fat 33.4% from sucrose	Male Rat Wistar
(la Fleur et al., 2007)	No change	NR	NR	37.4% from fat 14.8 from sucrose	Male Rat Wistar

Table 1. Continuation

(Mifune et al., 2015)	>amplitude	< Kcal during day compared to chow fed group	NR	60% kcal from fat	Male Rat Sprague-Dawly
(Hariri & Thibault, 2011)	NR	<Feeding at day specially from butter based pellet	NR	Pellet 65% kcal from either cannola oil or butter	Female Rat Sprague-Dawley
(Cunningham et al., 2016)	>during night	>Meal events during the night	Hypothalamus qPCR in whole hypothalamic punches no effect >BMAL1 in DIO mice under DD	Pellet 60% kcal from fat 16w	Male Mice C57BL/6J Mice C57BL/6J::LUC
(Wong et al., 2015)	> at day and night with the corn oil enriched	NR	NR	40% kcal from fat in olive and corn oil enriched pellets	Female Mice C57/Bl6
(Jang et al., 2012)	NR	< Feeding at day > Feeding at night	*Bmal1*, *Per2* and *Clock* in hypothalamus. No change	Pelllet 60% kcal from fat	Male Mice C57BL/6N

Effects of the hyper caloric diets over the rhythmic reward system

The exposure to a highly caloric diet can change locomotor activity and feeding patterns, but little is known about the clock gene expression in the reward related areas during a diet-induced obese state. Nevertheless, some studies have shown the influence of the hyper caloric hedonic properties of food on the behavioral rhythmic outputs. Under a paradigm of scheduled feeding where access to food was restricted to a short time frame, locomotor and feeding behavior of rats was synchronized to the precise moment of food access. In addition, the physiological

variables such as the release of hormones like corticosterone and insulin also follow the feeding time (Ángeles-Castellanos, Mendoza, & Escobar, 2007; Jorge Mendoza, Angeles-castellanos, & Escobar, 2005; Michael Verwey & Amir, 2012). This evidence highlights how the power of food access can modify behavior and that these anticipatory responses can be observed even in the absence of the main SCN clock. Within the scheduled feeding experiments, the metabolic as well as the rewarding properties of food cannot be dissociated, thus, they might be playing additive roles in the synchronization to food. Other studies have tried to dissociate the motivational aspect of food in the food restricted access paradigm. In those experiments, the animals were fed normal chow ad-libitum as well as a highly palatable food item, which was provided every day at the same hour. The behavioral outcomes from these studies done in both mice and rats, show that animals can entrain their behavior, developing an anticipatory activity, though to a lesser degree than when they are food restricted (Ángeles-Castellanos, Salgado-Delgado, Rodríguez, Buijs, & Escobar, 2008; Gallardo, Gunapala, King, & Steele, 2012; Hsu et al., 2010; J Mendoza et al., 2005; Merkestein et al., 2012). During this palatable food anticipation, the NAc, PFC and LH showed an increase in *Per1* (Ángeles-Castellanos et al., 2008). In a similar study, *Per2* was unchanged in the BNST and amygdala (M Verwey et al., 2007) of rats, suggesting that the effects of palatable food under a scheduled feeding depend on the clock-gene and brain area. The experiments discussed in this section used a palatable treat, given daily in a small amount that does not lead to animals becoming overweight. Therefore, no conclusions can be made so far about the effect of DIO on the rhythmic clock-gene expression in the reward system.

CONCLUSION

The effects of obesity in general physiology have been widely studied and a large amount of knowledge has been gathered about the changes within the brain produced by hyper caloric diets. Nevertheless, the changes in circadian outputs like locomotor activity and eating patterns are not reported in most of these studies. From the studies discussed in the present review, it appears that the access to a hyper caloric regime does not alter general locomotor activity to the same extent as the food intake rhythmicity. One possibility for this might be that the rhythmic locomotor output is more resistant to change due to the lack of effects in the SCN and in other hypothalamic areas, while the eating patterns guided by the food palatability might be changing together with the changes in the brain reward system (Figure 1).

No conclusion can be drawn at this point due to the fact that the effects of a hyper caloric diet on the rhythmic brain parameters is inconclusive for the hypothalamus and non-existent for the nuclei from the reward system. At level of the brain studies are region and system-specific and therefore, the findings might differ due to varying methodological approaches. Nevertheless a study looking at the broad spectrum of metabolomics and genomics supports the observations of altered gene expression in obesity (Eckel-Mahan et al., 2013). Showing how the relationship of the reward and metabolic systems integrate and intercommunicate with circadian function might be a step to gaining a better understand of the causes and consequences of obesity.

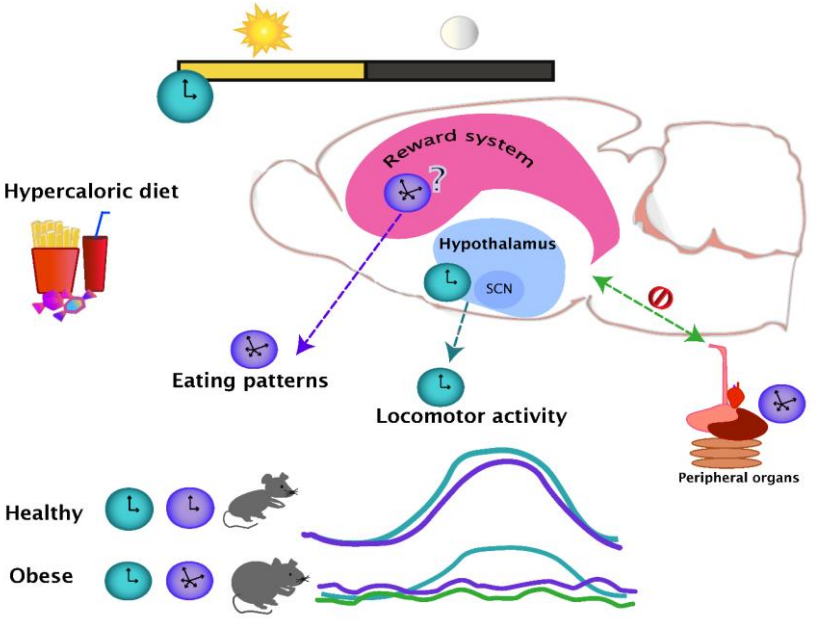

Figure 1. The day-night cycles set the regular oscillations of eating and locomotor activity, which are coupled, during a healthy state. Intake of high-caloric diets, leading in obesity, disrupts the eating daily patterns, producing small but frequent bouts of ingestion even during the normal resting period. The locomotor activity and eating pattern rhythms are uncoupled in an obese state. The effects of a high-caloric diet over the rhythmicity of the reward system are unknown but as the evidence suggest that the rhythmicity in the hypothalamus is mainly unaffected, the reward system might be influencing the disturbances of the daily eating patterns. In the diet-induced obese state, the rhythmicity of the peripheral organs are altered, causing an internal de-synchrony of central and peripheral oscillators.

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Chapter 3

Effects of a free-choice high-fat high-sugar diet on brain PER2 and BMAL1 protein expression in mice

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Effects of a free-choice high-fat high-sugar diet on brain PER2 and BMAL1 protein expression in mice

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Running title: Rewarding food impacts brain clocks

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Highlights

-Free access to a fat and sugar (fcHFHS) diet produces obesity and disrupts daily feeding behaviour in mice.

-The day-night expression of clock proteins is abolished in the lateral habenula, but not in the suprachiasmatic nucleus, of fcHFHS-exposed mice.

-Therefore, the exposure to a fcHFHS diet alters the normal day-night pattern among brain oscillators that modulate food intake.

Abstract

The suprachiasmatic nucleus (SCN) times the daily rhythms of behavioural processes including feeding. Beyond the SCN, the hypothalamic arcuate nucleus (ARC), involved in feeding regulation and metabolism, and the epithalamic lateral habenula (LHb), implicated in reward processing, show circadian rhythmic activity. These brain oscillators are functionally coupled to coordinate the daily rhythm of food intake. In rats, a free choice high-fat high-sugar (fcHFHS) diet leads to a rapid increase of calorie intake and body weight gain. Interestingly, under a fcHFHS condition, rats ingest a similar amount of sugar during day time (rest phase) as during night time (active phase), but keep the rhythmic intake of regular chow-food. The out of phase between feeding patterns of regular (chow) and highly rewarding food (sugar) may involve alterations of brain circadian oscillators regulating feeding. Here, we report that the fcHFHS diet is a successful model to induce calorie intake, body weight gain and fat tissue accumulation in mice, extending its effectiveness as previously reported in rats. Moreover, we observed that whereas in the SCN the day-night difference in the PER2 clock protein expression was similar between chow-fed and fcHFHS-fed animals, contrarily, in the LHb, this day-night difference was altered in fcHFHS-exposed animals compared to control chow mice. These findings confirm previous observations in rats showing disrupted daily patterns of feeding behavior under a fcHFHS diet exposure, and extend our insights on the effects of the diet on circadian gene expression in brain clocks.

Key words: suprachiasmatic; lateral habenula; circadian; clock genes; reward; palatable

Introduction

Obesity is a health problem that has increased over the last decades and it is associated with concomitant metabolic and cardiovascular diseases. The over-consumption of caloric diets has been identified as a principal factor in obesity development (Avena et al., 2008; Berthoud and Morrison, 2008) and therefore, different rodent models have been developed in order to have a better understanding on how highly-caloric food affects brain physiology and behavior. Nevertheless, some of these models have limitations concerning the presentation of the highly-caloric diet which is usually given chronically in one single pellet, having the animals in a forced-fed condition (Farley et al., 2003; Woods et al., 2003). However, in normal circumstances, humans don't follow a forced diet, and we even choose what we perceive as more pleasurable from our free-choice food environment. To solve these differences in experimental and real life situations, a free-choice diet has been used in rats as a model of diet-induced obesity where the caloric compounds of the diet (i.e., sugar and fat) can be chosen in addition to a nutritionally balanced chow diet (la Fleur et al., 2007). Besides inducing obesity, this free-choice feeding condition also induces a "snacking" behavior pattern, characterized by increased frequency of food bouts with similar total amount of food ingestion as compared to animals eating the chow control diet (la Fleur et al., 2014). This model, thus, represents the obese population that gain weight due to snacking palatable food items regularly. Moreover, it is different from other diet-induced obese models which induce larger meal consumption with over-time reduced meal frequency to compensate for increased weight gain (Furnes et al., 2009).

The feeding patterns of fcHFHS diet exposed animals have been related to an alteration of the daily rhythm of eating, which has been proposed as a contributing factor to the development of obesity (Kohsaka et al., 2007; Pendergast et al., 2013). Rats on the fcHFHS diet ingest sugar not only during the activity period (at night) but also during their normal resting period (at day), losing the day-night rhythm of

sugar intake (la Fleur et al., 2014). Interestingly, while the daily rhythm of sugar ingestion seems to be altered, the day-night intake of the regular chow diet and fat remains unchanged, with a main intake at night (la Fleur et al., 2014). Daily rhythms of behaviour and physiology are paced by the main circadian clock, the hypothalamic suprachiasmatic nucleus (SCN) which controls, among other variables, the daily rhythm of food intake (Coomans et al., 2013; Nagai et al., 1978). The SCN contains a molecular time-keeping mechanism which consists of positive and negative feedback loops of the clock-gene expression. Within this molecular machinery, the protein of the gene *Clock* dimerizes with the protein product of the *Bmal1* gene forming the positive loop. The dimerization of CLOCK-BMAL1 induces the transcription of genes from the negative loop like cryptochrome (CRY 1, 2) and period (PER 1-3) whose dimerization, in turn, inhibits the expression of the genes in the positive loop (Takahashi, 2015).

The clockwork molecular mechanism has been observed, beyond the SCN, in several areas throughout the brain and the peripheral organs (Guilding et al., 2009; Saini et al., 2015). In the brain, the clock activity has been reported in areas of homeostatic balance such as the Arcuate Nucleus (ARC) (Guilding et al., 2009), which besides regulating energy expenditure might well be involved in the circadian mechanisms controlling eating behaviour (Buijs et al., 2006). The habenula is an epithalamic structure involved in prediction and approach to a reward, as well as in regulation of emotional states like depression (Friedman et al., 2011; Proulx et al., 2014; Tian and Uchida, 2015). This area can be divided anatomically and functionally in two sub-areas: the medial habenula (MHb) and lateral habenula (LHb) where the latter is anatomically linked to the SCN (Zhang et al., 2009) and the dopaminergic ventral tegmental area (VTA) (Araki et al., 1988; Christoph et al., 1986; Matsumoto and Hikosaka, 2007). Moreover, within the LHb, the PER2 clock-protein has been described to oscillate in absence of the SCN inputs (Guilding et al., 2010). The processing of reward and the circadian characteristics of the LHb suggest that this area may be relevant when the animals have access to a palatable diet. Therefore, the first part of this study aimed to

evaluate whether mice under a fcHFHS diet develop hyperphagia and obesity similar to the results previously obtained in rats (la Fleur et al., 2014), and in a second part, to characterize the expression of the clock-proteins PER2 and BMAL1 in the SCN, LHb and the ARC of mice exposed to the fcHFHS diet.

Material and Methods

Animals and Housing

We used young adult (6-8 weeks old) male C57BL/6J mice weighting 22.1 ± 0.5 grams at the beginning of the study. Animals were housed individually in Plexiglas cages with food and water *ad libitum* before any feeding manipulation. Mice were kept under controlled conditions throughout all the experiment with a controlled temperature (21-23°C) and a light-dark (LD) cycle 12:12h (lights on at 7 am, zeitgeber time (ZT) 0 represent lights on) with dim red light (5 lux) at night. All experiments were performed in accordance with the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC) and the French Department of Agriculture (license no. 67-378 to JM).

Food intake assessment

After a week of habituation, mice were randomly divided into two groups and fed with two different diets. The control Chow group (n=12) had *ad libitum* access to regular chow food (SAFE, 105, U8400G10R. Augy, France. 2.85 kcal/g, where: 23% proteins, 65% carbohydrates and 12% fat) and tap water during the whole experiment. The free choice High-Fat High-Sugar (fcHFHS) group (n=11) was offered 4 different components in the diet separately: (1) regular chow food; (2) a bottle of tap water; (3) pellets made of fat (beef tallow, Vandemoortele, France; 9 kcal/g); (4) a bottle of water with 10% sugar (0,4 kcal/mL). Previous observations in our laboratory and by other groups showed that this was the most preferred concentration of sugar for C57BL/6J mice (Bainier et al., 2017; Feillet et al., 2017; Lewis et al., 2005). Both groups were kept on their respective diets during 6 weeks.

All animals were weighted weekly as well as the food components to evaluate ingestion. At the end of the first and sixth week of experiment, all the food components for both groups were measured during the 12h of the light and the dark period in order to have the day-night intake pattern.

Locomotor activity recordings

To measure activity-rest cycles we monitored locomotor activity of both Chow (n=10) and fcHFHS (n=11) animals by using infrared detectors placed above the cage. Data were recorded every 5 min. General locomotor activities were plotted as actograms. Clocklab software (Actimetrics, Wilmette, IL) was used to determine the activity profiles of each animal under different experimental conditions (Chow vs. fcHFHS) and changes in the amount of locomotor activity were evaluated.

Tissue sampling

After the sixth week of diet exposure, mice from both Chow (n=12) and fcHFHS groups (n=11) were divided and killed at two different time points around the 24h cycle; at either ZT4 (4h after lights on; n=6 per diet-group) or ZT16 (4h after lights off; n=5-6 per diet-group). These time points were chosen because a difference of high and low expression of clock-proteins can be found around these. Mice were deeply anesthetized with pentobarbital (80 mg/kg) and perfused intracardially with 50 mL of Phosphate Buffer Saline (PBS 0.1M) followed by 50 mL of Paraformaldehyde (PAF 4%). Brains were harvested and conserved overnight in PAF-4% and then transferred to 30% sucrose (Sigma-Aldrich, USA) for cryoprotection. Brains were sectioned with a cryostat to obtain 30 m thick slices at the level of the SCN (~Bregma -0.58mm), the ARC (~Bregma -1.82mm) and the LHb (~Bregma -1.82mm) according to the stereotaxic atlas of mouse brain (Paxinos & Franklin, 2004), and stored in a Watson solution (30% sucrose; 1% polyvinylpyrrolidone (PVP-40); 30% Ethylene glycol; 0.9% NaCl; 50mM Tampon Phosphate 0.1M pH 7,4) at -20 °C until immunohistochemistry processing.

Adipose tissue and hormonal determinations

Before perfusion, at day (ZT4; n=6 per group, Chow vs. fcHFHS) and night (ZT16; n=4 per group, Chow vs. fcHFHS), blood was collected from the left ventricle of the mice, into 15 ml Corning tubes containing 100 µl of 4% EDTA. Blood samples were centrifuged at 5000 rpm for 10 min, and plasma was stored at -80°C before determination of plasma leptin concentration. Leptin was determined through ELISA procedure using a mouse Leptin ELISA Kit (EZML-82K, Millipore, USA). The limit of sensitivity of the leptin assay was 0.05 ng/ml.
To determine the adiposity of mice the white adipose tissue from the abdominal area was rapidly dissected and immediately weighed (Chow, n=7; fcHFHS, n=6).

PER2 and BMAL1 immunolabeling

A series of brain slices containing SCN, ARC and LHb were rinsed 3 times for 5 min with PBS+0.05% tween. They were incubated overnight in PBS+0.3% tween 20 (Sigma-Aldrich, USA) + 5% goat serum and the rabbit anti-PER2 antibody (Alpha Diagnostic International) with a 1:1000 concentration during 24h. After the incubation period with primary antibody, the tissue was rinsed in PBS+0.05% tween and exposed to the secondary antibody anti-rabbit made in goat (Vector Laboratories, 1:500) in PBS+0.3% tween. Then, sections were rinsed in PBS+0.05% tween and incubated in PBS+0.3% tween and the Avidin Biotin complex (Vectastain Kit; Vector laboratories) for two hours. Sections were rinsed with PBS and placed 5 minutes in 3,3 Diaminobenzidine Tetraydrochloride (Sigma, DAB; 0.5 mg/mL) with 0.015% H_2O_2.
In a second series of tissue containing the same nuclei of interest, immunostaining against the BMAL1 protein was performed. We followed the same procedure as PER2 immunohistochemistry with the primary rabbit anti-BMAL1 antibody (Millipore AB2298, 1:5000) in PBS+0.3% tween and 5% donkey serum, followed by an incubation with a secondary antibody anti-rabbit made in donkey (Jackson Laboratories) and the incubation with the AB complex. Finally the same reaction

with DAB and H_2O_2 was conducted. At the end of DAB reaction, the tissue was rinsed with PBS and mounted in gelatinized slides. When dried, the slides were progressively dehydrated by submersion in alcohols with increasing concentration: 70%, 90%, 95%, 100%, and finally in toluene, then, a microscope coverslip was placed with mounting medium (Eukitt, Sigma-Aldrich, USA).

Cell counting

After immunolabeling, sections were visually inspected on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). Photomicrographs (10X) of rostral, medial and posterior levels of SCN, LHb and ARC were taken standardizing all lighting parameters on the microscope and the camera software (Viewfinder Lite, Olympus) to ensure consistent stable lighting throughout the image capture, and saved as images on a PC. High resolution digital images of representative SCN, ARC nuclei and habenular complex including both the LHb and the MHb, were taken with reference to the mouse brain stereotaxic atlas (Paxinos & Franklin, 2004). The images were analyzed with the ImageJ program (National Institute of Health, USA). Sections of the stained structures from both hemispheres were analysed from each animal (2 sections per animal). Contrast and brightness were adjusted in each image to set the difference with the background of the picture and the marked cells. The threshold to measure size and intensity of every particle was adjusted and all the pictures were treated with these parameters. The target area was selected manually and the result of the particle analysis was saved in an Excel file.

Statistical analysis

SigmaPlot (version 13.0) software was used for statistical analysis. For total body weight and caloric intake data over time, repeated measures ANOVA's were used followed by Bonferroni post-test to compare means. A 2-way ANOVA of repeated measures was used to determine the effects of *time* (ZT4 vs. ZT16) and *diet* (chow

vs. fcHFHS) on the caloric intake over the 24h of the Chow and fcHFHS diet groups, and to analyze the effects of time (ZT4 vs. ZT16) and component of the diet (chow, fat and sugar) on the caloric intake of the fcHFHS group. We used a *t*-test to evaluate the differences of the percentage of white adipose tissue between the chow and the fcHFHS-fed group. A 2-way ANOVA of independent measures was used to evaluate the effects of time and diet on PER2 and BMAL1 protein expression, and leptin concentrations. When the effects were significant, the analysis was followed by a Bonferroni post-hoc test. Significant differences were determined with an alpha ≤ 0.05.

Results

fcHFHS diet induces hypercaloric intake and body weight gain in mice

In the total caloric intake, mice under the fcHFHS diet ingested more calories than chow-fed control animals. The repeated measures ANOVA revealed significant differences on *diet* (Chow vs. fcHFHS; $F_{(1,167)}=122.4$; $p<0.001$) and time (weeks; $F_{(6,167)}=17.3$; $p<0.001$), as well as in the interaction of these two factors ($F_{(6,167)}=17.7$; $p<0.001$), from the first to the sixth experimental week (Figure 1A).

Body weight showed also differences between groups (Chow vs. fcHFHS) which were significant by the third week of diet exposure (time x diet, $F_{(6,167)}=15.08$; $p<0.001$); fcHFHS-fed animals gained more body weight than control chow-fed mice (Figure 1B). This difference was maintained until the end of diet exposure (Post-hoc, $p<0.05$; Figure 1B).Plasma leptin concentrations were measured at day (ZT4) and night (ZT16) to confirm the obese phenotype of the fcHFHS group, which were significantly higher in both time points (ZT4, 4.8 ± 0.8 ng/mL; ZT16, 9.2 ± 2.9 ng/mL) compared to the Chow group (ZT4, 2.7 ± 1.2 ng/mL; ZT16, 2.9 ± 0.4 ng/mL) after six weeks of diet exposure ($F_{(1,19)}=8.6$; $p=0.01$; Figure 1C). Finally, the percentage of abdominal fat accumulation of mice from both groups was calculated per total body weight, being significantly higher in the fcHFHS group (5.2, ± 0.3% fat/BW) compared to the control Chow group (2.2 ± 0.4%

fat/BW) ($t_{(11)}$=-4.9, *p<0.01*; Figure 1D). Thus, the effects of the fcHFHS diet in mice physiology are similar to those observed previously in rats (la Fleur et al., 2007).

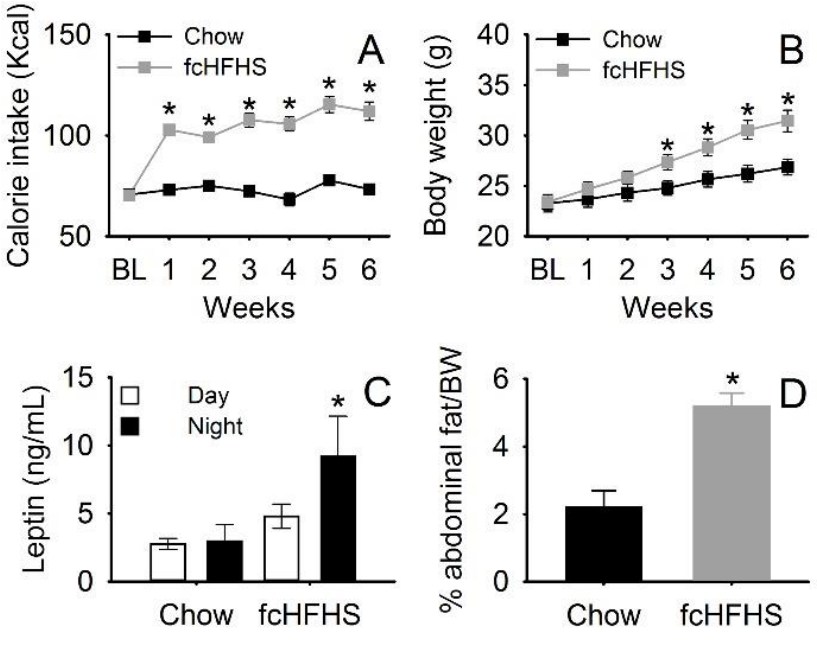

Figure 1. Exposure to the fcHFHS diet increases calorie intake and promotes body weight gain. (A) Total calorie intake of both Chow and fcHFHS groups during baseline (BL) and 6 experimental weeks. (B) Total body weight of control (Chow, n=12) group and mice under free choice high-fat-high-sugar (fcHFHS, n=11) diet. (C) Plasma leptin concentrations are significantly higher at night (ZT16) in mice after 6 weeks of fcHFHS (n=4-6 per time point) compared to Chow diet group (n=4-6 per time point). (D) Percentage of abdominal fat after 6 weeks of experiment with respect to their final weight is higher in fcHFHS diet mice (n=6) compared to Chow diet mice (n=7). Values represent the mean ± S.E.M. Asterisk indicates statistical differences (Post-hoc Bonferroni t-test) at *p<0.05*.

Day-night food intake pattern is affected in mice under a fcHFHS diet

At the first and sixth week of diet exposure, we measured the day-night pattern of food ingestion of the Chow and fcHFHS groups over 24h. We observed an overall higher caloric intake in the fcHFHS-fed mice and a day-night difference in both groups, ingesting more calories at nighttime at the first ($F_{(1,42)}$=33.9; *p<0.001*), and sixth week ($F_{(1,43)}$=18.9; *p=0.001*) of exposure, which were significantly higher in fcHFHS-exposed mice (first week, $F_{(1,42)}$=5.07; *p=0.04*; sixth week, $F_{(1,43)}$=46.2; *p<0.001;* Figure 2A and 2C). No differences in the interaction between diet x time factors were detected (first week, $F_{(1,42)}$=1.27; p=0.2; sixth week $F_{(1,43)}$=0.01; p=0.9). The day-night intake pattern of the fcHFHS-fed mice, during the first week, had an effect of the diet component ($F_{(2,65)}$=6.9; *p=0.005*) and time ($F_{(1,65)}$=12.1; *p=0.006*), with a clear day-night difference in calorie intake from regular chow food and fat, but not from sugar (Figure B).

At the sixth week of diet exposure, however, a significant day-night ($F_{(1,65)}$=9.28; *p=0.01*) intake pattern was observed for regular chow food ($F_{(2,65)}$=577.3; *p<0.001*), but not from fat nor sugar (Figure 2D).

Evaluating general activity, both groups showed similar daily rhythms in locomotion, with the highest activity during night time ($F_{(23,503)}$=44.3; *p<0.001*; Figure 2E and 2F). We did not, however, observe significant differences between groups in the daily rhythms of locomotor activity ($F_{(1,503)}$=0.08; p=0.7; Figure 2E and 2F).

PER2 and BMAL1 day-night expression in brain tissue of fcHFHS diet-fed mice

After 6 weeks of diet exposure, we assessed the expression of proteins PER2 and BMAL1 in the SCN, ARC and LHb. In the SCN, PER2 expression was higher at night (ZT16) in both Chow and fcHFHS-fed mice ($F_{(1,22)}$=41.17; *p<0.001;* Figure 3), and no effect of the diet ($F_{(1,22)}$=2.0; p=0.17; Figure 3) or interaction time x diet was observed ($F_{(1,22)}$=0.002; p=0.96*;* Figure 3). As for the SCN, PER2 in the ARC was higher during the night than during the day

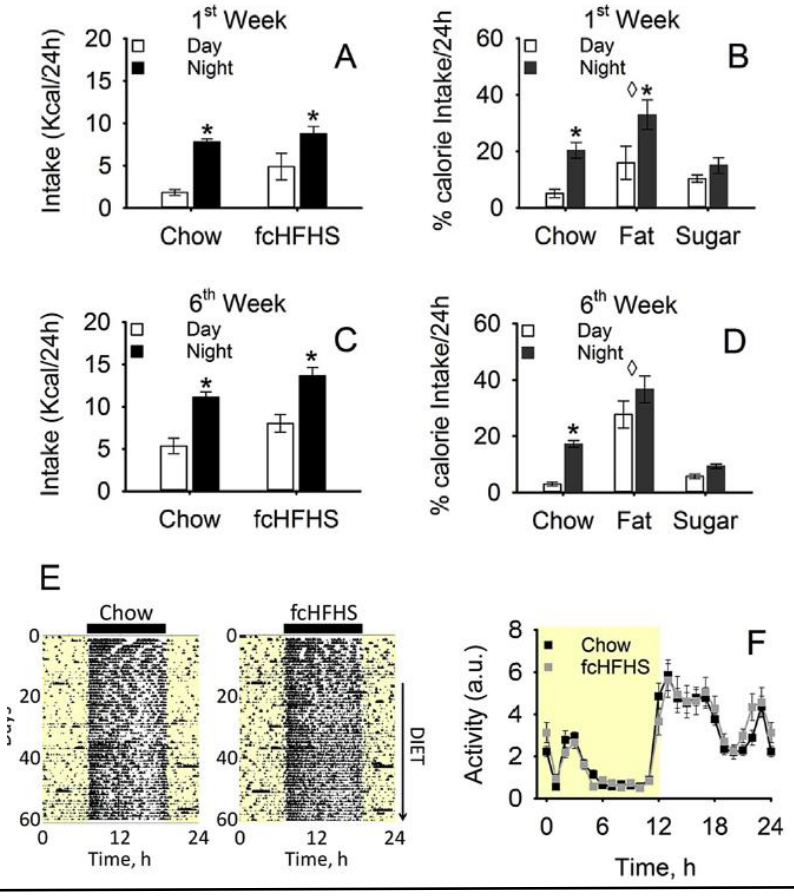

Figure 2. Day-night pattern of calorie intake of Chow and fcHFHS fed animals. Total calorie ingestion over 24h from the first (A) and the sixth (C) week of experiment during day (white bars) and night (black bars) for Chow and fcHFHS-exposed animals. Percentage of total caloric ingestion of the different components of the fcHFHS diet measured over 24h during day and night at the first (B) and the sixth (D) week of experiment. Values are the mean ± S.E.M. Asterisk indicates statistical differences (day vs. night) at *p<0.05* (Post-hoc Bonferroni t-test). Diamonds indicate a significant difference between diet components (Chow vs. Fat vs. sugar) at day and nighttime (*p<0.05,* Post-hoc Bonferroni t-test). (E) Representative actograms of the locomotor activity rhythms of a Chow and a fcHFHS fed mice (F) Activity profiles of the 24h rhythms of general locomotion of mice exposed to a chow or a fcHFHS diet during 6 weeks. Shaded yellow bars in both actograms and activity profiles represent light period.

in both Chow and fcHFHS groups ($F_{(1,19)}$=17.57; *p<0.001;* Figure 3), and there were no differences between the diets ($F_{(1,19)}$=2.14; p=0.16; Figure 3) or in the interaction between time x diet ($F_{(1,19)}$=0.26; p=0.61; Figure 3). Contrary to SCN and ARC, the PER2 protein expression in the LHb was higher at day (ZT4) than night (ZT16) in the chow-fed group (time, $F_{(1,19)}$=6.77; *p=0.01;* Figure 3), but this difference was blunted in the fcHFHS-fed mice in comparison to control Chow mice (time x diet, $F_{(1,19)}$=8.77; *p=0.009;* Figure 3). The PER2 protein expression in the MHb was also quantified but no effect of time ($F_{(1,15)}$=0.1; *p=0.7*), diet ($F_{(1,15)}$=0.12; *p=0.7*) or interaction between factors ($F_{(1,15)}$=0.9; *p=0.3*) was found.

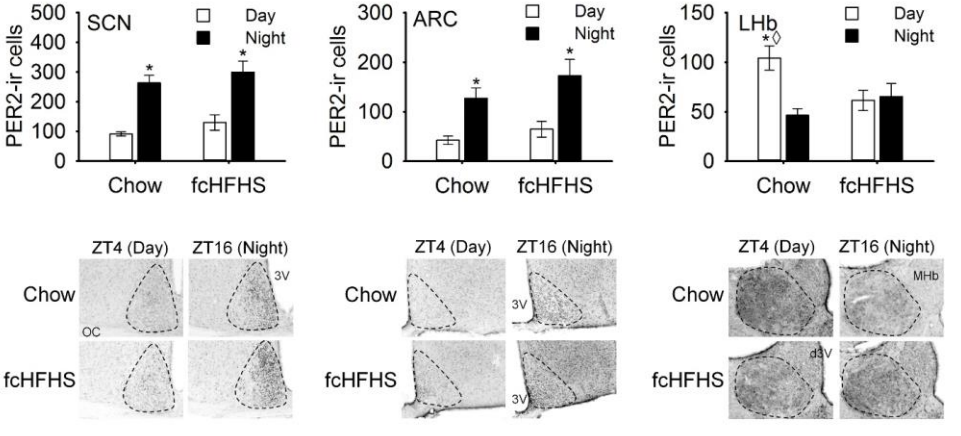

Figure 3. Day-night expression of the PER2 protein in Chow and fcHFHS fed animals. Day and night expression of PER2 of Chow (n=4-6 per time point) and fcHFHS (n=4-6 per time point) groups in the SCN, the ARC and the LHb. In the SCN and ARC nucleus the number of PER2 immunoreactive (ir) cells was higher at night than daytime in both Chow and fcHFHS-fed animals. Contrary, in the LHb PER2-ir cells was significantly higer at day (ZT4) than night time only in the chow-fed group. Representative images from PER2 immunolabeled tissue are shown under the graphs of each brain area respectively. Dotted black lines represent the area where PER2 was counted. Values are the mean ± S.E.M. Asterisk indicates statistical differences (day vs. night) at *p<0.05* (Post-hoc Bonferroni t-test). Diamond indicates a significant difference between groups (Chow vs. fcHFHS) at daytime (*p<0.05,* Post-hoc Bonferroni t-test). OC, optic chiasm; 3V, third ventricle; d3V, dorsal third ventricle; MHb, medial habenula.

The BMAL1 protein expression in the SCN was higher at night time compared to the day time in both chow and fcHFHS-fed mice ($F_{(1,19)}$=6.36; *p=0.02*; Figure 4). The post hoc analyses indicated that this difference was significant only in the fcHFHS group (Post-hoc test, *p<0.05*). There was no significant differences between the diets ($F_{(1,19)}$=0.0008; p=0.9; Figure 4) or an interaction time x diet ($F_{(1,19)}$=0.7; p=0.41; Figure 4). The same pattern was found in the LHb for BMAL1 expression, being higher at night ($F_{(1,17)}$=22.07; *p<0.001*; Figure 4), in both groups (post-hoc test, *p<0.05*), but with no differences of diet ($F_{(1,17)}$=0.53; p=0.47; Figure 4) nor an interaction time x diet ($F_{(1,17)}$=2.21; p=0.15; Figure 4). In the MHb no effects of time ($F_{(1,12)}$=0.56; p=0.4), diet ($F_{(1,12)}$=0.6; p=0.4) or interaction between factors ($F_{(1,12)}$=0.4; p=0.5) on BMAL1 protein expression were found. We performed the BMAL1 immunostaining in the ARC, however, the staining in this area was not optimal to be quantified and analyzed.

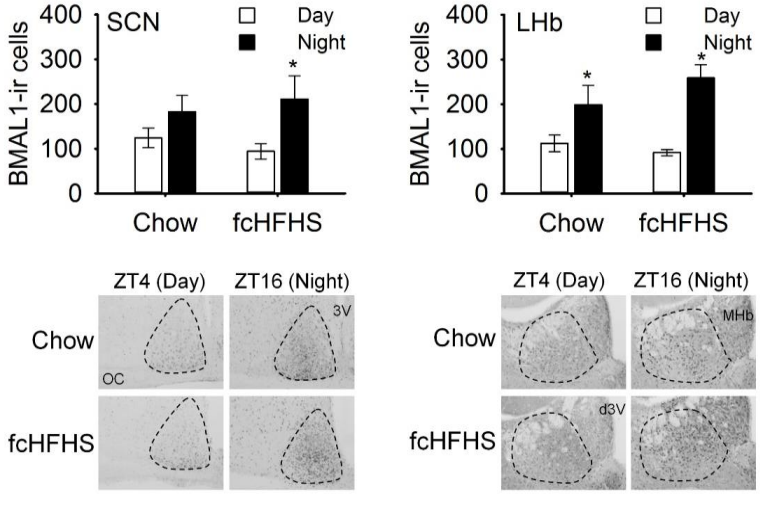

Figure 4. Day-night expression of the BMAL1 protein in Chow and fcHFHS fed animals. BMAL1 expression in SCN and LHb of Chow and fcHFHS groups showed a main number of positive cells at night time (ZT16). This effect was significant in the SCN of fcHFHS diet-exposed mice and in the LHb of both, chow and fcHFHS fed mice (*$p<0.05$, Post-hoc Bonferroni t-test). Values are the mean ± S.E.M. Representative images from BMAL1 immunolabeled tissue are shown under the graphs of each brain area respectively. OC, optic chiasm; 3V, third ventricle; d3V, dorsal third ventricle; MHb, medial habenula.

Discussion

In line with previous studies in rats, we here show that in mice the fcHFHS diet induces hyperphagia and enhanced body weight gain with an increase in adipose tissue stores and plasma leptin concentrations. Moreover, in the LHb the day-night difference in the expression of the clock-protein PER2, which was observed in chow-fed animals, was blunted when mice were fed with the fcHFHS diet.

Effects of fcHFHS on calorie intake and physiology

Our results show that the fcHFHS paradigm is effective for inducing calorie overconsumption that leads to an increase of body weight gain, abdominal fat accumulation and high blood leptin concentrations in mice. Like previous results observed in rats (la Fleur et al., 2014), day-night sucrose intake was similar in mice fed with fcHFHS diet, while chow food ingestion remained high during the night. The mice in this study ate more fat at night, however, the percentage of fat ingestion is higher during the day compared to chow food and this pattern differs to that of rats under a fcHFHS diet (la Fleur et al., 2014), which might indicate a species difference in food intake and metabolism. The mice of this study, however, were kept on the fcHFHS diet during 6 weeks, and it might be that when rats are exposed to the diet for more than 3 weeks, fat intake patterns could be altered as well.

Together with the earlier reported rat studies, we show clear effects of fcHFHS exposure on the daily intake of caloric food, but not for regular chow food. Although other studies did not present food items separately, our data go along with previous reports showing that when mice were fed with a high-fat single pellet, the daily food intake was altered under both LD and DD conditions, increasing food intake during the rest period (Branecky et al., 2015; Kohsaka et al., 2007; Mifune et al., 2015; Pendergast et al., 2013). In addition to altered feeding patterns, changes in locomotor activity have also been reported in some studies. Pendergast et al. (2013) showed altered locomotor activity rhythms after 2 weeks

of high-fat diet exposure, whereas Kohsaka et al. (2007) reported no increase in the locomotor activity during the light period of mice on a HF diet. We also recorded general locomotion in mice, but we did not observe differences between chow and fcHFHS animals. These different effects of HF-diets on locomotor activity rhythms might be due to age or to the composition of the diet. In fact, body weight gain and the disturbances of the daily ingestion patterns can be observed in a higher or lesser extent, depending on the nature of the fat sources (Buettner et al., 2007; Hariri and Thibault, 2011). The advantage of our model lies in the free choice for the animals to eat from what they might perceive as more rewarding, and as a consequence, each individual chooses the proportion of sugar and fat that produces the most pleasurable combination. Together, the behavioral results could indicate that the main SCN clock, synchronized by the LD cycle, keeps the locomotor activity in line to the light-dark cycle, whereas other central oscillators coordinate timing of rewarding food consumption.

Effects of fcHFHS on clock protein expression

The importance of *Per2* gene expression for energy balance is clear from studies with global *Per2* mutant mice. These mice, under normal chow food diet, show a decreased body weight gain and have disrupted lipid metabolism (Grimaldi et al., 2010). At the central level, *Per2* expression can be induced by environmental signals such as light in the SCN (Sosniyenko et al., 2009) and food restriction in diverse extra-SCN sites (Feillet et al., 2008; Mieda et al., 2006; Verwey et al., 2007). Under *ad libitum* chow feeding conditions and in a 12:12 LD cycle, PER2 protein expression in the SCN and the ARC has been reported to be higher at the dark period (Feillet et al., 2008; Field et al., 2000; Uchida et al., 2016). Similarly, we observed in the SCN and ARC of the Chow group, higher expression of PER2 at ZT16 compared to the expression at the light period (ZT4). As expected, the fcHFHS diet did not alter PER2 protein expression in the SCN. Others reports have shown that changes in the diet, and even changes in the schedule of feeding time of animals kept in similar LD conditions as in our study, do not modify *Per2* mRNA

or protein expression (Minana-Solis et al., 2009; Pendergast et al., 2013; Verwey et al., 2007). Our results regarding PER2 expression in the ARC showed no differences between both chow and fcHFHS diet. Interestingly, within the LHb we observed an interaction effect between time and diet revealing significant day-night differences in PER2 protein expression in the chow group, which was abolished when animals were exposed to a fcHFHS diet. This day-night difference in PER2 protein expression found in the control group agrees with the findings of previous studies in mice and rats (Guilding et al., 2010; Zhao et al., 2015). Therefore, our results suggest that the exposure to a highly palatable diet disrupts clock-gene expression in some brain clocks outside the SCN.

Palatable intake has been linked to dopamine signalling in cortico-limbic areas and the fcHFHS diet has also been shown to alter dopamine signalling (van de Giessen et al., 2012). Also described is a link between the dopaminergic system, BMAL1 and PER2 clock proteins. BMAL1 binds to the promoter of the monoamine oxidase A (MAO-A), a key enzyme for dopamine degradation, while a mutation of PER2 reduces the levels of this enzyme in the striatum (Hampp et al., 2008). Moreover, the dopaminergic depletion by a 6-OHDA treatment or the specific blocking of D2 receptors reduces PER2 expression in the striatum, but not in the SCN, of rats (Hood et al., 2010). Further studies are needed to determine whether clock-gene alterations observed in the LHb of mice fed with the fcHFHS diet are linked to changes in the dopaminergic system and whether these are causally linked to the (non-rhythmic) ingestion of palatable food.

It is known that *Bmal1* gene expression in the SCN is rhythmic (Honma et al., 1998; Maywood et al., 2003). Nevertheless, the expression of its protein product has been reported to be constitutive and non-rhythmic in mice (Ansari et al., 2009; von Gall et al., 2003; Wyse and Coogan, 2010). One study that aimed to test different antibodies against the BMAL1 protein reported higher levels at ZT0 compared to ZT12 in mice (LeSauter et al., 2012). Our analyses of BMAL1 expression at two time points reflected a significant difference with high levels at

ZT16 (nighttime) suggesting that there is a daily rhythm of this protein in the SCN. Wyse and Coogan (2010) reported that BMAL1 is not rhythmic in the SCN, but interestingly, they did find a rhythmic expression of this protein in the LHb with high levels during daytime. Our results regarding BMAL1 expression in the LHb of the Chow group agree with the previous report in the sense that we found a day-night difference. Nevertheless, Wyse and Coogan showed high BMAL1 expression during the day in young adult mice (around 4 months old), a similar age of the mice (at the end of manipulations) used in our study. However, our observations of higher BMAL1 expression during the nighttime resemble more to the expression pattern of this protein in old mice of around 16 months (Wyse and Coogan, 2010). Furthermore, we observed that the day-night difference in BMAL1 expression in the SCN and in the LHb was statistically higher in the fcHFHS-exposed mice according to the post-hoc analyses. This is especially interesting since we observed changes of PER2 in the LHb which might be relevant to hypothesize a possible de-synchronization of the molecular clock-machinery within the structure besides a desynchronization between nuclei such as the SCN.

Conclusion

The fcHFHS diet exposure in mice was successful to induce hyperphagia and obesity, as well as behavioral changes in the consumption of sugar, where the intake during day and night is the same as already shown in rats. Taken together, our data show a clear distinction between palatable and healthy chow feeding patterns. Within the brain areas involved in feeding behavior and reward, we detected an abnormal pattern of PER2 protein expression in the LHb of mice fed with the fcHFHS diet. These changes suggest that molecular clock mechanism in the LHb is altered when animals are exposed to a rewarding hypercaloric diet. These results are a step forward in the understanding on the relevance of coupling between brain circadian clocks to avoid the development of compulsive feeding and obesity.

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Chapter 4

A free-choice high-fat high-sugar diet alters day-night *Per2* gene expression in reward-related brain areas in rats.

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A free-choice high-fat high-sugar diet alters day-night *Per2* gene expression in reward-related brain areas in rats.

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Running title: fcHFHS diet and per2 gene expression in reward areas.

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Abstract

Under normal light-dark conditions, nocturnal rodents consume most of their food during the dark period. Diets high in fat and sugar, however, may affect the day-night feeding rhythm resulting in a higher light phase intake. *In vitro* and *in vivo* studies showed that nutrients affect clock gene expression. We therefore hypothesized that overconsuming fat and sugar will alter clock gene expression in brain structures important for feeding behavior. We determined the effects of a free-choice high-fat high-sugar (fcHFHS) diet on clock-gene expression in rat brain areas related to feeding and reward and compared them with chow-fed rats. Consuming a fcHFHS diet for 6 weeks disrupted day-night differences in *Per2* mRNA expression in the nucleus accumbens (NAc) and lateral hypothalamus but not in the suprachiasmatic nucleus, habenula and ventral tegmental area. Furthermore, short term sugar drinking, but not fat feeding, up-regulates *Per2* mRNA expression in the NAc. The disruptions in day-night differences in NAc *Per2* gene expression were not accompanied by altered day-night differences in the mRNA expression of peptides related to food intake. We conclude that the fcHFHS diet and acute sugar drinking affect *Per2* gene expression in areas involved in food reward; however, this is not sufficient to alter the day-night pattern of food intake.

Keywords: *Per2*, fat and sugar, clock-genes, obesity, reward, nucleus accumbens

Introduction

The suprachiasmatic nucleus (SCN) controls the circadian (24h period) rhythms in behavior and physiology (Coomans et al., 2013; Stephan & Zucker, 1972). In the SCN and in all cells of the body, a feedback loop of genes (known as clock genes) are expressed and repressed with a 24h period. The positive limb of the loop consists of the genes *Clock* and *Bmal1* of which the protein dimer promotes *Per* and *Cry* expression, and genes from the negative limb which protein products repress *Clock* and *Bmal1* activity (Takahashi, 2015). Environmental light is the main synchronizer for the SCN (Colwell, 2011), whereas other brain circadian clocks are more sensitive to internal hormonal and metabolic signals. Thus, feeding cues are also able to modify the day/night physiological variation. Circadian eating patterns can be altered by high fat diets (Branecky et al., 2015; Kohsaka et al., 2007; Pendergast et al., 2013) such as the free-choice High-Fat High-Sugar (fcHFHS) diet, consisting of the choice between tap water, chow-food, fat and sugar (la Fleur et al., 2014). Rodents exposed to a fcHFHS diet show smaller day-night differences in food intake. Especially intake of fat and sugar components of the diet does not show day-night variations, whereas the intake of the nutritionally balanced chow diet remains rhythmic with a higher intake in the dark period when animals are active (Blancas-Velazquez, la Fleur, & Mendoza, 2017; la Fleur et al., 2014). Moreover, we previously reported changes in the molecular clock properties of the Lateral Habenula (LHb) in fcHFHS diet-exposed mice, an area involved in reward related behavior, whereas clock-proteins in the Arcuate Nucleus, an important area for homeostatic feeding, were unchanged (Blancas-Velazquez et al., 2017). It remains however to be determined whether molecular clock-gene expression in food-related reward circuitry, like striatum and Lateral Hypothalamus (LH), are affected by a diet high in fat and sugar and if these effects are involved in disruption of the day/night feeding rhythm. We hypothesize that the obesogenic diet-induced disruption of day-night palatable intake is linked to nutrients (like fat and sugar) affecting the brain oscillators within the food reward circuitry. In the

present study, we exposed rats to a fcHFHS diet for 6 weeks and measured clock-genes and food related peptides gene expression in different reward-related brain areas. Subsequently, we evaluated the acute effects of sugar intake on *Per2* gene expression in the Nucleus Accumbens (NAc) of rats.

Materials and Methods

Male Wistar rats weighing ~250g were single-housed in Plexiglas cages in a temperature and light-controlled room with 21-23°C and a 12:12 h light:dark (LD)-cycle ZT0 at 7:00am (Zeitgeber Time: ZT0 onset of light and ZT12 when lights are off). Animals were fed with regular chow and water *ad libitum* during baseline. All experiments were approved by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (Amsterdam).

fcHFHS-diet effects on clock-gene and output-genes expression in feeding related areas: Rats were either fed chow (n=14) or the fcHFHS diet (n=14): tap water, chow-food, 30% sucrose-water bottle and a dish with fat (beef tallow, Vandemoortele, Belgium). Food intake was measured 3 times/week and 1 time/week at the beginning and the end of the day and night phases to assess the day-night food intake. Body weight was measured at least twice/week. After 6 weeks, rats from both groups were divided and euthanized in two different time points of day: ZT4 (day point) and ZT16 (night point) by sedation in a CO_2-chamber and immediately decapitated. Brains were quickly removed, frozen and stored at -80°C. Epidydimal and perirenal white adipose tissue (WAT) was dissected and weighted.

Sugar intake effect on Per2 mRNA expression in NAc: Rats were divided into 2 groups. During 7 days at ZT 10 (two hours before lights off), one group received an extra bottle of water (n=8) and the other group a bottle with 30% sugared-water (n=9) during 2,5 min to consume ~5kcal of sugar. To determine exact sugar intake, the bottle was weighted before and after drinking. Rats were sacrificed 30 min after

last water or sugar intake. Animals were sedated and decapitated and brains were harvested as described above.

mRNA extraction and quantitative real-time PCR: Punches from frozen brains were taken using a small needle dissecting NAc, SCN, Lateral Hypothalamus (LH), Habenula (Hb, containing both the medial and lateral parts), and Ventral Tegmental Area (VTA) according to the Paxinos Altlas (Paxinos & Watson, 2005). Tissue was placed in TRIzol (QIAGEN) and homogenized using an ULTRA THURRAX homogenizer (IKA, Germany). RNA extraction and RT-PCR was performed for *Per2*, *Bmal1*, *Vglut2*, *Orexin* (Wang et al., 2017)*, Cry1* (F primer: AAGTCATCGTGCGCATTTCA; R primer TCATCATGGTCGTCGGACAGA), *pre-pro-enkephalin* (F primer: CTTGTCAGAGACAGAACGGGT; R primer CCTTGCAGGTCTCCCAGATTT) as described previously (De Vries et al., 2017). Reference genes: Cyclophilin (F primer ATGTGGTCTTTGGGAAGGTG; R primer GAAGGAATGGTTTGATGGGT), β-Actin (F primer ACAACCTTCTTGCAGCTCCTC; R primer CTGACCCATACCCACCATCAC).

Statistics: All results are expressed as means±SEM. Statistical analysis was performed with Graphpad Prism. T-tests were performed for two group measures. Two-way ANOVA was performed to detect effects of diet, time or diet and time interaction on gene expression. When detecting an interaction effect, a Tukey's HSD post hoc test was performed. Results were considered statistically significant at $p<0.05$.

Results

During all 6 weeks of the experiment, fcHFHS-fed rats were hyperphagic, cumulatively consuming 3884 kcal±56.23, compared to 3041 kcal±50.39 ingested by the Chow group ($t_{(26)}=11.16$,$p<0.001$). Chow intake in the control group, and chow, fat and sugar intake in the fcHFHS diet group were significantly higher at night compared to day (Table 1). At the end of the experiment, fcHFHS-fed rats

were heavier and more obese than chow-fed rats (BW: 411.3g±4.2 vs 429.7g±4.9; $t_{(26)}$=2.38, *p<0.001;* WAT: (5.6g±0.2 vs 9.9g±0.5; $t_{(26)}$=8.34, *p<0.001*).

In all brain areas from the chow-fed group, *Per2* mRNA was higher at ZT16 (night) than at ZT4 (day). In fcHFHS-fed rats, however, this day-night difference was absent in the NAc and LH; i.e. no significant difference between day and night in animals fed the fcHFHS diet (Figure 1A). *Cry1* and *Bmal1* expression also showed significant day/night differences in most brain areas investigated (Table 1). Interestingly, the loss of day-night differences in the fcHFHS group was restricted to *Per2* (Table 1). We also measured day-night expression of *Vglut2* in all areas, *orexin* in the LH and *pre-pro-enkephalin* in NAc to investigate whether the observed changes in *Per2* were reflected in feeding-regulating genes. No significant changes were observed for *Orexin* (ANOVA: Diet $F_{(1,22)}$=2.83,p=0.1; Time $F_{(1,22)}$=0.008, p=0.9; Int. $F_{(1,22)}$=0.04,p=0.8), but *Vglut2* was altered in the LH and NAc of the fcHFHS-fed group (Table 1). In the LH, we observed an interaction effect, however, the posthoc analysis did not detect differences between night and day in the chow or in the fcHFHS group. In the NAc, *Vglut* mRNA was significantly lower at both day and night in fcHFHS diet-fed rats compared to chow-fed rats (Table 1). *Pre-pro-enkephalin* expression was higher during the light period in both chow-fed (0.052±0.002) and fcHFHS-fed (0.057±0.002) groups compared to the dark period (Chow, 0.044±0.003; fcHFHS, 0.042±0.002; Time $F_{(1,22)}$=27.0,p<0.001), but no significant diet or interaction effects were observed (Diet $F_{(1,22)}$=0.25,p=0.61; Interaction $F_{(1,22)}$=1.6,p=0.2).

Next, we determined the direct effect of sugar intake on *Per2* mRNA expression in the NAc and observed that *Per2* mRNA was significantly increased by sugar ingestion (4.7 kcal ± 0.1), compared to drinking water (Figure 1B).

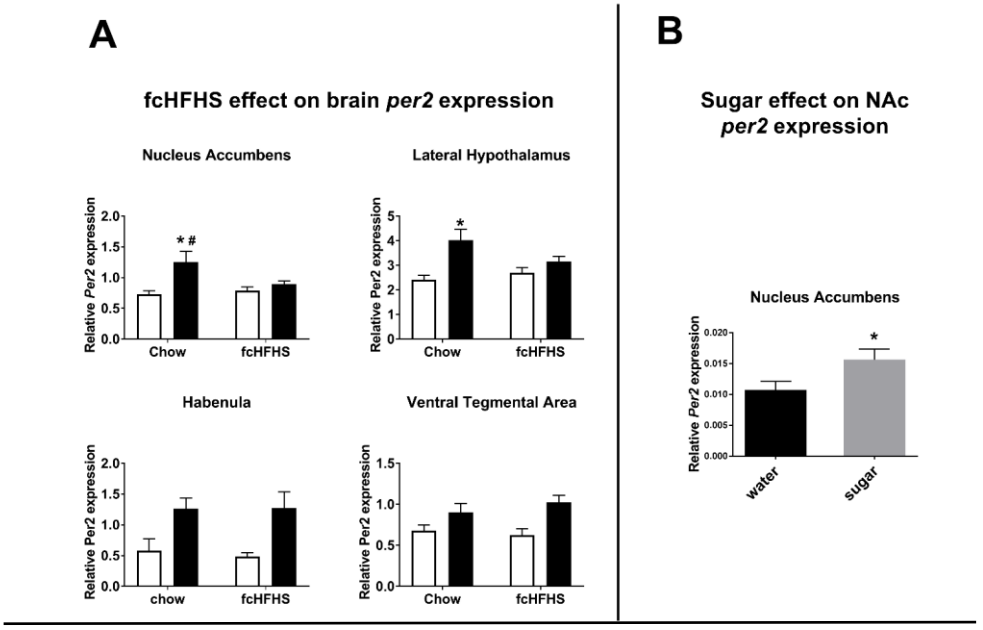

Figure 1. *Per2* mRNA expression in NAc and LH, but not Hb or VTA, is altered by fcHFHS diet exposure. (A) day (white bars) night (black bars; Time factor) expression of *Per2* in Chow diet vs fcHFHS diet groups (Diet factor). All the structures showed significant day-night variations, and when an interaction was observed * indicates a significant day-night difference of *Per2* expression; and # indicates a significant effect of diet (chow vs fcHFHS) on *Per2* expression at night. B) *Per2* mRNA expression in the NAc is significantly higher after sugar drinking compared to water drinking in chow-fed rats. * indicates a significant difference in *Per2* expression after water intake vs. sugar intake. Data is presented as mean ±SEM

Discussion

We show that the fcHFHS diet produced a specific disruption in day-night *Per2* expression in the NAc and LH, which was not observed for *Cry1* and *Bmal1* mRNA expression. In the LH and NAc, *Per2* mRNA disruption caused by the fcHFHS diet exposure coincided with alterations of *Vglut2* mRNA (Table 1), a marker of glutamatergic activity and excitatory neuronal functions (El Mestikawy, Wallén-Mackenzie, Fortin, Descarries, & Trudeau, 2011), suggesting a relation between the loss of daily *Per2* variation when consuming a fcHFHS diet and

changes in neuronal activity. In none of the brain areas studied, we observed a day-night difference in *Vglut2* expression. This could be due to the timing of sampling, missing the trough or peak, or to the neuronal heterogeneity in the studied areas. However, we did observe a clear overall diet effect on *Vglut2* mRNA in the NAc at both time points measured.

Table 1. Eating patterns from Chow fed and fcHFHS diet fed groups and mRNA expression from clock-genes *Cry1*, *Bmal1, Per2* and the *Vglut2* gene. The Upper part of the table shows feeding day-night patterns of chow and fcHFHS rats. Results of the 2way ANOVA comparing daytime (day, nigh) vs. diet component (chow, sugar, fat) on the percentage of caloric intake are shown for the fcHFHS group. Results from the t-test analysis of day-night intake are shown under every diet component for chow and fcHFHS groups. In the lower part of the table, the different brain areas are shown in columns. In rows are presented: the studied gene; the diet condition: chow/fcHFHS and; and daytime: day/night. Results from the 2 way ANOVA analysis (diet condition vs. daytime) per brain area are shown under every gene description. Significant statistical effects are highlighted in bold letters. Data is presented as mean ±SEM.

EATING PATTERNS								
	Chow		**fcHFHS**					
	chow		**Chow**		**Fat**		**Sugar**	
%	100		44,4 ± 1,4		13,5 ± 1,2		41,9 ± 2,2	
Day/night feeding	**day**	**night**	**day**	**night**	**day**	**night**	**day**	**night**
	16,2 ± 1,1	83,8 ± 1,1	15,7 ± 1,0	84,3 ± 1,0	8,4 ± 1,2	91,5 ± 1,2	22,1 ± 1,0	77,9 ± 1,1
2 way ANOVA			Time $F_{(1,78)}$=5894 p<0.001, Diet component $F_{(2,78)}$=0 p>0.99), Interaction $F_{(2,78)}$=76.6 p<0.001					
T test day vs night	$t_{(26)}$=44.9, *p<0.001*		$t_{(26)}$=49.3, *p<0.001*		$t_{(26)}$=48.1, *p<0.001*		$t_{(26)}$=36.2, *p<0.001*	

Table 1 continuation

GENE EXPRESSION							
Gene	**Group**	**Brain area**	**SCN**	**NAc**	**LH**	**Hb**	**VTA**
Cry1	**chow**	**day**	4,6 ± 0,3	1,9 ± 0,2	9,3 ± 0,5	5,8 ± 1,5	2,6 ± 0,3
		night	6,0 ± 0,5	2,4 ± 0,2	11,5 ± 1,4	9,4 ± 2,2	3,2 ± 0,1
	fcHFHS	**day**	4,8 ± 0,5	2,2 ± 0,2	8,4 ± 0,5	5,7 ± 0,9	2,6 ± 0,4
		night	6,5 ± 0,4	2,5 ± 0,2	10,9 ± 0,6	9,9 ± 1,9	2,9 ± 0,2
	2 way ANOVA	**INTER. DIET TIME**	F(1,23)=0.11; p=0.7 F(1,23)=0.65; p=0.4 **F(1,23)=14.2; p<0.01**	F(1,24)=0.4; p=0.5 F(1,24)=1.0; p=0.3 **F(1,24)=4.4; p<0.05**	F(1,21)=0.06; p=0.7 F(1,21)=0.8; p=0.3 **F(1,21)=9.5; p<0.01**	F(1,23)=0.03; p=0.8 F(1,23)=0.0; p=0.9 **F(1,23)=5.5; p<0.0**5	F(1,24)=0.1; p=0.7 F(1,24)=0.3; p=0.6 F(1,24)=3.4; p=0.07
Bmal1	**chow**	**day**	3,7 ± 0,1	1,9 ± 0,1	9,0 ± 0,6	1,8 ± 0,2	1,5 ± 0,1
		night	4,2 ± 0,4	1,6 ± 0,1	7,0 ± 0,5	1,7 ± 0,3	1,1 ± 0,1
	fcHFHS	**day**	4,4 ± 0,2	1,5 ± 0,3	8,9 ± 0,6	1,9 ± 0,2	1,3 ± 0,2
		night	3,9 ± 0,2	1,7 ± 0,1	6,6 ± 0,4	1,8 ± 0,3	1,1 ± 0,04
	2 way ANOVA	**INTER. DIET TIME**	F(1,24)=2.53; p=0.1 F(1,24)=0.7; p=0.4 F(1,24)=0.006; p=0.9	F(1,22)=2.47; p=0.1 F(1,22)=1.4; p=0.2 F(1,22)=0.4; p=0.4	F(1,22)=0.08; p=0.7 F(1,22)=0.3; p=0.5 **F(1,22)=16.5;p <0.01**	F(1,23)=0.001; p=0.9 F(1,23)=0.19; p=0.6 F(1,23)=0.03; p=0.8	F(1,24)=0.7; p=0.3 F(1,24)=1.1; p=0.2 **F(1,24)=9.1;p< 0.01**
Per2	**chow**	**day**	2,3 ± 0,2	0,7 ± 0,05	2,4 ± 0,1	0,5 ± 0,1	0,6 ± 0,07
		night	3,3 ± 0,4	1,2 ± 0,1	4,0 ± 0,4	1,2 ± 0,1	0,9 ± 0,1
	fcHFHS	**day**	3,0 ± 0,2	0,7 ± 0,06	2,6 ± 0,2	0,4 ± 0,06	0,6 ± 0,07
		night	3,3 ± 0,3	0,8 ± 0,05	3,1 ± 0,2	1,2 ± 0,2	1,0 ± 0,08
	2 way ANOVA	**INTER. DIET TIME**	F(1,24)=1.1;p=0.2 F(1,24)=1.0; p=0.3 F(1,24)=3.4;p=0.07	**F(1,22)=5.0;p< 0.03** F(1,22)=2.6; p=0.12 **F(1,22)=11.7;p <0.01**	**F(1,22)=4.3; p<0.04** F(1,22)=1.1; p=0.2 **F(1,22)=14.2;p <0.01**	F(1,23)=0.08; p=0.7 F(1,23)=0.05; p=0.8 **F(1,23)=14.9;p <0.01**	F(1,24)=0.9; p=0.3 F(1,24)=0.1; p=0.6 **F(1,24)=12;p< 0.01**
Vglut2	**chow**	**day**	2,2 ± 0,5	0,1 ± 0,02	21,6 ± 1,1	14,7 ± 2,5	11,6 ± 1,4
		night	2,9 ± 0,6	0,1 ± 0,03	26,8 ± 1,5	22,3 ± 3,7	10,5 ± 0,8
	fcHFHS	**day**	1,9 ± 0,2	0,06 ± 0,01	26,9 ± 2,7	16,6 ± 1,6	10,6 ± 1,6
		night	2,1 ± 0,4	0,07 ± 0,01	24,2 ± 1,4	19,0 ± 3,7	11,7 ± 0,6
	2 way ANOVA	**INTER DIET TIME**	F(1,24)=0.3; p=0.5 F(1,24)=0.9; p=0.3 F(1,24)=0.8; p=0.3	F(1,23)=0.2;p=0.6 **F(1,23)=7.1;p< 0.05** F(1,23)=0.0;p=0.9	**F(1,22)=4.3; p<0.05** F(1,22)=0.56; p=0.4 F(1,22)=0.45; p=0.5	F(1,23)=0.76; p=0.3 F(1,23)=0.06; p=0.8 F(1,23)=2.7; p=0.1	F(1,22)=0.83; p=0.3 F(1,22)=0.0; p=0.9 F(1,22)=0.0; p=0.9

Given the importance of glutamate in the NAc for dopamine signalling and the previously reported effects of high energy diets on dopamine receptor binding (Elsmarieke Giessen, Fleur, Bruin, Brink, & Booij, 2012), it might be that this reflects a dampening of neuronal activity of NAc dopamine neurons.

The changes in *Per2* mRNA expression, without changes in *Bmal1* and *Cry* mRNA in the NAc and LH of rats fed fcHFHS diet in this study are similar to previous results described in mice where the fcHFHS diet produced changes only in PER2 but not in BMAL1 protein expression in the lateral habenula (Blancas-Velazquez et al., 2017). Also, after chronic alcohol intake in mice, a specific *Per2* mRNA acrophase shift was observed in the liver while *Cry* and *clock* remained unaffected (Filiano et al., 2013). In vitro, the period length and acrophase of *Per2* mRNA expression in cultured hypothalamic neuronal cells are altered after glucose enrichment to the media, whereas *Bmal1* rhythmicity remained unaffected (Oosterman & Belsham, 2016). The specific alteration of *Per2* could indicate that this gene is more sensitive than other clock genes to changes in the physiological state (e.g. hypercaloric feeding or chronic alcohol intake), as for instance, the ablation of dopaminergic cells of the VTA decrease *Per2* mRNA expression as well as its protein product (Hood et al., 2010) which could reflect a direct response to the microenvironment independent of a clock-mechanism. On the other hand, it remains to be determined whether this specific *Per2* alteration might be due to an intra-cellular clock-gene de-synchronization that could be reflecting an aberrant clock function.

We also showed that acute sugar consumption when given at the end of the light period increased *Per2* mRNA expression in the NAc. Interestingly, mice with *ad libitum* access to a 5% and 10% sugared water solution consume it mainly during the night phase and this did not disturb *Per2* gene expression in the NAc (Bainier et al., 2017). Taken together, these data suggest that time of sugar intake is an important factor to produce *Per2* alterations in the NAc and that intake at the "wrong" time disturbs the day-night expression of this clock gene. Furthermore, we

observed in this study that rats with chronic access to the fcHFHS diet exhibited reduced *Per2* expression in the NAc and LH at night compared to the chow-fed rats. This could indicate that sugar ingestion, in behaviourally rhythmic animals, has to be accompanied with fat ingestion to produce the *Per2* reduction in NAc at night since in the experiment of Bainier et al 2017, where mice ingested only sugar (mainly during the night) *Per2* mRNA expression was similar compared to animals ingesting water. When chronically exposed to the fcHFHS intake which combines sugar and fat, also metabolic changes appear, including high basal blood glucose (la Fleur, Luijendijk, van Rozen, Kalsbeek, & Adan, 2011), thus it might be that this prolonged hyperglycemia impacts cell functioning and consequently, produces a clock-gene disruption in the NAc and LH, two areas with no self-sustained oscillations, in which normal rhythmicity could be overridden by abnormal physiological factors such as hyperglycemia. In line with such direct effects of glucose, the NAc and LH contain glucose-sensitive cells (Koekkoek, Mul, & la Fleur, 2017; Papp, Lukáts, Takács, Szalay, & Karádi, 2007).

Although we clearly show effects of the fcHFHS diet on *Per2* mRNA in NAc and LH, these changes were not accompanied by changes in feeding rhythm or expression of genes involved in feeding behavior. For example, *pre-pro-enkephalin* mRNA in chow-fed animals showed a clear difference between ZT4 and ZT16, but this was not affected by fcHFHS-diet feeding. Apparently the changes in *Per2* alone in these areas are not sufficient to induce changes in the daily feeding pattern. Of note, an overall *Per2* mutation in mice does result in loss of the daily rhythm in sucrose drinking (Bainier et al., 2017), pointing to a role for *Per2* in other areas of the brain (or body), or to developmental effects of *Per2* in feeding behaviour.

The LH has direct glutamatergic projections to the LHb (Stamatakis et al., 2016), which could have predicted changes in the Hb as well. We did not find, however, an effect of the fcHFHS diet on rhythmic *Per2* gene expression in the Hb. Possibly light is a stronger *zeitgeber* than food in the Hb, as there are close light inputs to

Hb (H. Zhao & Rusak, 2005) like is known for the SCN (which also still showed a day/night difference for clock genes). Earlier we showed, in mice, that PER2 protein in the LHb was affected by the fcHFHS diet (9), however, these mice showed clear changes in the daily feeding rhythm of fat and sugar. These results highlight the hierarchical organization of the circadian system; when disturbances are in "weak" brain oscillators (NAc and LH) this does not affect behaviour. It remains to be confirmed when a spontaneous change of feeding patterns towards day time does occur in rats, whether this would be accompanied by the same *Per2* disruptions in the LHb as shown for day-snacking mice. We cannot discard that disruptions of *Per2* in NAc and LH could reflect a progressive alteration of the circadian system and with more profound obese state, other areas like LHb would also be compromised.

In the present study, the fcHFHS diet did not result in high fat and/or sugar intake during the light period, as we had previously observed in mice and rats (Blancas-Velazquez et al., 2017; la Fleur et al., 2014). This discrepancy might be due to the amounts of sugar and fat consumed. In previous studies, mice and rats consumed more fat (>30%) than sugar (25%) when fed a fcHFHS diet. In the current experiment, rats consumed only 10% of their total caloric intake as fat, whereas sugar intake was higher than shown before. It is unclear what caused this difference in intake, however, it does point to a role for dietary intake on feeding patterns. Previously we observed that rats, consuming more than 30% fat on the fcHFHS diet, consumed 40% of their sugar intake during the light period (la Fleur et al., 2014). Nonetheless, when rats were exposed to only sugar *ad-libitum* in addition to chow (fcHS diet), sugar intake was mainly restricted to the dark period (la Fleur et al., 2014). The animals in the current experiment drank similar amounts of sugar as animals on the fcHS diet (la Fleur et al., 2014), thus, it could well be that although sugar can influence *Per2* in the reward circuitry, this is not sufficient to induce behavioural effects. This points to an additional factor linked to fat feeding that together with altered *Per2* expression mediates disruptions in palatable intake

patterns, but only when the total fat intake exceeds a minimum amount. It is clear that sugar intake or fat intake alone does not disrupt behavioural rhythms in rats (la Fleur et al., 2014).

Taken together, we show that the fcHFHS diet and acute sugar drinking affect *Per2* gene expression in areas involved in food reward. These *Per2* expression changes however were not sufficient to alter feeding-related peptides or feeding behavior.

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Conflict of interest statement

The authors report no conflict of interest.

Chapter 5

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Chapter 6

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Chapter 7

General discussion

General discussion

Obesity is a multifactorial disease with increasing prevalence and epidemic proportions in major parts of the world. The relative easy access to foods with high amounts of sugar and fat, perceived as highly palatable by the individual, are related to the obesity problem as this high availability is related to overeating which is one of the most prominent factors promoting overweight and obesity. In an effort to understand the causes and consequences of obesity, different diet-induced obesity (DIO) models in mice and rats have been used in experimental research. The use of diets in which all calories are condensed in a single pellet is a common practice. However, although these models are useful, they have limited translational value, as they do not replicate the wide variety in food choices humans are faced with. An alternative model is the use of different food items from which the animal is free to choose the most attractive ones. In previous studies, the use of the fcHFHS diet has shown its effectiveness to produce overeating, body weight gain and body fat accumulation in rats. Furthermore, this diet, providing access to different palatable nutrients (fat and sugar), caused an altered daily intake pattern in sugar intake, with 40% of total sugar intake spread over the light period (la Fleur et al., 2014). Interestingly, when rats have only one palatable nutrient (only sugar) as in the free-choice High-Sugar diet (fcHS; when animals have the choice between chow and a bottle of sugar water), sugar intake was mainly consumed during the dark period and thus did not show shifted sugar consumption towards the light period as shown for animals on a fcHFHS diet. The fcHFHS diet induced a-rhythmicity in sugar intake raised the hypothesis that the consumption of high caloric/palatable diets and the resulting obesity could involve the circadian system in some brain areas related to palatable food intake. This hypothesis was supported by previous studies in mice and rats showing similar disruptions of daily rhythms in feeding upon instatement of a high caloric diet and DIO, resulting in the total caloric intake showing a less pronounced difference between day and night (Kohsaka et al., 2007; Mifune et al., 2015; Pendergast et al., 2013). Differently to

the previous referred studies where a single high caloric pellet was used, the fcHFHS diet provides sugar and fat independently and as mentioned before, the feeding alterations in the fcHFHS-fed rats were observed specifically on sugar intake (la Fleur et al., 2014), a highly rewarding nutrient that stimulates the reward system. However, neither the studies using a single high caloric pellet nor the studies using the fcHFHS diet had studied the effects of the DIO on the rhythmic functions of reward-related brain areas.

Daily rhythmic behavioral outcomes in mice and rats after fcHFHS diet exposure

The aim of my thesis was to study the relation between a high caloric/palatable diet and the function of the circadian system. As a first approach to tackle the question of whether the elements of the molecular clock machinery were affected in brain areas related to feeding behavior, we characterized a mice model of DIO using the fcHFHS diet, as this was never used in mice before. Furthermore, characterization of the behavioral and physiological effects of the fcHFHS diet in wild-type mice would be a stepping stone for further use of this diet in genetically modified mice to study specific-gene deletions (e.g. clock-genes) and its effects on fcHFHS-diet ingestion. In **Chapter 3** of this thesis we described for the first time the effects of the fcHFHS-diet in mice (Blancas-Velazquez et al., 2017). Similar to rats, clear increases of hyperphagia, body weight and white adipose tissue accumulation were found compared to the chow-fed animals, but also several differences were observed as compared to the rat studies. In mice, the fcHFHS diet produced a more profound and rapid effect on body weight gain as compared to rats (Blancas-Velazquez, la Fleur, & Mendoza, 2017; Blancas-Velazquez et al, 2018, under revision) and mice consumed more fat overall, especially during the light period (la Fleur et al., 2014; la Fleur, van Rozen, Luijendijk, Groeneweg, & Adan, 2010). We observed reduced day-night differences in both sugar and fat intake in mice, which had not been observed in rats with the same fcHFHS diet. In order to compare changes in the brain observed in mice on a fcHFHS diet (**Chapter 3**), we also performed a new experiment in rats fed the fcHFHS diet, surprisingly, in this group

(described in **Chapter 4**) we did not observe the earlier reported clear intake of sugar in the light period. However, in this group of rats a spontaneous reduction of fat intake as compared to the other rat studies was observed (la Fleur et al., 2014). In mice and in rats, the sole exposure to a sugar solution, available *ad libitum* during the 24h of the day, does not modify their behavioral daily feeding patterns (Bainier et al., 2017; la Fleur et al., 2014), therefore, it is possible that the rats from the study reported in **Chapter 4** did not consume enough fat to produce physiological changes signaling to the brain, altering its rhythmic function and ultimately resulting in altered behavior.

Taken together, it is clear that the fcHFHS diet has more pronounced effects on the daily rhythms of palatable intake in mice compared to rats. The *Npas2* mutant mice study described in **Chapter 6** shows that the effects on fat and sugar intake when on a fcHFHS diet are reproducible in mice, as again the fcHFHS diet induced an almost equal intake of fat and sugar in the light and dark period as observed in **Chapter 3**. It thus seems that the daily rhythm in feeding behavior is more easily disrupted in mice than in rats, which might imply a differential regulation of food intake.

Not many studies have compared directly the feeding behavior of rats vs. mice. However, it has been shown that mice take more and relative smaller meals compared to rats, resulting in rats having more distinct feeding times whereas mice show feeding bouts continuously throughout the day (Rowland, Minaya, Cervantez, Minervini, & Robertson, 2015). In addition, when challenged by increases in the cost to obtain food (like having to press a lever) rats and mice also respond differently (Rowland et al., 2015). There are also some differences described in the anatomy and connectivity of feeding related systems between mice and rats. The anorexigenic neuropeptide melanin-concentrating hormone (MCH) system, for example, which cell bodies are located in the LH, is different between mice and rats. In mice there are MCH projections to the external part of the globus pallidus, especially to the parvalbumin neurons which receive strong

enkephalinergic input from the striatum, a connectivity that is not found in rats (Croizier et al., 2010). The differences in the neurotransmitter circuits linking the enkephalinergic system might explain the difference between mice and rat in their preference for fat, and maybe also sugar intake. In rats it was shown that infusing neuropeptide Y (NPY) in the NAc reduced pre-pro-Enkephalin within this nucleus and increased fat intake of rats on a fcHFHS diet (van den Heuvel et al., 2015). It is possible that differences in basal levels of neurotransmitter expression between mice and rats underlie the propensity to react differently to physiological changes caused by a high caloric diet and obesity. Even more, it is possible that the normal micro structure of feeding patterns (eg. meal/bout frequency, meal size) is different between species because of underlying metabolic, but also neuro-anatomical and functional differences.

Clock-gene expression in the brain of mice and rats exposed to the fcHFHS diet

It has been shown that clock gene expression responds to nutrients, for example, providing a single-pellet hypercaloric diet *ad libitum* alters clock-gene expression in the liver (Eckel-Mahan et al., 2013; Hatori et al., 2012; Kohsaka et al., 2007; Reznick et al., 2013; Yanagihara, Ando, Hayashi, Obi, & Fujimura, 2006). In this thesis, we focused on the effects of consuming a fcHFHS diet on clock-gene expression in the brain. Since, rats and mice on the fcHFHS diet showed increased day-time sugar consumption (in rats) or increased day-time fat and sugar consumption (in mice), but no alterations in the day-night pattern of chow ingestion (Blancas-Velazquez et al., 2017; la Fleur et al., 2014) we hypothesized that different brain areas were involved in signaling the ingestion of the different nutrients and that areas from the reward system would be specifically affected when animals consume the fcHFHS diet. We found indeed an effect in the fcHFHS-fed mice that spontaneously consumed a higher amount of calories during the day time and concomitantly their PER2 clock-gene expression in the LHb was altered. The LHb is a semi-autonomous brain circadian oscillator, involved in reward evaluation by modulating dopaminergic tone through its projections to the

midbrain tVTA/RMTg (Masasuke Araki, McGeer, & Kimura, 1988; Matsumoto & Hikosaka, 2007). In our mice experiment (**Chapter 3**), the fcHFHS diet consumption did not alter clock-gene expression in the SCN. The SCN is the main circadian clock of the body and indeed, the daily rhythm in locomotor activity was not affected and moreover, chow intake remained higher during the night and lower during the day. This suggests that the fcHFHS diet might induce a desynchronization of the LHb and the SCN in the snacking mice, with increased sugar and fat eating during the light period due to the altered habenula function, whereas the SCN might still be signaling rhythmicity for the homeostatic processes that require ingestion of the well-balanced nutritional chow which is an important protein source. In line with this notion we show similar patterns of clock gene expression in the ARC as in the SCN.

We expected to observe similar results, i.e., altered *Per2* mRNA expression in the LHb, in the rat experiment described in **Chapter 4.** However, we found clear day-night differences in clock gene expression measured in this area as well as in the SCN, independently of the diet. The unchanged *Per2* expression in the rat LHb might be explained by the finding that in this experiment (**Chapter 4**), rats did not show any day/night shifts in the ingestion of either of the nutrients from the fcHFHS diet. As discussed before, this unchanged behavior could be due to the abnormally low amount of fat these animals consumed. We did, however, observe differences in the NAc and LHA of the fcHFHS-fed rats, where the normal day-night *Per2* expression was altered. It has been shown that the NAc and LH are "weak" brain oscillators (i.e., they lack a self-sustained oscillation of *Per1* when they are cutured *ex-vivo*) (Abe et al., 2002a). Thus, the disruption of *Per2* expression rhythm in the NAc and LH in our rat experiment (**Chapter 4**), indicates that the rhythmic signals from the SCN can be overridden by the altered physiological status caused by the nutrients consumed. Although a specific role for *Per2* in the NAc and LH has not been described yet, there is evidence that mice lacking the *Per2* gene show depression like behaviors. They show increased

despair-related behavior which is accompanied by reduced monoamine oxidase a (MAOa), the dopamine degrading enzyme, in the NAc. Moreover, in wild-type mice there is a normal daily variation of MAOa in the NAc, which is down regulated in *Per2* mutant mice (Hampp et al., 2008). The LH shows daily expression of the neuropeptide orexin, that has an important role in the regulation of sleep, feeding and reward (Aston-Jones et al., 2009; Harris & Aston-Jones, 2006; Holtz et al., 2012; Sutcliffe & de Lecea, 2002). Rev-erbα is a nuclear receptor protein which is part of the molecular clock mechanism (Lowrey & Takahashi, 2011) and it has been shown to interact with the orexigenic system to modulate palatable food ingestion. Mice with a Rev-erbα KO show higher orexin cell activation after a palatable food (chocolate) ingestion (C?line A. Feillet et al., 2017). We could speculate that the changes on *Per2* expression observed in the NAc and LH could be related to changes in the rhythmic function of neurotransmitters within these nuclei, such as dopamine and orexin.

A possible mechanism for the *Per2* changes observed in LHb of mice (**Chapter 3**) and NAc and LH of rats (**Chapter 4**) could be changes in the extracellular microenvironment. Although we cannot make any firm conclusions yet based on the available data, it is of interest that within the LH and the NAc there are glucose-sensitive neurons, indicating possible effects of glucose from the diet on clock gene expression (Koekkoek et al., 2017; Papp et al., 2007). For example, the high caloric content from the combined sugar and/or fat diet might produce changes in the periphery that are also perceived directly by the brain and modify the expression of clock genes like *Per2*. In line with this notion, triglycerides injected intraperitoneally in mice are able to cross the blood brain barrier and modify the activity of leptin receptors in the hypothalamus (Banks et al., 2017), supporting the idea that the brain extra cellular environment is altered by changes in the periphery. Another possibility is that long term exposure to the fcHFHS diet modifies basal levels of different neurotransmitters, which subsequently could alter clock-gene expression. For instance, in young healthy individuals overconsuming

fat and sugar amounting to a 40% increase of overall caloric intake, serotonin transporter binding decreases, especially when the subjects consume the additional fat/sugar with a snacking pattern, i.e., eating the extra calories between the regular meals. These changes occur even when the individuals do the snacking during their active phase (Karin Eva Koopman et al., 2013). It is possible that even when the animals maintained their regular day-night difference in food intake, the micro-structure of food intake might be different between the chow- and diet-fed animals, thus provoking similar changes in the serotonin system as the previously observed in humans. Unfortunately, no micro-structure of feeding patterns is available for our mice and rat studies. It has been described that in obese mice and rats the dopaminergic system is compromised, as the obese animals show an abnormal binding/availability of the D2/D3 receptor in the striatum compared to the lean animals (Friend et al., 2017; E van de Giessen et al., 2012). Interestingly, dopamine is able to modulate PER2 expression in the striatum (Hood et al., 2010). Thus, chronic changes in different neurotransmitter systems after prolonged consumption of a high caloric/palatable diet might also be involved in the disruption of clock gene rhythms in the reward/feeding-related areas.

Lateral Habenula in feeding behavior

The LHb afferents and efferents have been described in great detail and these anatomical connections highlight a close relation to areas of the reward system, involving direct and indirect projections to the VTA that modulate dopamine release, and the feeding system, such as the LH (M. Araki, McGeer, & McGeer, 1984; Masasuke Araki et al., 1988). Nevertheless, little is known about its role in feeding behavior. Interestingly, besides its relation to the dopaminergic system, the LHb also provides input to the serotoninergic (5HT) system originating from the raphe nuclei (Pollak Dorocic et al., 2014). The 5HT system, which has an inhibitory role in feeding behavior (Simansky, 1995), shows daily variations and has been related to circadian functions of feeding behavior (Versteeg et al., 2015). A direct functional input from the LHb to the raphe nucleus has been shown with

LHb stimulation decreasing electrical activity of raphe neurons (Stern, WC; Johnson, A; Bronzino, J D; Morgane, 1979; Varga et al., 2003). Altogether, it is possible that the rhythmic function of the LHb exerts its effects through the modulation of serotoninergic system. Therefore, the acute inhibition of glutamatergic inputs to LHb could interfere with the normal communication of this area with either the VTA or the raphe nucleus, specifically during the night, and alter feeding behavior with a time-dependent effect as observed in the study of **Chapter 5**.

In 2016, Stamatakis et al. reported that inhibition of the glutamatergic inputs from the LH to the LHb produced an increased consumption of the palatable liquid Ensure (Stamatakis et al., 2016). Another study investigating the effects of the habenula on sugar consumption showed that rats with electrical stimulation of the LHb modified their motivation to work for sugar depending on the frequency of the stimulation. Ten Hz during 15 min increased sugar intake, whereas 100 Hz during 15 min did not produce increased lever presses for sugar (Friedman et al., 2011). This indicates that the LHb is involved in motivation for palatable food, dependent from its activity level. To our knowledge, this thesis is the first to report the effects of the type of diet on clock gene expression in the LHb. We showed that the alterations of the daily eating patterns in *mice* fed the fcHFHS diet were accompanied by a disruption of PER2 protein expression in the LHb (**Chapter 3**). However, in fcHFHS-fed *rats* we did not observe changes in mRNA expression, but we also did not observe changes in their daily eating patterns (**Chapter 4**). This may indicate that alterations of clock gene expression in the LHb are involved in the increased feeding during the light period. In **Chapter 5,** we show time-dependent effects of inhibiting glutamatergic input to the LHb. Moreover, previous studies have reported the involvement of the LHb in the approach to and avoidance of sugar (Friedman et al., 2011). Here we extended the scope of this work by showing an effect on the non-palatable chow consumption in rats after blocking glutamatergic input to the LHb. It has been shown that the LHb shows daily

variations in electrical activity (C Guilding et al., 2010; H. Zhao & Rusak, 2005), however the effects of glutamatergic inhibition in the LHb, shown in Chapter 5, were only observed at night and thus when electrically activity was shown to be low and thus not in line with the time-dependent effects of CNQX. It is more likely that at night there are signals that induce glutamate release into the habenula which is reduced with CNXQ resulting in changes in feeding behavior at night. It remains to be determined whether and which inputs are involved in the shown effects. The effect of glutamatergic receptor blocking in the LHb was also diet-component-dependent as it had an opposite effect on sugar and fat intake. Whereas sugar at night was reduced during glutamate receptor blockage, fat intake was increased. The downstream mechanisms underlying this effect remain unknown, we speculated that the reduced sugar and increased fat intake at night are the consequence of an induced preference for fat caused by the CNQX infusion. However, conditioned flavor preference experiments will have to be performed to determine whether the changes in fat and sugar intake are actually related to a preference shift to fat.

The primary suspect for the changes in food intake observed after pharmacological manipulation of the LHb with CNQX is the DA system. It has been shown that inhibition of glutamatergic function in the LHb during the light phase, by a local infusion of the AMPA receptor antagonist LY293558, increased DA release in the NAc and PFC of rats (Lecourtier et al., 2008). In our study (**Chapter 5**) we infused an AMPA/kainate receptor antagonist in the LHb during both the light and dark phase. Based on the Lecourtier et al. study and the increased dopaminergic tone observed, we expected a higher food intake during the day time, however, the effects on food intake were only observed during the dark phase. Therefore, possibly another neurotransmitter system is involved in the changes in food intake after the glutamatergic blockade in the LHb. As mentioned before, the LHb has also wide projections to the raphe nucleus and LHb stimulation modifies electrical activity in the raphe nucleus (Stern, WC; Johnson, A; Bronzino, J D; Morgane,

1979; Varga et al., 2003). Moreover, there are serotonergic receptors in the VTA and their activation inhibits normocaloric food intake, whereas their inhibition increases palatable food intake. Although these effects are dependent on the type of 5HT receptor (Pratt et al., 2016), they do suggest that activity within the LHb-raphe-VTA system might be altered when altering LHb function with CNQX.

In **Chapter 5** we showed an apparent switch of preference from sugar to fat after the night time administration of CNQX, however, extra experiments are needed to clarify if it is indeed a preference switch or whether it is a specific motivation for fat intake. Moreover, it would be important to dissociate if the LHb is directing intake specifically to sugar or fat or whether its action on food intake is caused by the combination of sugar and fat. Separate groups of rats fed only sugar and chow or only fat and chow are needed to get conclusive answers on this point. Clearly, in future experiments more specific techniques, such as optogenetics, are needed to more specifically target different cell groups and projections to and from the habenula in order to unravel the mechanism by which the LHb might be affecting food intake. Of special interest would be the specific ablation of clock genes within the LHb, especially *Per2* which seems to be involved in the proper functioning of this nucleus and engaged in the behavioral changes observed after exposure to a fcHFHS diet (**Chapter 3**).

As obesity is a growing health problem, understanding its causes and consequences is important to be able to develop preventive strategies or interventions to reverse its deleterious effects on health. The increased availability of highly processed palatable food is related to obesity, as its easy access in the western lifestyle and the frequent food advertisements produce a higher intake of these palatable foods (Juul & Hemmingsson, 2015; Louzada et al., 2015). It has been shown that both food composition and meal frequency can affect the brain and energy metabolism. For example, more frequently consuming fat and sugar (as compared to consuming bigger meals with fat and sugar) induced more fat accumulation in the liver and altered the brain serotonin system (Karin E. Koopman et al., 2014; Karin Eva Koopman et al., 2013). There is also evidence pointing towards the advantages of restricting feeding to the active phase of the organism. Moreover, in humans it has been observed that narrowing the feeding time from >14h to 10 -12 h a day has beneficial results on health, such as body weight reduction and higher subjective measurements of "wellness" (Gill & Panda, 2015). Thus, a better understanding of the mechanisms linking the circadian system and palatable food intake might provide novel strategies to battle overeating and obesity,

In this thesis we reviewed data on how consuming high caloric palatable diets may produce altered day-night patterns of food ingestion in animal models. We have shown that, especially in mice the fcHFHS diet can alter daily feeding patterns by shifting the ingestion of the palatable diet components to the normal resting phase (**Chapter 3**). These changes were accompanied by alterations in the clock gene *Per2*. It is possible that in the rats that did not alter their feeding patterns, but still showed clock gene disruptions in their reward system, we observed a progressive alteration of the circadian timing system with the "weak" brain oscillators being more prone to changes, whereas in the obese mice with changes in both feeding behavior and PER2 in the LHb also the "strong" brain oscillators became engaged. Thus, our results highlight the hierarchic organization of the circadian timing

system and a possible progressive deregulation of this system depending on the severity of the obese state.

Altogether, data from humans and animal models suggest that the consumption of high caloric/palatable diets can produce changes to eating patterns and that especially snacking behavior has adverse health consequences, even more when performed during the normal resting phase. We also show that even when a behavioral disruption is not present, clock genes in the brain reward system may already be engaged. Although the consequences of this disruption are not yet understood, reduction of fat and sugar intake as well as avoiding snacking patterns, especially during the resting phase, might be a good way to keep our circadian and metabolic system healthy.

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Chapter 8

Summary

Résumé de la thèse

Samenvatting

Thesis Summary

Obesity is a health problem reaching epidemic levels. An important factor for the development of obesity is overeating especially that of palatable food with high amounts of fat and sugar. In mammals, feeding behavior, among different biological functions, has a periodical variation of 24h. These endogenous rhythmic functions with a period close to 24h are known as circadian rhythms and are intrinsically generated in the body by the circadian system. Located in the hypothalamus, the suprachiasmatic nucleus (SCN) is the main biological clock of the body which coordinates rhythmic functions of other brain and peripheral oscillators. The SCN and virtually all cells of the body contain a feedback loop of genes known as the clock-genes, *clock* and *Bmal1* form the positive limb of the feedback loop whereas *Per* and *Cry* are part of the negative limb. These genes form a loop of expression and repression mechanism with a period of ~24h and regulate the rhythmic expression of clock-controlled genes inducing rhythms in cell physiology.

Brain areas implicated in regulation of food intake, including those in the reward system, which modulate food preference reinforcing palatable food intake, also show circadian variations in their physiology. Some studies have shown that ingestion of high caloric foods with high amounts of fat and/or sugar alter behavioral rhythms such as in general locomotor activity and food intake. However, the effects of high caloric/palatable food ingestion on the circadian activity of the reward system are not well known. The objective of this thesis was to study the relationship between high caloric/palatable food ingestion and its relationship to the brain circadian system.

First, in **Chapter 2** we carried out a review focused on the effects of high caloric/palatable diets on general locomotor activity and daily food intake rhythms of rats and mice. The effects of the high caloric/palatable diets on the rhythmic activity of brain regions controlling eating behavior were also examined. The

results showed that these kinds of diets, (either in presentation of single pellets containing all the high caloric nutrients, or presenting the different nutrients separately) can alter the rhythms of general activity and disrupt the temporal patterns of food intake. It should be noted that the eating patterns are more easily affected by the high caloric/palatable diets than the general locomotor activity. However, we found little research on the effects of these regimes on the rhythmic activity of the brain's reward system.

A high caloric/palatable diet that has already being described in rats as a diet-induced obesity model is the free-choice High-Fat High-Sugar (fcHFHS) diet. This diet allows free access to 4 different diet components: a bottle of water, standard chow pellets, a bottle of water with 30% sugar concentration, and saturated fat. Offering the different nutrients separately is a way of replicating the human condition in which people are able to freely choose the most attractive foods, something that does not happen when a high caloric/palatable diet is presented as one single pellet. In previous work in rats, the fcHFHS diet model led to a change in temporal dietary patterns, specifically for sugar ingestion: there is a high sugar consumption during the light period (rest phase), characterized by intake spread over the entire light period and making up 40% of total sugar intake.

In **Chapter 3** we used for the first time the fcHFHS diet in mice in order to describe the effects on rhythmic behavior (locomotion, food intake). Compared to the chow-fed mice, animals fed with the fcHFHS diet during 6 weeks increased caloric intake, as well as body weight, and adiposity. After 6 weeks, these mice also showed a disrupted temporal pattern of feeding, they consumed more chow food at night but sugar and fat consumption was similar between day and night. We explored the effects of the diet on brain clock-gene expression. Using immunocytochemistry techniques, we determined the expression of the clock proteins BMAL1 and PER2 in the brain of chow-fed and fcHFHS diet-fed mice at two different times: 4 hours after the start of the light phase and 4 hours after the start of the dark phase. The expression of the 2 clock-gene proteins was quantified

in SCN, the arcuate nucleus (ARC) and the lateral habenula (LHb) in both groups. In the SCN and ARC, the expression of PER2 showed differences between day and night and was not altered by the diet. In contrast, the LHb of the control chow-fed group showed a day-night variation in the expression of PER2, that was absent in the fcHFHS diet-fed group. The results of this study suggest that a disruption of the day-night patterns of food intake in the fcHFHS fed diet group may be linked to changes in clock function in the LHb.

Although the rhythm of food intake during fcHFHS diet feeding had already been described in rats, the consequences on clock-gene expression in reward-related areas had not been reported. Therefore, in **Chapter 4**, we used the fcHFHS diet model in rats with the aim of studying the effects of this diet on brain clock-gene expression. The fcHFHS diet-fed rats of this experiment showed day-night differences for the ingestion of all diet components, these unexpected results were accompanied by an overall decrease in fat intake. We analyzed the expression of different clock-genes including *Per2*, *Cry1* and *Bmal1* in the nucleus accumbens (NAc), SCN, LHb, the ventral tegmental area (VTA) and lateral hypothalamus (LH). Despite the absence of changes in daily patterns of food intake in the fcHFHS diet-fed group, analysis of gene expression by real-time polymerase chain reaction (qPCR) showed alterations in *Per2* mRNA expression, in NAc and LH, but not in LHb. Interestingly, only *Per2,* and not any of the other clock-genes studied, was modified. These results showed an effect of the fcHFHS diet on *Per2* mRNA expression in some areas of the reward system even when a behavioral rhythm alteration was not observed. The clock-gene expression in the LHb of these rhythmic rats was not altered, pointing to a relation between behavioral and LHb rhythmicity.

The results of studies in mice and rats in this thesis pointed to a possible role of the LHb in rhythmic regulation of food intake. Thus, in **Chapter 5** we proposed the hypothesis of a role for LHb in the temporal control of food intake. We used a pharmacological approach to block the glutamatergic input to the LHb in rats by

infusing an AMPA/kainate glutamatergic receptor antagonist (CNQX) into the LHb. Rats were operated to implant guided cannulas towards the LHb. Rats were randomly divided into two groups, a chow-fed and a fcHFHS diet-fed group. Infusions of CNQX or saline (as a control treatment) were performed at two time points (during the day or night) in both experimental groups. Food intake was measured one hour after the injection of either CNQX or saline. The chow-fed rats injected only saline showed higher food consumption during the night compared to day time, while rats receiving CNQX, showed a reduction in food consumption at night. Interestingly, different to the chow-fed group, the total caloric consumption of the fcHFHS diet-fed rats was increased by CNQX infusion into the LHb at night. We next analyzed intake of each caloric nutrient of the fcHFHS diet after treatment infusion (saline vs. CNQX), and observed that the overall increase in caloric intake on the fcHFHS diet was driven by a clear increase in fat intake, which was most pronounced in the dark period. Interestingly, the sugar intake was reduced by infusing the glutamatergic antagonist but only during the night. These results point to a role for the LHb in modulating both palatable and nutrition balanced intake in a time-dependent manner.

Finally, in **Chapter 6** we performed an experiment using mutant mice lacking the *Npas2* clock-gene which is a homologue of *clock*. *Npas2* is an important gene for rhythmic functioning of forebrain areas. Mice lacking this gene show a decreased behavioral sensitivity to cocaine compared to wild-type mice. Since the mutant *Npas2* gene mice have reduced sensitivity to non-natural rewards such as cocaine, we hypothesized that *Npas2* mutant mice would show a reduction in motivation to ingest the high caloric/palatable nutrients of the fcHFHS diet. We used *Npas2*::PER2Luc mice. These mice lack the *Npas2* gene and have a luciferase reporter fused to the *Per2* gene which was used to measure *ex-vivo* PER2 protein oscillations in ARC and SCN. Wild-type and *Npas2*::PER2Luc mice were divided into two groups: fed with a chow or the fcHFHS diet during 6 weeks. The fcHFHS diet produced hyperphagia and enhanced weight gain in both wild-type and *Npas2*

mutant mice compared to the control chow-fed groups. There were no differences between genotypes on the day-night patterns of food intake of the different diet components. The analysis of *ex vivo* PER2 oscillations did not show differences between genotypes or diets in the SCN. Only in the ARC, the period of PER2 was shortened in the *Npas2* mutants compared to the wild-type mice but this was independent of the diet (chow vs fcHFHS). Thus, the shortened PER2 period in the ARC did not have consequences for rhythms in behavior, suggesting that "stronger" brain oscillators may be directing daily rhythms of food intake in these mice.

The results of the present thesis indicate a relationship between high caloric/palatable food intake on the brain circadian system, where the ingestion of fat and sugar leading to obesity can produce a deregulation in day/night expression of clock-genes, especially *Per2,* in reward-related brain regions even in the absence of a disruption in theday-night food intake pattern. Our results point to a role for the LHb in the time regulation of palatable food intake since *Per2* in this area remains unaltered when the eating patterns are rhythmic. And, when eating patterns were altered in mice, consuming more palatable food during the day, PER2 expression in the LHb was disrupted. Furthermore, the results in rats where the blocking of LHb glutamatergic inputs have a time-dependent effect on chow and palatable food intake agree with the hypothesis of the LHb as a brain oscillator able to modulate daily variations of food intake.

Résumé de la thèse

L'obésité est un problème de santé qui atteint un niveau épidémique. Un facteur important pour le développement de cette maladie est la prise excessive d'aliments palatables qui ont généralement un haut contenu calorique. Chez les mammifères, le comportement alimentaire, parmi plusieurs fonctions biologiques montre une variation périodique d'environ 24h, et les cycles biologiques de cette durée sont qualifiés de circadiens, un mot d'origine latine, *circa*: autour et *dies*: jour. Ces variations circadiennes, le comportement alimentaire inclut, sont régulées par le noyau suprachiasmatique (SCN) la principale horloge biologique. D'autres noyaux cérébraux impliqués dans la régulation de la prise alimentaire et dans le circuit de la récompense, qui module la préférence alimentaire en renforçant la consommation de nourriture palatable, ont une capacité circadienne. Certaines études ont montré que la consommation de nourriture hypercalorique grasse et/ou sucrée peut altérer les comportements rythmiques au niveau de l'activité générale ou de la prise alimentaire. Cependant, les effets de cette nourriture sur la rythmicité circadienne des noyaux du circuit de la récompense, impliqués dans la prise alimentaire, restent peu connus.

L'objectif de la présente thèse a été d'étudier les conséquences physiologiques d'un régime alimentaire calorique/hédoniques ainsi que les effets de ce régime sur le système circadien. Premièrement dans le **chapitre 2** nous avons référencé les études concernant les effets de régimes hypercaloriques/hédoniques chez le rat et la souris (rythme de l'activité locomotrice et prise alimentaire). Les effets du régime hypercalorique/hédonique sur l'activité circadienne des régions cérébrales contrôlant le comportement alimentaire ont également été examinés. Les résultats de ces études ont montré qu'un régime hypercalorique/hédonique (soit en présentation d'un pellet contenant tous les nutriments hypercaloriques/hédoniques, soit en présentant les différents nutriments séparément) peut produire dans certains

cas, une altération du rythme d'activité général et également influencer les patterns temporels de prise alimentaire. Il est à remarquer que de manière générale, dans la prise alimentaire c'est la variable rythmique comportementale la plus affectée par les régimes hypercaloriques/hédoniques.

Par ailleurs, il y a un manque de recherche des effets de ces régimes sur l'activité rythmique des régions du circuit de la récompense; en effet ce point est important pour comprendre le mécanisme de dérégulation journalière de la prise alimentaire, cette dernière n'étant plus entraînée par un besoin énergétique mais plutôt par une motivation hédonique.

Un modèle de régime hypercaloriques/hédoniques déjà décrit chez le rat, le « free-choice High-Fat High-Sugar » (fcHFHS) permet le libre accès au rat à 4 composants du régime: une bouteille d'eau; pellets de nourriture standard; une bouteille d'eau avec une concentration de sucre à 30%; et de la graisse saturée. L'objectif d'offrir les nutriments séparément est de reproduire les conditions d'un humain pouvant choisir les aliments les plus attirants, ce qui n'arrive pas quand le régime induisant l'obésité consiste en un pellet unique. Le modèle de fcHFHS avait amené chez le rat, un changement des patterns alimentaires journaliers, spécifiquement concernant la prise de sucre: il y avait une consommation de sucre plus importante pendant la période de lumière (phase de repos de l'animal), ce qui diminuait la différence jour-nuit quant à la prise de ce composant du régime.

Dans le **chapitre 3** nous avons utilisé le modèle de fcHFHS pour le valider chez la souris et étudier les effets de ce régime sur le comportement circadien mais aussi au niveau cérébral, dans des structures impliquées dans la prise alimentaire. En augmentant l'apport calorique chez la souris, ce régime a induit une augmentation de la masse corporelle et du tissu adipeux en comparaison aux souris nourries avec un pellet standard normocalorique. Les souris nourrie avec le régime fcHFHS après 6 semaines ont montré un pattern temporel avec plus d'ingestion de l'aliment normocalorique pendant la nuit mais par contre, la consommation de sucre et de

graisse était similaire entre le jour et la nuit. Avec la technique d'immunohistochimie, nous avons déterminé l'expression des protéines horloges BMAL1 et PER2 à deux moments différents de la journée: 4h après le début de la phase lumineuse et 4h après le début de la phase d'obscurité. L'expression des 2 protéines horloges été quantifiée dans le SCN, le noyau arqué (ARC) dans hypothalamus et l'habenula latérale (LHb) dans l'épithalamus chez les groupes contrôles et le groupe nourri avec le régime fcHFHS. Dans le SCN et l'ARC, l'analyse de l'expression de PER2 a montré des différences entre le jour et la nuit mais pas d'effets du régime, par contre, dans la LHb, le groupe contrôle a montré une variation temporelle de PER2 qui était absente dans le groupe du régime fcHFHS. Ces résultats montrent que la perturbation des patterns jour-nuit de la consommation d'aliments hypercaloriques/hédoniques est liée au fonctionnement de la LHb.

Chez le rat, même si le rythme du comportement de prise alimentaire pendant le régime fcHFHS avait été décrit, les conséquences au niveau des gènes-horloge n'avaient pas été étudiées, c'est pourquoi, dans le **chapitre 4**, nous avons utilisé le modèle de fcHFHS avec l'objectif d'analyser l'expression de gènes horloges dans le cerveau des rats soumis à ce régime. Les résultats de cette expérience n'ont pas montré une dérégulation comportementale des patterns jour-nuit dans la prise alimentaire des nutriments du régime. Nous avons analysé l'expression de différents gènes horloge dont *Per2*, *Cry1* et *Bmal1* dans le noyau accumbens (NAc), le SCN, la LHb, l'aire tegmentale ventrale (VTA) et l'hypothalamus latéral (LH). Malgré l'absence de changements comportementaux des rythmes de prise alimentaire dans le groupe fcHFHS, l'analyse de l'expression génétique par la technique de réaction en chaîne par polymérase en temps réel (qPCR) a montré des altérations de l'expression de *Per2* dans le NAc et LH, mais pas dans la LHb. Fait intéressant, seul l'expression de *Per2* et non des autres gènes horloges étudiés ont été modifiés. Ces résultats montrent que même en l'absence d'altération du rythme de prise alimentaire, la seule ingestion d'aliments hypercaloriques/hédoniques peut

modifier l'expression de *Per2* dans certaines régions du circuit de la récompense. Les résultats concernant la LHb où aucune altération n'a été trouvée suggèrent que le pattern jour-nuit de la prise alimentaire est lié à la rythmicité circadienne de la LHb.

Ces résultats chez la souris et le rat ont mis en évidence un rôle possible de la LHb dans la régulation des rythmes comportementaux de prise alimentaire. Dans le **chapitre 5** nous avons proposé l'hypothèse de l'implication de la LHb dans le contrôle circadien de prise alimentaire. Une approche pharmacologique a été utilisée chez les rats pour bloquer la fonction glutamatergique de la LHb en infusant un antagoniste des récepteurs glutamatergiques AMPA/kainate dans la LHb. Des rats ont été opérés pour implanter des canules guide vers la LHb. Les rats ont été sélectionnés aléatoirement en deux groupes, un groupe avec un régime normocalorique et un groupe nourri avec le régime fcHFHS. Les infusions du CNQX ou de solution saline (groupe contrôle) ont été effectuées à deux moments de la journée (jour vs. nuit) dans les deux groupes expérimentaux, et la consommation de nourriture a été mesurée une heure après l'injection soit de CNQX soit de solution saline. Les rats soumis au régime normocalorique ont montré une prise alimentaire plus élevée pendant la nuit, alors que les rats recevant le CNQX, bloquant les afférences glutaminergiques, ont montré une réduction de consommation d'aliment dans la nuit. On peut noter que la totalité de la consommation calorique des rats dans le régime fcHFHS n'a pas été affectée par l'infusion de CNQX dans la LHb, contrairement aux observations chez les rats nourris avec l'aliment normocalorique. Néanmoins, quand nous avons analysé la prise de chaque nutriment calorique du régime fcHFHS, nous avons observé une diminution de la prise de sucre lors de l'infusion de CNQX pendant la nuit. Contrairement, au sucre, la consommation de graisse a montré une tendance à être plus importante pendant la nuit. Il n'y a pas eu d'effet de l'infusion de CNQX sur la prise d'aliment normocalorique. Ces résultats surlignent qu'en effet, la LHb peut moduler la prise alimentaire des aliments normo et hypercaloriques, le blocage du

fonctionnement du système glutamatergique de la LHb a un effet lié au temps de la journée.

Finalement, dans le **chapitre 6** nous avons effectué une expérience chez la souris mutante chez qui il manque le gène horloge *Npas2*. Ce gène est important pour le fonctionnement rythmique du cerveau où se trouvent les aires du circuit de la récompense. Ces souris mutantes ne montrent pas la même réponse comportementale à la cocaïne comme les souris sauvages, et cette évaluation diminuée de la récompense a généré l'hypothèse que ce gène horloge pourrait être impliqué dans la consommation d'aliments hypercaloriques/hédoniques. Puisque les souris mutantes du gène *Npas2* ont une sensibilité diminuée vers les drogues comme la cocaïne, nous nous attendions à une réduction de la motivation pour consommer les nutriments hédoniques du régime fcHFHS. Nous avons utilisé des souris Npas2:PER2 luciférase, pour mesurer *ex-vivo* les oscillations de la protéine PER2 dans l'ARC et le SCN, des régions impliquées dans consommation alimentaire. Les souris sauvages et les souris Npas2:PER2 luciférase ont été réparties dans deux groupes: un avec le régime normocalorique et un autre avec le régime fcHFHS. Après 6 semaines les souris sauvages et mutantes de *Npas2* nourries avec le régime fcHFHS étaient hyperphagiques avec une prise de masse augmentée. Il n'y a pas eu de différences entre les génotypes concernant le pattern jour-nuit du comportement alimentaire de nourriture hédonique. L'analyse des oscillations de PER2 *ex-vivo* n'a pas montré de différences entre les génotypes ni entre les régimes dans le SCN. Dans l'ARC la période de l'activité de PER2 était diminuée dans le groupe des souris mutantes par rapport aux souris sauvages et cette réduction était indépendante du régime auquel les souris ont été exposées. Cette réduction n'a pas eu d'effets sur le comportement, indiquant qu'il existe des oscillateurs cérébraux qui régulent de façon plus importante la rythmicité de la prise alimentaire.

L'ensemble des résultats de la présente thèse indiquent qu'il existe une relation entre la régulation de la prise d'aliments hypercaloriques/hédoniques et le système

circadien faisant intervenir le gène horloge *Per2* dans les régions cérébrales du circuit de la récompense. Il est aussi à noter que la LHb peut être impliquée dans la régulation des comportements rythmiques comme la prise d'aliments hédoniques puisque quand il n'y a pas d'altération du rythme de la prise alimentaire. De plus l'activité circadienne de la LHb reste inaltérée. En revanche, quand les rythmes de la LHb sont altérés la souris consomme plus d'aliments palatables pendant le jour. Le blocage de l'activité glutamatergique dans la LHb chez le rat montre un effet selon le moment de la journée, ce qui est en accord avec l'hypothèse de la participation de ce noyau sur l'activité circadienne de la prise alimentaire d'aliments (normocaloriques et hypercaloriques/palatables).

Samenvatting

Obesitas vormt een groot gezondheidsprobleem met epidemiologische proporties. Een belangrijke factor betrokken bij de ontwikkeling van obesitas is de overconsumptie van vooral vet en suiker. Vele biologische processen in zoogdieren, waaronder ook eetgedrag, laten variatie zien over de 24 uur. Deze 24-uurs variatie, zoals in eetgedrag, wordt gecoördineerd door de suprachiasmatische nucleus (SCN), de centrale lichaamsklok, een hersenkern die zich bovenop de kruising van beide oogzenuwen bevindt en onderdeel is van de hypothalamus. In de SCN en in bijna alle cellen van het lichaam, bevinden zich klokgenen met een feedback-loop. Deze feedback-loop van expressie en repressie mechanismen genereert een ritme van ongeveer 24 uur. Hierin vormen c*lock* en *Bmal1* de positieve arm van de feedback-loop en *Per* en *Cry* vormen samen de negatieve arm van de feedback-loop. Samen reguleren ze ritmische expressie van de door de klok gecontroleerde genen betrokken bij de fysiologie van de cel.

Andere hersenkernen betrokken bij eetgedrag, zoals bijvoorbeeld beloningsgebieden betrokken bij de voedselmotivatie en evaluatie of iets lekker is, laten ook een 24-uurs variatie zien. Studies toonden aan dat het nuttigen van hoogcalorische diëten met grote hoeveelheden vet en suiker een verandering te weeg brengen in 24-uurs ritmes in bijvoorbeeld locomotor activiteit en voedselopname. Echter, de effecten van hoogcalorische vet-suikerrijke diëten op de ritmiek van het beloningssysteem is nauwelijks onderzocht. Het doel van dit proefschrift was dan ook om te onderzoeken wat de relatie is tussen hoogcalorische diëten en het circadiane systeem in het brein.

In **hoofdstuk 2** wordt een overzicht gegeven van de in de literatuur beschreven effecten van een hoogcalorisch dieet met veel vet en suiker op dagelijkse ritmes in locomotor activiteit en eetgedrag in zowel ratten als muizen. Daarnaast worden ook de effecten van het hoogcalorisch vet-suikerrijk dieet op de ritmische activiteit van

verschillende hersengebieden beschreven. Dit overzicht laat zien dat verschillende diëten (zowel die waarbij een pellet wordt gegeven met daarin een hogere concentratie vet en suiker, als wel die diëten die bestaan uit verschillende voedselnutriënten die separaat genuttigd kunnen worden) een invloed hebben op de algemene locomotor activiteit en ook een verstoring laten zien in de 24-uurs ritmiek van voedselopname. Wel is het zo dat hoogcalorisch vet-suikerrijke diëten eerder de 24-uurs ritmiek in eetgedrag verstoren dan de algemene locomotor activiteit. Verder vonden we bijna geen publicaties over de effecten van dit soort diëten op de ritmische activiteit van beloningsgebieden in de hersenen.

Het vrije-keuze hoog-vet hoog-suiker dieet oftewel het free-choice high-fat high-sugar (fcHFHS) dieet geeft dieren ongelimiteerd toegang tot verzadigd vet en een fles suikerwater naast de normale voeding die bestaat uit een pellet (chow, onderhoudsvoer) en kraanwater. Dit dieet, waarbij er dus een vrij keuze is om nutriënten los van elkaar te eten, is een manier om het humane eetgedrag beter na te bootsen, met keuze vrijheid tussen verschillende aantrekkelijke (hoogcalorische) soorten voeding. Dit is wezenlijk anders dan veel andere modellen waarbij voer wordt verrijkt met vet en suiker en aangeboden als één homogene pellet. Dit dieet is reeds succesvol toegepast in ratten en leidt in kort tijdsbestek tot een toename in lichaamsgewicht en vetmassa doordat dieren veel meer calorieën nuttigen vergeleken bij dieren met een controle chow dieet. Uit eerder werk is gebleken dat dit fcHFHS dieet model leidt tot veranderingen in de 24-uurs ritmiek van voedselinname en vooral die in suikeropname, waarbij dieren blootgesteld aan het fcHFHS dieet, regelmatig suiker drinken gedurende de lichtperiode (wanneer ratten inactief zijn), zodat ruim 40% van alle suikerinname in de inactieve fase (d.w.z. gebruikelijke slaapperiode) plaatsvond.

In **hoofdstuk 3** wordt beschreven hoe we het fcHFHS dieet voor het eerst gebruiken in muizen om te kijken naar de effecten van dit dieet op gedrag en fysiologie. Door hun hogere calorie inname werden de dieren die blootgesteld werden aan het fcHFHS dieet snel zwaarder en hadden meer vetmassa dan de

dieren die alleen chow tot hun beschikking hadden. Na 6 weken, lieten muizen op het fcHFHS dieet ook verstoorde 24-uurs eetpatronen zien waarbij de chow inname nog gewoon ritmisch was met de meeste inname tijdens de nacht (actieve periode), maar met een gelijke inname van vet en suiker gedurende de nacht en de dag, resulterende in aritmische eetpatronen voor de smakelijke vet en suikercomponenten van het dieet. Daarna werden de effecten van het 6-weken fcHFHS dieet op de expressie van klokeiwitten in verschillende hersengebieden onderzocht. Door middel van immunocytochemie hebben we de dagelijkse veranderingen in BMAL1 en PER2 bestudeerd in chow gevoede en fcHFHS dieet gevoede dieren op 2 tijdspunten verspreid over de licht-donkercyclus: 4 uur na de start van de lichtfase en 4 uur na de start van de donkerfase. De eiwitexpressie werd gekwantificeerd in de SCN, de nucleus arcuatus (ARC) en de laterale habenula (LHb). In de SCN en de ARC liet PER2 eiwitexpressie een duidelijk verschil zien tussen dag en nacht en dit verschil werd niet beïnvloed door het fcHFHS dieet. In de LHb was echter alleen in de chow gevoede groep een verschil tussen dag en nacht zichtbaar. In de dieren op het fcHFHS dieet was geen verschil tussen PER2 eiwit expressie in de licht en de donkerfase. Deze experimenten laten dus zien dat de verstoring in eetritmiek (van vooral vet en suikerinname) wanneer dieren worden blootgesteld aan een fcHFHS dieet, gekoppeld zou kunnen zijn aan veranderingen in klokeiwitexpressie in de laterale habenula.

Ook al waren de veranderingen in 24-uurs ritmiek in voedselinname tijdens een fcHFHS dieet al beschreven voor ratten, de consequenties van een fcHFHS dieet voor klokeiwitexpressie in beloningsgebieden in de hersenen waren nog niet beschreven in de rat. Daarom hebben we, in **hoofdstuk 4**, ratten blootgesteld aan het fcHFHS dieet en gekeken naar de effecten van dit dieet op klokgenexpressie in verschillende hersengebieden betrokken bij eetgedrag. Echter in dit rattenexperiment werden geen verstoringen in de dag-nacht ritmiek in voedselopname waargenomen in de dieren op het fcHFHS dieet. Deze onverwachte uitkomst ging gepaard met lagere vetinname in de dieren op het fcHFHS dieet

vergeleken met eerdere experimenten waarbij wel verstoringen in dag-nacht ritmes werden waargenomen. We analyseerden de klokgenexpressie van onder andere Per2, Cry2 en Bmal1 in de nucleus accumbens, SCN, LHb, ventral tegmental nucleus (VTA) en de laterale hypothalamus (LH). Ondanks het feit dat we geen verstoringen in eetgedrag vonden, werden veranderingen in Per2 mRNA expressie gezien in de NAc en de LH maar niet in de LHb en VTA. Deze veranderingen werden alleen gevonden voor het Per2 gen en niet voor de andere bestudeerde klokgenen. Dus deze resultaten laten veranderingen zien in Per2 mRNA expressie in sommige beloningsgebieden in de hersenen zonder dat er sprake is van gedragsveranderingen. Het feit dat juist nu in de LHb geen veranderingen in klokgenexpressie werden gevonden terwijl dit wel eerder het geval was, in dieren met een verstoord eetpatroon, duidt op een eventuele rol voor Per2 veranderingen in de LHb in fcHFHS dieet-geïnduceerde effecten op de 24-uurs ritmiek van vet en suikerinname.

De resultaten zoals beschreven in hoofdstukken 3 en 4 duiden op een rol voor de LHb in het dag-nacht ritme in vet-suiker inname. Daarom hebben we in **hoofdstuk 5** de hypothese getest dat de LHb een rol speelt in de tijdsafhankelijke controle van voedselinname (en specifiek van vet en suiker). Ratten ondergingen een operatie om bilateraal canules te plaatsen gericht op de LHb. Ratten werden willekeurig ingedeeld in twee groepen: een chow gevoede groep en een fcHFHS dieet gevoede groep. Infusies met CNQX of saline (als controle groep) werden uitgevoerd op 2 tijdstippen, 1 gedurende de dag en de andere gedurende de nacht, en voedselopname werd gemeten 1 uur na de injectie van CNQX of saline. CNQX blokkeert de glutamaat receptor en infusie van CNQX in de LHb remt dus de activatie van LHb neuronen. De chow gevoede ratten lieten meer voedselinname zien gedurende de donkerperiode dan gedurende lichtperiode wanneer ze met saline werden geïnjecteerd, met CNQX injecties daarentegen was de inname hetzelfde in de licht als in de donkerperiode, waarbij er een significante afname was van voedselinname in de donkerperiode. Tegenovergestelde effecten werden

gezien in de ratten op het fcHFHS dieet. Hier was er sprake van een verhoogde totale inname na injectie van CNQX en dit verschil in inname was vooral het resultaat van een verhoogde vetinname na CNQX, terwijl de suikerinname in de donkerperiode zelfs gereduceerd was ten opzichte van de saline geïnjecteerde groep. Samengenomen laten deze experimenten een duidelijke rol zien voor de LHb in voedselopname, zowel voor gezonde chow als de meer smakelijke vet en suiker, afhankelijk van het tijdstip van de dag.

Tot slot, beschrijft **hoofdstuk 6** een experiment met muizen die deficiënt zijn voor het NPAS2 klokgen. Dit is een homoloog van het '*clock'*-gen. Terwijl het *clock* gen vooral belangrijk is in de centrale klok, speelt NPAS2 voornamelijk een rol in gebieden in de voorhersenen. Eerder onderzoek heeft laten zien dat muizen deficiënt voor NPAS2 lagere sensitiviteit hebben voor de belonende effecten van cocaïne in vergelijking met wild type muizen. Deze verlaagde sensitiviteit voor beloning leidde tot de hypothese dat NPAS2 mutante muizen een verminderde motivatie of voorkeur zouden hebben voor de vet en suiker componenten van het fcHFHS dieet. Door gebruik te maken van NPAS2::PER2Luc muizen was het mogelijk om zowel effecten van NPAS2 deficiëntie te bestuderen als ook de PER2 oscillaties te meten. PER2Luc muizen hebben namelijk een luciferase reporter in het Per2 gen wat gebruikt kan worden om *ex vivo* PER2 eiwit oscillaties in afzonderlijke hersengebieden te meten. Wild type en Npas2:PER2Luc muizen werden verdeeld over twee groepen: blootgesteld aan een chow dieet of aan het fcHFHS dieet voor 6 weken. Het fcHFHS dieet resulteerde in overeten en meer gewichtstoename in zowel wild type als NPAS2::PER2Luc muizen in vergelijking met het chow dieet. Er was geen verschil tussen beide genotypes in de ritmiek van de voedselinname. De analyse van *ex vivo* PER2 oscillaties liet ook geen effecten van genotype of van dieet zien in de SCN. Alleen in de ARC was de periode van PER2 verkort in de NPAS2 mutante groep vergeleken met de wild type muizen, maar dit verschil was onafhankelijk van het dieet. Daarnaast had de verkleining van de periode in PER2 in de ARC geen gevolgen voor de gedragsritmes. Het kan

dus goed zijn dat een andere, sterkere breinoscillatoren voedselinname beïnvloeden in deze muizen.

De resultaten van dit proefschrift bevestigen een reciproke relatie tussen hoogcalorisch vet-suikerrijk eten en het circadiane systeem in de hersenen. Naast dat het obesitas veroorzaakt, zien we hier dat consumptie van veel vet en suiker ook resulteert in verstoring van het dag-nacht-ritme in klokgenexpressie. Vooral de Per2 expressie in beloningsgebieden in de hersenen is gevoelig voor vet-suikerrijk eten, zelfs zonder detecteerbare veranderingen in eetgedrag. Onze resultaten wijzen naar een rol voor de LHb in de regulatie van tijdsafhankelijk eetgedrag omdat Per2 in dit gebied wel veranderde wanneer circadiane eetpatronen waren verstoort maar niet veranderde wanneer het gedrag niet veranderde, zoals we zagen in het ratten experiment beschreven in hoofdstuk 4. Verder laten we effecten zien van het blokkeren van de glutamaat input naar de laterale habenula op eetgedrag en dat dit effect tijdsafhankelijk is wat de hypothese ondersteunt dat de laterale habenula betrokken is bij de dagelijkse variatie in eetgedrag.

Appendices

PhD portfolio

Publications

Acknowledgments

Phd Portfolio

Name PhD candidate: Aurea Susana Blancas Velazquez

PhD period: October 2013-March 2018

Name PhD supervisors: J.Mendoza, S.E la Fleur

Communications (Posters)

Blancas-Velazquez A, Eggels L, Foppen E, Kalsbeek A, Mendoza J & la Fleur S.E. (2017) Time-dependent effect of lateral habenula inhibition on food intake in rats fed a regular-chow or a palatable free-choice hypercaloric diet. **XV Congress of the European biological rhythms society (EBRS)**. Amsterdam, The Netherlands.

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Communications (Oral)

Blancas-Velazquez A, Mendoza J, la Fleur S. (2017) Relation between a hypercaloric free-choice high-fat high-sugar diet and the circadian system (Elevator pitch). **Amsterdam Gastroenterology & Metabolism (AG&M) retreat**. Garderen, The Netherlands.

Blancas-Velazquez A, la Fleur S, Mendoza J. (2017, 2014). Implications of the circadian system in the control of compulsive feeding behaviors. **Neurotime Annual meeting**. Amsterdam, The Netherlands.

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Blancas-Velazquez A. (2015) Behavioral measurements in chronobiology. **Neurotech seminars**. Strasbourg (67), France.

PhD training

Trainee day XV European Biological Rhythms Society Congress	2017
Laboratory animal course (Article 9) KNAW	2015
Systematic reviews of animal studies e-learning course SYRCLE	2015
Neurex meeting “Circadian rhythms and sleep homeostasis: Two inseparable processes	2014
NUTRIBRAIN Summer school Université de Bordeaux	2014
Neurex course “Cholinergic tone: where from, what for? Physiological modes of cholinergic signalling	2014
Neurex course Metabolomics: A set of new analytical technologies to study the world of small molecules.	2014
Neurex course “The Cognitive Thalamus”.	2013

Publications

Peer reviewed in thesis

Blancas-Velazquez A, Sage-Ciocca D, la Fleur SE, Mendoza J (2018) Eating behavior in NPAS2 clock gene mutant mice under a free choice high-fat high-sugar diet condition. ***Submitted.***

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Blancas-Velazquez A, la Fleur SE, Mendoza J Effects of a free-choice high-fat high-sugar diet on brain PER2 and BMAL1 protein expression in mice. ***Appetite.*** 2017 Oct 1; **117**: 263-269. (2017) doi:1016/j.appet.2017.07.002.

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fnins.2017.00023.

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Feillet CA, Bainier C, Mateo M, ***Blancas-Velazquez A***, Salaberry NL, Ripperger JA, Albrecht U, Mendoza J. Rev-erbα modulates the hypothalamic orexinergic system to influence pleasurable feeding behaviour in mice. ***Addict Biol*** **22**:411–422. (2017) doi: 10.1111/adb.12339

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Aurea Susana BLANCAS VELAZQUEZ

Nourriture palatable, gènes horloges et le circuit de la récompense

Résumé

Cette thèse a étudié l'interaction entre l'obésité induit par la diète et la physiologie fcHFHS) avec un régime gras et sucrée ou un régime normocalorique chez la souris et le rat. Le régime fcHFHS a modifié le profile journalier de prise alimentaire et l'expression de la protéine PER2 dans l'habenula latérale (LHb) chez la souris. Cependant chez le rat, ni la prise alimentaire ni l'expression du gène *Per2* dans la LHb n'ont changé, mais des altérations ont été observées dans le noyau accumbens. Chez les rats nourris avec une diète standard ou fcHFHS, le blocage glutamatergique dans la LHb induit une altération de la prise alimentaire dépendant du temps du blocage. Finalement, nous avons étudié le comportement alimentaire chez des souris contrôle et mutantes du gène horloge *Npas2* nourris avec le régime fcHFHS. Le comportement alimentaire, néanmoins, était similaire entre les deux génotypes. Les résultats indiquent une relation entre le type de diète et une expression anormale des gènes horloges dans le circuit de la récompense, ainsi comme un rôle important de la LHb dans la prise alimentaire.

Mot clefs : circuit de la récompense, circadien, obésité, gènes horloge

Abstract

This thesis studied the interaction between diet-induced obesity and the 24h variations in behavior and physiology paced by the circadian system. Mice and rats were fed with a free choice high-fat high-sugar diet (fcHFHS). In mice, fcHFHS diet changed day-night eating patterns and PER2 clock-protein expression in the Lateral Habenula (LHb), a food-reward related area. In rats, no feeding patterns or clock-gene changes in LHb were found, however, *Per2* gene expression was disrupted in the Nucleus Accumbens, which is indirectly connected to LHb. When blocking pharmacologically the glutamatergic functioning of the LHb, food intake was altered in both chow and fcHFHS-fed rats in a time-dependent manner. Finally, we tested the influence of *Npas2* clock-gene on the disruption of rhythmic behavior produced by the fcHFHS-diet using *Npas2* mutant and WT mice. Both genotypes, however, displayed similar altered eating patterns caused by the fcHFHS diet. Our findings indicate a relationship between nutrient type and an abnormal clock-gene expression in food reward-related areas, and an important role for the LHb in feeding behavior.

Key words: reward system, circadian, obesity, clock-genes.