Sleep-wake behaviors
resulting from early life obesity
Hedonic and homeostatic signal contributions

Dissertation

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

vorgelegt von

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München, September 2015

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Dissertation eingereicht am: 16. September 2015

Mündliche Prüfung am: 20. Juni 2016

ERKLÄRUNG

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Abstract

Overconsumption of palatable foods causes obesity, which affects an increasing number of adolescents. Previous studies have suggested that early-life obesity may permanently alter hedonic and homeostatic mechanisms regulating feeding behaviors, and may therefore increase the risk of medical conditions in later life. While depressive disorders and sleep disturbances often occur in association with obesity, treatment efficacy of these disorders appears to be worsened by the obese state. Hence, a better understanding of the mechanism linking these three conditions might provide contact points for innovative pharmacotherapy and obesity prevention strategies. In this thesis, we aimed to investigate how early-life obesity impacts on sleep-wake regulation and mood-related behaviors during aging. We further aimed to explore possible mechanisms linking obesity, sleep disturbances and depressive disorders. This relationship was here examined in the context of obesity during peripubertal development.

Our mouse model of peripubertal diet-induced obesity (ppDIO) was generated by feeding male C57BL/6N mice a high-fat diet (HFD) between postnatal days 28 and 70. Termination of HFD was followed by standard normal chow (NC). To monitor sleep-wake behaviors, EEG/EMG recordings were performed at different ages (10, 12, 24 and 52 weeks). Further, neurotransmitter and neuropeptide levels in particular brain areas related to reward, feeding and sleep-wake regulation were evaluated. Depression-like behaviors and the activity of the hypothalamic-pituitary-adrenal axis were examined as well. Mice that were maintained on NC served as controls.

A history of ppDIO increased nocturnal sleep time and decreased sleep quality in mice that were still exposed to HFD (10 weeks) and in mice aged 52 weeks. These changes were accompanied by decreased serotonin levels in the lateral hypothalamus, a brain region that is an important integrator of feeding, motivation and sleep-wake behaviors. Interestingly, both food deprivation (at 52 weeks) and an intraperitoneal injection of PYY_{3-36} (at 10 and 52 weeks), the latter being a peripheral satiety hormone, corrected nocturnal sleep time in ppDIO mice. Further, ppDIO mice displayed elevated basal corticosterone levels and increased depression-like behaviors during aging.

Cessation of HFD and re-exposure to NC resulted in hypophagia, body weight loss and a dramatic reduction in sleep time during the active phase, compared to those in control mice. Concomitant with HFD withdrawal, dopamine levels in the nucleus accumbens (a reward-related brain region) were reduced, while serotonin levels in the lateral hypothalamus were

increased in ppDIO mice. However, the expression of hunger signals in the lateral hypothalamus and arcuate nucleus was increased in ppDIO mice similarly to conditions of fasting in control animals.

In conclusion, ppDIO exerted significant effects on the long-term regulation of sleep-wake and depression-like behaviors. Our results suggest that reduced serotonergic tone underlies sleep disturbances and depressive disorders associated with obesity. During weight loss following HFD withdrawal, serotonergic tone increases, which might have contributed to increased vigilance. Further, we indicated the lateral hypothalamus and the nucleus accumbens as possible brain regions linking obesity with sleep disturbances. A dysfunction in the nucleus accumbens response to healthy diets may impede with strategies aimed at body weight loss in the obese. Lastly, $PYY_{3\text{-}36}$ has the potential to ameliorate sleep disturbances triggered by ppDIO.

Introduction

1 The regulation of food intake

Food intake is regulated by homeostatic needs and hedonic drives (Fig. 1). Both components are crucial for survival from an evolutionary perspective; homeostatic signals sustain energy balance, while hedonic drives are important for motivated behaviors. In addition, ingestive behaviors are under circadian control and can be influenced by other factors, such as emotions and stress. Interactions of these components are complex and will be explained in more detail in the following sections.
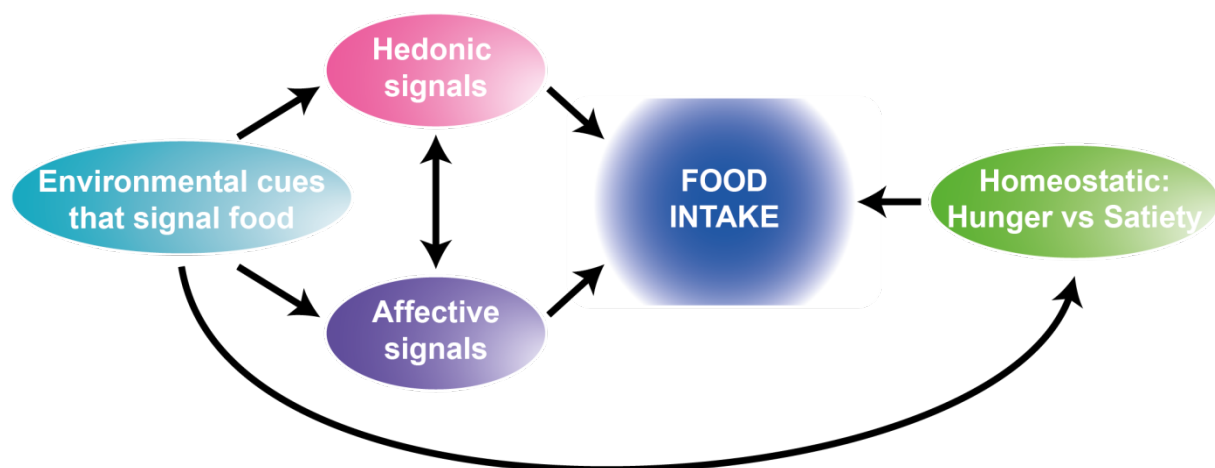
Figure 1: Signals that regulate food intake.

Homeostatic signals convey information about the energy status of the body to the brain and represent the main regulators of food intake. Also hedonic (e.g. food palatability and reward characteristics of food) and affective (e.g. stress) aspects can drive food intake. Environmental cues that may facilitate food intake include circadian and contextual (e.g. food advertisements) signals. Adapted from Johnson, 2013.

1.1 Homeostatic influence on food intake

Homeostatic regulation of food intake involves a wide network of brain regions including hypothalamic and brainstem nuclei, such as the lateral hypothalamus (LH), the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH), dorsomedial

hypothalamus (DMH), paraventricular hypothalamic nucleus (PVN), nucleus tractus solitarius (NTS) and the area postrema (AP). In these brain regions, so called first-order and second-order neurons are located. To regulate ingestive behaviors, first-order neurons sense energy availability, energy stores and the digestive status of the body through humoral and neural signals. They convey this information to second-order neurons, which induce food intake and suppress behaviors other than feeding by directly modulating the activity of other brain regions.

1.1.1 First-order neurons in the regulation of food intake

The hypothalamic ARC contains first-order neurons expressing appetite-promoting (orexigenic) neuropeptides neuropeptide Y (NPY) and agouti-related protein (AGRP), but also appetite-suppressing (anorexigenic) neurons that express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) (Schwartz *et al.*, 2000). These first-order neurons receive peripheral signals and act reciprocally to elicit or suppress food intake (Fig. 2; Schwartz *et al.*, 1996; Cheung *et al.*, 1997).

Because of its adjacent location to the median eminence, which possesses fenestrated capillaries, the ARC is not entirely protected by the blood-brain barrier (Peruzzo *et al.*, 2000). Therefore, feeding and satiety signals that are circulating in the blood can directly influence the neuronal activity of the ARC. Among the peripheral satiety signals, long-term signals such as leptin and insulin can be differentiated from short-term signals including peptide tyrosine-tyrosine (PYY), glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK). While leptin and insulin need to be produced in the adipose tissue (Y. Zhang *et al.*, 1994) and in hepatic β-cells (reviewed in Zou et al., 2014), respectively, short-term satiety signals are released immediately from the gastrointestinal tract upon ingestion of a meal (Sam *et al.*, 2012).

The only orexigenic peripheral hormone described to date is ghrelin. It is produced in the stomach and gastrointestinal tract (Kojima *et al.*, 1999; Korbonits *et al.*, 2004) and opposes the actions of satiety hormones on ARC first-order neurons.
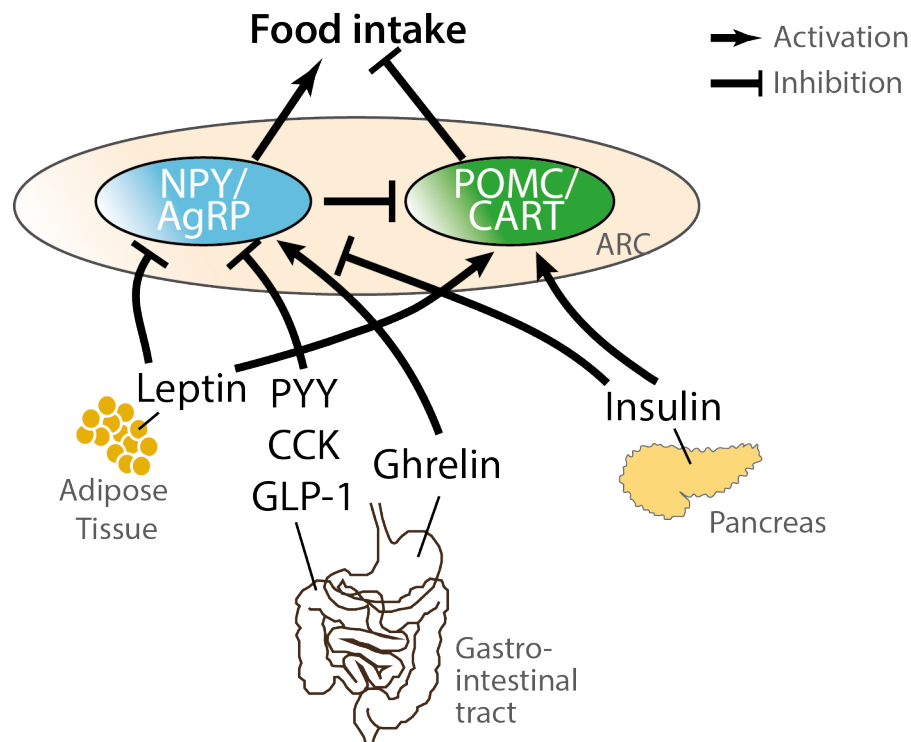
Figure 2: Scheme of peripheral satiety and feeding cues signaling to first-order neurons in the arcuate nucleus.

Anorexigenic POMC/CART and orexigenic NPY/AGRP neurons receive inputs from peripheral satiety signals such as PYY, CCK, GLP-1, leptin and insulin and the feeding signal ghrelin. POMC/CART and NPY/AGRP neurons inhibit or facilitate food intake, respectively.

An alternative route of these feeding and satiety signals to the brain involves the NTS and AP. These areas receive neural inputs from the gastrointestinal tract and digestive viscera (e.g. liver) both directly via the vagus nerve, where receptors for PYY and ghrelin are expressed, and via circulating hormones due to their fenestrated capillaries (Young, 2012). NTS and AP form a simple reflex arc with the dorsal motor nucleus of the vagus to control gut motility and secretion, as well as meal size (Young, 2012). The NTS and AP further project to CNS regions including the ARC to modulate ingestive behaviors.

1.1.2 Second-order neurons in the lateral hypothalamus

ARC first-order neurons send projections to various brain regions; one of the most important projections in terms of food intake regulation is the LH. Early studies showed that lesions of the LH resulted in death by starvation and dehydration (Anand

& Brobeck, 1951a, 1951b; S. D. Morrison *et al.*, 1958). By contrast, electrical
stimulation of the LH stimulated food intake and increased physical activity (Delgado
& Anand, 1953; Mogenson & Morgan, 1967). Hence, the LH has been designated as
"feeding center".

The LH constitutes orexin- and melanin concentrating hormone (MCH)–containing
neurons (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998; Vaughan *et al.*, 1989). Both
orexin and MCH neurons stimulate food intake, which was shown by icv infusions of
these peptides (Lubkin & Stricker-Krongrad, 1998; Griffond & Risold, 2009, 2009; Qu
et al., 1996; Rossi *et al.*, 1997). However, ablation of MCH or orexin neurons results
in different phenotypes; orexin knockout mice are obese, whereas MCH knockout
mice are lean and develop rather late-onset obesity (Shimada *et al.*, 1998;Willie *et
al.*, 2008). These differences stem from divergent roles of orexin and MCH in energy
expenditure; icv injections of orexin increased oxygen consumption (Funato *et al.*,
2009), while icv-injected MCH decreased it, suggesting that MCH is important for
energy conservation (Asakawa *et al.*, 2002).
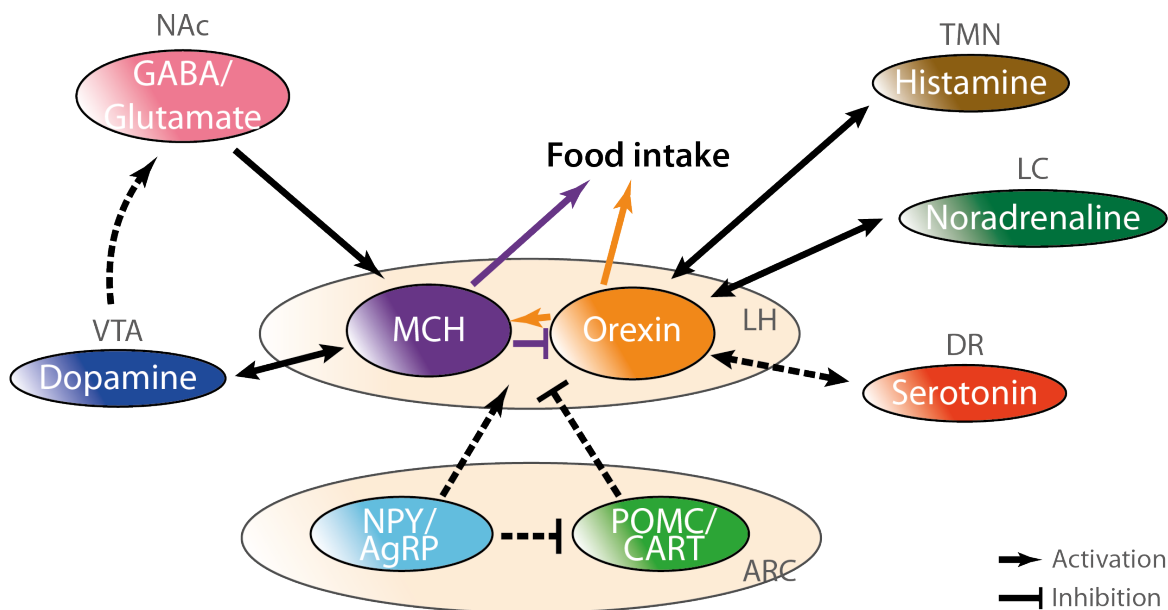
Figure 3: Schematic projections to and from neurons in the lateral hypothalamus related to the reward
system, food intake and sleep-wake behaviors.

MCH and orexin neurons receive projections from ARC first-order neurons. The LH integrates
information about energetic needs, arousal and reward via projections to and from the VTA (ventral
tegmental area), NAc (nucleus accumbens), TMN (tuberomammillary nucleus), LC (locus coeruleus)
and DR (dorsal raphé nucleus). Adapted from Sakurai, 2005.

Furthermore, orexin and MCH neurons play divergent roles in sleep-wake and reward-associated behaviors. Both innervate and receive projections from brain regions involved in arousal, reward-associated brain regions (Fig. 3) and also in memory and learning, such as the hippocampus and amygdala (Peyron *et al.*, 1998; Horvath *et al.*, 1999). A more detailed description of how orexin and MCH interact with the reward system and sleep-wake centers will follow in the sections 1.2 and 2.3, respectively. Moreover, orexinergic fibers project to the ARC and inhibit POMC/CART neurons, while stimulating NPY/AGRP neurons to facilitate food intake.

It is worth mentioning that, besides the LH, NPY/AGRP and POMC/CART neurons innervate other second-order neurons located in the PVN, VMH, DMH and other brain areas to regulate food intake (Elmquist *et al.*, 1999).

1.1.3 Hypothalamic dopamine and serotonin actions

Studies in the 70s and 80s have shed light on the potent food intake suppressing effects of dopamine and serotonin when injected into the LH (Leibowitz & Rossakis, 1979; Parada *et al.*, 1988; Hoebel *et al.*, 1989). Increased serotonin and dopamine concentrations in the LH have also been reported to be proportional to meal size (Schwartz *et al.*, 1989; Fetissov *et al.*, 2000). Further evidence was provided by drug treatments that affected serotonergic signaling, e.g. fluoxetine, a selective serotonin reuptake inhibitor, and d-fenfluramine, a serotonin receptor agonist. Both drugs increased serotonergic transmission and resulted in suppression of food intake (Le Feuvre *et al.*, 1991).

Accordingly, the LH was determined as a terminal field for both dopaminergic (Jacobowitz & Palkovits, 1974; Leibowitz & Brown, 1980; Parada *et al.*, 1988) and serotonergic (Vertes, 1991; Jalewa *et al.*, 2014) projections.

Serotonergic projections to the LH originate mainly from the DR (Vertes, 1991; Jalewa *et al.*, 2014). However, the exact source of dopamine release into the LH is not clear. It is still controversial whether the LH receives dopaminergic projections from the VTA (Kizer *et al.*, 1976; Leibowitz & Brown, 1980; Swanson, 1982) and PVN, whereas projections from dopamine-producing neurons in the zona incerta

(A13 group, Björklund & Lindvall, 1984; Wagner *et al.*, 1995) and the ARC (Ershov *et al.*, 2005) are strongly supported.

1.2 Hedonic control of food intake

1.2.1 Food consumption and the brain reward system

Food is a natural reward. Thus, food consumption is reinforcing and leads to a repetition of its ingestion. Both consumption of palatable foods and drugs of abuse engage the brain reward system to enable their reinforcing properties (Lutter & Nestler, 2009; Berridge, 2009). The reward system is embedded in the limbic system and its most extensively studied neural correlate is the mesolimbic pathway, which consists of dopaminergic projections ascending from the VTA to the NAc through the medial forebrain bundle (Dahlstrom & Fuxe, 1964;Bjorklund & Dunnett, 2007).

Several studies have shown that food intake leads to an increase in dopamine levels and turnover in the VTA and NAc (Hernandez & Hoebel, 1988; Yoshida *et al.*, 1992; Bassareo & Di Chiara, 1999; Roitman *et al.*, 2008). This rise in NAc dopamine concentrations is comparable to other rewards (Schultz *et al.*, 1993; Carelli, 2002), indicating that food can be equally reinforcing as drug abuse (Corsica & Pelchat, 2010; Koob *et al.*, 1992). Conversely, the dopamine response in the NAc decreases when aversive liquids such as quinine are consumed (Roitman *et al.*, 2008). The activation of the mesolimbic pathway is thought to be involved in generating the motivation to obtain food rewards (Schultz, 1997). Therefore, dopamine in the mesolimbic pathway plays a role in motivated behaviors that promote food seeking ('wanting' of a reward, Berridge, 2009).

The palatability of food can lead to its consumption regardless of the energetic status of the body (Johnson, 2013). This effect of palatable food can be driven by the sensory perception of food, such as sight and smell (Sorensen *et al.*, 2003), or by factors such as previous experience (developing food preferences and aversions) and conditioning to specific food cues (e.g. by advertisements) (Berridge, 2009; Johnson, 2013). This "hijacking" of homeostatic regulation by hedonic drive may lead

to overconsumption of palatable foods and may be the main cause for the development of obesity. The contribution of the hedonic systems to the development of obesity will be discussed further in the section 3.

1.2.2 Cross-talk between homeostatic and hedonic regulation of feeding

Based on the observation that food deprivation enhances the rewarding property of food (Berridge & Valenstein, 1991; Cameron *et al.*, 2014), a close connection between homeostatic and hedonic regulation of food intake can be inferred. One major link is provided by extensive reciprocal projections between the NAc and the LH (Phillipson & Griffiths, 1985; Usuda *et al.*, 1998; Urstadt & Stanley, 2015).

Increased inhibition of NAc disinhibits LH neurons, resulting in increased food consumption (Kelley *et al.*, 2005). Administration of orexin into the NAc led to increased feeding together with an increase in locomotor activity (Thorpe & Kotz, 2005). Valdivia et al. showed that acute high-fat diet (HFD) exposure induced c-Fos expression in the LH and VTA, and the majority of HFD-responsive LH neurons projected to the VTA. Furthermore, c-Fos induction in the VTA was attenuated following a pre-treatment with an orexin receptor-1 antagonist (Valdivia *et al.*, 2014). Thereby, orexin neurons facilitate dopamine release into the NAc (Fig. 4, Patyal *et al.*, 2012; Vittoz & Berridge, 2006; Espana *et al.*, 2011).

Further, MCH injection into the NAc promotes feeding, whereas antagonism of its receptor (MCHR1) suppresses feeding (Georgescu *et al.*, 2005). MCH appears to promote dopamine release into the NAc and thus contributes to the consumption of palatable liquids (Domingos *et al.*, 2013).

In summary, the actions of orexigenic peptides, such as MCH, NPY and ghrelin, may be mediating the hedonic drive, either via the VTA or the NAc. Hence, a hedonic drive facilitates feeding on palatable foods and should be integrated with homeostatic signals (Fig. 4). However, the question still remains why the termination of palatable food consumption fails to be executed far beyond energetic demands (see the section 3).
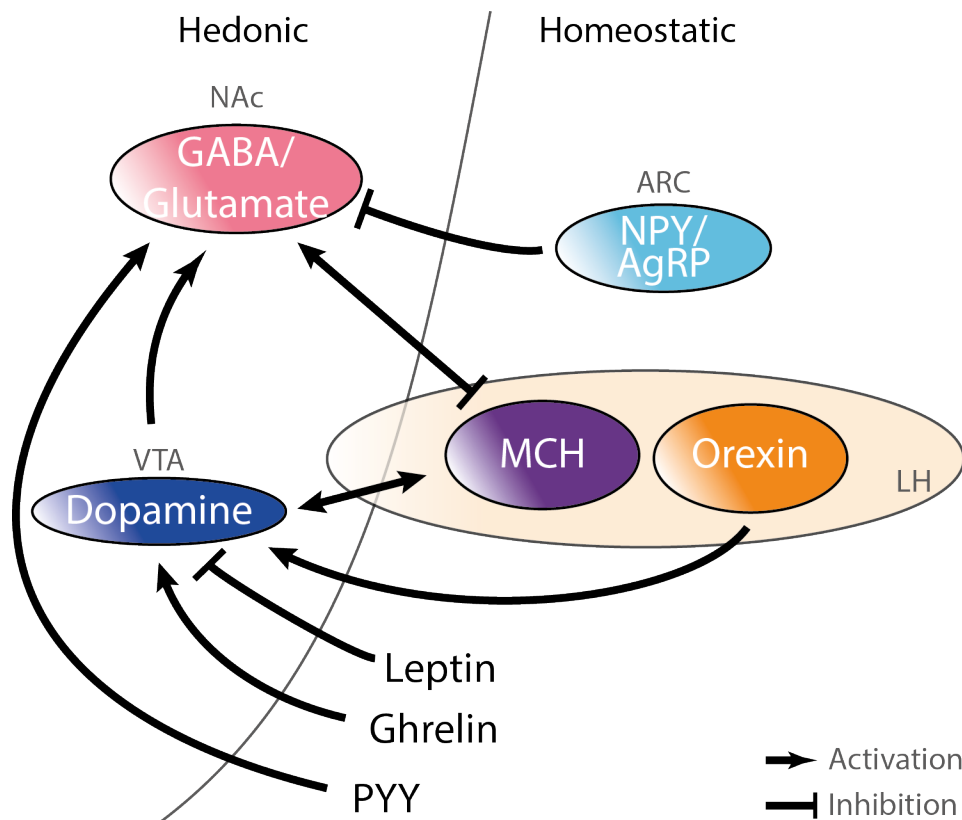
Figure 4: Scheme of interactions between homeostatic and hedonic modulators of food intake.

Peripheral cues such as leptin, ghrelin and PYY, signal to the VTA and NAc to modulate hedonic aspects of feeding. While leptin inhibits firing of VTA dopamine neurons and decreases food intake, ghrelin activates their firing, thereby increasing food consumption (Hommel *et al.*, 2006; Fulton *et al.*, 2006; Abizaid *et al.*, 2006; Naleid *et al.*, 2005). Paradoxically, application of satiety factor PYY$_{3-36}$ onto striatal slices increases the synthesis and release of dopamine (Adewale *et al.*, 2005, 2007), and its peripheral injection potentiated amphetamine-induced locomotor activity (Stadlbauer *et al.*, 2014) suggesting an induction of a hyperdopaminergic state by PYY (Stadlbauer *et al.*, 2015). ARC NPY neurons project to the NAc, where they reduce neuronal firing via NPY1R activation, resulting in increased feeding on fat-rich foods (van den Heuvel *et al.*, 2015).

1.3 Stress and food intake

The stress response, or the so called fight-or-flight-response, is elicited by circumstances that are harmful or even life-threatening to an individual. Energetic resources need to be diverted to muscles, heart and brain, while other behaviors such as sexual activity or libido, digestion, food intake or appetite are suppressed (Chrousos & Gold, 1992).

The key effectors of the stress response are the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary system. The latter mediates the fast stress response and originates from the locus coeruleus in the brainstem. Its

activation leads to the release of norepinephrine and epinephrine from the adrenal medulla. The principal regulator of the HPA axis is corticotropin-releasing hormone (CRH), which is produced in the PVN and stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH further stimulates the release of glucocorticoids, e.g. cortisol (humans) or corticosterone (rodents, amphibians, birds etc.) from the zona fasciculata of the adrenal cortex. Several feedback loops prevent prolonged, deleterious activation of the HPA axis (Chrousos & Gold, 1992).

Furthermore, CRH neurons in the PVN are second-order neurons in the regulation of food intake and exhibit reciprocal connections with ARC first-order neurons (X. Y. Lu *et al.*, 2003; Schwartz *et al.*, 2000; Heinrichs & Richard, 1999). CRH released from the PVN has anorexigenic actions, possibly to divert resources from other functions such as feeding and digestion towards stress coping (Heinrichs *et al.*, 1993). However, one hour following peripheral CRH injection, an increase in food intake is observed in humans and the amount of ingested food is proportional to the magnitude of cortisol release (George *et al.*, 2010). This suggests a role for cortisol in appetite stimulation after acute stress. Indeed, glucocorticoids appear to stimulate appetite (Santana *et al.*, 1995). Centrally, chronic glucocorticoid administration increased NPY, but decreased CRH expression, which together facilitate feeding (Zakrzewska *et al.*, 1999). Interestingly, during chronic stress, the ingestion of palatable, calorie-dense foods is preferred over healthy foods. This may serve as a coping mechanism, because consumption of highly palatable food leads to reward-mediated negative feedback onto the HPA axis (Pecoraro *et al.*, 2004; Foster *et al.*, 2009; Finger *et al.*, 2011).

In summary, food intake can be also modulated by stress hormones, i.e. CRH and cortisol. During chronic stress, prolonged HPA axis activation results in the overconsumption of palatable "comfort" foods, which may contribute to the etiology of obesity (see the section 3).

2 Sleep and wakefulness

2.1 Fundamentals of Sleep

Sleep is a rapidly reversible state of reduced responsiveness to external stimuli, decreased motor activity and reduced metabolism (Siegel, 2009). Even though sleep occurs similarly across different species including homoeothermic animals, the exact mechanisms regulating our sleep-wake behaviors are still poorly understood. Also the function of sleep is still mysterious; but the fact that individuals engage in sleeping despite the reduced protection against predators or the inability to mate or to eat during sleep, emphasizes the fundamental importance of sleep (Allada & Siegel, 2008).

Several studies have proposed some of the functions of sleep. For example, sleep was shown to be important for energy conservation (Walker & Berger, 1980), memory consolidation (Stickgold, 2005; Diekelmann & Born, 2010), neuronal plasticity (Tononi & Cirelli, 2006) and metabolic clearance (Xie *et al.*, 2013). Further, total sleep deprivation for several days has been shown to lead to death, pinpointing the vital importance of sleep (Everson *et al.*, 1989).

Since the first successful recording of the electroencephalogram (EEG, Berger, 1929), we know that sleep is a heterogeneous state. Based on EEG and electromyogram (EMG) recordings, five different vigilance states can be distinguished in humans (modified guidelines: Iber *et al.*, 2007; original guidelines: Rechtschaffen & Kales, 1968); wakefulness, non-rapid eye movement sleep (NREMS), which itself consists of three stages, S1, S2 and slow-wave sleep (SWS), and rapid-eye movement sleep (REMS). In rodents, S1, S2 and SWS cannot be clearly distinguished from one another. Therefore, in rodents three distinct vigilance states can be recognized; wakefulness, NREMS and REMS (Fig. 5).

A Wakefulness

B NREMS

C REMS

250 µV

1 sec
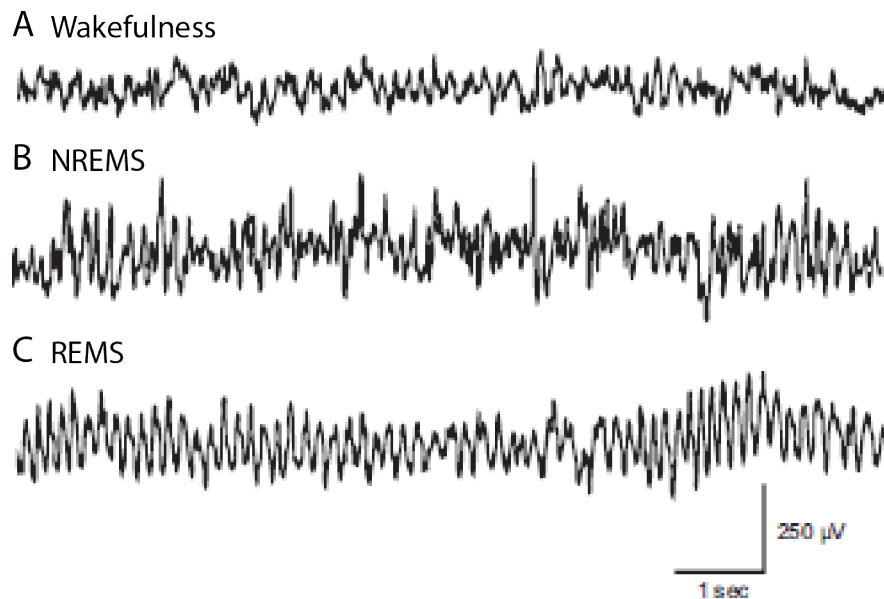
Figure 5: Exemplary EEG traces during different vigilance states in a rat.

(A) Wake EEG is characterized by low-voltage, high-frequency oscillations. (B) NREMS EEG is predominated by low-frequency, high-voltage delta waves. (C) REMS EEG traces typically consist of theta frequencies, and closely resemble wake EEG. Adapted from Brown *et al.*, 2012.

During wakefulness, cortical activity is high and produces a desynchronized EEG pattern with high-frequency and low-amplitude oscillations. Wake EEG spans mixed frequencies of 15-30 Hz (beta range) and above 30 Hz (gamma range). EEG patterns during wakefulness depend on the degree of attention; tasks that require higher levels of attention are represented by higher EEG frequencies, while during quiet waking the EEG oscillation shifts to lower frequencies (Steriade, 2006).

When vigilance level shifts from wakefulness to NREMS, EEG waves become even slower and their amplitude increases, representing an increasing cortical synchronization. EEG patterns during NREMS are predominated by high-voltage, low-frequency waves (< 4 Hz, delta range). NREMS is followed by REMS, a sleep stage that is accompanied by cortical activity similarly seen during wakefulness including low-voltage, but slower waves (6-9Hz; theta range). Differences in EMG activity provide a distinction of REMS from wakefulness; while EMG activity is high during wakefulness, it is virtually none during REMS, reflecting the mucle atonia that accompanies the REMS state.

Throughout the resting phase, several sleep cycles occur that contain a specific sequence of sleep states. A human sleep cycle lasts for 90 to 110 minutes, starting with shallow sleep (NREMS S1) and continuing to deeper vigilance states, passing

though NREMS S2 to SWS. SWS is also known as deep sleep and is characterized by specific EEG and EMG properties, as described above, but also by the high threshold that is necessary to awaken an individual from this sleep state. After SWS, REMS is entered, completing one sleep cycle. Afterwards, the cycle starts again from shallow sleep and passes through all the vigilance states.

In contrast, sleep cycles in rodents have a duration from a few minutes up to 12 minutes per cycle. Most rodents are nocturnal animals including mice and rats; most of their sleep occurs during the light period, whereas they are mostly active during the dark period. A rodent sleep cycle also starts with NREMS and ends with REMS (Tobler, 1995).

Humans sleep cycles are repeated four to five times per night, whereas in mice, sleep cycles are frequently intruded by bouts of wakefulness. Hence, mice exhibit an episodic (polyphasic) sleep pattern, while human sleep is monophasic (Tobler, 1995).

2.2 The regulation of sleep and wakefulness

2.2.1 Circadian and homeostatic regulation of sleep-wake behaviors

Sleep occurs in a timely organized manner; humans sleep during the night and are awake during the day, while nocturnal rodents sleep mostly during the light period (80 %), and to some extent also during the dark period (20 %, Tobler, 1995). This rhythmicity of sleep-wake behaviors is regulated by the circadian system, in which the suprachiasmatic nucleus (SCN) serves as the key pacemaker (Achermann & Borbely, 2003; Borbely, 1982).

SCN pacemaker activity is generated by sequential expression of clock genes that are regulated by networks of transcriptional-translational feedback loops within the clock machinery (Jin *et al.*, 1999; Reppert & Weaver, 2002). In addition, SCN activity synchronizes with external cues called Zeitgeber, such as light. This process is called entrainment and allows for re-adjustment to environmental changes and for an adherence to an ultradian 24-hour rhythm (Bellet & Sassone-Corsi, 2010).

The SCN controls sleep-wake behaviors through the subparaventricular zone (J. Lu *et al.*, 2001), which projects to the DMH (Chou *et al.*, 2003; Deurveilher & Semba,

2005). In turn, the latter projects to wake-promoting and sleep-promoting regions and conveys rhythmic information from the SCN (Chou *et al.*, 2003).

In addition to the rhythmicity of sleep-wake behaviors given by the circadian oscillation, homeostatic processes regulate sleep (Achermann & Borbely, 2003; Borbely, 1982). Prolonged wakefulness accumulates the pressure to sleep. Once asleep, more intense slow waves in the EEG are found during NREMS, which decrease over the course of a sleeping period (Achermann & Borbely, 2003). Adenosine was suggested to be one of the mediators reflecting sleep pressure, as it accumulates in the extracellular space during prolonged wakefulness and decreases during sleep (Porkka-Heiskanen *et al.*, 1997).

In fact, homeostatic and circadian processes interact, and they are both required for operating normal sleep-wake cycles. The interaction of these processes is explained by the two-process model advocated by Borbely (Fig. 6; Borbely, 1982). Only when sleep pressure is high enough and the circadian timing is appropriate, sleep can take place. Both act on specific sleep-wake regulatory systems, as will be described in the following sections.
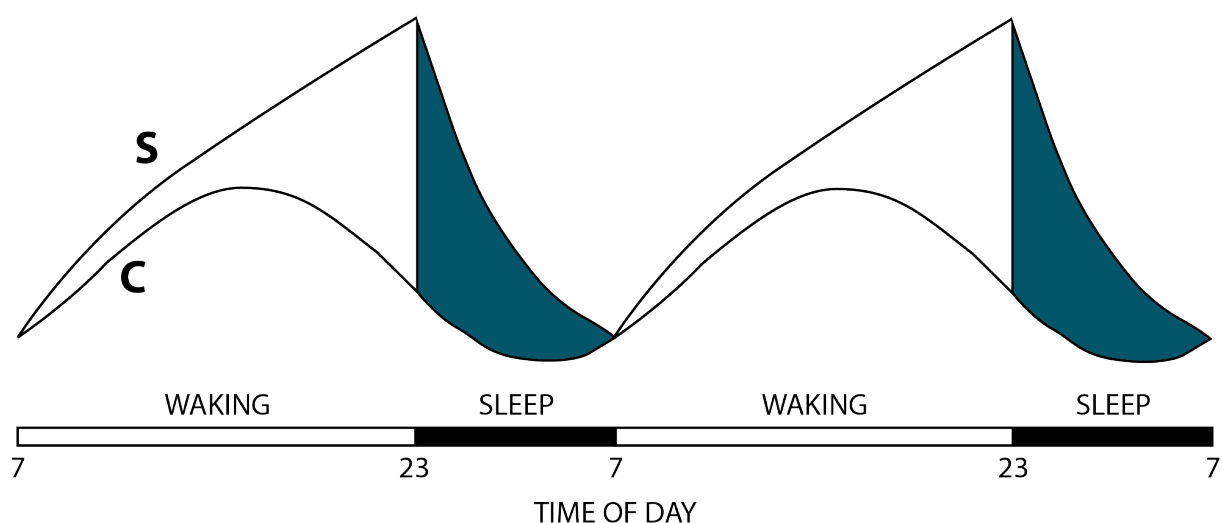
Figure 6: The two process model of sleep regulation.

Sleep is regulated by two processes: 1) Process S (upper curve), which represents the homeostatic sleep drive, increases exponentially during wakefulness and signals an urge to sleep once a specific threshold is crossed. 2) Process C (lower curve) represents the circadian rhythm, which inhibits sleep during the day. When the circadian inhibition of sleep decreases during the night (nadir of process C) and the sleep pressure is high enough (peak of process S), sleep can occur. Adapted from Borbély and Achermann, 2000.

2.2.2 Neural mechanisms of wake-to-sleep transitions

A large number of brain regions have been accounted for the regulation of wakefulness, NREMS or REMS. Wake- and NREMS-promoting regions appear to mutually inhibit one another, thus explained by a flip-flop switch model responsible for the transition from wakefulness to NREMS and vice versa. A further mutual inhibition between REMS-on and REMS-off nuclei was suggested, which interprets how the transition from NREMS to REMS is mediated (Saper *et al.*, 2010).

Wake-promoting systems

Wakefulness is maintained by the activities of a network of nuclei in the brainstem, hypothalamus and thalamus (Fig. 7). Early studies showed that lesions within the brainstem ascending reticular activating system (ARAS) results in marked somnolence (Lindsley *et al.*, 1949), while its electrical activation produces EEG desynchronization similar to arousal (Moruzzi & Magoun, 1949). These brainstem arousal nuclei comprise the noradrenergic LC, dopaminergic VTA, serotonergic DR and median raphe nuclei (MR) and cholinergic pedunculopontine and laterodorsal tegmental nuclei (PPT, LDT), which project to LH, basal forebrain, thalamic relay nuclei etc. and ultimately induce EEG activation (Saper *et al.*, 2010).

While brainstem arousal nuclei fire most actively during wakefulness and decrease their firing during NREMS, they display different firing patterns with respect to REMS; cholinergic PPT and LDT neurons fire essentially as rapidly during REMS as during wakefulness, whereas monoaminergic nuclei (LC, DR, MR) cease firing during REMS (el Mansari *et al.*, 1989; Aston-Jones & Bloom, 1981; Kocsis *et al.*, 2006; Takahashi *et al.*, 2010). However, lesions to single arousal nuclei do not cause a complete destruction of arousal, suggesting a complexity within the wake-promoting system (Saper *et al.*, 2010).
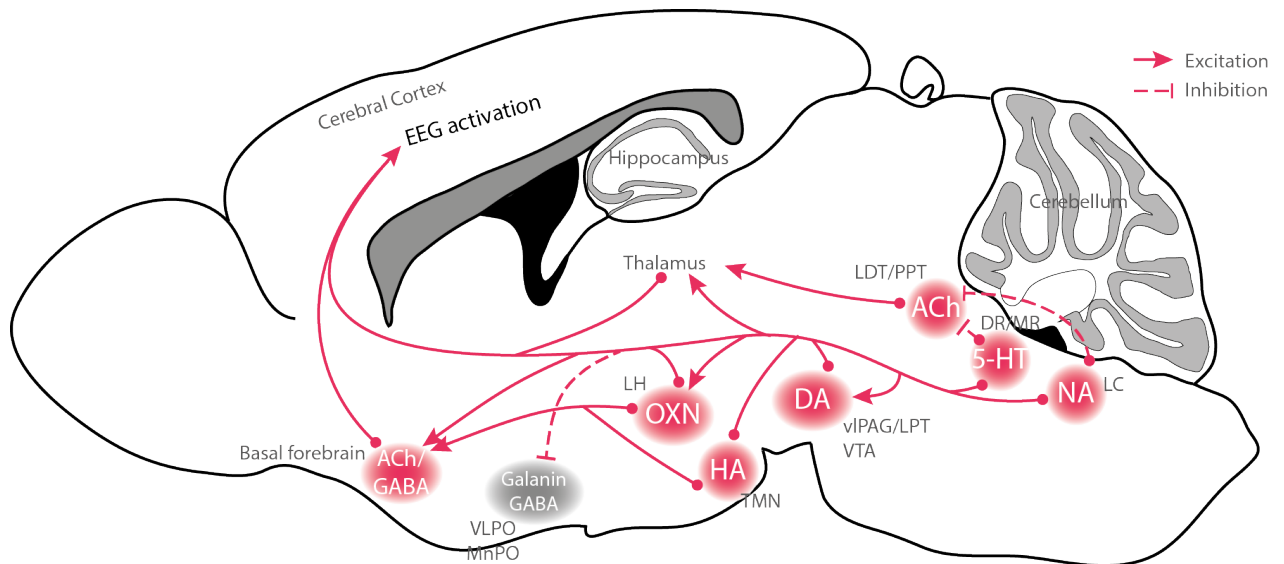
Figure 7: Schematic representation of the wake-promoting network.

Brainstem monoaminergic (DR, MR, LC, VTA, vlPAG, LPT) and cholinergic arousal nuclei (LDT, PPT) project to the LH, basal forebrain and thalamus to induce wakefulness. In addition, hypothalamic nuclei (LH, TMN) innervate brainstem and forebrain regions and evoke arousal. To maintain wakefulness, sleep-active neurons (VLPO, MnPO) are inhibited by the wake-promoting network. Wake-promoting nuclei and their projections are depicted in red. Dashed lines represent inhibitory inputs. Sleep-active VLPO and MnPO are given in gray. Adapted from Saper et al., 2010;

Similarly to monoaminergic nuclei, orexinergic neurons in the LH fire predominantly during active wakefulness, especially when the animal is exploring the environment or during motivated behaviors as described in section 1 (Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005). They virtually halt firing during NREMS and REMS (Lee *et al.*, 2005). Optogenetic stimulation of orexin neurons or icv application of orexins induces arousal from sleep (A. R. Adamantidis *et al.*, 2007; Piper *et al.*, 2000). Loss of orexin neurons destabilizes wakefulness and causes recurrent intrusions of NREMS and REMS during activity phases (Hara *et al.*, 2001).

In addition to their direct projections to the cerebral cortex, wake-promoting cell groups also project to the thalamus, where they modulate the activity of thalamic relay neurons, intralaminar and related nuclei that form thalamocortical projections. The rhythmic bursting activity of thalamic relay neurons contributes to cortical synchronization. However, the firing pattern of thalamocortical neurons is further modulated by reciprocal interactions with GABAergic reticular thalamic neurons. Firing of the latter hyperpolarizes thalamocortical neurons, thereby decreasing their excitability by wake-promoting cell groups and other sensory inputs. This results in

synchronous burst firing of thalamic relay neurons that in turn produce waves of excitation in cortical neurons, as reflected by slow waves in the EEG (Steriade, 2003; Steriade *et al.*, 1993). In contrast, when inputs from reticular thalamic neurons are low, thalamocortical neurons are in their transmission mode and relay sensory information to the cortex contributing to high-frequency EEG oscillations typical of wakefulness (Steriade, 2003; Steriade *et al.*, 1993).

NREMS-promoting systems

When falling asleep, activities of the wake-promoting system are suppressed by sleep-promoting nuclei that include the ventrolateral pre-optic nucleus (VLPO) and median pre-optic nucleus (MnPO, Fig. 8). During an epidemic of encephalitis lethargica around the time of World War I, Von Economo observed that pathological lesions in the preoptic region produced insomnia (Economo, 1926). Later, the lesion was found to correspond to sleep-active VLPO and MnPO neurons; they fire faster during sleep and their discharge rate may reflect sleep depth (Szymusiak *et al.*, 1998; Suntsova *et al.*, 2002). MnPO neurons, but not VLPO neurons, increase their firing rate prior to sleep onset and may play a role in the initiation of sleep, while both are essential for sleep maintenance (Suntsova *et al.*, 2002; Saper *et al.*, 2010). Further, MnPO and VLPO activity is homeostatically regulated, because Fos expression in these areas increases in response to sleep deprivation (Gvilia *et al.*, 2006).

Both VLPO and MnPO contain GABA as neurotransmitter and project to the brainstem and hypothalamic arousal nuclei to exert their inhibitory influence on wake-promoting networks (Sherin *et al.*, 1998; Gong *et al.*, 2004; Uschakov *et al.*, 2007). In addition to GABA, VLPO neurons also contain galanin, which is an inhibitory neuropeptide (Sherin *et al.*, 1998; Sherin *et al.*, 1996). VLPO and MnPO receive inhibitory projections from wake-promoting regions, thus, providing a mechanism for fast sleep-wake and wake-sleep transitions (Gallopin *et al.*, 2000; Saper *et al.*, 2010).

Furthermore, MCH neurons, which are located in the LH and zona incerta adjacent to wake-promoting orexin neurons, increase their firing during NREMS and are most active during REMS (Hassani *et al.*, 2009; Verret *et al.*, 2003). Icv injections of MCH

increase REMS dose-dependently; at some doses also NREMS enhancement was observed (Verret *et al.*, 2003). MCH injection into NREMS-promoting regions such as VLPO increased NREMS (Benedetto *et al.*, 2013), while VLPO microinjection of its counterpart orexin induced wakefulness (Mavanji *et al.*, 2015). Optogenetic activation of MCH during the dark period produced NREMS (Konadhode *et al.*, 2013; Tsunematsu *et al.*, 2014). However, when MCH neurons were optogenetically activated during NREMS, a transition into REMS was induced, and their activation during REMS prolonged REMS episodes (Jego *et al.*, 2013; Tsunematsu *et al.*, 2014). However, the exact role of MCH in NREMS and REMS promotion is still controversial.
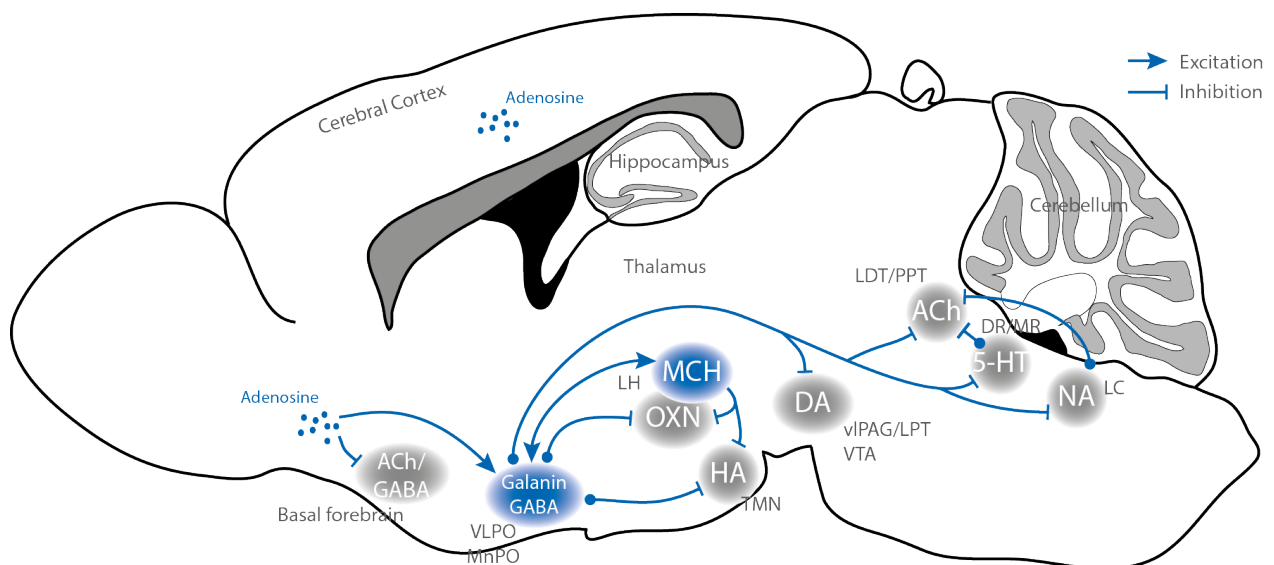
Figure 8: Schematic representation of the NREMS-promoting network.

Sleep-inducing molecules such as adenosine increase during prolonged wakefulness and provide a signal for the initiation of sleep. Sleep-active nuclei (blue) such as the VLPO, MnPO, and MCH-expressing neurons in the LH and zona incerta are excited by adenosine and suppress wake-active nuclei (grey). This mechanism drives the switch from wakefulness to NREMS and maintains NREMS. Adapted from Saper et al., 2010.

Interestingly, lesions of the ventral striatum (NAc) increased time spent awake (Qiu *et al.*, 2010). Previous reports have suggested that the NAc may be a key region for the arousing effects of caffeine (J. P. Zhang *et al.*, 2013). Projections from NAc to arousal nuclei such as the LH are mainly GABAergic (Baldo *et al.*, 2004). Thus, the NAc may represent a further NREMS-promoting region, but its exact role in sleep-wake behaviors is still poorly understood.

REMS-promoting systems

REMS was discovered in the 1950s and its regulatory mechanism was extensively studied ever since (Aserinsky & Kleitman, 1953). Based on increased neuronal activities associated with REMS, initial studies implicated PPT and LDT with the generation of REMS (Aston-Jones & Bloom, 1981; Takahashi *et al.*, 2010; Hobson *et al.*, 1975; Trulson *et al.*, 1981). However, lesions within these cell groups produced only limited reductions in REMS. During REMS rebound in response to sleep deprivation, only few Fos-positive cells were found in these regions (Lu *et al.*, 2006; Blanco-Centurion *et al.*, 2007; Boissard *et al.*, 2002), whereas a stronger neuronal activation was observed in sub-laterodorsal nucleus (SLD), precoeruleus region (PC) and medial parabrachial nucleus (MPB) (Boissard *et al.*, 2002). Pharmacological activation of the SLD produced REMS-like behavior (Boissard *et al.*, 2002), while its lesion suppressed REMS. Neurons in these nuclei were thus termed REM-on neurons (Lu *et al.*, 2006).

Furthermore, these REM-on neurons exhibit reciprocal connections with neurons in the ventrolateral periaqueductal gray (vlPAG) and lateral pontine tegmentum (LPT), (Lu *et al.*, 2006). The latter appear to be REM-off neurons, because both their pharmacological inhibition and their lesion increase REMS (Lu *et al.*, 2006). They send GABAergic projections to SLD, PC and MPB, providing a mutual inhibition between REM-on and REM-off neurons. This mutual inhibition may serve as neural basis of the NREMS-to-REMS transition (Lu *et al.*, 2006; Saper *et al.*, 2010). Furthermore, these neurons receive inputs from LH and VLPO (Lu *et al.*, 2006), which may contribute in general to the termination or maintenance of sleep, respectively.

2.3 Sleep and metabolism

Sleep-wake behaviors are closely associated with other behaviors such as food intake. For example, when food availability is scarce, periods of prolonged wakefulness are necessary, to possibly maximize food-seeking behavior. Hence, a

period of fasting substantially increased arousal in rats. During the refeeding period, sleep rebounds occurr in a comparable manner as during recovery sleep following sleep deprivation (Borbely, 1977; Danguir & Nicolaidis, 1979). Refeeding also increases slow-wave activity, which demonstrates that energetic needs are able to override homeostatic sleep pressure (Shimizu *et al.*, 2011). However, ablation of orexin neurons in the LH abolished fasting-induced arousal (Yamanaka *et al.*, 2003), suggesting a role of orexin in the coupling of feeding and arousal. Indeed, orexin injections increase both food intake and arousal as already mentioned in previous sections (Piper *et al.*, 2000; Lubkin & Stricker-Krongrad, 1998).
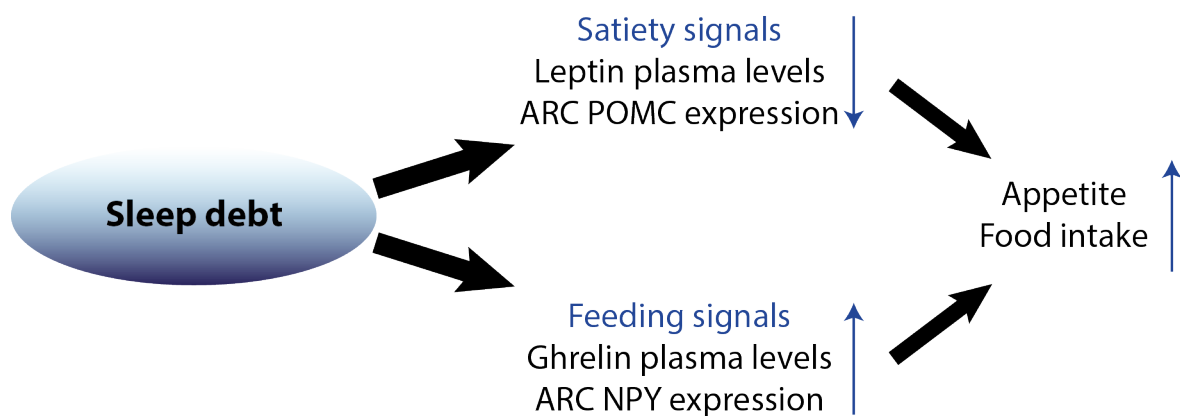
Figure 9: Sleep debt and its effects on food intake.

Sleep debt in humans and rodents decreases levels of satiety signals, but increases concentrations of feeding signals. These processes result in a net feeding signal. Thus, chronic sleep restriction is thought to confer a higher risk of weight gain. Adapted from Laposky et al., 2008 and Kim *et al.*, 2015.

Conversely, sleep restriction impacts dramatically on energy balance leading up to a condition where weight gain is favored (Fig. 9, Taheri *et al.*, 2004; Laposky *et al.*, 2008; Kim *et al.*, 2015). Glucose homeostasis was also affected by sleep restriction; glucose tolerance and insulin sensitivity were decreased in sleep-restricted subjects resulting in an increased risk of developing type II diabetes. Sleep restriction further activated the HPA axis and increased cortisol levels (Spiegel *et al.*, 1999).

Furthermore, peripheral satiety cues such as leptin and PYY may influence sleep-wake behaviors. Leptin administration increased SWS and decreased REMS in rats (Sinton *et al.*, 1999), while food intake was suppressed. PYY administration yielded similar results in rats (Akanmu *et al.*, 2006). These findings are in line with the idea that feeding and sleeping behaviors are under mutual control (A. Adamantidis & de Lecea, 2008; Morselli *et al.*, 2012).

However, the effects of orexigenic factors on sleep yielded contradictory results. While ghrelin consistently promoted feeding, its effects on sleep showed high variability between different studies and between rodents and humans (Weikel *et al.*, 2003; Obal *et al.*, 2003; Szentirmai *et al.*, 2007; Kluge *et al.*, 2010). Similarly, the orexigenic peptide NPY showed conflicting results on sleep (Szentirmai & Krueger, 2006; Zini *et al.*, 1984; Antonijevic *et al.*, 2000).

Thus, the study here attempted to clarify the mechanistic relationship underlying the mutual inhibition between feeding and sleeping.

3 Obesity

3.1 Epidemiology and health consequences of obesity

The body mass index (BMI) is an estimate of body fat percentage of an individual with a BMI of 18.5 – 25 kg/m² considered healthy (Keys *et al.*, 1972). BMI below or above the "healthy" range indicates underweight and overweight, respectively. In this respect, obesity is often defined as a BMI > 30 kg/m². A BMI outside the healthy range increases the risk of various medical conditions and early mortality.

Despite this fact, during the past 30 years the incidence of overweight and obesity has been rising at an alarming rate. Obesity is not exclusive to adults any longer, and is now more frequently observed in young children and adolescents. Over 35 % of the U.S. population between five and 17 years are overweight or obese. In Germany, 22.6 % of boys and 17.6 % of girls are overweight or obese, according to the latest available estimates (The Organisation for Economic Co-operation and Development, 2012).

Nowadays, in addition to genetic predispositions and a lack of physical exercise, weight gain is promoted by the obesogenic environment. Easy and quick ways to access food high in carbohydrates and fats demand higher resistance by individuals. However, the consequences are dramatic; considerable evidence indicates a causal relationship between obesity and diabetes mellitus type 2, cardiovascular diseases, hypertension, insulin resistance and metabolic syndrome in adults (Westphal, 2008), and thus, early mortality (Pischon *et al.*, 2008). Furthermore, there is evidence that obesity is even associated with depressive disorders (Akbaraly *et al.*, 2009; Rosmond *et al.*, 1996). Kloiber et al. showed that antidepressant drug treatment efficacy negatively correlates with patient BMI; depressed patients with a BMI > 25 exhibited slower treatment response than those with lower BMI (Kloiber *et al.*, 2007). Therefore, it is important to understand the biology underlying obesity and to find treatment options for obese people.

3.2 Mouse models of obesity

Obesity in humans results from a combination of environmental, genetic and behavioral factors. To understand the etiology of obesity, and to improve treatment options and weight loss strategies, scientists have studied rodent models mimicking human obesity (Mavanji *et al.*, 2012; Yazdi *et al.*, 2015).

Many obesity rodent models are based on single-gene mutations. The probably most extensively studied mutant models concern defective leptin signaling such as ob/ob mice (Ingalls *et al.*, 1950; Y. Zhang *et al.*, 1994), db/db mice (Chen *et al.*, 1996; et al.,Chua *et al.*, 1996) and obese Zucker rats (Zucker, 1961). Leptin-deficiency and leptin-resistance cause hyperphagia and obesity and may even lead to the development of core features of the human metabolic syndrome, which is characterized by abdominal obesity, impaired glucose tolerance and insulin sensitivity, high blood pressure and elevated blood triglyceride levels (de Artinano & Castro, 2009).

However, one has to keep in mind that only a few cases of human obesity exhibit a monogenic cause. To address polygenic predispositions to obesity, Levin and colleagues selected rats from an outbred population of Sprague-Dawley rats, based on their propensity to gain excess weight when exposed to a high energy and high fat diet. About half of these rats tended to remain lean and were thus termed obesity-resistant, while the other half developed overweight (obesity-prone rats) (Levin *et al.*, 1997). This model resembles human obesity in many ways, including the display of insulin resistance (Levin *et al.*, 1997).

On the other hand, different dietary schemes also have to be considered when mimicking human obesity in animal models. Obesity in humans usually is the consequence of overconsumption of palatable foods, while healthy foods are neglected (Kenny, 2011). Therefore, a comparison between rodents fed a palatable diet and those fed a healthy diet might be more suitable. To this end, rodents can be fed a standardized palatable diet, which is high in fat and carbohydrates (HFD, Black *et al.*, 1998). Within six weeks, HFD-feeding leads to severe diet-induced obesity (DIO) along with hyperglycemia and hyperinsulimia as compared to control mice that are maintained on standard chow (Black *et al.*, 1998). The use of C57BL/6 mouse

strain is advantageous in this respect, as these mice are prone to develop obesity when fed a HFD.

Alternatively, rodents can be fed a diet composed of daily varying palatable snacks, e.g. chocolate, shortbread cookies etc. ("cafeteria diet", Danguir, 1987). This approach also leads to hyperphagia and accelerates weight gain. However, as compared to the standardized HFD, monitoring the daily caloric intake of the cafeteria diet proved to be problematic and thus, results are difficult to reproduce. Nevertheless, both models exhibit several parallels to human obesity, including a comorbidity with type II diabetes (C. D. Morrison *et al.*, 2009), cardiovascular disease (Ayer *et al.*, 2015), depression-like behaviors (Sharma & Fulton, 2013) and sleep-wake disturbances (Jenkins *et al.*, 2006). The relationship between obesity and sleep disturbances will be discussed in the following section.

3.3 Sleep-wake behavior in obesity

As compared to diabetes and cardiovascular disease, sleep disturbances are often overseen medical disorders in obese subjects. Many obese individuals complain about sleep disturbances including frequent awakenings during the night and numerous shifts from one sleep stage to another, a phenomenon termed sleep fragmentation (Fig. 10; Vgontzas & Kales, 1999; Vgontzas *et al.*, 2008; Vgontzas *et al.*, 1998). This results in non-refreshing sleep and produces excessive daytime sleepiness (EDS), which decreases life quality and impairs daily performance.

Studies in rodent obesity models shed light on the possible role of orexin in sleep disturbances in the obese. In Levin's obesity-prone rats, orexin function was reduced and active phase NREMS time was increased (Teske *et al.*, 2006; Mavanji *et al.*, 2010). Also obese Zucker rats showed orexin deficiency and an abnormal distribution of NREMS and REMS between the active and resting phase, e.g. with more NREMS during the dark phase. Furthermore, these obese animal models both showed sleep fragmentation (Mavanji *et al.*, 2012). Similarly, leptin-deficient, leptin-resistant, cafeteria-diet fed and DIO animals showed a sleep phenotype with a loss of proper circadian distribution of sleep with more nocturnal NREMS and REMS time (Laposky *et al.*, 2006; Danguir, 1987; Jenkins *et al.*, 2006).
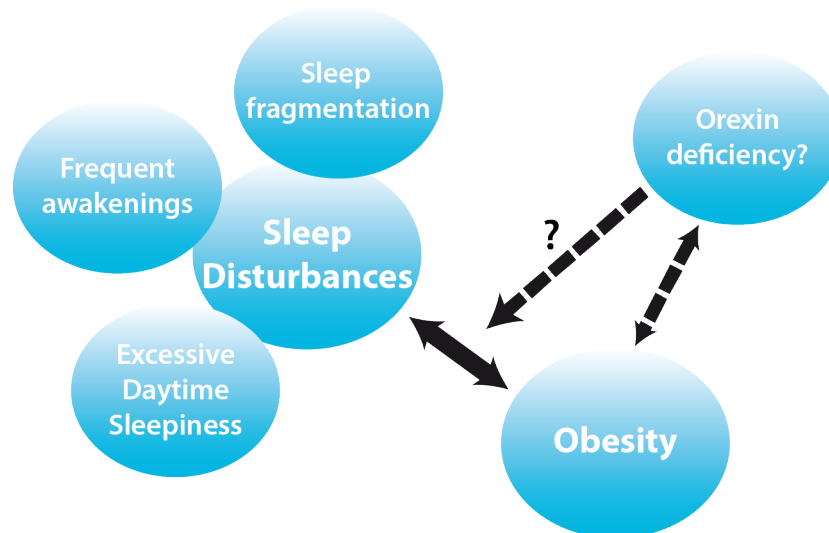
Figure 10: A scheme of associations between sleep disturbances and obesity.

Obese individuals display sleep disturbances such as sleep fragmentation, frequent awakenings during the night and excessive daytime sleepiness. In turn, sleep disturbances promote weight gain as described in the previous section. Deficiencies in orexin signaling may link obesity with sleep disturbances.

Except for the cafeteria-fed rats, all the other mouse models for obesity mentioned in the section 3.2 reproduced sleep fragmentation, which is typical of human obese subjects. In conclusion, to investigate the relationship between sleep disturbances and obesity, the DIO mouse model might be the more appropriate choice, because this mouse model translates all the sleep disturbances observed in obese humans and is based on overconsumption of palatable foods. Orexin deficiency is prevalent in the obese and was suggested to associate obesity with sleep disturbances. This hypothesis was tested in the present study.

3.4 Food intake regulation affected by obesity

Evidence accumulates that both homeostatic and hedonic regulators of food intake undergo adaptive changes during prolonged intake of palatable foods that ultimately result in obesity (Alsio *et al.*, 2012).

In human and rodent obesity, leptin levels are elevated, which is consistent with the finding that peripheral leptin concentrations are proportional to fat stores (Fig. 11; Considine *et al.*, 1996; Kohsaka *et al.*, 2007). However, a central and peripheral

leptin resistance emerges during obesity, which appears to diminish the efficacy of leptin signaling (T. L. Martin *et al.*, 2006). In addition, a central and peripheral insulin resistance is evident in obese subjects, contributing to increased risk of type II diabetes (Arase *et al.*, 1988; Scarlett & Schwartz, 2015). Moreover, basal and post-prandial PYY concentrations are decreased in obesity (Batterham *et al.*, 2003; Karra *et al.*, 2009). In contrast to the aforementioned satiety signals, PYY signaling appears to be intact in obesity and can still elicit a suppression of food intake (R. D. Reidelberger *et al.*, 2008).

Further, ghrelin concentrations are decreased in obese humans (Tschop *et al.*, 2001). Similarly to leptin and insulin, peripheral ghrelin fails to influence food intake in DIO mice, suggesting a ghrelin resistance in obesity (Perreault *et al.*, 2004; Briggs *et al.*, 2010).
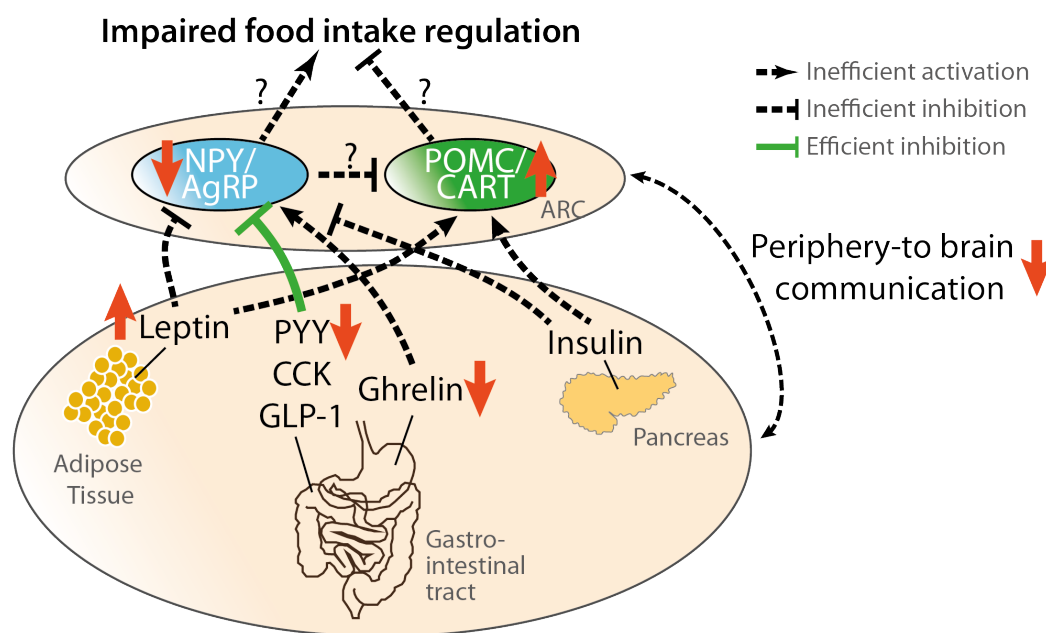
Figure 11: Scheme of periphery-to-brain signaling in obesity.

In obesity, levels of anorexigenic factors are increased, while concentrations of orexigenic factors are reduced. However, anorexigenic signaling is not efficient due to decreased central and peripheral sensitivity. Even though levels of peripheral PYY are low, its signaling pathway appears to be intact in the obese.

Also central alterations were observed in obesity. NPY and AGRP mRNA expression in the ARC was reduced in the DIO mouse model, whereas POMC and CART mRNA expression was increased (Kohsaka *et al.*, 2007). These findings suggest an

aberrant CNS response to peripheral satiety and feeding cues in obesity resulting in a loss of control over energetic needs.

With respect to LH second-order neurons, the number of orexin-positive cells is decreased in DIO mice and the circadian expression of prepro-orexin mRNA expression is blunted (Fig. 12, Kohsaka *et al.*, 2007; Nobunaga *et al.,* 2014). Interestingly, prepro-orexin mRNA expression is inversely correlated with daily NREMS amounts in DIO mice, further supporting the LH as a center modulating both food intake and sleep-wake behaviors (Tanno *et al.*, 2013). In contrast, the number of MCH-positive cells in the LH was not affected in DIO mice (Nobunaga *et al.*, 2014).
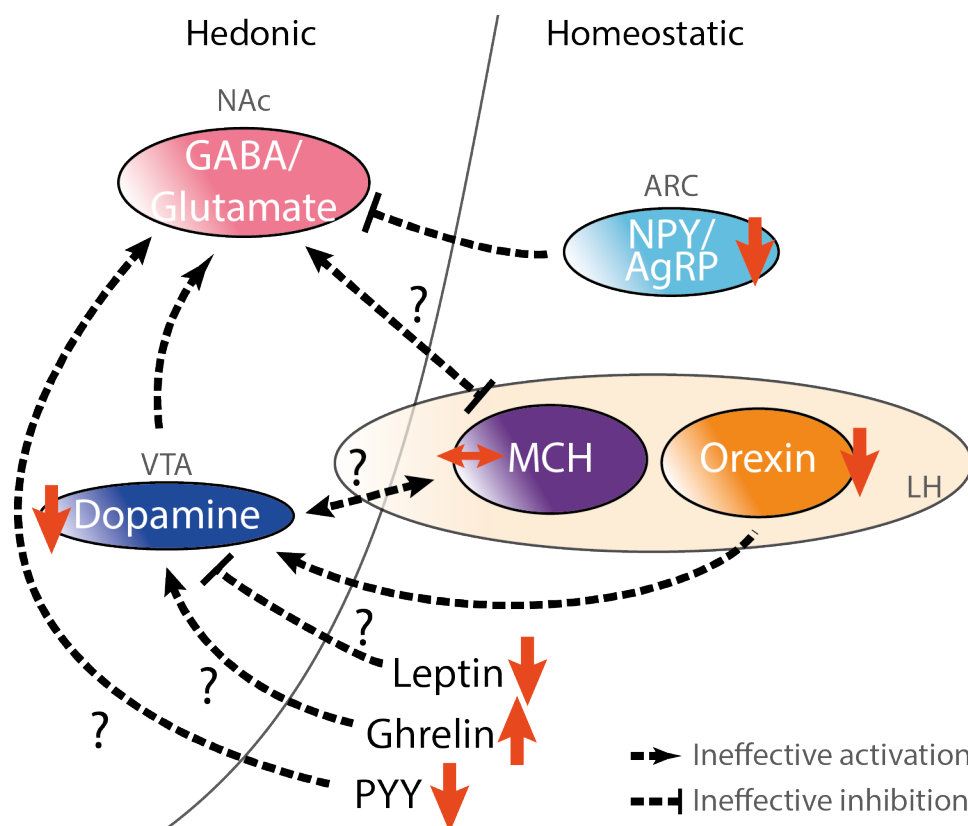
Figure 12: Scheme of central homeostatic and hedonic signal interaction on food intake regulation in obesity.

A hypodopaminergic tone was suggested to drive overconsumption on palatable foods in obesity and may be responsible for overwriting homeostatic satiety signals. However, the interaction of the depicted factors driving overconsumption in obesity is still poorly understood.

Altogether, the changes in food intake regulatory centers signal a satiated state in obesity. Nevertheless, obese rodents still ingest the same total amount of food in grams as control mice after overcoming an initial hyperphagic response to the

obesogenic diet (Kohsaka *et al.*, 2007). One possible explanation for this behavior is a hedonic hyperdrive on food intake, which may be mediated by opioid and endocannabinoid signaling (Berridge *et al.*, 2010). In addition, obesity is accompanied by a hypo-dopaminergic state in the NAc and VTA, areas regulating hedonic aspects of feeding; extracellular dopamine levels were decreased in the NAc and VTA in obese rodents (Geiger *et al.*, 2008; Li *et al.*, 2009; Vucetic *et al.*, 2012; Sharma & Fulton, 2013). In slice preparations from obesity-prone rats, electrically induced dopamine release from NAc, dorsal striatum and prefrontal cortex was attenuated (Geiger *et al.*, 2009). Obese rats further displayed reduced expression of tyrosine hydroxalase, vesicular monoamine transporter-2 and dopamine transporter in the NAc and VTA (Geiger *et al.*, 2008; Li *et al.*, 2009; Vucetic *et al.*, 2012; Sharma & Fulton, 2013).

Based on these findings, it was suggested that prolonged exposure to palatable foods may increase the hedonic set point (hence, a stronger hedonic stimulus is needed to activate the reward system) and may drive the overconsumption of palatable foods to compensate for the perceived reward deficit (Egecioglu *et al.*, 2011). However, this hypothesis has received strong criticism in recent studies, because reduced dopaminergic signaling in obesity was associated with a decrease in motivated behaviors rather than alterations in hedonic perception of foods (Berridge *et al.*, 2010; Harb & Almeida, 2014). To shed light on this controversy, the present study examined whether chronic exposure to palatable foods may alter setpoints in homeostatic and hedonic components of food intake regulation.

3.5 Weight loss strategies and therapies

Concerning the increased risk of medical severity and early mortality in obese subjects, appropriate weight loss strategies are of major importance. Weight loss strategies mostly involve lifestyle modifications, including an increase in physical activity, dietary restriction, healthy food choices and behavioral treatment (Blomain *et al.*, 2013). The latter comprises behavior change techniques to promote physical activity and sustained compliance with healthy eating regimens.

However, physiological adaptations during weight loss rather promote weight regain than weight loss (Fig. 13). As mentioned previously, a decrease in striatal D2 receptors was observed in obese individuals suggesting that increased intake of palatable foods is required to compensate for the perceived reward deficit (Egecioglu *et al.*, 2011). Abstinence from palatable diet and/or decreased meal size and frequency may result in a reward deficit that may also counteract weight loss attempts in obese individuals.
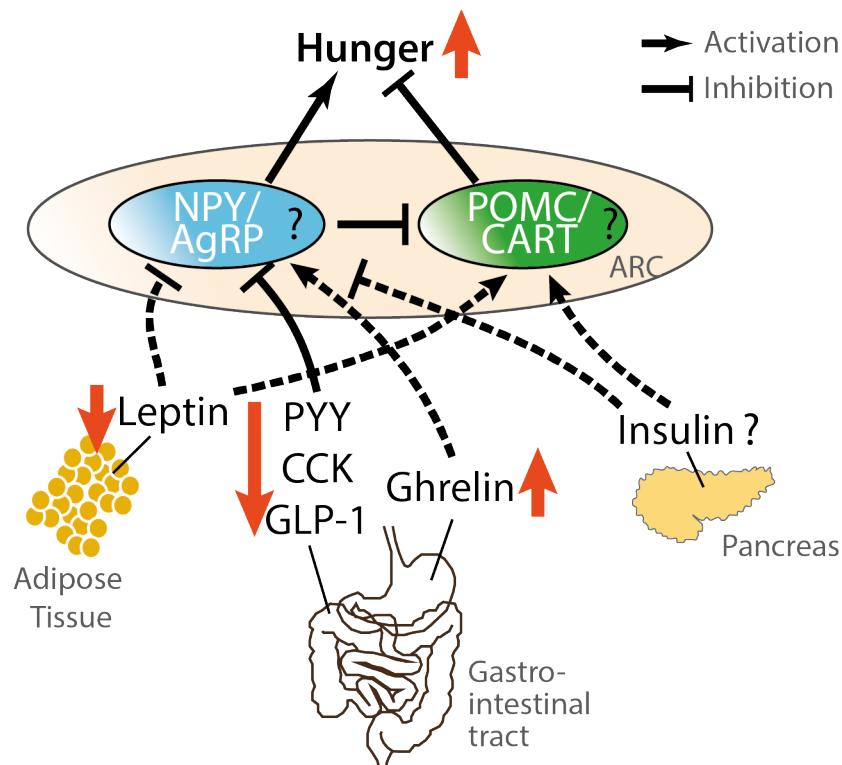
Figure 13: Scheme of possible interactions between central homeostatic and hedonic signals regulating food intake during weight loss in obese subjects.

During the challenge of weight loss, physiological adaptations that cause hunger occur. For example, peripheral satiety signals such as leptin, PYY, CCK and GLP-1 decrease, while the feeding signal ghrelin increases. These changes can persist for nearly one year in humans and may oppose weight loss attempts. Dashed lines indicate ineffective signaling. Figure based on Cummings *et al.*, 2002; Essah *et al.*, 2010; de Luis *et al.*, 2007; Sumithran *et al.*, 2011

Due to these contradicting factors, the above mentioned weight loss strategies have brought only limited success in the past. Therefore, the use of anti-obesity drugs was suggested (Heal *et al.*, 2013; Greenway, 2015). Several CNS-acting drugs were developed to treat obesity, including some altering serotonergic function. For example, fenfluramine and lorcaserin reduce food intake and result in weight loss through activation of 5-HT$_{2C}$ receptors (Vickers *et al.*, 2001;Smith *et al.*, 2006; C. K.

Martin *et al.*, 2011). However, both drugs provoke adverse side effects, such as hallucinations at high doses, as well as increased cellular proliferation in the heart valve and subsequent inelasticity and loss of function (Heal *et al.*, 2013). Therefore, these drugs are no longer approved for anti-obesity treatment in Europe.

Alternatively, drugs that stimulate the peripheral satiety machinery could be used (Fig. 13). For example, chronic intermittent PYY_{3-36} administration in DIO rats with ad libitum access to high-fat diet and liquids produced a significant body weight loss (R. D. Reidelberger *et al.*, 2008). In contrast, leptin treatment had no effect on body weight or food intake in DIO rats. The use of other satiety factors including GLP-1 was also shown to be effective (R. Reidelberger *et al.*, 2012). Hence, the application of satiety peptides such as PYY_{3-36} and GPL-1 or their analogues may be new promising treatment options for obesity. Whether they may ameliorate health consequences associated with obesity is again still poorly understood and was thus subject to this study.

4 The peripubertal period

4.1 Definition of puberty and its distinction from adolescence

Puberty and adolescence mark periods of transition between childhood and adulthood. Despite their frequent synonymous use, there are clear differences between the terms adolescence and puberty; while adolescence refers to the time of behavioral maturation including the acquisition of adult social, emotional and cognitive behaviors (Sisk & Zehr, 2005), puberty implies an endocrinological event that commences with the activation of hypothalamic-pituitary-gonadal axis concluding with reproductive maturation (Sisk & Foster, 2004).

Girls usually enter puberty at 10 or 11 years of age, and puberty onset occurs about one year later in boys. The onset of adolescence is more difficult to designate and is usually defined by puberty onset. However, the adolescent period continues beyond puberty, i.e. until the early twenties in humans. In rodents, puberty is initiated at around 4 weeks of age, and sexual maturity is reached at approximately postnatal day (P) 35, when vaginal opening occurs in females, and when penile and testicular development is completed in males (Ojeda & Urbanski, 1994); reproductive behaviors typically emerge 1-2 weeks thereafter (Sisk & Zehr, 2005). The term "adolescence" is anthropomorphic and should not be applied to rodents. In light of the experimental designs used in this thesis, we will simply refer to the "peripubertal period" (P28-70), since dietary treatments were initiated a few days before and continued after the usual age of puberty.

In addition to gonadal hormone changes, peripuberty is a particular period when dramatic remodeling occurs in the nervous system (Spear, 2000). The dynamic changes in brain anatomy and neurotransmission and their interaction with environmental influences are thought to sculpt adult behaviors. Accordingly, sleep-wake behaviors also change dramatically during adolescence and might be subject to programming by external or internal stimuli. This section will briefly review the interaction between brain reorganization and behavioral maturation during peripuberty.

4.2 Brain reorganization and sleep changes during peripuberty

During the peripubertal period, the brain undergoes structural and functional changes. During this period, a surge of synapse and receptor production occurs in rats (Andersen, 2003). This initial overproduction is followed by "synaptic pruning", a process that eliminates idle synapses while preserving frequently used ones according to the "use it or lose it"-principle (Feinberg *et al.*, 1990; Feinberg & Campbell, 2010). Similar age-related changes in synaptic density during adolescence were found in the postmortem frontal cortex of humans (Huttenlocher, 1979). These synaptic pruning events are generally thought to translate into changes in the gray matter volume, which increases at puberty onset and decreases after the completion of reproductive maturation. In parallel, axon myelination increases during puberty, which leads to an expansion of the white matter volume (Giedd *et al.*, 1999; Paus *et al.*, 2001). These changes are suggested to increase speed and efficiency of neuronal networks resulting in improved cognitive capabilities in adolescence (Giedd *et al.*, 2012).

Interestingly, cortical reorganization is reflected in changes in sleep EEG during development (Fig. 14, Feinberg & Campbell, 2010). Along with decreasing synaptic density during adolescence, sleep depth, as reflected in NREMS SWA, declines. This relationship is partly explained by the principle that the amplitude of EEG power depends on the degree of connectivity of cortical neurons and thus synchronicity of neuronal firing; the stronger the connection between these neurons was made, e.g. high-degree neuronal connectivity during childhood, the higher the synchronization of neuronal firing occurs, i.e. the larger EEG power during NREMS (Feinberg & Campbell, 2010). Due to synaptic pruning during adolescence, neuronal connectivity decreases to the adult level, resulting in smaller EEG power in adolescence than during childhood (Feinberg & Campbell, 2010; Huttenlocher, 1979).

A Human

B Rat
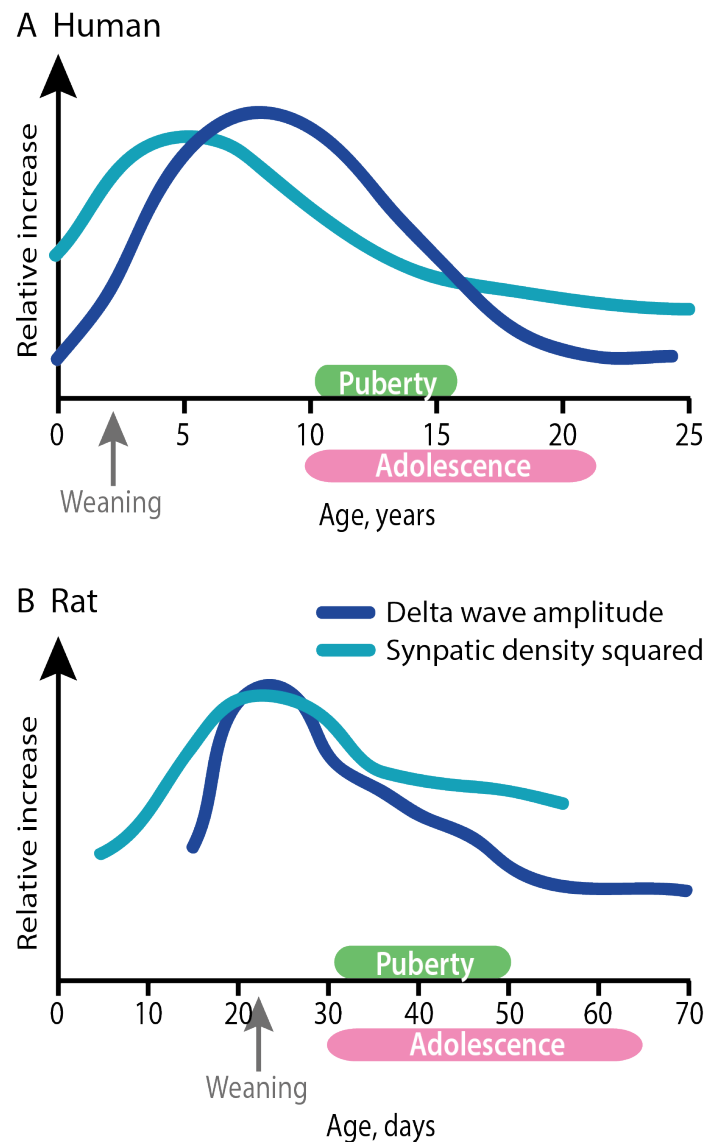
Figure 14: Developmental timeline of relative delta power as compared to frontal grey matter volume and chronotype in humans and rats.

(A) In humans, the developmental changes in frontal grey matter are accompanied by declines in relative delta power during NREMS. (B) In rats, rises and declines in frontal grey matter and NREMS delta power follow similar patterns. Schematic adapted from Hagenauer & Lee, 2013.

Furthermore, homeostatic sleep pressure decreases during adolescence; 36 hours of total sleep deprivation leads to a slower buildup of homeostatic sleep pressure during wakefulness in more mature adolescent subjects as compared to younger subjects (Jenni *et al.*, 2005). Accordingly, sleep behaviors change during adolescence; time spent asleep becomes shorter and circadian distribution of sleep is shifted during adolescence. While children sleep as much as 13-16 hours per day during the first years of their lives, the amount of sleep decreases during puberty, reaching sleep durations of 9 hours per night. Moreover, a delay in bedtime occurs in adolescents,

which is probably due to a lower physiological drive to sleep around the hours of typical bedtimes (Taylor *et al.*, 2005). However, during the day, daytime sleepiness emerges, even under controlled laboratory conditions and when sleep time is not restricted (Carskadon *et al.*, 1980).

Similarly to humans, peripubertal rodents show decreased amounts of NREMS and REMS and a resistance to sleep deprivation with lower SWA buildup (Sieck *et al.*, 1976; Hagenauer & Lee, 2013; Alfoldi *et al.*, 1990). Data on changes in SWA during development are somewhat inconclusive in rodents (Hagenauer & Lee, 2013). In addition, in developing rats a reorganization of diurnal sleep patterns occurs between P21 and P30; at P21, sleep amounts are evenly distributed between the light and dark period, whereas at P30, the largest amounts of sleep occur during the light period (Gvilia *et al.*, 2011).

In conclusion, adolescence/peripuberty marks a period in life that is characterized by dramatic changes in brain structure and function, which directly influence sleep EEG and behaviors.

4.3 Remodeling of neurotransmitter systems during peripuberty

In addition to structural brain changes, several neurotransmitter systems are reorganized during the peripubertal period, such as the dopamine and serotonin system. In the rat, the establishment of dopaminergic markers such as tyrosine hydroxylase (TH) activity, dopamine transporters and dopamine content, is completed between postnatal days 28 and 35 (Broaddus & Bennett, 1990). Similarly, dopamine D1 and D2 receptor density and binding increase linearly until P40 in the NAc, striatum and other dopamine projection sites, and undergo a developmental decline towards adulthood (Rao *et al.*, 1991; Pardo *et al.*, 1977; Gelbard *et al.*, 1989; Tarazi *et al.*, 1998). These changes in dopamine signaling in NAc and striatum suggest a developmental fine tuning of motor and reward-associated behaviors (Teicher *et al.*, 1995; Tarazi & Baldessarini, 2000). Alterations in the development of the dopaminergic system are thought to contribute to the increasing prevalence of schizophrenia and other psychiatric disorders and to the development of substance abuse during post-puberty (Trotman *et al.*, 2013).

It was further shown that serotonin brain concentrations decrease progressively during brain maturation (Lauder & Bloom, 1974; Lauder, 1990). In addition, serotonin transporters increase until adulthood in the NAc and striatum (Tarazi *et al.*, 1998). These findings suggest that the establishment of some behaviors that require proper serotonin function, such as sleep-wake behaviors and mental health may depend on appropriate development of serotonergic circuits during peripuberty. The following section will propose possible factors that may alter serotonergic or dopaminergic signaling during peripuberty that elicits behavioral changes.

4.4 Peripuberty; a window of sensitivity for the development of adult behaviors

More than 40 years ago, Scott et al. hypothesized that there are multiple steps in the organization of the nervous system, and that these periods of rapid neural development may represent sensitive windows for behavioral development (Scott *et al.*, 1974). Given the profound remodeling of the nervous system (see the previous section), the peripubertal period may exemplify such a sensitive one in life (Patchev *et al.*, 2014).

In this respect, environmental influences during puberty may remain as long-term traits in behaviors that depend on dopaminergic or serotonergic signaling. For example, *ad libitum* access to sucrose solution during P30-46 selectively decreased motivation in the adult rat (Vendruscolo *et al.*, 2010). This effect was absent when the treatment period was shifted to adulthood. Furthermore, brief exposure to a HFD during the third postnatal week (reflecting the juvenile period) programmed dietary preferences later in life. This short exposure to a palatable diet altered dopamine signaling in the NAc and led the researchers to postulate that exposure to palatable foods or liquids during early life may have long-lasting effects on reward function even when body weights are not influenced (Teegarden *et al.*, 2009). Moreover, although the development of the NPY system is largely completed by the time of puberty onset (Grove & Smith, 2003), peripubertal HFD-feeding was shown to permanently decrease ARC NPY expression (Ferretti *et al.*, 2011).

Conversely, a critical amount of body fat is required for puberty to be initiated (Kennedy & Mitra, 1963; Frisch, 1972; Fernandez-Fernandez *et al.*, 2006; Martos-Moreno *et al.*, 2010). Hence, puberty is accompanied by a developmental

hyperphagia and increased consummatory behavior (Spear, 2000; Soliman *et al.*, 2014).

Consummatory behaviors and the increasing prevalence of peripubertal overweight appear to affect puberty onset. In fact, the onset of puberty has slightly advanced in the last few decades, an effect that is especially seen in girls (Wang, 2002). This effect was reproduced in studies where rodents were fed a high-fat diet (Kirtley & Maher, 1979; Ramaley, 1981; Castro-Gonzalez *et al.*, 2015). Importantly, precocious puberty has been associated with the development of psychopathology (Adriani & Laviola, 2004). For example, early maturing females and boys are more susceptible to develop eating disorders (Kaltiala-Heino *et al.*, 2001), depression (Kaltiala-Heino *et al.*, 2003) or substance abuse disorders (Adriani & Laviola, 2004), further highlighting the peripubertal period as a phase of life during which the mental health trajectory can be influenced by environmental stimuli such as diet.

Questions and Approaches

Earlier studies have shown that obesity is associated with sleep disturbances and depressive disorders. Because brain and body undergo dynamic remodeling during the peripubertal period, obesity during this developmental time window may increase the risk of sleep disturbances and depressive disorders later in life. Furthermore, brain mechanisms linking obesity and sleep disturbances are not fully elucidated and it is not clear whether increased body weight per se or whether the ingestion of palatable diets causes disturbed sleep. Therefore, this thesis aimed to investigate how peripubertal diet-induced obesity impacts on sleep-wake, emotional and feeding behaviors later in life. We further elaborated on mechanisms of sleep-wake actions in response to the removal of a palatable obesogenic diet.

We specifically asked the following questions:

1. Does peripubertal diet-induced obesity (ppDIO) influence sleep-wake behaviors later in life? Which brain mechanisms are involved in and how can we ameliorate possible sleep disturbances? (Study 1)

2. How does a history of ppDIO alter the arousal response to fasting? (Study 2)

3. Can depression-like behaviors and elevated HPA axis activity be developed along with sleep disturbances by a history of ppDIO? (Study 3)

4. Can we alleviate sleep disturbances in ppDIO mice by withdrawing the high-fat diet and replacing it by healthy food? How do food intake regulatory regions (hedonic and homeostatic systems) respond to acute high-fat diet withdrawal? (Study 4)

Materials and Methods

1. Animals

In the present study, male C57BL/6N mice were used. The animals were obtained from the animal facility of the Max Planck Institute of Biochemistry, Martinsried, Germany, at 21 days of age (postnatal day 21, P21). Mice were housed in groups of four in transparent type-2 polycarbonate cages (macrolone, 25.5 cm x 19.5 cm x 13.8 cm) in the animal facility of the Max Planck Institute of Psychiatry (MPI-Psy), Munich, Germany.

Throughout the study, mice were kept under constant environmental conditions with a 12h light-dark cycle (lights on at 08:00 a.m., lights off at 08:00 p.m., approximately 100 lux during the light period). Animals were maintained at a temperature and humidity of 22°C ± 1°C and 50 ± 10 %, respectively. All animal experiments conducted in this thesis were approved by the local commission for the Care and Use of Laboratory Animals of the State Government of Upper Bavaria.

2. Experimental Design

To achieve peripubertal diet-induced obesity (ppDIO, Fig. 15), one group of animals was fed a high-fat / high-carbohydrate diet (HFD, D12451, 4.73 kcal / g, ResearchDiets Inc., New Brunswick, NJ, USA) between P28 and P70. From P71, all animals were maintained on standard laboratory food (normal chow, NC, 1320, 2.844 kcal / g, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany).
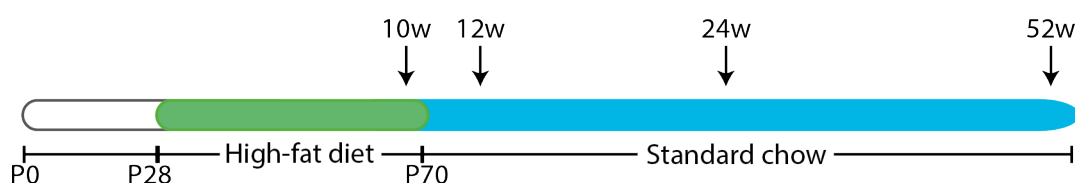
Figure 15: Experimental design showing a time line of diet regimens in ppDIO mice.

ppDIO mice were fed a HFD between P28 and P70 and were then switched to standard chow. Control mice were maintained on standard chow. Experiments were performed in ppDIO and control mice when animals reached different ages (10, 12, 24 and 52 weeks).

Control animals were maintained on NC throughout the experimental period. Body weights and food intake of all animals were measured weekly. All animals had *ad libitum* access to water and the food (unless stated otherwise), which was provided freshly every week.

2.1 Study 1 and 2

These studies were conducted to examine the effects of a ppDIO history on sleep-wake and depression-like behaviors during aging.

Study 1: To investigate whether a history of ppDIO impacts on sleep-wake behaviors during aging, sleep-wake behaviors were recorded in 38 ppDIO mice and 30 control at 10 w, 24 w and 52 w of age. During these recordings, animals were left undisturbed (baseline recording). In addition, in ppDIO and control mice aged 10 and 52 w, sleep-wake and feeding behaviors were recorded following PYY_{3-36} and vehicle injections and neurotransmitter and neuropeptide levels were examined at the end of the study. Furthermore, we applied a 24 hour fasting and re-feeding challenge on 52 w old ppDIO and control mice to investigate whether sleep disorders can be ameliorated by fasting. For recordings of sleep-wake behaviors during fasting and re-feeding, six control and seven ppDIO mice were used.

Study 2: To evaluate depression-like behaviors and HPA axis activity in ppDIO mice, a forced swim test was conducted and corticosterone concentrations were compared in 40 ppDIO and 39 control mice at 12 w, 24 w and 52 w of age. These tests were performed in the EMOLAB of the MPI-Psy. Mice were transferred to the EMOLAB one week prior to testing to allow them to acclimatize to the new environment.

After experimental testing, animals were left undisturbed for three days and were then sacrificed at ZT8.

Table 1: Overview of experimentation performed in ppDIO and control mice in studies 1 and 2 at different ages.

Evaluated parameters	10 w	12 w	24 w	52 w
Baseline sleep-wake behaviors	x	x	x	x
PYY$_{3-36}$ effects on sleep	x			x
Neurotransmitter + neuropeptide analysis	x			x
Fasting effects on sleep-wake behaviors + monoamine levels	x (study 3)			x
FST + HPA axis activity		x	x	x

2.2 Study 3

In this study, we examined the effects of withdrawal from HFD on sleep-wake behaviors and body weights. We further analyzed underlying changes in hedonic and homeostatic components of food intake regulation. To this end, ppDIO mice were tested under the following conditions:

- chronic peripubertal HFD exposure (*ad lib* HFD)
- *ad lib* HFD followed by HFD withdrawal and switch to NC (Fig 16A)
- HFD re-feeding following the HFD-NC switching (Fig 16A)

Separate groups of mice were used for sleep recordings and for the analysis of changes in neurotransmission per each experimental condition.
In addition, fasting and *ad lib* NC feeding conditions were compared in control mice (Fig. 16B).

Figure 16: Experimental design of Study 3.
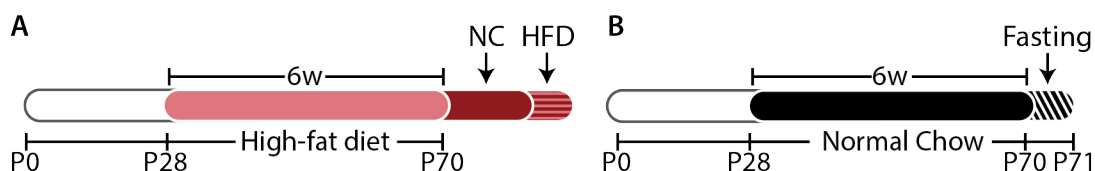
A) ppDIO mice were fed a HFD for six weeks and then HFD was withdrawn acutely and replaced by NC. For sleep-wake analysis, NC feeding lasted for two weeks. After these two weeks, ppDIO mice were re-exposed to HFD during the dark period and sleep-wake behaviors were analyzed. For neurotransmitter and neuropeptide analysis, the HFD-NC switch was limited to 24 hours. A short

(1 hour) HFD re-feeding was subjected to one group before sacrifice. **B**) Control mice were food deprived (fasted) from NC for 24 hours. Under these conditions, one batch of animals was tested with respect to sleep-wake behaviors during fasting, while the other batch was used for neurotransmitter and neuropeptide analysis. Modified from Gazea *et al.*, submitted to Front Neurosci.

3. Surgery for EEG/EMG electrode implantation

One week prior to surgeries, animals were moved to sound-attenuated recording chambers, where they were single-housed in custom-made transparent Lucite® cages (length x width x height: 25 x 25 x 25 cm).

Surgeries were performed as described in previously (Kumar *et al.*, 2015). Animals were anesthetized using an inhalation vapor system (Drägerwerk AG, Lübeck, Germany) with an isoflurane/oxygen mixture (DeltaSelect GmbH, Dreieich, Germany) and were fixed to a stereotactic apparatus (Stoelting Co., Wood Dale, USA) with a heating pad below their body to maintain body temperature during surgery. At the beginning of the surgery, animals received meloxicam (0.5 mg/kg, Metacam, Braun Melsungen AG, Melsungen, Germany) subcutaneously (s.c.) to reduce postoperative pain. In addition, to stabilize blood flow and breathing, the animals received atropine sulfate s.c. (0.5 mg/kg, Atropinsulfat, Braun, Melsungen AG).

Once fixed in the stereotactic frame, the mouse head was shaved and disinfected using 70 % ethanol. Then, a 1-cm long incision was made on the scalp and the connecting tissues on top of the skull were removed carefully. For the implantation of EEG recording electrodes, small holes were drilled through the cranial bone using a dental drill (KaVo-5 Type EWL4970; Kaltenbach und Voigt Elektronisches Werk GmbH, Leutkirch, Germany). The EEG electrodes were made of 7 mm long gold wires with ball-shaped ends, which served to avoid irritation of the brain tissue and to increase the surface for EEG acquisitions. Four EEG electrodes were placed epidurally (location of electrodes is depicted in Fig. 17). Two EMG electrodes (12 mm long gold wires with ball-shaped ends) were inserted into the neck muscles. The electrodes were soldered to an 8-pin minisocket connector (BCP socket connector, Compona, Switzerland). The connector and the electrodes were fixed to the skull with dental acrylic resin (Paladur, Heraeus Kulzer, Hanau, Germany).

At the end of the surgery, the incision sites were sutured and the animals were placed back into the home cage. An 8-pole recording cable was plugged into the

socket connector and was fixed with the dental acrylic resin. The recording cable was attached to an electric swivel system (Type SW-921.18; Precisor Messtechnik, Munich, Germany). The weights of the swivel and the recording cable were counterbalanced using a mechanical device. This system allowed mice to move freely around the cage without restriction. Mice were allowed to recover from surgery for two weeks.
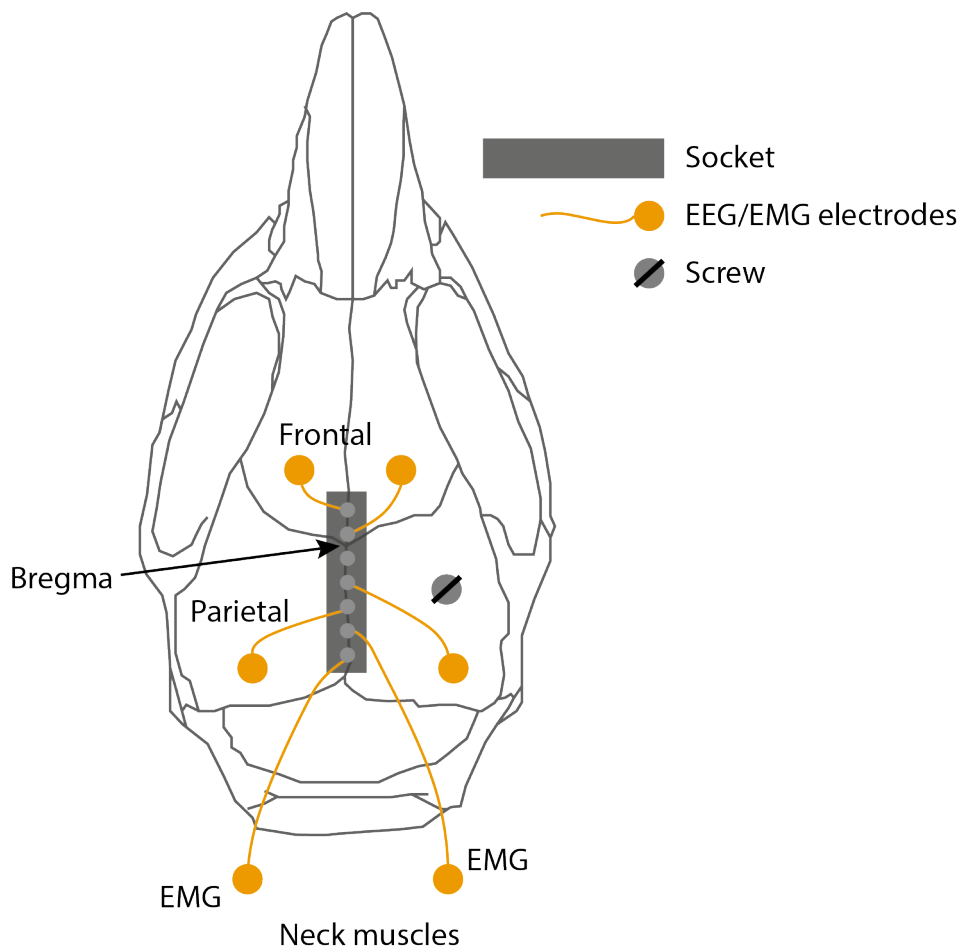

Figure 17: Dorsal scheme of the mouse skull with the location of EEG and EMG electrodes.

The location of holes for the implantation of frontal and parietal EEG electrodes is indicated as orange circles on the skull. EMG electrodes were inserted into the neck muscles. Skull adapted from www.informatics.jax.org.

4. EEG/EMG recordings and sleep data analysis

4.1 Recordings of EEG/EMG signals

After recovery from surgery, baseline EEG/EMG recordings were performed in all mouse groups for 48 hours. EEG and EMG signals were amplified (10,000 fold) and filtered (EEG: 0.25-64 Hz; EMG: 175-1,000 Hz). EMG signals were rectified using the root mean square. Both EEG and EMG signals were digitized by an analog-to-digital converter (NI-USB-6343-X-series, National Instruments, Austin, TX, USA) and were fed into a LabVIEW-based software (National Instruments) that was designed for mouse sleep EEG/EMG data acquisition (EGEraVigilanz, SEA, Cologne, Germany). Recordings were obtained at a sampling rate of 128 Hz and were stored on a computer for subsequent offline processing. The last 24 hours of the baseline recordings were used for analysis, while the first 24 hours served as a backup recording.

4.2 Sleep-wake data analysis

EEG signals were first automatically analyzed with the aid of a Fast Fourier transformation (FFT), which performs a power density analysis across pre-defined EEG frequency bands (delta: 0.5-5 Hz, theta: 6-9 Hz, sigma: 10-15 Hz, beta: 16-29 Hz and gamma: >30 Hz). Thereby, the FFT helps a semiautomatic classification of sleep-wake vigilance states (NREMS, REMS or WAKE) that should be determined every four seconds. This semi-automatic classification algorithm was applied based on a report by Louis *et al.*, 2004 and assigns each epoch one of the vigilance states based on the combination of an EMG threshold and the NREMS and REMS formulas that are provided by the FFT analysis. This semi-automatically scored data was inspected visually and was corrected whenever necessary. Vigilance states are given as percentage per hour or per six hours ± SEM.

Slow-wave activity (SWA) was obtained from epochs that were scored as NREMS and was calculated by normalizing the absolute power of the frequency range between 0.5-4 Hz during NREMS by the average total EEG power from all the

vigilance states. Further, the transition frequencies from one vigilance state to another were analyzed and are given as total number of transitions per six hour bins.

4.3. Experimental procedures under EEG/EMG recordings

4.3.1 Food challenges

In Study 1 and 3, 10 and 52 w old ppDIO and control mice were food deprived for 24 hours starting at light onset. Corresponding baseline EEG/EMG signals were obtained the day before food deprivation was executed. To avoid feeding on food crumbs that may be distributed inside the cage, fresh cages were provided 24 hours prior to the baseline acquisition. Following 24 hours of fasting, NC or HFD was returned to the animals at light onset and food intake was monitored during different time points (1, 3, 12 and 24 hours).

An additional group of 10 w old ppDIO mice was switched from HFD to NC starting at dark onset (Fig. 16A). During the HFD-NC switch, EEG/EMG signals were recorded during the first 48 hours, while food intake and body weights were monitored daily. A subset of mice received a follow-up recording two weeks after the HFD was replaced by NC. Further, this group of ppDIO mice received a HFD re-feeding for 12 hours during the dark period. Sleep was monitored continuously.

Separate groups of mice were used for neurotransmitter and neuropeptide analysis. In these groups, all the above described food challenges were started at ZT8 and lasted for 24 hours. Notably, for neurotransmitter analysis, HFD re-feeding was performed for only one hour after HFD was switched to NC for 23 hours. Tissues were collected as described in section 6.

4.3.2 PYY$_{3-36}$ injections

Mouse PYY$_{3-36}$ was purchased from Bachem (H6042, Bachem AG, Bubendorf, Switzerland). To habituate mice to the injection procedure, mice were given intraperitoneally (i.p.) 150 µl of sterile 0.9 % NaCl (Braun Melsungen AG, Melsungen,

Germany) three to four times prior to real treatment. All injections were performed 15 minutes prior to dark onset. PYY_{3-36} was dissolved in 0.9% NaCl and was injected at a dose of 60 µg/kg body weight at a volume of 150 µl. After habituation was completed, each mouse received two injections on two consecutive days; one injection of saline (vehicle, injection control) and one injection of PYY_{3-36}. The order of the injections was performed in a randomized fashion. EEG/EMG signals were recorded 23 hours post-injections, and food intake was monitored 1, 3, 12 and 24 hours after the injections.

5. Behavioral tests

5.1 Forced Swim test

Porsolt's Forced Swim test (FST, Porsolt *et al.*, 1977) was used to examine depression-like behaviors in ppDIO and control mice. For the FST, four glass cylinders were filled with 1.75 liter of 23-25° C warm water. Each mouse was placed into the water for six minutes and was recorded using a web cam. Mice were always tested in groups of four. After each run, mice were dried gently using a towel and placed back into their home cages. Cylinders were filled with fresh water after each run.

Swimming behaviors were scored visually (offline) after FSTs were completed. An animal was considered inactive when it was floating passively in the water with as little movements as possible only to maintain its balance and to keep its head above the water surface. Usually, animals were swimming vigorously during the first two minutes of the test. Therefore, the first two minutes were discarded from the analysis and only the last four minutes of the FST were counted into behavior analysis. The time floating is given as % time ± SEM. Furthermore, the latency to the first floating episode was determined (sec ± SEM).

5.2 Open Field test

The Open Field test was used to assess exploratory activity and general locomotion in ppDIO and control mice (previously described in Dedic *et al.*, 2012). The round open field arena had a diameter of 58 cm with 40 cm high walls, and its center was illuminated with 50 lux. Mice were placed in the corner of the arena, and their activity inside the arena was recorded for 5 min using the ANY-maze Video Tracking System V4.99m (Stoelting Co., Wood Dale, IL, USA). The distance traveled in meters was automatically scored by the ANY-maze software and is given as meters ± SEM.

6. Tissue and sample collection

6.1 Tail blood sampling

Mice were fixed gently underneath a towel, leaving only the tail accessible. The lateral tail vein was cut using a scalpel, and several drops of blood were collected in a 300 µl Eppendorf tube. Mice were returned to their home cages after this procedure.

Blood samples were left to coagulate at room temperature (RT) for two hours. Afterwards, blood samples were centrifuged at 4° C for 15 min at 8000 rpm. Serum (supernatant) was pipetted into a fresh vial and was stored in a deep freezer (-80° C) until further processing.

6.2 Brain collection

All mice in this study were sacrificed at ZT8. Mice were anesthetized using isoflurane and killed by decapitation. Brains were removed quickly and snap frozen in isopentane (DeltaSelect GmbH, Dreieich, Germany), which was stored on dry ice to reach temperatures below -60° C. Afterwards, brains were kept in dry ice for one more hour until completely frozen. Finally, the brain samples were stored in a deep freezer (-80° C).

6.3 Brain tissue processing

For RNA *in situ* hybridization, coronal sections were made from brains of ppDIO and control mice using a cryostat (Leica, Germany). The sections at the level of the LH and ARC were prepared with a thickness of 10 µm (Paxinos & Franklin, 2001; Allen-Brain-Atlas, 2015). All sections were stored at -20° C for later use in radioactive RNA *in situ* hybridization.

For neurotransmitter and neuropeptide measurements, Palkovit's Punch technique was used to obtain cylindrical tissue samples of the NAc, LH, VTA and DR of the same brains (Fig. 18, Palkovits, 1983). A sample corer of 0.5 mm diameter was used (Fine Science Tools GmbH, Heidelberg, Germany). Tissue samples were made by pushing the sample corer approximately 500 µm deep into the brain tissue, which was fixed by cryostat holder, followed by a lever movement. The bilateral tissue samples were collected in 1.5 ml Eppendorf tubes. The samples were stored at -80° C until they were further processed for HPLC measurements.
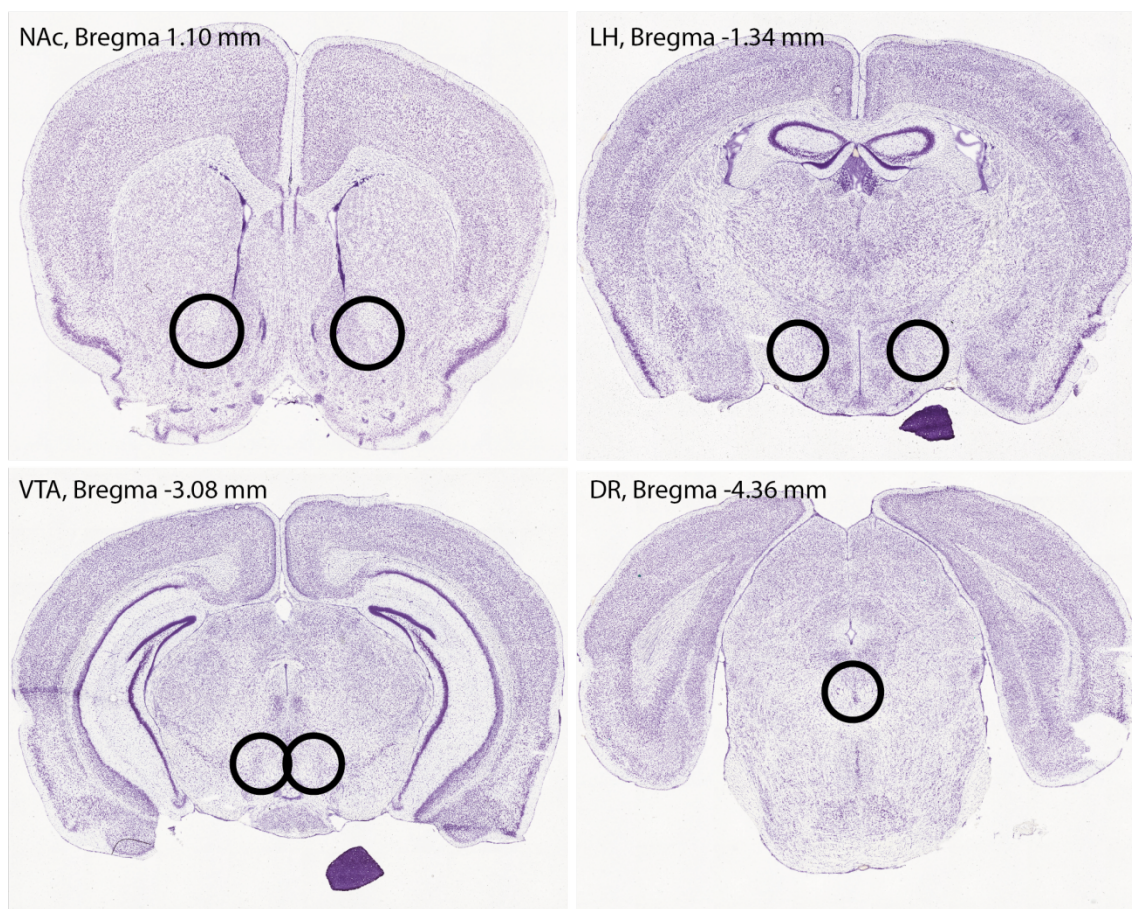

Figure 18: Location of NAc, LH, VTA and DR tissue punches.

The approximate location of the tissue punches is illustrated as black circles on respective sections containing NAc, LH, VTA or DR. Nissl stained coronal sections were adapted from the Allen-Brain-Atlas, 2015.

7. Radioimmunoassay (RIA)

7.1 Principle

Radioimmunoassay (RIA) is a method to determine concentrations of ligands such as hormones or allergens in blood samples or tissue lysates in vitro using an antigen-antibody reaction. This technique was demonstrated first by Yalow & Berson, 1960. To measure the concentration of a ligand, a specific volume of the ligand in solution is mixed with a defined concentration of radioactively labeled antigen. These antigens are usually labeled with radioactive isotopes of iodine such as ^{125}I, which can be introduced into tyrosine residues of proteins. Subsequently, an antibody against the specific antigen is added, leading the unlabeled antigen and the radioactive antigen to compete for the binding sites of the antibody. Increasing amounts of unlabeled antigen in the mixture displace the radioactive antigen from the antibody binding sites resulting in lower amounts of radioactive antigen binding to the antibody. A second antibody directed against the first is added and precipitates the antigen-antibody complex. After centrifugation and removal of the supernatant, only the antigen-antibody complexes remain, of which the radioactivity can be measured by a Gamma counter. To transform this measure into a unit that calculates the actual concentration of the desired ligand, samples of known antigen concentrations are assayed as well, providing reference values to generate a standard curve. The standard curve then allows us to calculate the concentration from each unknown sample.

7.2 Corticosterone RIA

For the detection and quantification of corticosterone concentrations in serum samples, the Corticosterone Double Antibody RIA kit was used (MP Biomedicals

Germany GmbH, Eschwege, Germany). Samples were diluted 1:50 (morning samples) or 1:200 (evening and stress samples) using a steroid diluent provided by the RIA kit. 50 µl of the diluted samples, controls and standards were pipetted in duplicates into RIA test tubes. Subsequently, 100 µl radioactively labeled corticosterone (Corticosterone-^{125}I) was added to each tube. After adding 100 µl anti-corticosterone antibody, samples were mixed vigorously and were left to incubate for two hours at RT. Afterwards, 250 µl secondary antibody solution was added to each tube. Again, samples were mixed carefully. This step was followed by a centrifugation of the tubes for 15 min at 2500 rpm. The supernatant was removed and the radioactivity contained by the pellet was measured in the Gamma counter (2470 WIZARD^2 Automatic Gamma Counter, PerkinElmer Inc., Rodgau, Germany). Corticosterone concentrations were analyzed by the aid of StatLIA Quantum Premium software for RIA assays (Brendan Technologies Inc., Carlsbad, CA, USA). Repeated assays were conducted when the error coefficient obtained from the duplicates reached more than 15 %. The detection limit was between 12.5 and 1000 ng/ml.

7.3 Orexin RIA

The concentration of orexin A extracted from tissue samples of bilateral LH was measured by the orexin A RIA kit (Phoenix Pharmaceuticals Inc, USA). LH tissue samples were processed as described in the section 8.2. After extraction from tissue homogenates, the supernatant was lyophilized by a centrifugal concentrator at 4°C. The lyophilized samples were re-suspended in a 50 µl RIA buffer, which was provided by the RIA kit. Subsequently, 50 µl of the primary antibody (from rabbit) against orexin A was added. Samples were mixed and incubated over night at 4°C. Afterwards, 50 µl radioactively labeled orexin A (^{125}I-orexin) was added to each tube containing our unknown samples or known standard samples. Samples were mixed again and incubated over night at 4°C. Then, 50 µl of normal rabbit serum and 50 µl of the secondary antibody (goat anti-rabbit) were further addded into each tube. Samples were mixed well and left at RT for 90 min. Finally, adding 250 µl of the RIA buffer stopped the reaction. After centrifugation at 4°C for 20 min (3,000 rpm), the supernatant was aspirated carefully. The radioactivity of the pellet was measured

using a Gamma counter, and the concentration of orexin A was calculated using StatLIA Quantum Premium Software, as described above. The detection limit of this RIA assay ranged from 10 to 1280 pg/ml.

8. High-performance liquid chromatography for the determination of neurotransmitters

8.1 Principle

High-performace liquid chromatography is a sensitive technique to separate, detect and quantify chemical components of low concentration in biological samples. The separation of the chemical components is achieved by injecting the liquid sample into a column filled with a stationary phase, which retains the components in the sample and a mobile phase made up of an eluent which detaches the components from the stationary phase depending on their physical and chemical properties. Identification of molecules is achieved by electrochemical detection following separation of the components in the column and depends on the elution time a molecule is released from the stationary phase (retention time). The following sections provide a detailed description of HPLC measurements.

8.2 Sample preparation

25 µl of 0.1 M perchloric acid was added to NAc, DR and VTA tissue samples, while 50 µl of 0.1 M perchloric acid were applied onto LH samples. Subsequently, tissue samples were sonicated, and centrifuged for 15 min at 4° C and 14000 rpm. The supernatant was transferred into fresh tubes. For the LH, two aliquots of supernatant were prepared. Supernatants were stored in a deep freezer (-80° C) for HPLC measurements.

The pellet was used to measure protein concentrations of the tissue samples. Pellets were re-suspended in 25 µl 3N NaOH and stored in a freezer (-20° C).

8.3 Quantification of monoamine contents

Concentrations of dopamine, serotonin, and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) were determined by reverse-phase HPLC with electrochemical detection (UltiMate3000 / CoulochemIII, ThermoFischer, USA) in the microdialysis core facility of the MPI-Psy (Anderzhanova *et al.*, 2013). A citrate - phosphate mobile phase containing 8.5 % of acetonitrile with the pH set at 3.00 was used. The mobile phase was prepared using reagents of analytical grade (Carl Roth GmbH or MERCK KGaA, Germany). Components (monoamines) of the tissue extracts were separated on an analytical column (C18, 150 mm×3 mm, 3 µm, YMC Triart, YMC Europe GmbH, Germany) at a flow rate of 0.5 ml/min. The potentials of the working electrodes were set at -75 mV, +220 mV and the guard cell potential was set at +350 mV. HPLC measurements provided separate peak areas for each monoamine detected in the samples by the guard cell. The peak area was used to calculate the monoamine concentrations with the aid of an external standard curve. The detection limits for all compounds ranged from 0.032 to 0.050 nM. Monoamine concentrations were adjusted by the sample protein content, as described in the following section.

8.4 Normalization to protein content (Lowry Assay)

The protein content in NAc, DR, VTA and LH samples was determined by the Lowry method (Lowry *et al.*, 1951). To this end, samples were diluted 1:50 in double-distilled water to yield a final volume of 150 µl. Each sample dilution was prepared in duplicates. A standard measurement using five freshly prepared dilutions of 1 mg/ml BSA protein was always included in each assay. Furthermore, two more vials containing 150 µl water served as blank.

After the preparation of all the dilutions, 750 µl Lowry reagent was added to each tube. Tubes were mixed well and incubated for 15 min at RT. Subsequently, 75 µl Follin reagent was added. Samples were again mixed and incubated in the dark at

RT for one hour. Finally, 300 µl sample was pipetted in a 96-well plate, and the absorbance was measured at 750 nm using a spectrophotometer. With the aid of the known standard samples, we computed the concentration of each unknown sample using (Gen5 DataAnalysis Software, BioTek Instruments Inc., USA).

The concentration values given by the Lowry Assay were used to adjust the neurotransmitter data from the HPLC. Therefore, neurotransmitter concentrations are given as µmol/mg protein ± SEM.

9. RNA in situ hybridization

9.1 Principle

To achieve radioactive RNA *in situ* hybridization, oligonucleotides or riboprobes can be used that are complementary to the intrinsic RNA. Hence, oligo- and riboprobes can hybridize proportionally to the intrinsic RNA, for example in fixed tissue sections. To visualize the amount of hybridized RNA a radioactive label can be used; ^{35}S-dATP for oligonucleotides and ^{35}S-UTP for riboprobes. Here, we made use of radioactively labeled oligonucleotides to detect the expression intrinsic RNA by hybridization. Radioactively labeled probes have the advantage of providing qualitative and also quantitative results. In the following sections, the RNA in situ hybridization procedure is explained in detail.

9.2 Oligonucleotide Design

Orexin and MCH oligonucleotides were designed by selecting sequences comprising maximally 50 nucleotides within the coding sequence of the respective gene (GenBank, http://www.ncbi.nlm.nih.gov/genbank/). The selected sequences had to fulfill the following criteria (according to Erdtmann-Vourliotis *et al.*, 1999):

- GC content: 48 – 62 %

- Length: 42 – 54 nucleotides
- no hairpin formation
- no self-annealing properties

The accuracy of the sequence was validated using the nucleotide analyzer by www.geneinfinity.org. Furthermore, the specificity of the oligonucleotide to the gene of interest was verified using the nucleotide BLAST by NIH (http://blast.ncbi.nlm.nih.gov/).

Afterwards, the oligonucleotides (custom-made, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were tested by means of RNA in situ hybridization on brain sections that included the structure where the RNA of interest is supposed to be expressed (for orexin and MCH: lateral hypothalamus). Negative control sections that should not express the respective RNA were run in parallel. Oligonucleotides that yielded strong signals with a minimum amount of background binding were used for further RNA in situ hybridizations on experimental sections (Table 2). NPY oligonucleotide sequence was kindly provided by Dr. V. Patchev.

Table 2: Oligonucleotide sequences for radioactive RNA in situ hybridization.

Complementary sequences and GenBank reference number are given for prepro-orexin, pro-melanin-concentrating hormone and neuropeptide Y oligonucleotides.

Name	Complimentary sequence	GenBank reference
Prepro-orexin	5'AGCAGCGTCACGGCGGCCCAGGGAACCTTT GTAG	AF041242.1
pro-melanin-concentrating hormone	5'CAACATGGTCGGTAGACTCTTCCCAGCATAC ACCTGAGCATGTCAA	NM_029971.2
Neuropeptide Y	5'GTCCTCTGCTGGCGCGTCCTCGCCCGGATT GTCCGGCTTGGAGGGGTA	Provided by Dr. V. Patchev

9.3 Oligonucleotide labeling

Oligonucleotides were labeled using ^{35}S-dATP (NEG734H250UC, PerkinElmer Inc., Rodgau, Germany) by polyadenylation of the DNA 3' ends. The labeling was prepared in autoclaved 1.5 ml Eppendorf tubes on ice, which were pre-filled with 32

µl sterile water. Polyadenylation was achieved by adding 10 µl of 5 x tailing buffer (Invitrogen, Karlsruhe, Germany) and 5 µl of ^{35}S-dATP to 1 µl of 5 µM oligonucleotide. After mixing, 100 units of terminal deoxynucleotidyl transferase (TdT) (Invitrogen, Karlsruhe, Germany) were added to catalyze polyadenylation, and the mixture was incubated at 37° C for 15 to 30 minutes. Subsequently, the reaction was terminated by 20 µl TE buffer and 2 µl tRNA (25mg/ml, Yeast tRNA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) on ice. The tRNA served to occupy the binding sites of the TdT enzyme. The labeled oligonucleotide was purified using the QIAquick Nucleotide Removal kit (QIAgen GmbH, Hilden, Germany).

The labeling efficacy was evaluated before testing by counting a small aliquot of the labeled probe using a beta counter. A good incorporation yielded between 500.000 and 1.500.000 cpm/µl. Probes that fulfilled this criterion were used for in situ hybridization.

9.4 Hybridization procedure

Sections mounted on glass slides were taken out of the -20° C freezer and were kept at RT until the condensate was dried out entirely. All the following steps were carefully operated using sterilized materials (including utensils and solutions). Ingredients of the solutions are depicted in the appendix.

Sections were fixed in 4 % formaledhyde (prepared in 1 x PBS) for 5 min at RT. After a short wash in 1 x PBS, sections were incubated in freshly prepared 0.25 % acetic anhydride in 0.1 M TEA/HCl for 10 min. Then, the sections were dehydrated using an ascending ethanol series as follows: 1 min 70 % ethanol, 1 min 80 %, 2 min 96 % and 1 min in 100 % ethanol. To reduce the possible background hybridization to lipids, the dehydrated slides were then placed into chloroform for 5 min. After a short washing step in 100 % and 96 % ethanol for 1 min, sections were left at RT to dry.

In the meantime, the hybridization mixture (hybridization buffer and 20.000 cpm / µl oligonucleotide) was prepared. 2 µl of 5 M 1,4-Dithiothreitol (DTT; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added per 100 µl hybridization mix. After placing the sections in moisturized hybridization trays, 25 µl of the hybridization mix

were applied onto each section. Glass slides were covered with cover slips. Hybridization trays were sealed and incubated at 37° C overnight for a maximum of 18 hours.

The next day, slides were removed from the hybridization trays and placed into 1 x SSC. After removing cover slips in 1 x SSC, a high-stringency wash was performed to remove background hybridization and free oligonucleotides from the slides. This was achieved by washing the slides in a 1:1 pre-warmed mixture of formamide and 4 x SSC at 40° C in a water bath for 15 min. This washing step was repeated three more times. Subsequently, slides were washed twice in 1 x SSC at RT for 30 min each. A wash in distilled water for 10 min followed to remove residual SSC. Slides were then dehydrated in 70% ethanol and 100 % ethanol (1 min each) and dried at RT.

To visualize the labeling, slides were placed horizontally in film cassettes. A carbon-14 radioactivity standard with a range of 0-35 nCi/mg (glass slide, American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was added to each cassette. A 35 x 43 cm large film (Carestream Biomax, MR Film, High Resolution, Radioisotope & Chemiluminescent, Kodak) was applied onto the slides and the cassettes were closed. The films were handled in the dark room under red light to avoid white light exposure of the films. The cassettes were kept sealed for 20 to 24 hours (orexin and MCH RNA in situ hybridization) or for 3 days (NPY) at 4° C. Afterwards, the cassettes were opened in the dark room and the films were developed in developing solution (Kodak Developer and Replenisher) for 1 min. After washing the film in water for 1 min, the film was fixed for 1 min (Kodak Fixer and Replenisher), and then dried at RT.

9.5 Quantification

To quantify orexin, MCH and NPY mRNA expression digitally, signals on the films were scanned using the CanoScan 9950F (Canon Deutschland GmbH, Krefeld, Germany). Optic density of the signals was measured using Image J (1.48v, NIH, USA) by carefully circling the area containing the signals (Fig. 19). Optic density of each reference spot (carbon-14 radioactivity standard) was also measured and was equated with its respective radioactivity level. The obtained fit function was used to

calculate the amount of radioactive signal per mg tissue [nCi / mg]. Values of orexin, MCH and NPY mRNA expression are given as nCi / mg ± SEM.

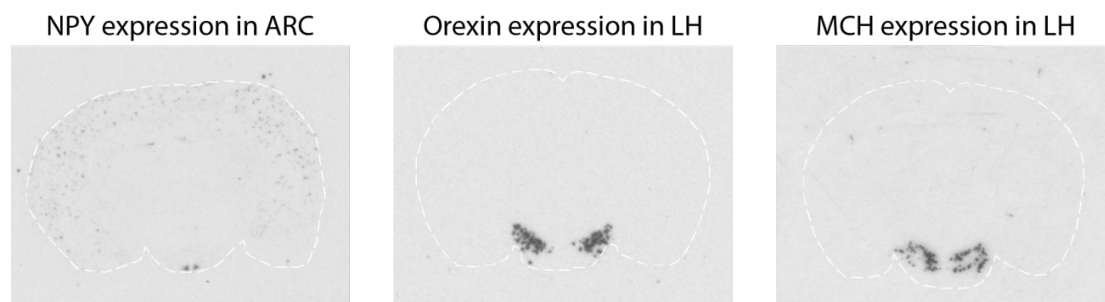| NPY expression in ARC | Orexin expression in LH | MCH expression in LH |

Figure 19: Examples of radioactive RNA in situ hybridization signals of NPY, orexin and MCH expression.

From left to right: NPY expression in the Arc, orexin expression in the LH and MCH expression in the LH. Dashed white lines outline section silhouettes.

10. Statistical analysis

To compare diet, age or treatment effects on sleep-wake behaviors, RNA expression etc., statistical analysis was performed using GraphPad Prism (Version 6.01, GraphPad, San Diego, CA) or SPSS (SPSS 18, Chicago, IL). Firstly, Grubb's test based on the extreme studentized deviate method was used to detect significant outliers in our data sets (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Significant outliers were substituted by the mean of a data set. Subsequently, a Kolmogorov-Smirnov normality test was performed. When a data set passed the normality test (when $p>0.05$), an analysis of variance (ANOVA), a multivariate ANOVA (MANOVA) or a student's t-test was used for statistical analysis, as appropriate. One-way and two-way ANOVAs were followed by Sidak's multiple comparison's test, while Bonferroni post-hoc comparisons were used after MANOVAs. Further, when data sets did not pass normality tests, non-parametric statistical tests were used for the analysis, i.e. the Kruskal-Wallis test followed by Dunn's multiple comparisons test or a Mann-Whitney U-test. Furthermore, in Study 3, an analysis of covariance (ANCOVA) was performed to test whether group differences in swimming behaviors during the FST persist when body weights are partialled out. $P<0.05$ was considered as significant. All values are given as mean ± SEM.

Furthermore, adjusted energy intake was calculated based on the study by Austad & Kristen using the following formula, which adjusts body mass to a species-specific coefficient for body size: adjusted energy intake = food intake [kcal]/body weight $[g]^{0.568}$ (Austad & Kristan, 2003).

PYY_{3-36} and fasting/re-feeding effects on vigilance states and food intake were compared to the vehicle condition (0.9% NaCl) and the *ad libitum* feeding condition, respectively. Relative changes were computed using the following formula (kindly provided by the head of the research group statistics, A. Yassouridis, MPI-Psy): relative change = $((y^2 + 0.000005)/(y^2 + x^2 + 0.00001))*200$, with y being the variable obtained after PYY_{3-36} or fasting/re-feeding and x the variable following vehicle or *ad libitum* feeding. Values are given as percentage change.

Results

1. Programming of sleep-wake and ingestive behaviors by ppDIO

Recent reports have shown that alterations in sleep-wake behaviors seen in obese humans are also displayed by DIO mice. However, these reports have focused on adult DIO and acute effects of HFD withdrawal on sleep following DIO. The long-term effects of DIO induced specifically during puberty are unknown. Because body and brain undergo dynamic remodeling during puberty, it is very likely that environmental challenges leave a permanent mark. To elucidate whether ppDIO leads to life-long changes in sleep-wake profiles, EEG/EMG recordings were performed in ppDIO and control mice at different ages (10 w, 24 w and 52 w). While 10 w old ppDIO mice were still exposed to HFD, the other age groups had been switched to normal laboratory food following six weeks of peripubertal HFD exposure. Furthermore, mechanisms linking obesity with sleep disturbances are not well understood. Therefore, I analyzed changes in neurotransmitter and neuropeptide levels in areas related to food intake and sleep-wake regulation in our ppDIO mouse model. I further explored a role of PYY as a treatment option for sleep disturbances in obesity at the end of this section.

1.1 Sleep in mice following peripubertal diet-induced obesity during aging

1.1.1 Effects of ppDIO and aging on NREMS and REMS time under baseline conditions

All mice recorded in our study exhibited a circadian distribution of NREMS, REMS and wakefulness across the 24-hour recording period. Highest amounts of NREMS and REMS occurred during the 12-hour light (resting) period and lower amounts during the 12-hour dark (active) period (Fig. 20).
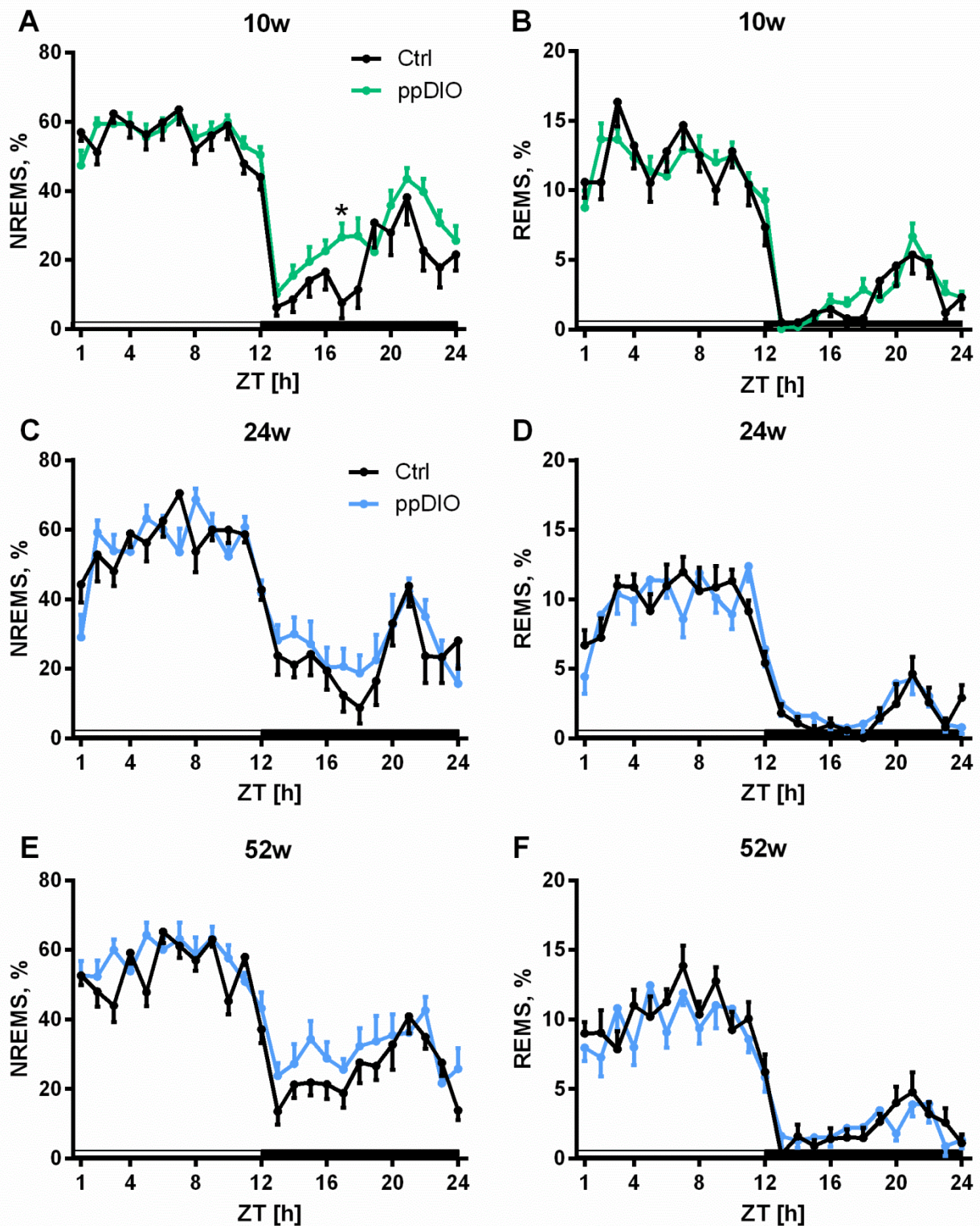
Figure 20: Circadian distribution of 24-hour NREMS and REMS in ppDIO and control mice during aging.

For figure legend please refer to the next page.

Figure 20: Circadian distribution of 24-hour NREMS and REMS in ppDIO and control mice during aging.

Data points represent time spent in NREMS (left panel) or REMS (right panel) during one hour in percentage ± SEM. Zeitgeber time (ZT) 0 to 12 refers to the light (resting) period (white bars above the x-axis), while ZT 13 to 24 refers to the dark (active) period (black bars). Data from control mice are drawn in black (10 w: n=11; 24 w: n=8; 52 w: n=11). ppDIO mice still receiving HFD (10 w: n=18) are depicted in green, while ppDIO mice that were switched to NC (24 w: n=10; 52 w: n=10) are shown in blue. Two-way ANOVA showed that ppDIO significantly increased NREMS in the 10 w ($F_{(1, 27)}$ = 9.776, p = 0.0042) and 52 w old group ($F_{(1, 19)}$ = 6.753, p = 0.0176). NREMS increases in 10w ppDIO mice were significantly dependent on time of day ($F_{(23, 621)}$ = 1.574, p = 0.0434). Sidak's post-hoc test was performed to detect group differences per hour. *P<0.05. Modified from Gazea et al., submitted to PNAS.

In 10 w old mice, NREMS time was significantly elevated in ppDIO mice throughout the recording (Fig. 20A, $p < 0.05$). In addition, post-hoc analysis showed that 10w old ppDIO mice displayed significantly higher amounts of NREMS time at ZT 17 as compared to 10 w old control mice ($p < 0.05$). Also in 52 w old mice, a history of ppDIO increased NREMS time significantly (Fig. 20E, $p < 0.05$), whereas this effect was not seen in 24 w old ppDIO mice (Fig. 20C). REMS time was not affected by ppDIO in any age group (Fig. 20, right panel).

To determine which time windows were affected by ppDIO, we divided the 24-hour recording period into 6-hour bins as a next step (Fig. 21). This analysis showed that ppDIO affected NREMS mainly during the dark period; 10 w old ppDIO mice spent significantly more time in NREMS during ZT 13-18 and ZT 19-24 (depicted as ZT 18 and 24 in Fig. 21, respectively) as compared to controls of the same age ($p < 0.05$). Similarly, 52 w old ppDIO mice exhibited elevated NREMS time during ZT 13-18 ($p < 0.05$), but not during ZT 19-24. REMS time was not influenced by ppDIO.
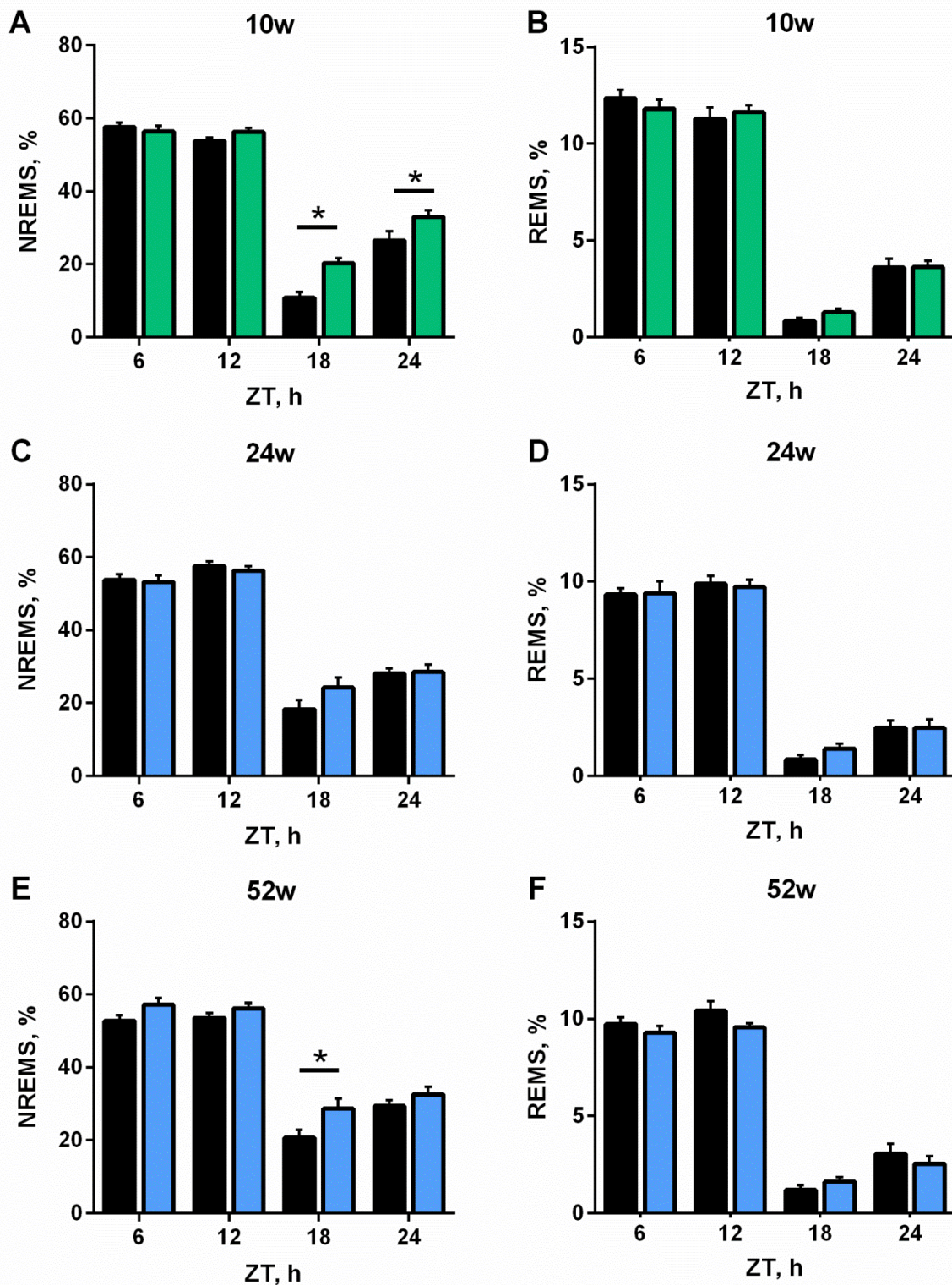
Figure 21: Six-hour averaged percentage spent in NREMS and REMS in ppDIO and control mice during baseline.

Time spent in NREMS (left panel) and REMS (right panel) during six hour intervals are given as mean percentage ± SEM. Two-way ANOVA showed that peripubertal diet affected NREMS in 10 w old mice (F (1, 27) = 9.776, p = 0.0042) and that this effect interacted significantly with time (F (3, 81) = 4.570, p = 0.0052). Peripubertal diet also increased NREMS in 52 w old mice significantly (F (1, 19) = 6.753, p = 0.0176). Sidak's multiple comparison's test was used to identify group differences within each time interval. *P<0.05. Modified from Gazea et al., submitted to PNAS.

Because significant effects were mostly found during the six-hour interval spanning ZT 13-18 (first half of the dark period), we chose this time window to address the interaction between age and peripubertal diet (Fig. 22). We found a significant increase in NREMS time in ppDIO mice as compared to controls, especially in 10 w and 52 w old ppDIO mice (Fig. 22A, $p < 0.05$). Furthermore, amounts of NREMS were increasing with age ($p < 0.05$). Peripubertal HFD also increased REMS time during ZT13-18 (Fig, 22B, $p < 0.05$). However, REMS amounts did not change during aging.
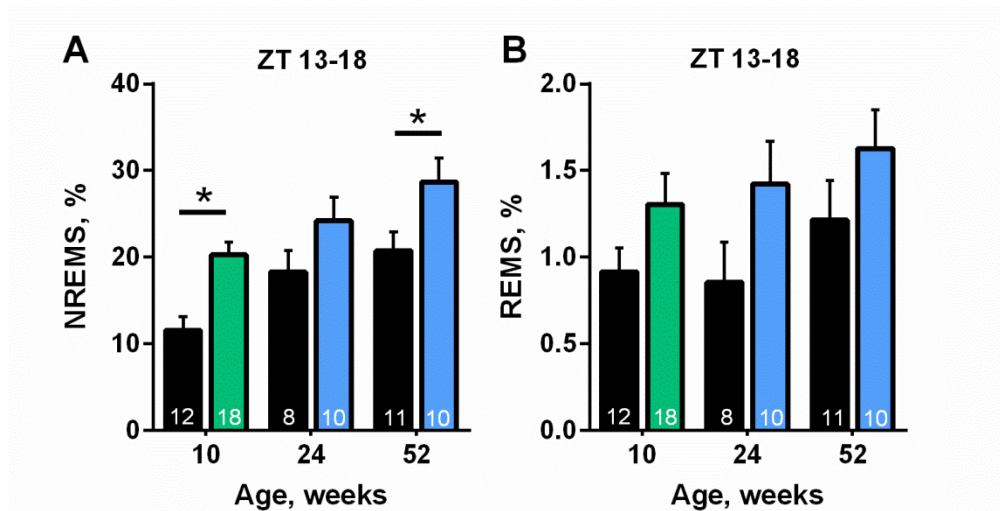

Figure 22: Comparison of sleep during ZT13-18 in different age groups.

Each bar represents time spent in NREMS (A) or REMS (B) during six hours between ZT13 and 18 in percentage ± SEM. Animal numbers per age and diet are given at the bottom of each bar. Two-way ANOVA showed significant effects of peripubertal diet ($p < 0.0001$) and age ($p = 0.003$) on NREMS. REMS was also influenced by ppDIO ($p = 0.0107$). Group differences were calculated using Sidak's multiple comparisons test. *$P < 0.05$. Modified from Gazea et al., submitted to PNAS.

1.1.2 Effects of ppDIO and aging on sleep architecture under baseline conditions

Obese individuals suffer from frequent awakenings during the night as described in the introduction. Therefore, we addressed the question whether our mouse model reproduces such a sleep phenotype and whether a history of ppDIO may program changes in sleep architecture during aging. To this end, we analyzed transitions between the three different vigilance states per 6-hour intervals during baseline

recordings in 10 w and 52 w ppDIO and control mice (Fig. 23). The group of 24 w old mice was not included in this analysis due to their insignificant sleep phenotype.

With respect to transitions from NREM to WAKE and vice versa, peripubertal HFD increased the number of transitions in a time-dependent manner (Fig. 23A and B); 10 w old ppDIO mice exhibited significantly more transitions from NREM to WAKE and WAKE to NREM at ZT7-1; ($p < 0.05$), ZT13-18 ($p < 0.05$) and ZT19-24 ($p < 0.05$) as compared to control mice of the same age. This difference was not evident in 52 w old mice. In ppDIO mice the NREM-WAKE transitions decreased significantly from 10 w to 52 w of age at ZT1-6 ($p < 0.05$) and ZT7-12 ($p < 0.05$). By contrast, in control mice the number of transitions was increasing with age, especially at ZT13-18 ($p < 0.05$) and ZT19-24 ($p < 0.05$).

Transitions from NREM to REM and from REM to WAKE were not significantly different between the diet groups (Fig. 23C and D). However, in ppDIO mice the number of these transitions during the light period decreased with age (ZT1-6, ZT7-12, $p < 0.05$). An age-related influence on REM-transitions was not observed in control mice.
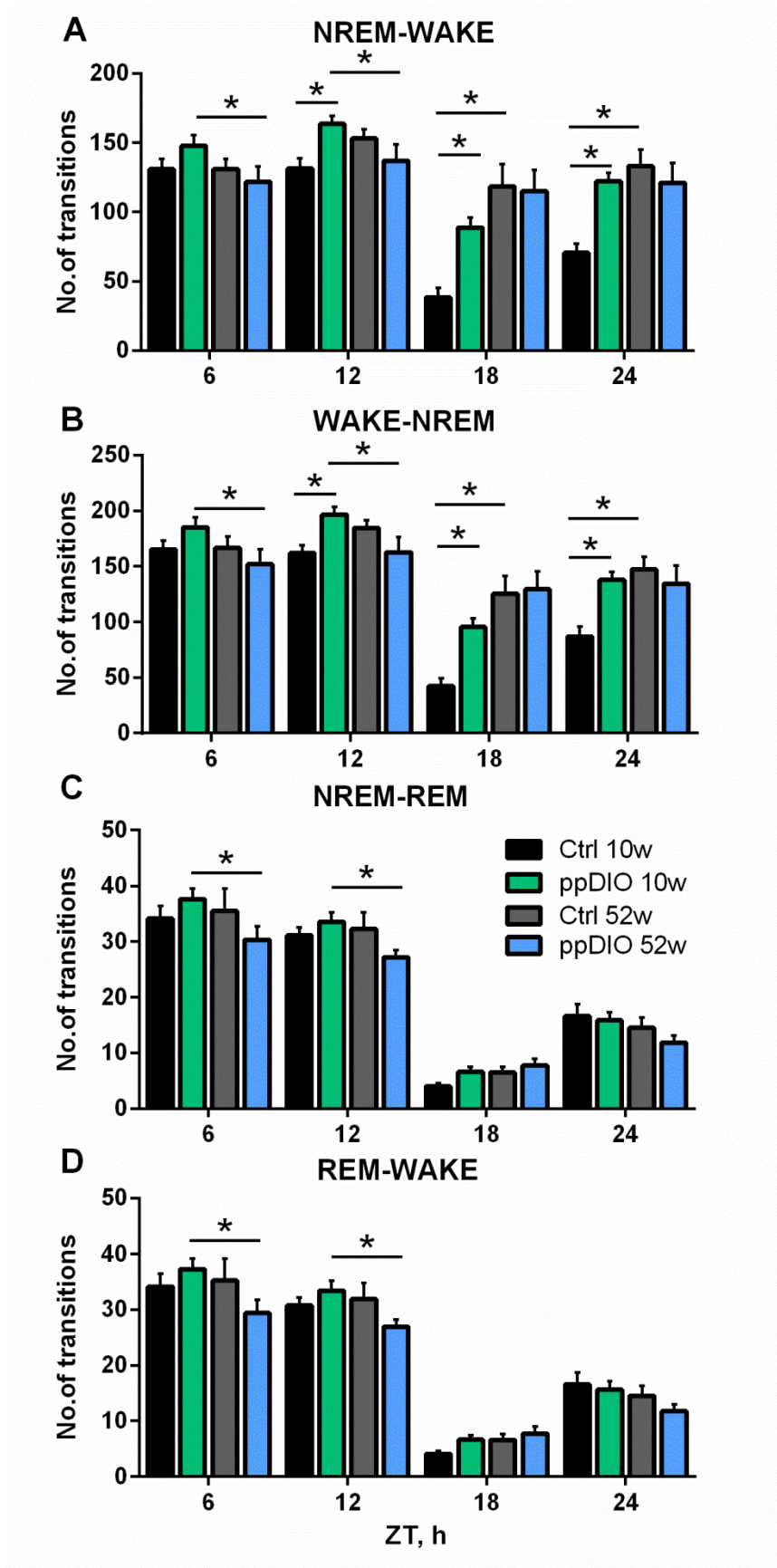
Figure 23: Vigilance transitions in 10 w and 52 w old ppDIO and control mice.
For figure legend please refer to the next page.

Fig.23 Vigilance transitions in 10 w and 52 w old ppDIO and control mice.

Each bar represents the total number of transitions during a six-hour period for NREM to WAKE (A), WAKE to NREM (B), NREM to REM (C) and REM to WAKE (D). 10w old ppDIO mice (n=18) are shown in green, 52w ppDIO mice in blue (n=10), 10w controls (n=11) in black and 52w controls (n=11) in grey. Repeated-measures MANOVA with peripubertal diet, age and ZT as factors, revealed a significant interaction between these factors for NREM-WAKE ($F_{(1, 46)}$ = 9.266, p = 0.004) and WAKE-NREM transitions ($F_{(1, 46)}$ = 9.599, p = 0.003). The transitions from NREM-REM ($F_{(1, 46)}$ = 3.146, p = 0.083) and REM-WAKE transitions ($F_{(1, 46)}$ = 3.48, p = 0.069) interacted marginally with peripubertal diet and age. Post-hoc tests were Bonferroni adjusted. *P < 0.05. Modified from Gazea et al., submitted to PNAS.

1.1.3 Slow-wave activity in ppDIO mice

We further compared differences in sleep quality, if any, between control and ppDIO mice at 10 and 52 w of age. Slow-wave activity (SWA) during NREMS serves as an index of homeostatic control, because SWA is supposed to accumulate during a waking period and it declines during sleep. Therefore, SWA is normally highest at the beginning of the light period and can be used as a measure of sleep quality as well. In both 10 w and 52 w old ppDIO mice, SWA during the resting period was significantly decreased as compared to control mice of the same age (Fig. 24, p < 0.05). The effect on SWA activity at 52 w was depending on time. This finding suggests that sleep quality was decreased in mice with a history of ppDIO.
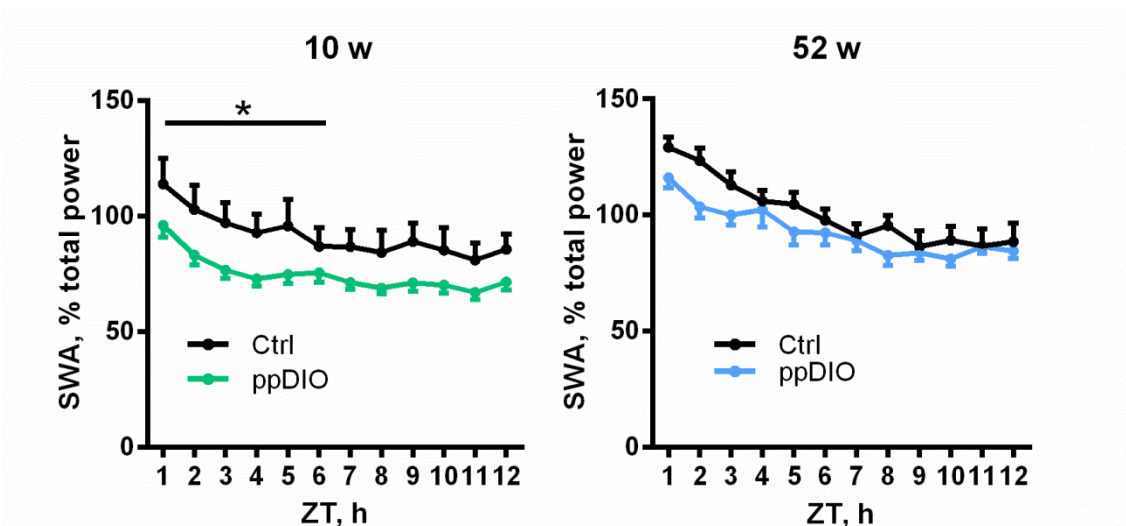

Figure 24: Slow-wave activity during NREMS in 10 w and 52 w old ppDIO and control mice.

For figure legend please refer to the next page.

Fig.24 Slow-wave activity during NREMS in 10 w and 52 w old ppDIO and control mice.

Data points represent hourly changes in SWA during NREMS in percentage ± SEM. Zeitgeber time (ZT) 0 to 12 refers to the light (resting) period. Data from ppDIO mice still receiving HFD (10 w: n=17) are depicted in green, while ppDIO mice that were switched to NC (52 w: n=7) are shown in blue. Control mice are drawn in black (10 w: n=9; 52 w: n=7). Two-way ANOVA showed that SWA was significantly decreased in ppDIO mice at 10 w (diet effect: $F_{(1, 24)}$ = 4.869, p = 0.0372) and at 52 w as compared to controls (diet*time interaction: $F_{(11, 132)}$ = 1.904, p = 0.0441).Sidak's post-hoc test was performed to detect group differences per hour. *P<0.05. Modified from Gazea et al., submitted to PNAS.

1.3 Impact of ppDIO on life-time body weights and energy intake

Following the observation that ppDIO leads to life-long alterations in sleep-wake behaviors, we hypothesized that body weights and food intake regulation may also be affected by a history of ppDIO. Therefore, we determined weekly body weight and energy intake profiles in ppDIO and control mice during aging (Fig. 25). The groups depicted in Fig. 25 were used as 52 w old group in this section.

Body weights increased significantly during aging in both ppDIO and control mice (Fig. 25A, p < 0.05). In addition, a history of ppDIO amplified body weight gain during aging (p < 0.05). More specifically, body weights were significantly higher in ppDIO mice at nine and ten weeks of age (p < 0.05), reflecting the time points of five and six weeks of HFD exposure, respectively.

Based on the assumption that the maintenance and the attainment of a specific body weight require a distinct amount of energy intake, we normalized weekly caloric intake to body weights in ppDIO and control mice. Using this normalization, we aimed to estimate whether food intake behaviors were matching body weight requirements in ppDIO mice as compared to control animals. We found a significant elevation in energy intake during the first five weeks of HFD exposure in ppDIO mice as compared to NC-fed mice (Fig. 25B, p < 0.05). However, during the sixth week of HFD exposure adjusted energy intake did not differ significantly between ppDIO and control mice.
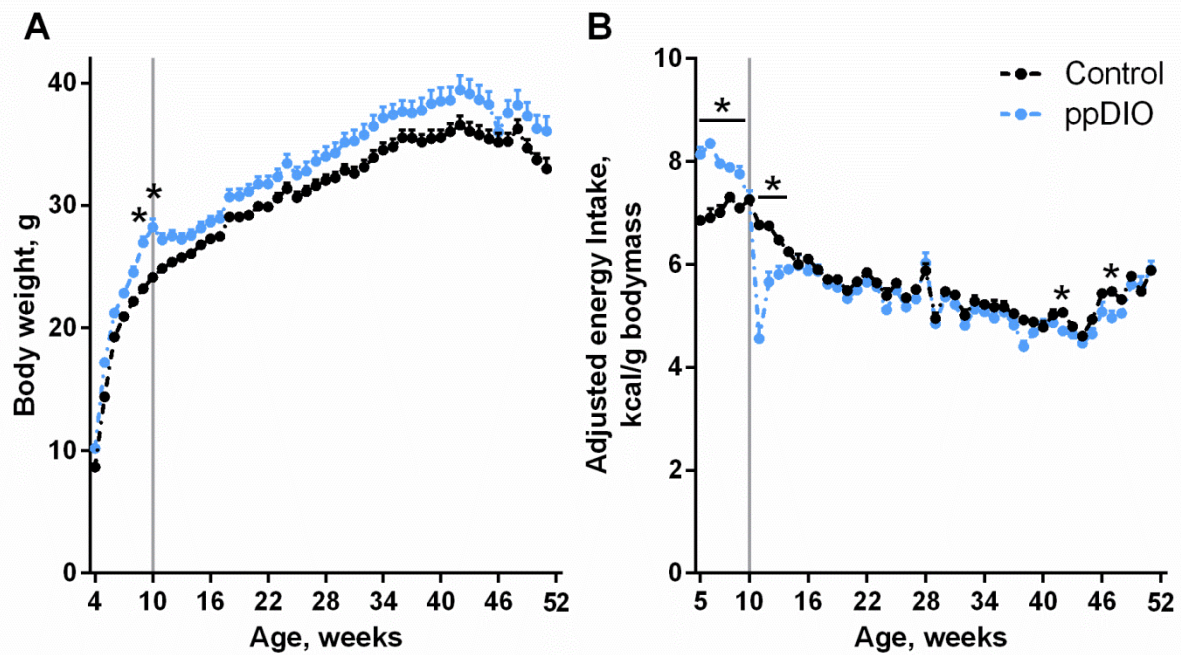
Figure 25: Body weights and adjusted energy intake in ppDIO and control mice during aging.

Body weights (A) are given as mean gram per week ± SEM. Adjusted energy intake (B) is also depicted per week and given as mean $kcal/g^{0.568}$ body weight ± SEM. Control mice are depicted in black (n=15) and ppDIO mice in blue (n=16). Two-way ANOVA showed that body weights were affected significantly by diet ($F_{(1, 1392)}$ = 241.7, $p < 0.0001$) and age ($F_{(47, 1392)}$ = 149.1, $p < 0.0001$), but not by their interaction. Adjusted energy intake was dependent on the interaction of age with peripubertal diet ($F_{(47, 288)}$ = 12.09, $p < 0.0001$), as well as by each factor separately (diet: $F_{(1, 288)}$ = 27.66, $p < 0.0001$; age: $F_{(47, 288)}$ = 119.2, $p < 0.0001$). Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea et al., submitted to PNAS.

Interestingly, after switching HFD with NC, ppDIO mice exhibited a significant hypophagia as shown by decreased adjusted energy intake during weeks 11 to 13 of life ($p < 0.05$). Afterwards, adjusted energy intake was not significantly different between the two diet groups, except for weeks 38 and 47 ($p < 0.05$, mice were transferred to other rooms in the animal facility).
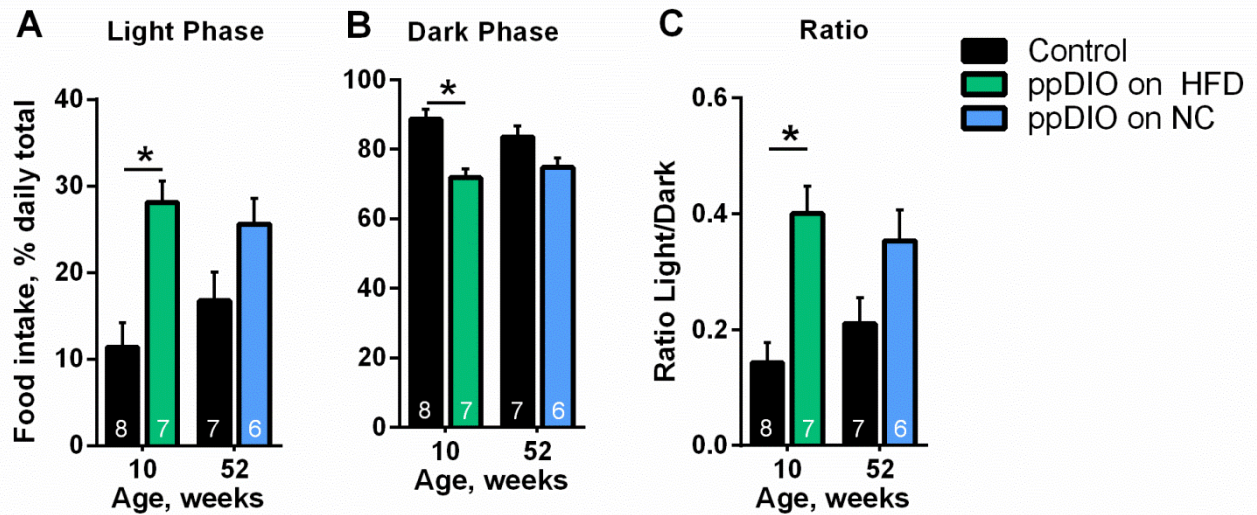
Figure 26: Food intake during the light/dark period in ppDIO and control mice at 10 w and 52 w of age.

Food intake during the light (A) and dark (B) period and the ratio of food eaten during the light versus dark (C) are given as percentage of daily total ± SEM. Two-way ANOVA showed that food intake was affected significantly by peripubertal diet regimen during the resting (F (1, 24) = 18.9, p = 0.0002) and the active phase (F (1, 24) = 19.59, p = 0.0002). The ratio of light/dark food intake was also influenced by peripubertal diet regimens (F (1, 24) = 20.18, p = 0.0002). Sidak's multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea et al., submitted to PNAS.

Previous reports suggested that circadian rhythms are disrupted in obese humans and rodents and may promote food intake during the wrong time of day. To address this relationship, we compared the amount of food consumption during the light and the dark period in 10 w and 52 w ppDIO with that in control mice.

ppDIO mice displayed higher relative food intake during the light period (Fig. 26A, p < 0.05) and lower relative food intake during the dark period (Fig. 26B, p < 0.05). 10 w old ppDIO mice exhibited an imbalance in the food intake ratio as compared to controls (p < 0.05), because their food consumption was significantly shifted from the active to the resting phase (p < 0.05). In 52 w ppDIO a trend for an imbalance was observed, but statistical analysis did not reveal significant group differences.

1.4 Central adaptations underlying ppDIO-induced changes in sleep-wake behaviors

1.4.1 Adaptations in ARC first-order and LH second-order neurons

To understand which central mechanisms may have contributed to programming of sleep-wake behaviors by ppDIO, we examined the mRNA expression of peptides that are involved in food intake regulation. We focused our analysis on 10 w and 52 w old ppDIO and control mice, because these two age groups exhibited significant sleep changes. We hypothesized that signaling in centers involved in sleep-wake and food intake regulation was altered by DIO during peripuberty and that these changes may re-appear later in life.

First, we determined whether first-order neurons in the ARC were affected by ppDIO. For this purpose, we performed radioactive RNA *in situ* hybridization of NPY in the ARC (Fig. 27A). Quantification of the NPY signal showed that 10 w old ppDIO mice displayed a significantly lower NPY mRNA expression than control mice of the same age. This effect was not apparent in the 52 w old group.

Next, to investigate whether changes in the lateral hypothalamus may have contributed to ppDIO-induced changes in sleep-wake behaviors, we quantified mRNA expression of MCH and orexin in the lateral hypothalamus by radioactive RNA *in situ* hybridization (Fig. 27B and C). We observed a significant decrease in LH MCH expression in 52 w old ppDIO mice as compared to controls (Fig. 27C, $p < 0.05$).

Orexin expression was not significantly affected by diet or age (Fig. 27A).
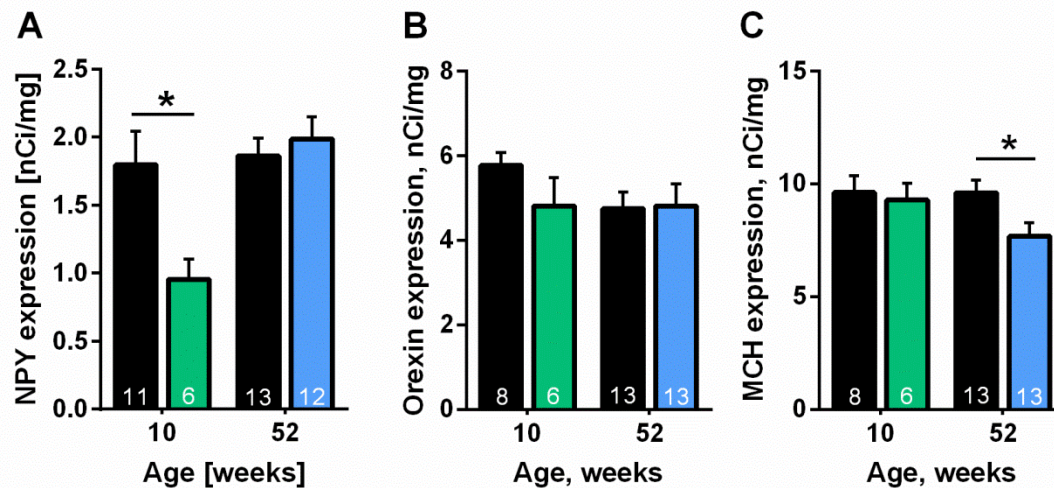
Figure 27: Changes in neuropeptide expression related to food intake regulation in 10 w and 52 w old ppDIO and control mice.

ARC NPY (A), LH orexin (B) and MCH (C) mRNA expression were determined by radioactive RNA in situ hybridization. Two-way ANOVAs showed that impacted significantly on NPY mRNA expression (F $(1, 38) = 8.067$, $p = 0.0072$) and that diet interacted significantly with the age effect (F $(1, 38) = 6.274$, $p = 0.0167$). Two-way ANOVA did not show any changes in orexin and MCH mRNA expression by diet or age, but Sidak's post-hoc test showed significant group differences MCH expression in 52 w old mice. *$P < 0.05$. Values are given as mean ± SEM. Modified from Gazea et al., submitted to PNAS.

1.4.2 Adaptations in neurotransmitter systems

Catecholamines have been implicated in the regulation of both feeding and sleep-wake behaviors. In addition, especially the dopamine system undergoes remodeling during peripuberty. To examine whether signaling of dopaminergic and serotonergic systems were changed by ppDIO, we measured concentrations of neurotransmitters and their metabolites by HPLC.

We first determined dopamine and serotonin levels in the LH (Fig. 28 and 29). Our measurements showed that serotonin was significantly reduced in the LH of 52 w old ppDIO mice as compared to 52 w old controls (Fig. 28A, $p < 0.05$). The concentration of serotonin and its metabolite 5-HIAA increased significantly with age ($p < 0.05$), but peripubertal diet regimens did not affect 5-HIAA concentrations in the LH (Fig. 28B). Further, serotonin turnover decreased significantly during aging (Fig. 28C, $p < 0.05$).
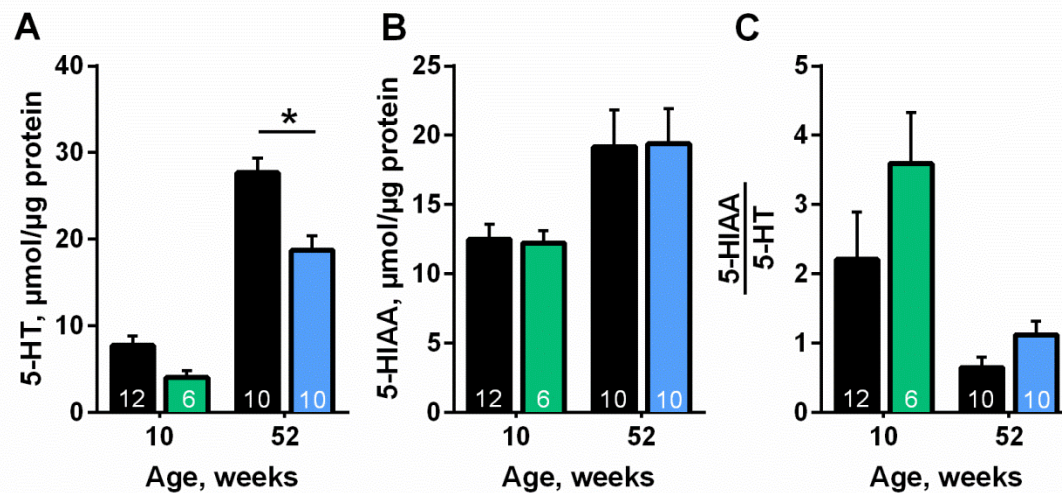
Figure 28: Changes in the LH serotonin system of 10 w and 52 w old ppDIO and control mice.

Concentrations of (**A**) serotonin (5-HT) and (**B**) its metabolite 5-HIAA in the LH were determined by HPLC from the tissue homogenates. Serotonin (**C**) turnover was calculated as described above. Values are given as mean ± SEM. Two-way ANOVAs showed that age ($F_{(1, 34)}$ = 132.3, $p < 0.0001$) and diet ($F_{(1, 34)}$ = 17.49, $p = 0.0002$) altered 5-HT levels in the LH significantly. Age affected 5-HIAA concentrations ($F_{(1, 34)}$ = 10.33, $p = 0.0029$) and 5-HT turnover ($F_{(1, 34)}$ = 14.75, $p = 0.0005$) significantly. Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea et al., submitted to PNAS.

In addition, concentrations of LH dopamine and its metabolites DOPAC and HVA increased significantly during aging (Fig. 29A, C and D, $p < 0.05$). Dopamine turnover was not influenced by age or diet (Fig. 29B). Peripubertal diet regimens did not impact significantly on the LH dopamine system.
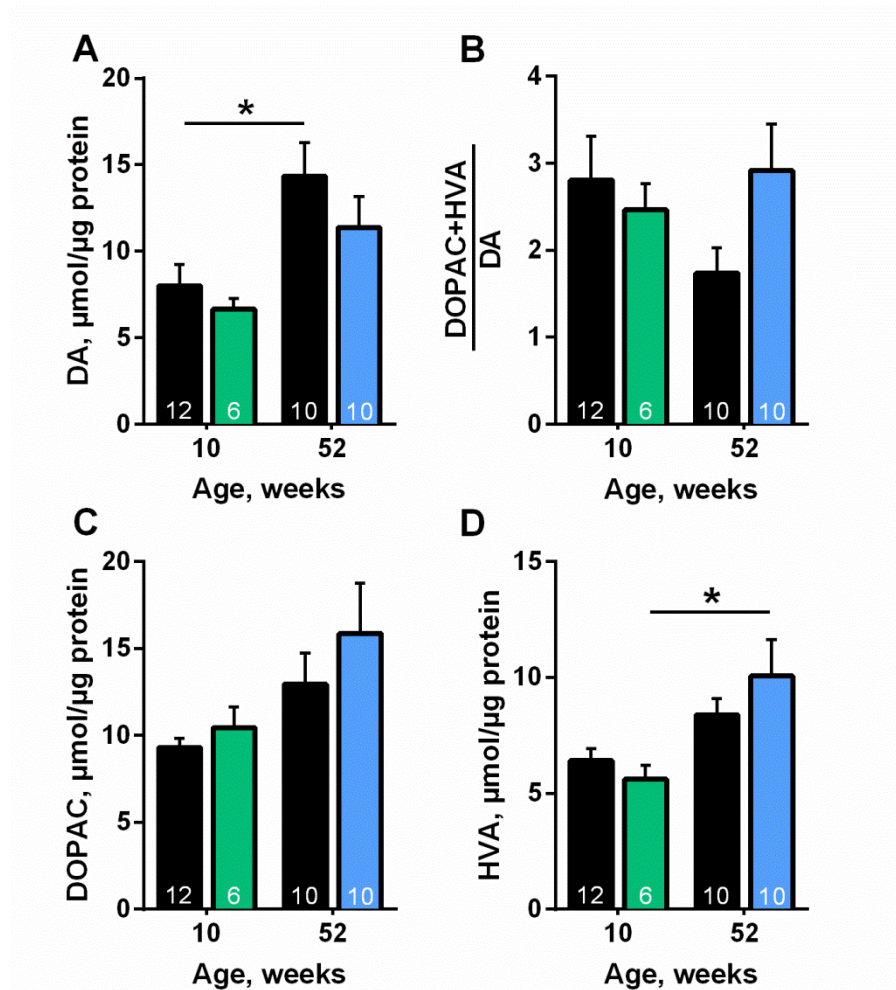
Figure 29: Changes in the LH dopamine system of 10 w and 52 w old ppDIO and control mice.

Concentrations of (**A**) dopamine (DA) and its metabolites DOPAC (**C**) and HVA (**D**) in the LH were determined by HPLC from the tissue homogenates. Dopamine (**B**) turnover was calculated as described above. Values are given as mean ± SEM. Two-way ANOVAs showed that age impacted significantly on levels of DA ($F_{(1, 34)} = 11.16$, $p = 0.002$), DOPAC ($F_{(1, 34)} = 5.644$, $p = 0.0233$) and HVA ($F_{(1, 34)} = 9.906$, $p = 0.0034$) in the LH. Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea et al., submitted to PNAS.

Based on the changes that we observed in LH serotonin transmission, we continued our analysis in the dorsal raphe nucleus (DR). The DR is the major production site of brain serotonin and sends projections to the LH.

Similarly to the LH, serotonin concentrations increased significantly in the DR during aging (Fig. 30A, $p < 0.05$). However, the age-related increase in serotonin was absent in 52 w old ppDIO mice as compared to 10 w old ppDIO group, contributing to the significantly lower serotonin levels than controls of the same age ($p < 0.05$). However, this difference was not reflected by Tph2 mRNA expression, the rate-limiting enzyme in serotonin synthesis (Fig. 30B). Tph2 expression was significantly

reduced in 10 w ppDIO mice as compared to age-matched controls (p < 0.05), whereas there were no significant differences in the 52 w old group, resulting in a significant increase in Tph2 mRNA expression in ppDIO mice during aging (p < 0.05).
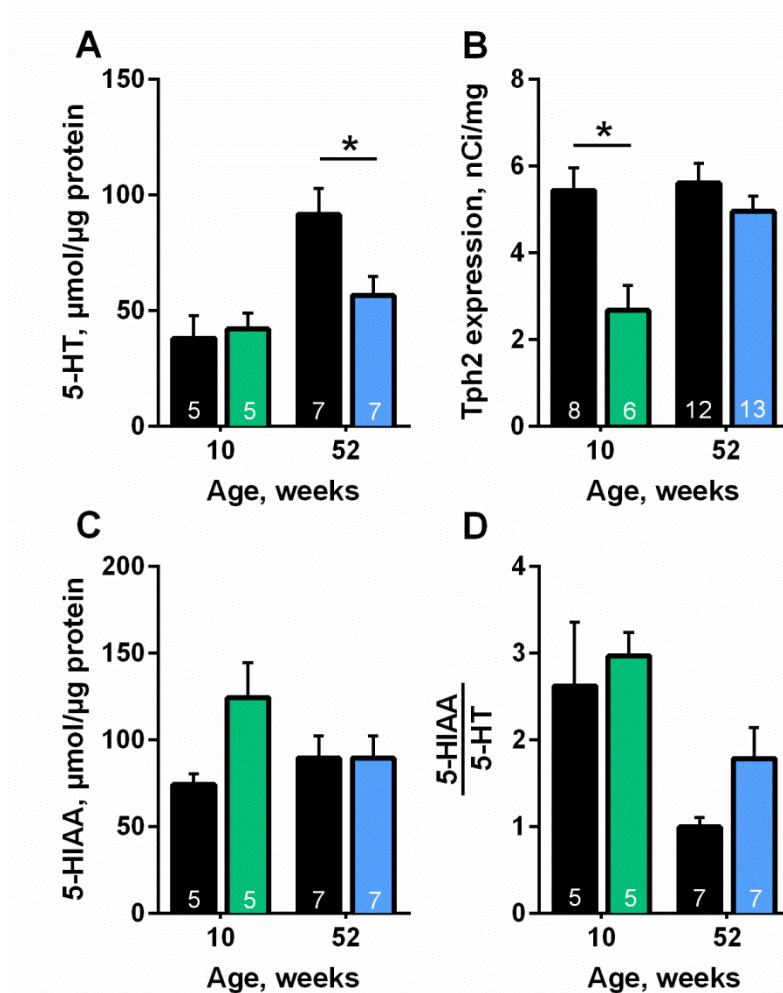
Figure 30: Changes in the serotonin system in dorsal raphe nucleus of 10 w and 52 w old ppDIO and control mice.

Concentrations of (**A**) serotonin (5-HT) and (**C**) its metabolite 5-HIAA in the DR were determined by HPLC from the tissue homogenates. Serotonin (**D**) turnover was calculated as described in the graph. Tph2 mRNA expression (**B**) was examined by radioactive RNA *in situ* hybridization on DR sections. Values are given as mean ± SEM. Two-way ANOVAs showed that age significantly impacted on 5-HT levels ($F_{(1, 20)}$ = 12.03, p = 0.0024), Tph2 mRNA expression ($F_{(1,35)}$ = 6.846, p = 0.013) and 5-HT turnover ($F_{(1, 20)}$ = 12.85, p = 0.0019) in the DR. Tph2 mRNA expression was also affected significantly by peripubertal diet ($F_{(1, 35)}$ = 13.09, p = 0.0009) and the interaction between diet and age ($F_{(1,35)}$ = 5.067, p = 0.0308). Sidak's multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea et al., submitted to PNAS.

5-HIAA concentrations in DR were not influenced significantly by ppDIO or age (Fig. 30C). However, serotonin turnover decreased significantly with age in DR (Fig. 30D, $p < 0.05$).

Due to their major implication in the hedonic aspects of food intake regulation, we further determined dopamine levels in the VTA and NAc (Fig. 31 & 32).

Figure 31: Changes in the VTA dopamine system of 10 w and 52 w old ppDIO and control mice.

Concentrations of (**A**) dopamine (DA) and its metabolites DOPAC (**C**) and HVA (**D**) in the VTA were determined by HPLC from the tissue homogenates. Dopamine (**B**) turnover was calculated as described in the graph. Values are given as mean ± SEM. Two-way ANOVAs revealed that VTA DA turnover was altered significantly age ($F_{(1,28)} = 6.709$, $p = 0.0151$), diet ($F_{(1,28)} = 5.863$, $p = 0.0222$) and the age*diet interaction ($F_{(1,28)} = 6.348$, $p = 0.0177$). Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea et al., submitted to PNAS.

10 w old ppDIO mice had significantly lower dopamine levels in the VTA as compared to control mice of the same age (Fig. 31A, $p < 0.05$). DOPAC and HVA concentrations were not significantly altered by peripubertal diet regimens or age (Fig.

31C and D). Opposite to dopamine levels, dopamine turnover was significantly increased in ppDIO mice aged 10 w as compared to age-matched controls (Fig. 31B, $p < 0.05$). This difference did not re-appear in the 52 w group.

Furthermore, we observed an age-dependent decrease in NAc dopamine concentrations (Fig. 32A, $p < 0.05$) and levels of its metabolites DOPAC (Fig. 32C, $p < 0.05$) and HVA (Fig. 32D, $p < 0.05$). However, the peripubertal diet regimen itself did not have a significant effect on the dopamine system in the NAc. Dopamine turnover was not influenced by diet or age (Fig. 32B).
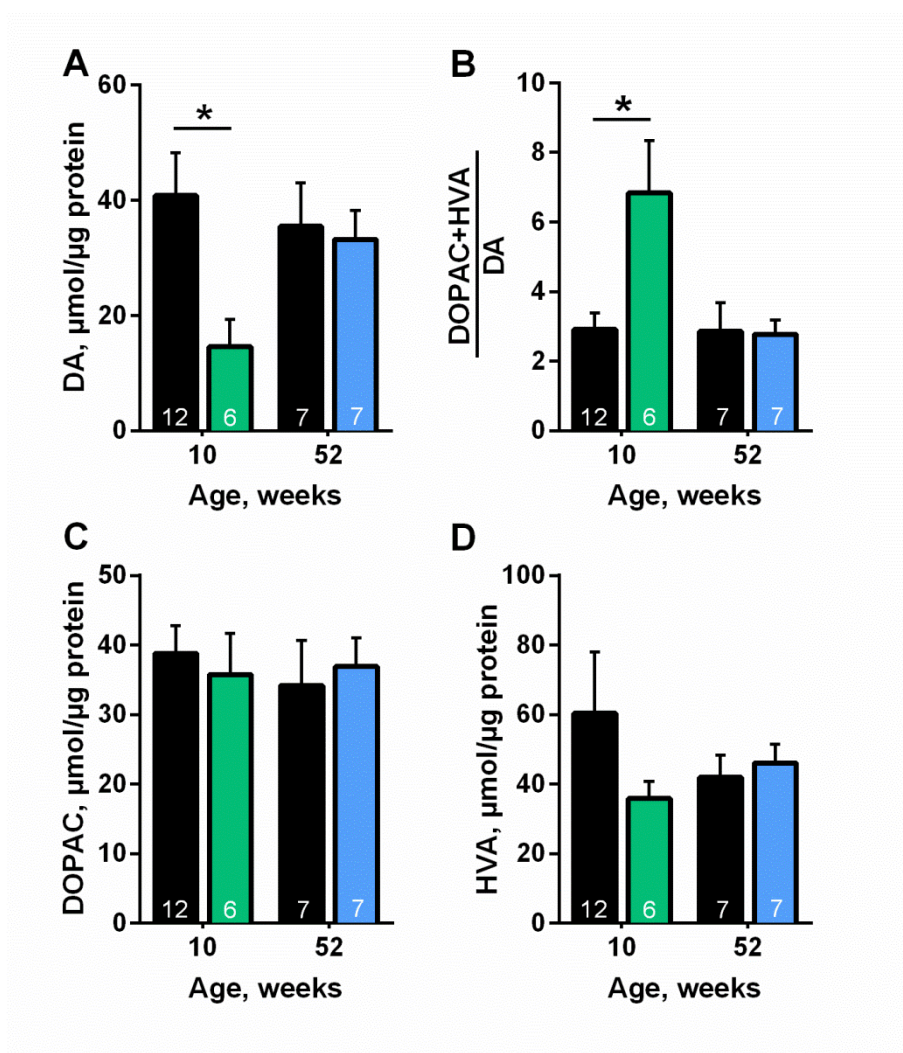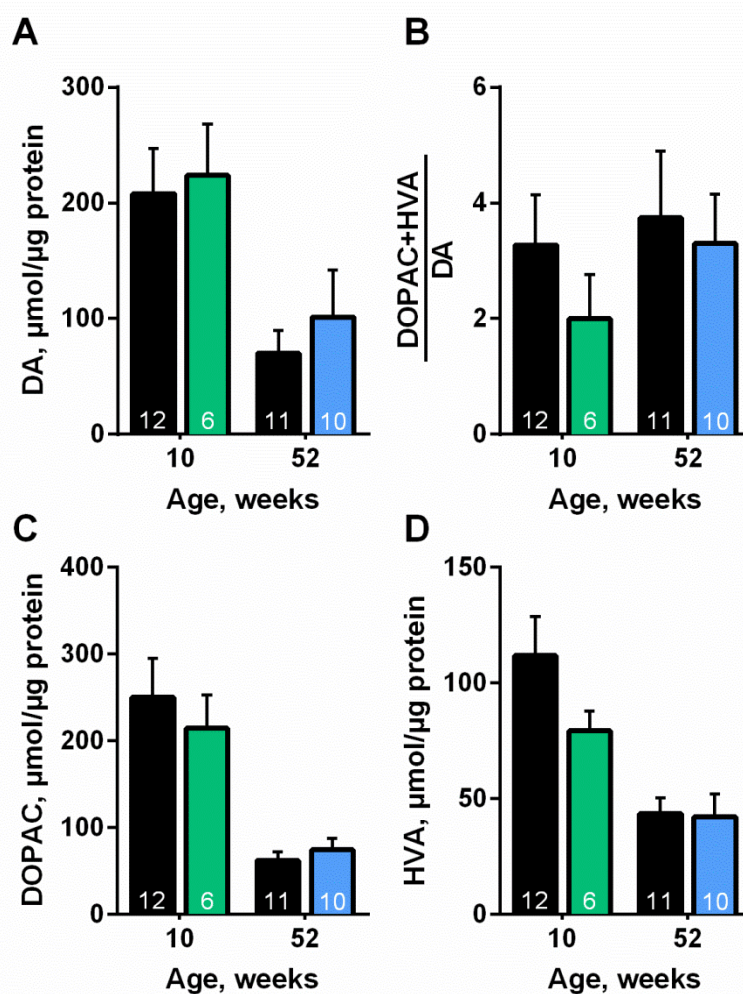

Figure 32: Changes in the NAc dopamine system of 10 w and 52 w old ppDIO and control mice.

Concentrations of (**A**) dopamine (DA) and its metabolites DOPAC (**C**) and HVA (**D**) in the LH were determined by HPLC from the tissue homogenates. Dopamine (**B**) turnover was calculated as described above. Values are given as mean ± SEM. Two-way ANOVAs revealed that age significantly influenced concentrations of NAc DA (F (1, 35) = 11.97, p = 0.0014), DOPAC (F (1, 35) = 24.87) = p < 0.0001) and HVA (F (1, 35) = 16.60, p = 0.003). Peripubertal diet did not impact on the NAc dopamine system. Sidak's multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea et al., submitted to PNAS.

1.5 Interventions to ameliorate sleep disturbances in ppDIO mice

1.5.1 Fasting and re-feeding effects on sleep in ppDIO and control mice at 52 w of age

Fasting elicits food-seeking behaviors and thereby, increases wakefulness. Therefore, we investigated, whether fasting is able to reduce sleep time in 52 w old ppDIO as efficiently as in control mice. We performed EEG/EMG recordings during the period of 24 hours of fasting and subsequent re-feeding and compared changes to the respective baselines.

After the food was removed from the food tray at light onset, all mice displayed normal sleep patterns during the light period (Fig. 33A, B, C and D). In contrast, during the dark period, fasting induced a dramatic reduction in NREMS time in both control ($p < 0.05$) and ppDIO mice ($p < 0.05$, Fig. 33A, B). After calculating the differences relative to baseline levels during 6-hour bins, a more pronounced reduction in NREMS time appeared in control as compared to ppDIO mice during the dark period (Fig. 33C, $p < 0.05$ at ZT19-24). REMS time was significantly reduced in control ($p < 0.05$), but not in ppDIO mice during fasting in the dark period. The relative REMS reduction during fasting was not significantly different between the diet groups when baseline differences were taken into account (Fig. 33D).

Re-feeding decreased NREMS time during the light period in both control ($p < 0.05$) and ppDIO mice ($p < 0.05$) as compared to baseline and fasting conditions (Fig. 33A). REMS time was suppressed in both diet groups during the light period as compared to the baseline condition (Fig. 33B, $p < 0.05$). However, the relative differences of NREMS and REMS during re-feeding between the diet groups were not significantly different (Fig. 33E, F). Regarding REMS, upon re-feeding a further reduction in REMS time was evident in control mice during the resting phase ($p < 0.05$), but not in ppDIO mice (Fig. 33B). A rebound of REMS during the dark period appeared similarly in both diet groups ($p < 0.05$).
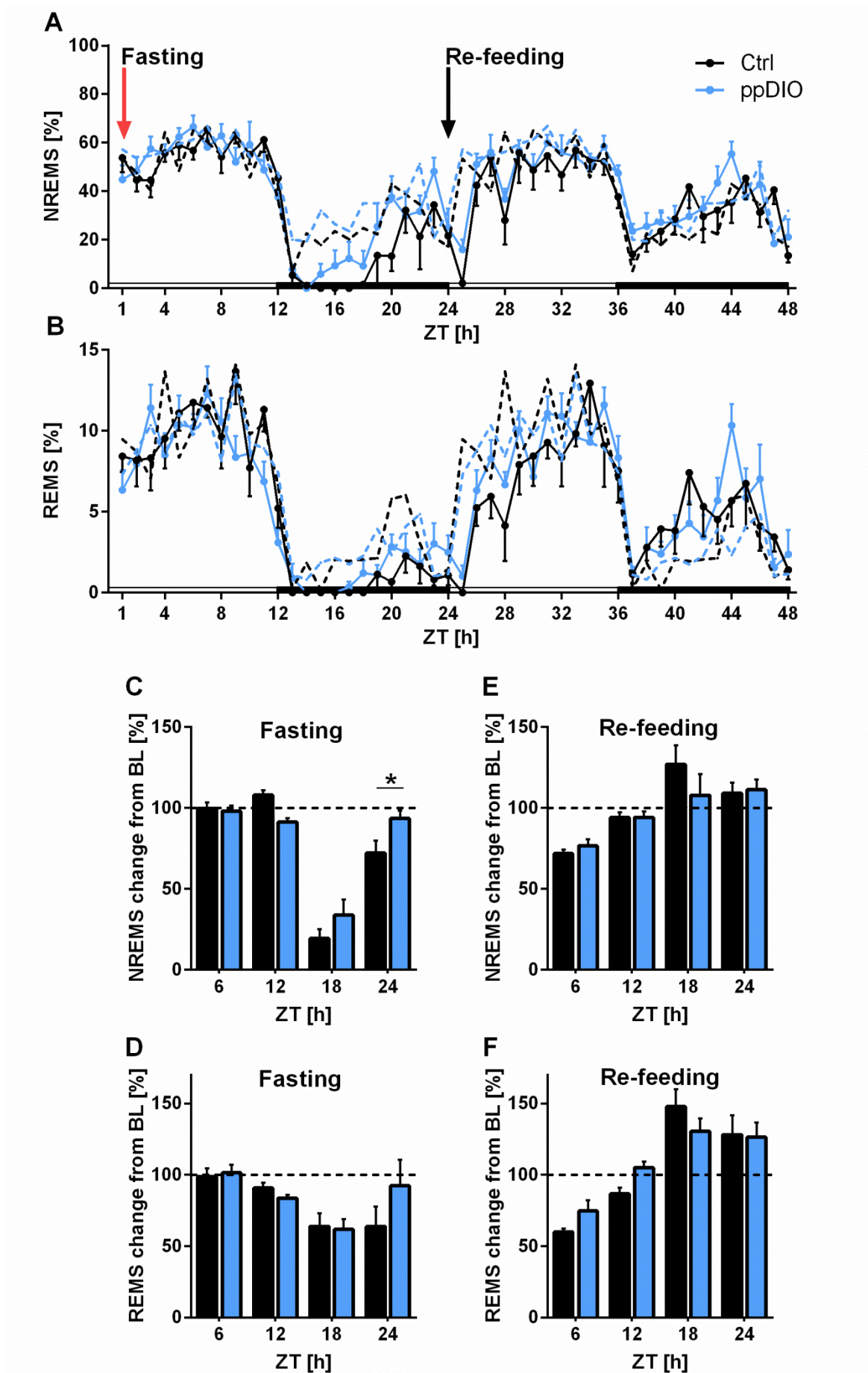
Figure 33: NREMS and REMS time during fasting and re-feeding in 52 w old ppDIO and control mice.

NREMS (A) and REMS (B) time is given in percentage per hour ± SEM during 24 hours of fasting and subsequent 24 hours of re-feeding in 52 w old ppDIO (n=7) and control mice (n=6). Respective baseline profiles are plotted by dotted lines. (C-F) Relative differences from baseline, calculated as six-hour means ± SEM of NREMS during fasting (C), re-feeding (E) and REMS during fasting (D), re-feeding (F). Two-way ANOVA showed that fasting and re-feeding induced different NREMS time responses in ppDIO and control mice during a 48-hour recording (A, diet x time interaction: $F_{(47,517)}$ = 17.25, $p < 0.0001$; diet effect: $F_{(1, 11)} = 5.086$, $p = 0.0455$), while REMS was not significantly affected in this comparison. Furthermore, fasting reduced NREMS time more dramatically in controls than in ppDIO mice (C, two-way ANOVA; diet x time interaction: $F_{(3, 33)} = 4.19$, $p = 0.0128$). Sidak's multiple comparisons test was used for post-hoc analysis. *$P<0.05$. Modified from Gazea *et al.*, submitted to PNAS.

Following 24 hours of fasting, both control and ppDIO mice aged 52 w lost weight (control mice -2.9 ± 0.43 g vs. ppDIO mice: -2.8 ± 0.27 g). The weight loss per se was not significantly different between the diet groups (data not shown).

1.5.2 PYY$_{3-36}$ rescues sleep disturbances in ppDIO mice

PYY$_{3-36}$ was previously suggested as pharmacotherapy accompanying weight loss strategies in humans. Its potent anorexigenic effects in the obese were reported previously and promised its potential as anti-obesity drug. To determine whether PYY$_{3-36}$ could also restore sleep-wake behaviors in ppDIO mice, we injected PYY$_{3-36}$ or vehicle i.p. prior to dark onset and performed EEG/EMG recordings and monitoring food intake after the injections. The main effects of PYY$_{3-36}$ were observed during the first hour following the injection. Therefore, only the first post-injection hour is depicted in Fig. 34.

In 10 w old mice, initial differences in NREMS time under vehicle conditions were abolished following injections of PYY$_{3-36}$ (Fig. 34A, $p < 0.05$). Such equalization occurred, because PYY$_{3-36}$ decreased NREMS time significantly in ppDIO mice ($p < 0.05$). However, PYY$_{3-36}$ promoted NREMS time in 10 and 52 w old control animals.

Along with 10 w old ppDIO mice, significant differences in NREMS amount between 52 w ppDIO and control mice under vehicle conditions ($p < 0.05$) were diminished following PYY$_{3-36}$ treatment (Fig. 34B). This effect was mainly due to increased NREMS time in controls and decreased NREMS time in ppDIO mice aged 52 w, as seen in the 10 w old group.
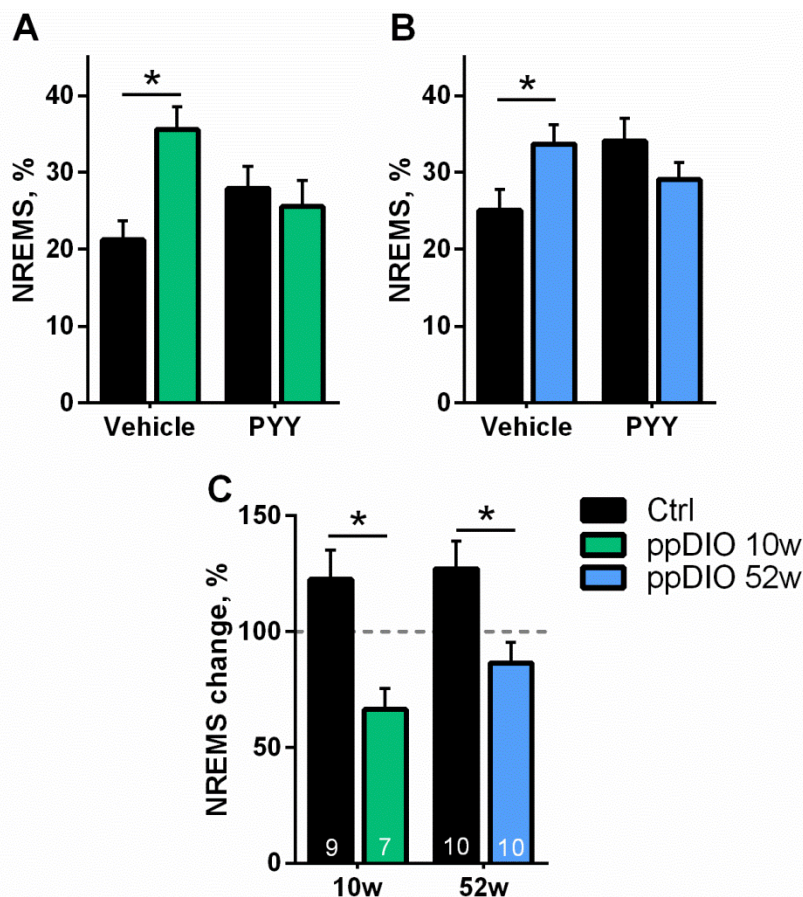
Figure 34: PYY$_{3-36}$ effects on NREMS time during the first hour of the active phase in 10 w and 52 w old ppDIO and control mice.

NREMS amounts are given as mean percentage per hour ± SEM in 10 w (**A**) and 52 w old (**B**) ppDIO and control mice. The change intensity of NREMS following PYY$_{3-36}$ as compared to vehicle injections is given as mean percentage ± SEM (**C**). Two-way ANOVA revealed a significant diet and treatment interaction in 10 w (F (1, 14) = 14.72, p = 0.0018) and 52 w (F (1, 18) = 8.3, p = 0.0099) mice. Change intensity of NREMS time was dependent on peripubertal diet (F (1, 32) = 19.19, p = 0.0001). Sidak's multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea et al., submitted to PNAS.

To further illustrate the efficacy of PYY$_{3-36}$ treatment, we calculated the change intensity of NREMS time following PYY$_{3-36}$ relative to vehicle injections (Fig. 34C). This type of analysis showed that PYY$_{3-36}$ affected NREMS time depending on the dietary background; both 10 w and 52 w old ppDIO mice displayed a significant suppression of NREMS time in response to PYY$_{3-36}$, whereas controls showed an increase in NREMS time reaching levels above 100 % of the vehicle condition (p < 0.05). Food intake behaviors were equally suppressed by PYY$_{3-36}$ treatment irrespective of peripubertal diet regimen or age (data not shown), exemplifying that specifically NREMS time is differentially influenced by PYY$_{3-36}$ treatment in ppDIO and control mice.

2 Neuroendocrine and behavioral responses to stress in mice with a history of ppDIO

Obesity is associated with alterations in HPA axis activity and is often accompanied by depressive symptoms. Since puberty is a time when stress systems are still developing, DIO during peripuberty may impact on the establishment of the HPA axis. To investigate whether ppDIO may program HPA axis activity and stress-related behaviors, we compared basal and stress-induced serum levels of corticosterone in ppDIO versus control mice during aging. In addition, we examined stress-coping behaviors in our ppDIO model.

2.1 Changes in HPA axis activity in ppDIO mice during aging

To investigate whether a history of ppDIO affects HPA axis activity, we compared corticosterone (CORT) serum concentrations of mice aged 12 w, 24 w and 52 w. The basal levels were examined from tail blood samples at around ZT0 and ZT12.

CORT concentrations at ZT0 and ZT12 were higher when mice were aged (Fig. 35A, B, $p < 0.05$). 52 w old ppDIO mice showed elevated CORT levels at ZT12 as compared to 52 w old controls in the post-hoc test ($p < 0.05$). Further effects of peripubertal diet on basal corticosterone levels were not found.

In response to a six-minute forced swim test (FST, performed between ZT2 and 4), dramatic increases in CORT levels were evident as compared to baseline conditions (Fig. 35C, compare to Fig. 35A). Again, CORT stress responses became significantly higher when animals got older ($p < 0.05$). Depending on age, peripubertal diet regimens impacted on stress-related CORT concentrations ($p < 0.05$); 12 w old ppDIO mice displayed lower CORT levels than controls after stress, while 24 w old ppDIO mice exhibited a higher CORT response than controls, both of which may contribute to the overall statistical interaction between diet and age.

Two hours after termination of the FST, CORT concentrations were still significantly upregulated according to age (Fig. 35D, p < 0.05). However, there were no significant differences between the two dietary groups.
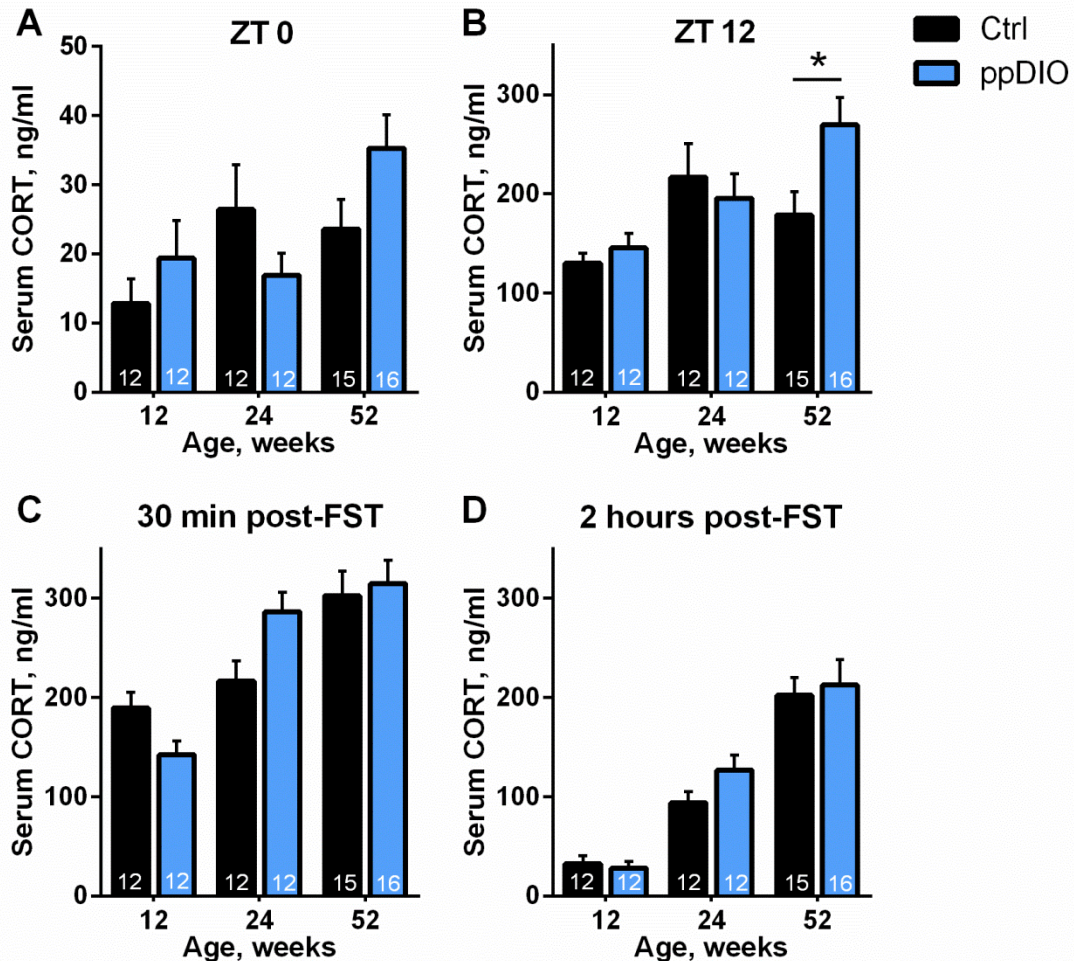
Figure 35: Corticosterone levels under baseline conditions and following forced swim test during aging.

CORT serum concentrations are given as ng/ml ± SEM at ZT0 (A), ZT12 (B), 30 minutes (C) and 2 hours (D) following a forced swim test. Age affected CORT concentrations significantly during all four conditions (two-way ANOVA, ZT0: $F_{(2, 73)} = 4.133$, $p = 0.0199$; ZT12: $F_{(2, 73)} = 6.811$, $p = 0.0019$; FST-30': $F_{(2, 73)} = 23.61$, $p < 0.0001$; FST-2h: $F_{(2, 73)} = 54.20$, $p < 0.0001$). Peripubertal diet interacted significantly with age to influence CORT concentrations 30 minutes after the FST (C, ppDIO x age interaction: $F_{(2, 73)} = 3.546$, $p = 0.0339$). Sidak's multiple comparisons test was used to determine group differences. *P<0.05. Modified from Gazea *et al.*, submitted to PNAS.

2.2 Stress coping behavior in the forced swim test in ppDIO mice during aging

To investigate whether altered HPA axis activity in ppDIO mice is reflected in stress coping behaviors during the FST, we analyzed swimming behaviors of ppDIO mice in comparison to controls (Fig. 36). In the FST, inactive time, representing the time spent floating, increased significantly during aging (Fig. 36A, $p < 0.05$). 24 w old ppDIO mice spent more time inactive in the FST than age-matched controls ($p < 0.05$). Overall, ppDIO mice were more inactive during the FST ($p < 0.05$).
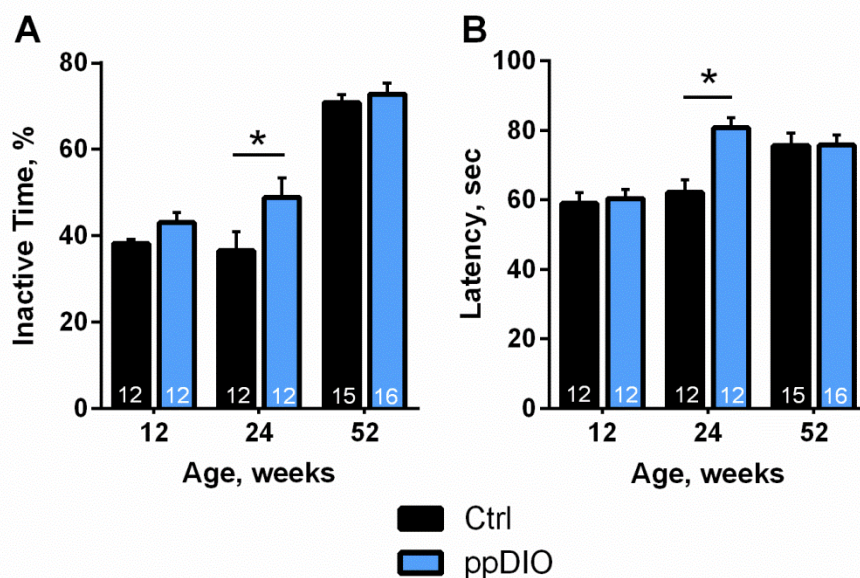
Figure 36: Stress coping behavior in the forced swim test in ppDIO and control mice during aging.

Time spent inactive (A) in percentage ± SEM and latency to being inactive / floating (B) in sec ± SEM in 12 w, 24 w and 52 w old ppDIO and control mice during an FST. Two-way ANOVA showed that inactive time and latency to floating increased significantly during aging (inactive time: $F_{(2, 73)}$ = 73.37, $p < 0.0001$; latency: $F_{(2, 73)}$ = 12.67, $p < 0.0001$). In addition, both parameters were increased further by a history of ppDIO (inactive time: $F_{(1, 73)}$ = 6,649, $p = 0.0119$; latency: $F_{(1, 73)}$ = 6,143, $p = 0.0155$). Latency to floating was further dependent on the interaction between diet and age ($F_{(2, 73)}$ = 4,800, $p = 0.011$). Sidak's multiple comparisons test served as post hoc test. *$P<0.05$. Modified from Gazea *et al.*, submitted to PNAS.

Also the latency to the first floating episode was elevated in ppDIO mice (Fig. 36B, $p < 0.05$), especially in 24 w old ppDIO mice ($p < 0.05$). The latency increased with age in both diet groups ($p < 0.05$).

Interestingly, inactive time correlated with body weights in 52 w old ppDIO mice (Fig. 37A, p < 0.05), while it did not correlate with body weights of 12 w and 24 w old ppDIO mice or control groups of any age (Fig. 37B, 52 w old controls).
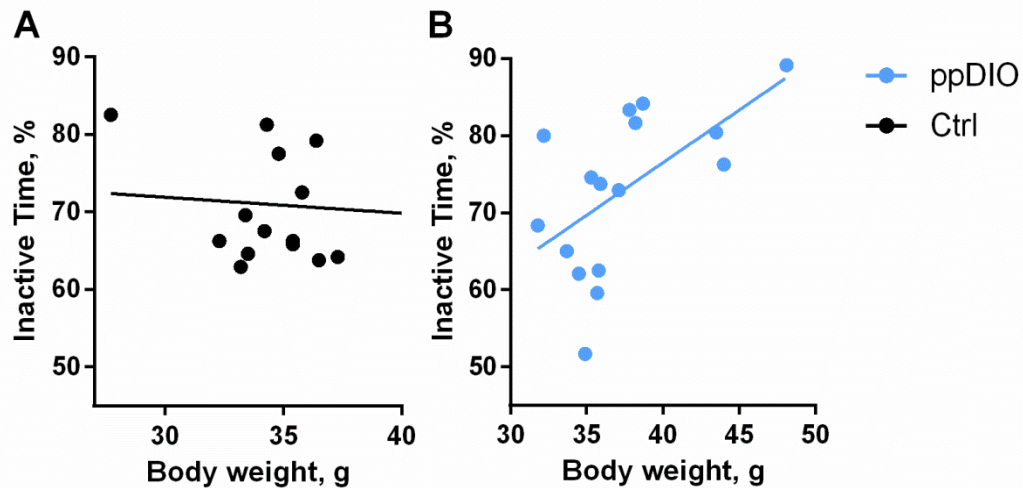
Figure 37: Time spent inactive in the forced swim test against body weights in 52 w old ppDIO and control mice.

Correlation of time spent inactive in the FST (y axis, given in percentage as in Fig. 28) with body weights (x axis, given in grams) in 52 w old control (**A**) and ppDIO mice (**B**). FST inactive time correlated with body weights in 52 w old ppDIO mice (p = 0.0183, r² = 0.3375). Modified from Gazea *et al.*, submitted to PNAS.

To overcome a potential confounding of our FST data, we used an ANCOVA to re-evaluate the data when body weights were partialled out. Also without the contribution of body weights age and peripubertal diet experience increased the time spent inactive and the latency to the first immobility in the FST (p < 0.05).

2.3 Exploratory activity in ppDIO and control mice during aging

To examine whether exploratory (locomotor) activity was generally altered in mice with a history of ppDIO, we performed an open field test. Distance traveled decreased significantly with age independent of the diet group (Fig. 38, p < 0.05). Peripubertal diet regimens did not alter distance traveled in the open field test. Other parameters that can be extracted from the open field test, such as time spent in the

center of the arena, were not significantly influenced by ppDIO either (data not shown). In contrast to the FST, body weights did not correlate with the distance traveled in the open field test (data not shown).

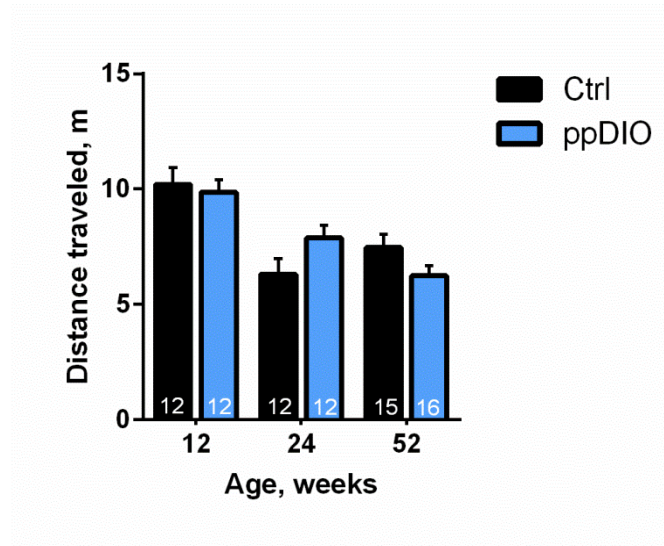**Figure 38:** Distance traveled in the open field test in ppDIO and control mice during aging.

Distance traveled is given in meters ± SEM. In the open field test, the traveled distance decreased significantly during aging in both diet groups (two-way ANOVA, $F_{(2, 73)} = 17.59$, $p < 0.0001$). Sidak's multiple comparisons test served as post hoc test. Modified from Gazea *et al.*, submitted to PNAS.

3. High-fat diet withdrawal: the impact on sleep and feeding regulatory mechanisms

Previously, it was reported that a dietary switch from high-calorie palatable foods to healthier balanced foods triggers adaptive changes in food intake regulators in the brain of obese rodents and humans. These changes would counteract weight loss. However, the exact central mechanism driving obese animals or humans to resume the overconsumption of palatable foods during the period of abstinence is still poorly understood. Furthermore, from the viewpoint of the results of Study 1 showing that chronic HFD exposure led to increased nocturnal sleep time in ppDIO mice, we aimed to examine whether HFD withdrawal rescues sleep disturbances occurring in ppDIO mice and further to determine brain regions that may provide those causal changes. Specifically we questioned:

1. How does HFD withdrawal affect ingestive and sleep-wake behaviors?

2. What are the adaptive changes in neurotransmitter and neuropeptide systems in feeding and sleep-wake regulatory centers?

3. Does HFD withdrawal trigger adaptations that are similarly elicited by fasting?

4. Does HFD re-exposure reset neurotransmitter/neuropeptide systems and sleep-wake behaviors of ppDIO mice to a condition before they experienced HFD withdrawal?

To seek answers to these questions, we compared behavioral responses (sleep, feeding) and neuropeptide/neurotransmitter levels in ppDIO mice at the end of a 6-week HFD exposure with those in another group of ppDIO mice that were withdrawn from HFD followed by exposure to NC. Mice that were maintained on NC during peripuberty served as controls. Controls were also *ad libitum* fed with NC or were fasted.

3.1 Changes in body weights and food intake after HFD withdrawal in ppDIO mice

First, we determined changes in body weights and food intake in ppDIO mice when HFD was replaced by NC. The dietary switch from HFD to NC significantly reduced

body weights in ppDIO mice within 24 hours of withdrawal, but not after 48 hours (Fig. 39A, p < 0.05). In controls, fasting produced a ten-fold greater body weight loss than HFD withdrawal in ppDIO mice (Fig. 39B, p < 0.05).
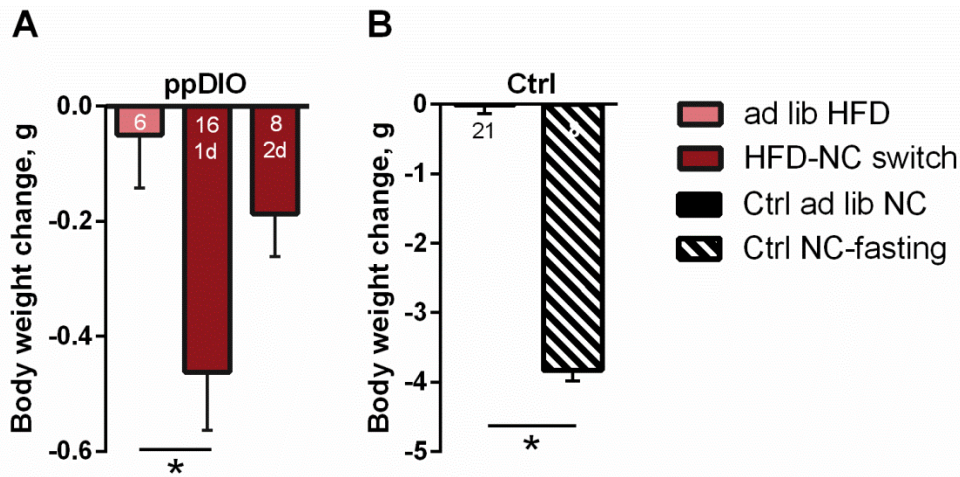

Figure 39: Body weight changes following HFD withdrawal in ppDIO mice and fasting in controls.

Daily body weight changes in gram in (**A**) ppDIO mice following *ad libitum* HFD exposure (mean value of three days, n = 6), one (1d, n = 16) or two days (2d, N = 8) of HFD withdrawal in ppDIO mice and (**B**) in control mice following *ad libitum* NC exposure (n = 21) and fasting (n = 8). Body weights were significantly reduced by the HFD-NC switch (one-way ANOVA, $F_{(2, 27)}$ = 4.01, p = 0.0299) and by fasting (unpaired t-test, t=17.57 df=27, p < 0.0001). Values are given as mean gram ± SEM. Dunnett's multiple comparisons test was used for post-hoc analysis in ppDIO mice. *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.
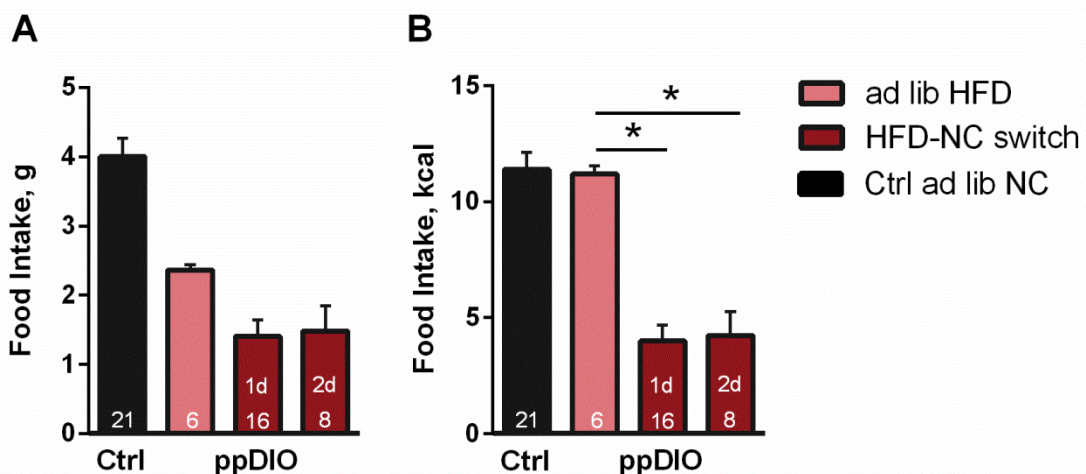
Figure 40: Food intake following HFD withdrawal in ppDIO mice.

Food intake is given in gram (A) or in kcal (B) during 24 hours of different dietary treatments in ppDIO. Food intake in control mice is depicted as a reference value in the graph. While food intake expressed in gram was not significantly affected by the diet switch, caloric intake was reduced significantly (Kruskal-Wallis test, K = 14.02, p = 0.0009). Values are given as mean gram/kcal ± SEM. Dunn's multiple comparisons test was used for post-hoc analysis in ppDIO mice. *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.

The amount of food intake was not significantly affected by the dietary switch (Fig. 40A, Kruskal-Wallis test, n.s.), but in terms of calories, energy intake was significantly reduced by the HFD-NC switch in ppDIO mice (Fig. 40B, p < 0.05). The latter difference was still evident after 48 hours following the dietary switch (p < 0.05).

3.2 Nocturnal time spent awake increases during HFD withdrawal in ppDIO mice and during fasting in controls

When ppDIO mice were switched from HFD to NC at ZT8, we observed a decrease in NREMS time during the subsequent dark period. The reduction in NREMS time reached significance during the second half of the dark period following withdrawal from HFD (Fig. 41A, p < 0.05).

To compare whether fasting in control mice would induce similar sleep changes to those after the HFD-NC switch in ppDIO mice, we monitored nocturnal sleep-wake behaviors in control mice during 24 hours of fasting. Fasting significantly decreased sleep time in controls during the dark period as compared to *ad libitum* NC feeding (Fig. 41B, p < 0.05).
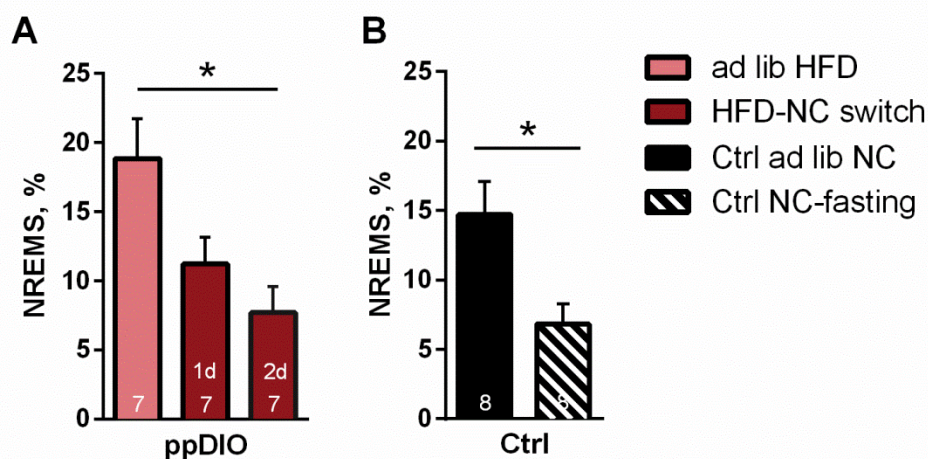
Figure 41: Changes in nocturnal NREMS time following the HFD to NC switch in ppDIO mice and fasting in control mice.

(**A**) Mean NREMS time during ZT13-18 (± SEM) in ppDIO mice at the end of a six-week HFD exposure (HFD), during the first dark period after the switch from HFD to NC (1d) and during the second dark period following the switch (2d). (**B**) Mean NREMS time during ZT13-18 (± SEM) in control mice during *ad libitum* access to NC (ad lib NC) and during fasting. As described in previous

sections, ppDIO mice displayed elevated NREMS time when maintained on HFD for six weeks as compared to controls (unpaired t-test, t=2.376, df=15, p = 0.0313). The switch from HFD to NC reduced NREMS time significantly in ppDIO mice (RM one-way ANOVA, F (1.299, 7.795) = 8.620, p = 0.0156). Fasting reduced NREMS time significantly in control mice (paired t-test: t=4.884, df=7, p = 0.0018). *P < 0.05. Dunnett's multiple comparisons test was used for post-hoc analysis in ppDIO mice. Modified from Gazea *et al.*, submitted to Front Neurosci.

We further addressed the question whether decreased nocturnal sleep time persisted for a longer time period after HFD withdrawal. After two weeks of NC-feeding, active-phase NREMS and REMS time were still significantly decreased in ppDIO mice as compared to 12 w old controls (Fig. 42, p < 0.05) and 10 w old ppDIO mice (p < 0.05). Hence, nocturnal sleep time was continuously decreased in ppDIO mice after HFD withdrawal for at least two weeks.

Interestingly, control mice showed a significant developmental increase in sleep time from 10 w to 12 w of age, whereas the withdrawal from HFD elicited an opposite direction in regard to age-related sleep enhancement (p < 0.05). REMS time also showed albeit a non-significant increase during aging in control mice.

We next examined whether acute HFD re-feeding may reverse these changes in ppDIO mice. To address this question, sleep recordings were performed when ppDIO mice were exposed to NC for two weeks and then re-exposed to HFD for 12 hours, with the HFD exposure starting at dark onset. As we expected, HFD re-feeding increased NREMS time significantly in ppDIO mice when compared to NC-2w exposure (Fig. 43, p < 0.05). REMS time was not significantly affected (data not shown).
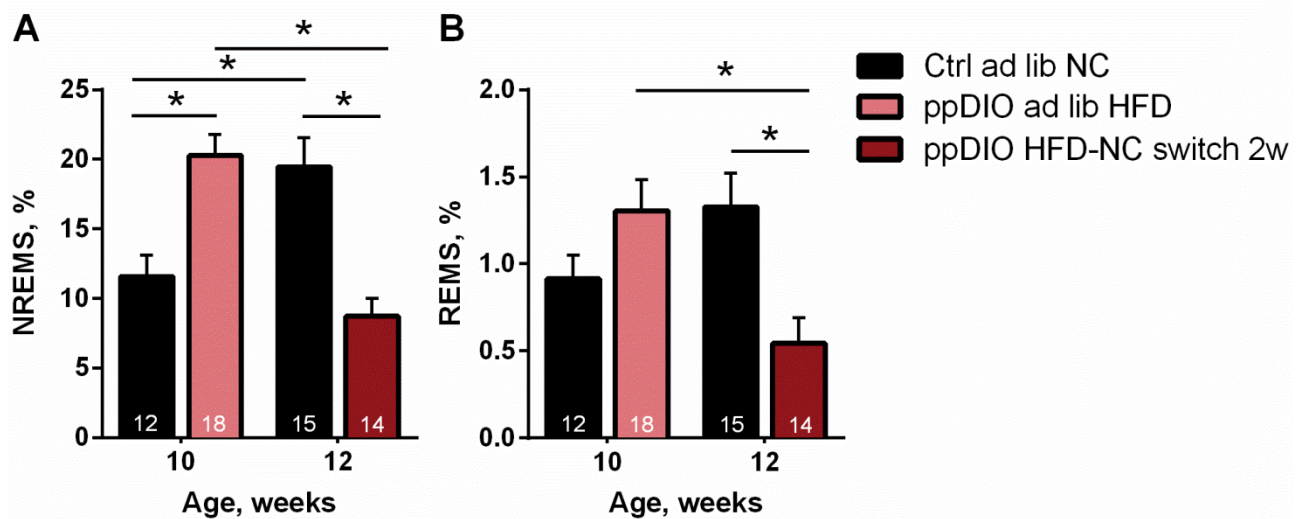
Figure 42: HFD withdrawal for 2 weeks resulted in NREMS and REMS reduction in ppDIO mice.

NREMS (A) and REMS (B) amounts are given as mean percentage during ZT13-18 ± SEM in ppDIO and control mice at 10 w and 12 w of age. At 10 w, ppDIO mice were still exposed to HFD, while at 12 w, they were withdrawn from HFD and fed NC for two weeks. Peripubertal diet regimen interacted significantly with age in terms of NREMS time (two-way ANOVA, $F_{(1, 55)} = 33.25$, $p < 0.0001$) and REMS time (two-way ANOVA, $F_{(1, 55)} = 11.39$, $p = 0.0014$). Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea *et al.*, submitted to Front Neurosci.
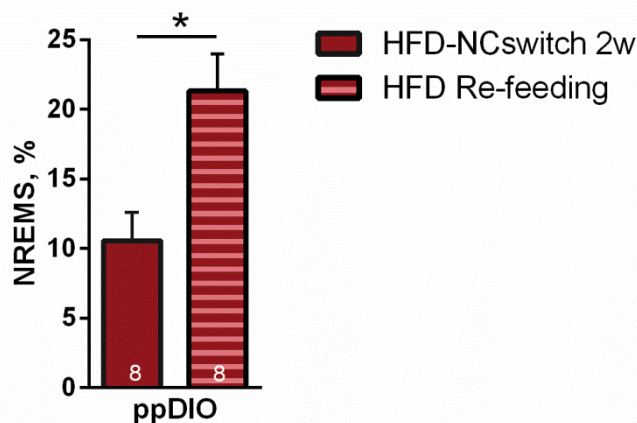
Figure 43: Changes in NREMS time during HFD re-feeding following the HFD-NC switch in ppDIO mice.

Mean NREMS time during ZT13-18 (± SEM) in ppDIO mice fed NC for two weeks and re-exposed to HFD during the dark period. HFD re-feeding significantly increased NREMS time in ppDIO mice (paired t-test, t = t=3.712 df=7, $p = 0.0075$). *$P < 0.05$. Modified from Gazea *et al.*, submitted to Front Neurosci.

3.3 Neural adaptations after HFD withdrawal or fasting in brain regions conducting homeostatic and reward-associated regulation

We hypothesized that HFD withdrawal may alter neurochemical signaling in brain regions that are involved in the homeostatic and hedonic control of food intake. Therefore, we compared neurotransmitter and neuropeptide levels among three groups of ppDIO mice: 1) HFD *ad libitum* fed (ad lib HFD), 2) HFD-withdrawal followed by NC-exposure for 24 hours (HFD-NC switch) and 3) one hour of HFD re-feeding after 23 hours of HFD-NC switch (HFD Re-feeding). As a control for homeostatic activation of food intake behaviors, two control groups were included as well: 1) NC *ad libitum* fed (Ctrl ad lib NC) and 2) fasting for 24 hours (Ctrl NC-fasting).

3.3.1 Expression of neuropeptides that promote food intake

Firstly, we analyzed neuropeptide expression in areas related to food intake regulation, such as the ARC and LH. Switching from HFD to NC significantly upregulated NPY expression in the ARC of ppDIO as compared to *ad libitum* HFD-fed animals (Fig. 44A, $p < 0.05$). Similarly, fasting elevated NPY expression significantly in control mice (Fig. 44D, $p < 0.05$). In addition, orexin expression increased after fasting in controls (Fig. 44F, $p < 0.05$). Interestingly, the diet challenges did not induce significant changes in orexin and MCH expression in the LH in ppDIO mice (Fig. 44B and C).
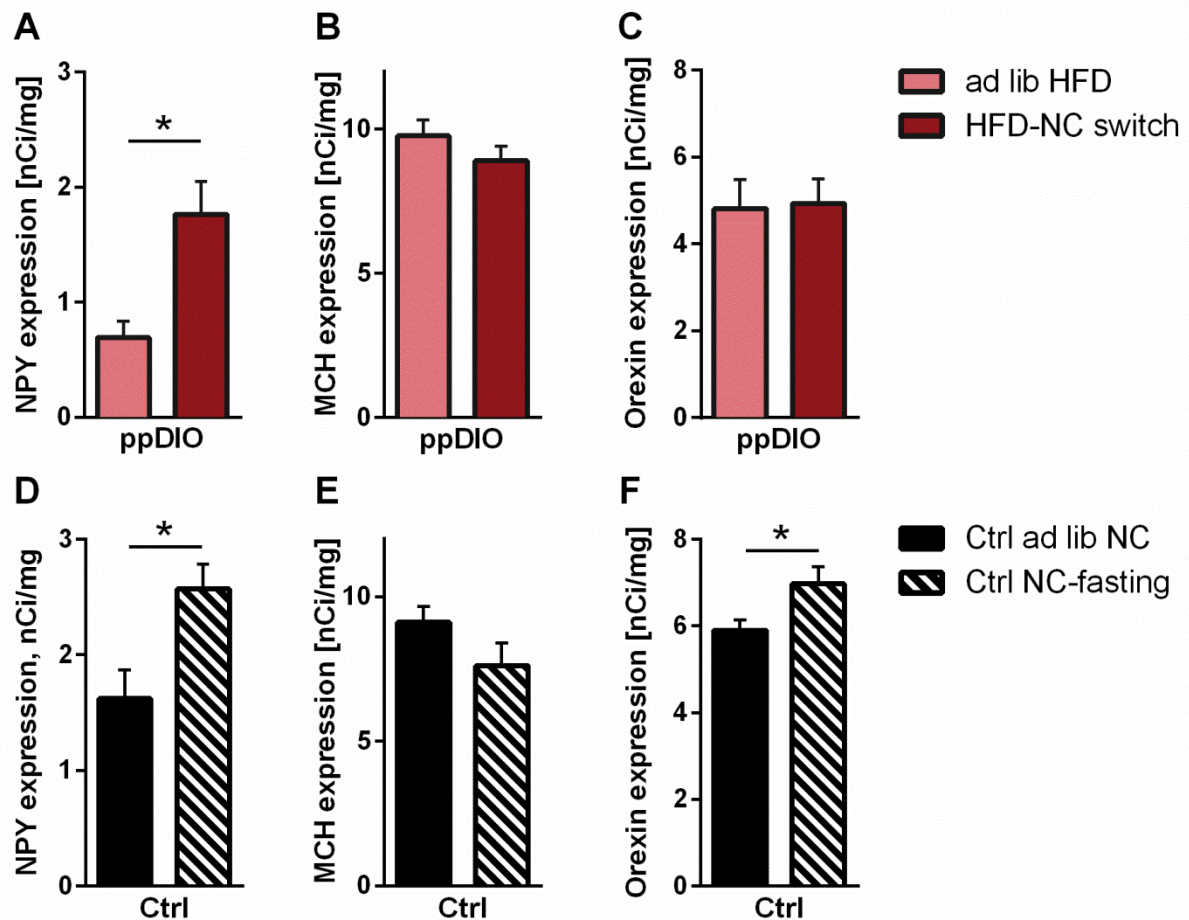
Figure 44: Changes in neuropeptide levels in the hypothalamus of ppDIO and control mice following HFD withdrawal and fasting, respectively.

mRNA expression of NPY in the ARC (**A, D**), MCH in the LH (**B, E**) and orexin in the LH (**C, F**) were determined by radioactive RNA *in situ* hybridization. Values are given as mean ± SEM. In ppDIO mice, withdrawal from HFD increased NPY mRNA expression (**A**, unpaired t-test, t=3.056, df=12, p = 0.01), whereas orexin and MCH expression were not significantly affected by the diet change. In control mice, fasting increased NPY (**D**, unpaired t-test, t=2.625, df=16, p = 0.0184) and orexin expression (**F**, unpaired t-test, t=2.420 df=17, p = 0.027). *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.

However, orexin peptide levels extracted from the LH tissue showed a different pattern than orexin mRNA expression; HFD withdrawal increased orexin concentrations in the LH of ppDIO mice (Fig. 45A, p < 0.05), similarly to the increase seen in controls after fasting (Fig. 45B, p < 0.05). Interestingly, HFD re-feeding led to the return of orexin concentrations to *ad libitum* HFD levels in ppDIO mice (Fig. 45A).
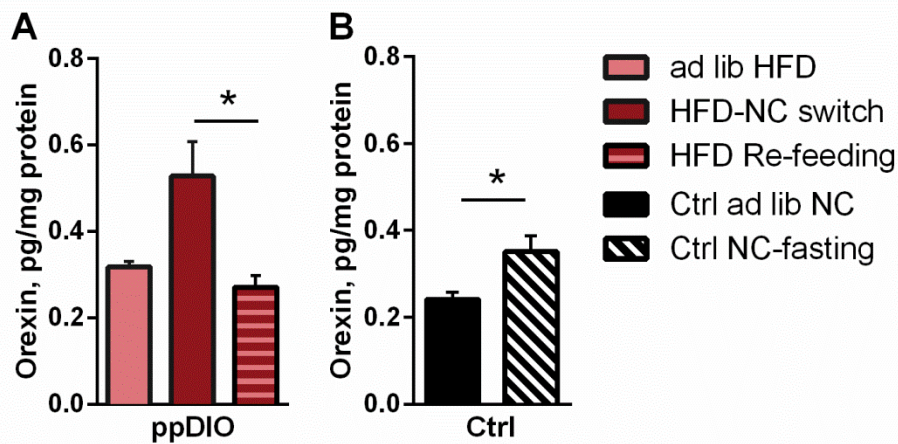
Figure 45: Changes in LH orexin concentrations of following HFD withdrawal in ppDIO mice and fasting in controls.

Orexin peptide concentrations in the LH of ppDIO mice after HFD withdrawal and re-feeding (**A**) and after fasting in control mice (**B**) were determined by RIA from the tissue homogenates. Values are given as pg/mg protein ± SEM. In ppDIO mice, withdrawal from HFD increased orexin concentrations in the LH as compared to ad libitum HFD exposure or HFD re-feeding (Kruskal-Wallis test, K = 7.892, p = 0.0134). Similarly, in control mice, fasting increased orexin concentrations (Kolmogorov-Smirnov test, D = 0.75, p = 0.0112). Dunn's multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.

3.3.2 Serotonin and dopamine neurotransmission in brain regions regulating homeostatic and hedonic impacts of food intake

To further understand how HFD withdrawal impacts on sleep and feeding behaviors, we continued our analysis in the LH, VTA, DR and NAc.

In the LH, the switch from HFD to NC significantly increased serotonin concentrations (Fig. 46A, p < 0.05) as compared to chronic HFD exposure. LH dopamine levels did not differ significantly between ppDIO mice that were maintained on HFD vs. NC (Fig. 46B), but its turnover was significantly increased in ppDIO mice exposed to one day of NC as compared to HFD-fed ppDIO mice (Fig. 46D, p < 0.05). Serotonin turnover was significantly increased when HFD was returned for one hour as compared to 24 hours of NC feeding (Fig. 46C, p < 0.05). Further, HFD re-feeding reduced dopamine turnover in ppDIO mice to *ad libitum* HFD feeding levels (Fig. 46D, p < 0.05). In controls, none of these parameters in the LH were altered in response to fasting (data not shown).
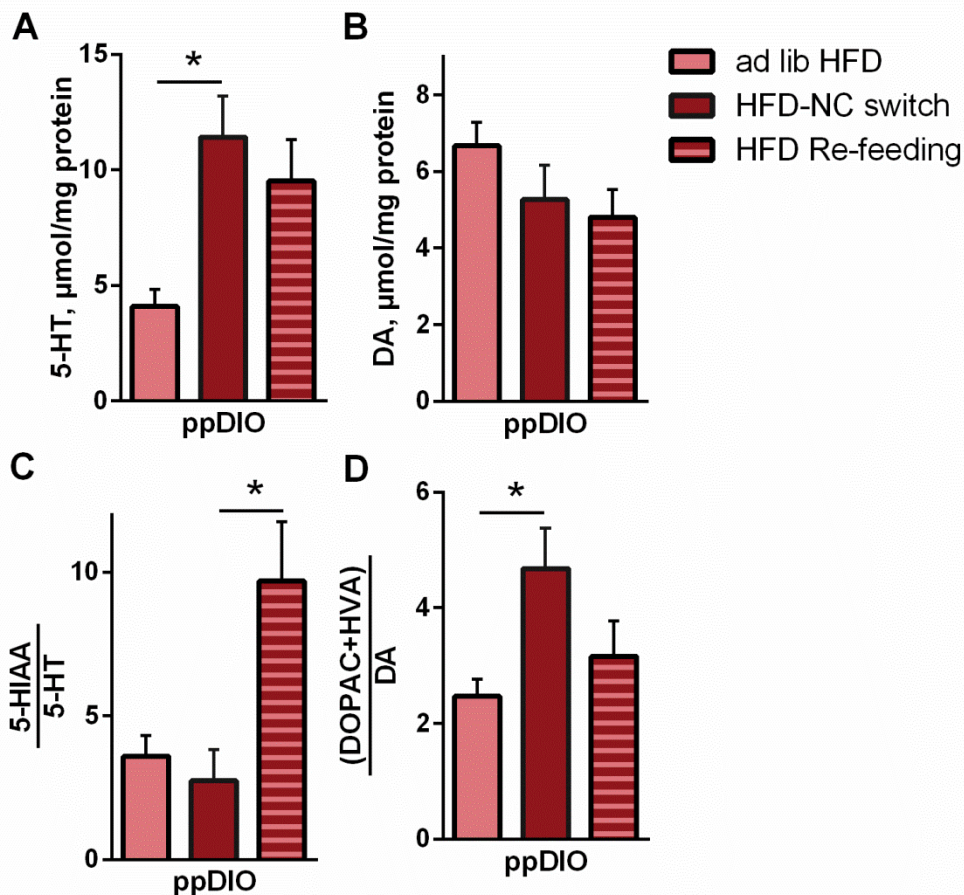
Figure 46: Changes in the LH dopamine and serotonin system of ppDIO mice following HFD withdrawal and re-feeding.

Concentrations of (**A**) serotonin (5-HT) and (**B**) dopamine (DA) in the LH were determined by the HPLC system from the tissue homogenates. Serotonin (**C**) and dopamine turnover (**D**) ratios were calculated as described in the graph. Values are given as mean ± SEM. Serotonin concentrations were significantly up-regulated by HFD withdrawal and re-feeding (one-way ANOVA, $F_{(2, 17)} = 5.390$, $p = 0.0154$), whereas serotonin turnover was only up-regulated when HFD was returned for one hour (Kruskal-Wallis test, $K = 9.583$, $p = 0.0041$). Dopamine concentrations were not affected by the dietary challenges. Dopamine turnover was increased after HFD withdrawal and returned to ad lib HFD levels after HFD re-feeding (one-way ANOVA, $F_{(2, 18)} = 3.689$, $p = 0.0454$). Dunnett's (parametric) or Dunn's (parametric) multiple comparisons test was used for post-hoc analysis. *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.

In the NAc, HFD withdrawal reduced dopamine concentrations significantly in ppDIO mice (Fig. 47A, $p < 0.05$). One hour of HFD re-feeding was sufficient to restore the drop in dopamine concentrations after HFD withdrawal (Fig. 47A, $p < 0.05$). Dopamine turnover was not significantly affected by the dietary challenges (Fig. 47B). Control mice did not exhibit changes in dopamine concentrations or turnover after fasting (Fig. 47C, D).
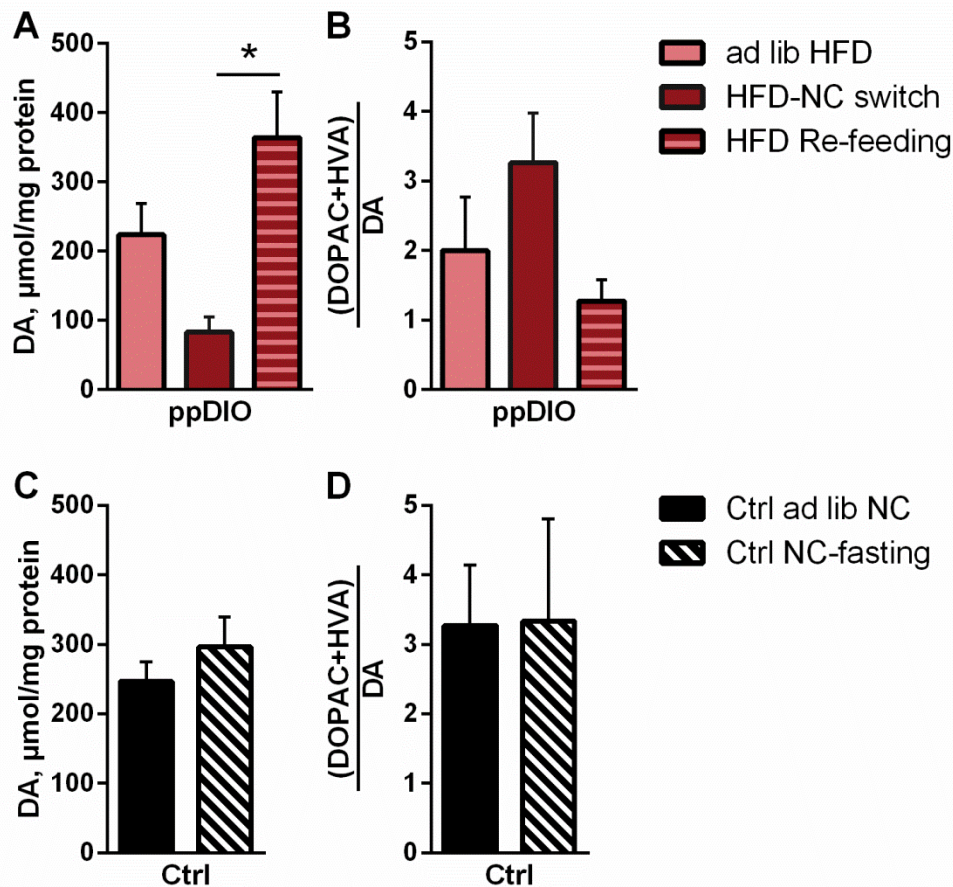
Figure 47: Changes in the NAc dopamine levels following HFD withdrawal and re-feeding in ppDIO mice and fasting in control mice.

Dopamine concentrations (**A, C**) and turnover (**B, D**) in the NAc of ppDIO (**C, D**) and control mice (**A, B**) were determined by the HPLC system from the tissue homogenates. Values are given as mean ± SEM. HFD withdrawal decreased and 1h of HFD re-feeding significantly increased dopamine concentrations (one-way ANOVA, $F_{(2, 18)} = 10.03$, $p = 0.0012$), while dopamine turnover was not significantly affected. Sidak's multiple comparisons test was used for post-hoc analysis. *$P < 0.05$. Modified from Gazea *et al.*, submitted to Front Neurosci.

Based on the changes in NAc dopamine contents, we also analyzed dopamine concentrations in the VTA, the main source of NAc dopamine. Both, the HFD-NC switch and one hour of HFD re-feeding significantly increased dopamine concentrations in the VTA as compared to chronic HFD-feeding (Fig. 48A, $p < 0.05$). On the other hand, dopamine turnover was deceased significantly as compared to mice that had uninterrupted access to HFD (Fig. 48B, $p < 0.05$).
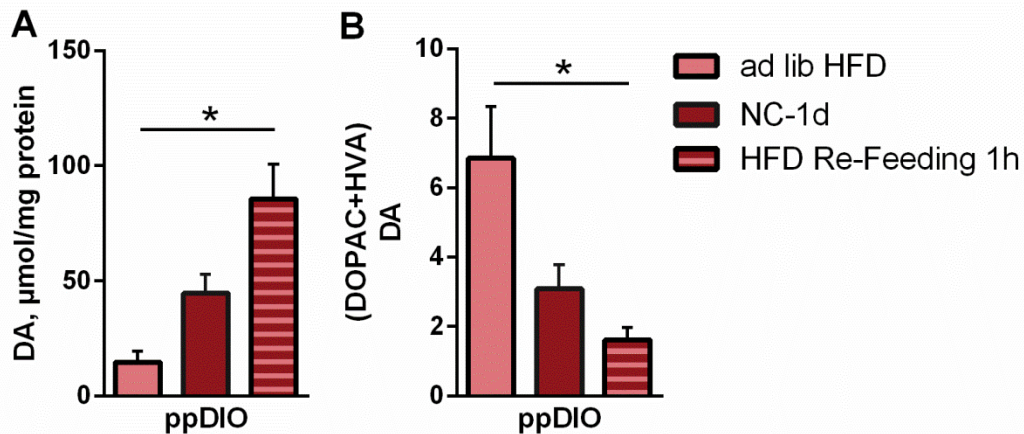
Figure 48: Changes in the VTA dopamine levels in ppDIO mice following HFD withdrawal and re-feeding.

Dopamine concentrations (**A**) and turnover (**B**) in the VTA were determined by the HPLC system from tissue homogenates. Values are given as mean ± SEM. HFD re-feeding for 1h significantly increased dopamine concentrations (Kruskal-Wallis test, K = 12.20, p = 0.0004), while dopamine turnover ratio was significantly decreased (Kruskal-Wallis test, K = 10.69, p = 0.0017). *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.
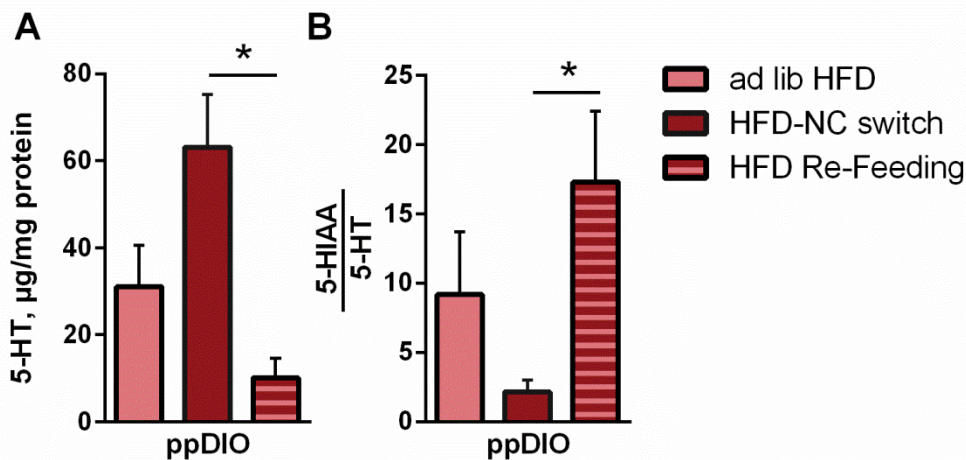
Figure 49: Changes in the DR serotonin level in ppDIO mice following HFD withdrawal and re-feeding.

Serotonin concentrations (**A**) and turnover (**B**) in the DR were determined by the HPLC system from the tissue homogenates. Values are given as mean ± SEM. HFD re-feeding for 1h significantly decreased serotonin concentrations (Kruskal-Wallis test, K = 10.38, p = 0.0021), whereas it increased serotonin turnover significantly (Kruskal-Wallis test, K = 10.05, p = 0.0028). *P < 0.05. Modified from Gazea *et al.*, submitted to Front Neurosci.

Because we found differences in serotonin concentrations in the LH, we further analyzed serotonin concentrations and turnover in the DR (Fig. 49). The switch from HFD to NC increased serotonin concentrations in the DR (Fig. 49A, p < 0.05).

Conversely, serotonin turnover was significantly decreased after the HFD-NC switch (Fig. 49B, $p < 0.05$). One hour of HFD re-feeding significantly reduced serotonin concentrations in the DR (Fig. 49A, $p < 0.05$) and elevated serotonin turnover as compared to the HFD-NC switch (Fig. 49B, $p < 0.05$). Thus, HFD-refeeding restored up-regulated serotonin concentrations in the DR following HFD withdrawal. In control animals, fasting did not impact on serotonergic signaling in the DR (data not shown).

Discussion

1 The impact of ppDIO on sleep-wake and depression-like behaviors during aging

1.1 ppDIO elevates nocturnal sleep time

Previous studies have shown that rodents become significantly overweight when fed a high-fat/high-carbohydrate diet (HFD) for at least six weeks (Black *et al.*, 1998). These diet-induced obese (DIO) animals display sleep fragmentation and prolonged sleep time during their active phase (Jenkins *et al.*, 2006; Tanno *et al.*, 2013). The current study reproduced these findings in peripubertal diet-induced obese (ppDIO) mice. Further, elevated nocturnal NREMS time re-appeared at 52 w of age in mice with a history of ppDIO, even though HFD feeding was terminated at 10 w and was replaced by a healthier, normal-calorie food variant during aging (standard laboratory food, NC).

Increases in nocturnal sleep time as seen in DIO mice may correspond to excessive daytime sleepiness (EDS) in obese humans. Previous findings support this idea (Jenkins *et al.*, 2006; Vgontzas *et al.*, 1998). EDS usually emerges due to insufficient or non-refreshing nocturnal sleep in humans. In our study, 10 and 52 w old ppDIO mice presented an EDS-like phenotype, which was accompanied by lower slow-wave activity (SWA) during the resting phase in 52 w old ppDIO mice and sleep fragmentation in 10 w old ppDIO mice. SWA is often used to estimate sleep depth, with higher SWA values reflecting a stronger cortical synchronization and greater sleep depth. Conversely, low SWA reflects poor sleep quality (Achermann & Borbely, 2003). In ppDIO mice, the EDS-like phenotype might have occurred because sleep during the light period was relatively shallow, thus an extended nocturnal sleep time may serve as a compensatory mechanism. However, according to the two-process model by Borbely (Borbely, 1982), an accumulation of SWA builds up during periods of prolonged wakefulness, which in rodents occurs during the dark period. Hence,

increased sleep time during the active phase may eliminate the build-up of homeostatic sleep pressure in ppDIO mice (Achermann & Borbely, 2003). This creates a vicious circle, in which lower sleep quality during the resting phase results in increased nocturnal sleep time and vice versa.

These findings raise a question of which mechanism may underlie the changes in SWA and nocturnal sleep time as observed in ppDIO mice. The reduced ability of ppDIO mice to stay awake during the dark period may be caused by different mechanisms; an over-activity of sleep-promoting areas, such as the VLPO, or a decreased activity of wake-promoting areas, such as the LH and DR. Alterations in the activity of either of these areas will impact on the activity of the other brain areas due to their extensive reciprocal projections. Based on previous studies on DIO rodent models, orexin neurons in the LH were suggested as a link between obesity and sleep time (Rolls *et al.*, 2010). LH orexin neurons are involved in the maintenance of wakefulness (A. R. Adamantidis *et al.*, 2007; Hara *et al.*, 2001) and promote food intake by integrating information about metabolic needs (Sakurai, 2005). Acute and chronic HFD consumption impacts on orexinergic function (Tanno *et al.*, 2013; Valdivia *et al.*, 2014). While acute HFD exposure activates orexin neurons (Valdivia *et al.*, 2014), chronic HFD feeding in DIO animals results in decreased orexin mRNA expression (Kohsaka *et al.*, 2007; Nobunaga *et al.*, 2014). In this regard, Tanno and colleagues showed that increased NREMS levels in DIO mice were inversely correlated with LH expression of orexin mRNA (Tanno *et al.*, 2013). In our ppDIO mouse model, orexin mRNA expression was unaltered, whereas serotonin concentrations in the LH were decreased, suggesting a decreased modulation of orexin neurons by serotonin. The present study demonstrates that serotonergic dysfunction may link obesity with sleep disturbances. The following section will outline how alterations in serotonergic neurotransmission may underlie elevated nocturnal sleep time in ppDIO mice.

1.2 A role of serotonin in elevated nocturnal sleep time

To explore possible mechanisms that contributed to the observed sleep phenotype in ppDIO mice, we analyzed neurotransmitter levels in brain regions related to sleep-

wake and food intake regulation. As mentioned above, serotonin levels in the LH were significantly decreased in ppDIO mice aged 10 w and 52 w as compared to controls of the same age. We postulated that changes in neurotransmitter signaling that appear in ppDIO mice at both 10 and 52 w of age may underlie elevated nocturnal sleep time and decreased SWA as seen in both groups. Therefore, the reduction in LH serotonin observed in both 10 w and 52 w old ppDIO mice may be one of the factors responsible for decreased wakefulness during the active phase and/or for decreased sleep quality during the resting phase.

Serotonergic projections to the LH originate mainly from the dorsal raphe nucleus (DR; Sakurai, 2005). DR serotonergic cells fire most actively during wakefulness, decrease firing during NREMS and cease firing during REMS (Trulson *et al.*, 1981; Monti, 2010). In fact, serotonin is mostly released from the DR into different brain regions during wakefulness and at its lowest level during REMS (Monti, 2010). Based on these earlier findings, serotonin is considered to promote wakefulness and inhibit REMS. Hence, the observed reduction of serotonin signaling in wake-promoting areas such as the LH may facilitate the occurrence of NREMS and REMS in ppDIO mice. A possible dysregulated firing of these serotonergic neuronal populations during NREMS might influence the proper generation of slow oscillations by disrupting cortical synchronization, and may hence decrease SWA as seen in our ppDIO mouse model.

However, the role of serotonin in the regulation of sleep-wake behaviors is far more complex. Seven different serotonin receptor classes have been described ($5-HT_{1-7}$;Monti, 2010). All 5-HT receptors are G-protein coupled receptors, except for the $5-HT_3$ receptor, which is a 5-HT-gated cation channel. Activation of $5-HT_{1B}$, $5-HT_{2A}$, $5-HT_{2B}$ and $5-HT_{2C}$ receptors promotes wakefulness and suppresses REMS, probably via activation of wake-promoting neuronal populations (Monti & Jantos, 2008, 2015; Monti, 2010). By contrast, activation of the $5-HT_{1A}$ receptor facilitates the occurrence of NREMS, possibly by inhibiting orexin neurons and by decreasing serotonin release (Tabuchi *et al.*, 2013; Muraki *et al.*, 2004). Thus, the influence of serotonin on sleep-wake behaviors depends on the availability of serotonin receptors in wake-promoting areas. Previous studies reported that DIO rats showed increased binding to $5-HT_{1A}$ in DR (Park *et al.*, 1999). Higher availability of the $5-HT_{1A}$ receptor in the DR may lead

to increased auto-inhibition onto serotonin neurons, which may result in lower DR serotonin levels and Tph2 mRNA expression as observed in our ppDIO mice.

In the LH, the expression of 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors was identified (Collin *et al.*, 2002; Park *et al.*, 1999; Tabuchi *et al.*, 2013). In our study, nocturnal NREMS time was increased by a history of ppDIO, while REMS time increased in proportion to NREMS. The expression of 5-HT$_{1A}$ receptors might be upregulated in the LH and its activation may promote NREMS via hyperpolarization of orexin neurons (Muraki *et al.*, 2004) in ppDIO mice. However, given the low concentrations of serotonin in the LH, it is rather unlikely that an overstimulation of the 5-HT$_{1A}$ receptor occurred in ppDIO mice. Intriguingly, many studies have posited a role of 5-HT$_{2C}$ receptors in the promotion of wakefulness by serotonin. Pharmacological activation of this receptor promotes wakefulness and suppresses NREMS (Nonogaki, 2012). Because our ppDIO mice show lower levels of serotonin in the LH along with increased NREMS time, we propose that serotonin fails to sufficiently activate 5-HT$_{2C}$ receptors on orexinergic neurons in this model to promote wakefulness. Treatment of ppDIO mice with substances that increase serotonergic tone such as selective serotonin re-uptake inhibitors (SSRIs) may be capable of ameliorating sleep disturbances. In line with this hypothesis, SSRIs are potent anti-obesity drugs through their effect on the mediation of satiety (Heal *et al.*, 2013). Simultaneously, SSRIs are frequently used to treat depressive disorders. The following section will further elaborate on this interaction.

1.3 Obesity and mood disorders – serotonin dysregulation as a common determinant

Mental disorders often first occur during peripuberty (Merikangas *et al.*, 2010). Indeed, several risk factors for the development of depression were discovered in peripuberty, for example stressful events during early life (Patchev *et al.*, 2014). Here we propose that peripubertal diet-induced obesity is an additional risk factor for future depression-like behaviors. This idea is supported by our results showing decreased central serotonin levels in ppDIO mice.

Decreased serotonin levels are dominantly associated with depressive disorders in humans (Coppen, 1969; Coppen *et al.*, 1967; Owens & Nemeroff, 1994), even though this dogma has received strong criticism in recent literature (Andrews *et al.*, 2015). We found lower serotonin levels in ppDIO mice as compared to controls, which were reflected in a passive stress-coping style, as measured by increased inactive (floating) versus decreased active (swimming, struggling) behaviors in a forced swim test (FST).

The FST is a frequently used behavioral test that examines depression-like or stress-coping behaviors in rodents (Porsolt *et al.*, 1977). This test was originally invented to detect the efficacy of antidepressant drugs, because potential drugs decrease immobility time in the FST (Porsolt *et al.*, 1977). This test is further used to evaluate the effects of behavioral or neurobiological challenges in basic research, thereby classifying immobility as behavioral despair and thus depression-like (Mineur *et al.*, 2006). We also made use of this test to examine possible depression-like behaviors in the ppDIO model. In addition to a depression-like phenotype in ppDIO mice, we also observed a positive correlation between body weight and inactive time in the FST, specifically in 52 w old ppDIO mice. Others have shown that both age and body weight increase inactive behaviors in the FST (Bogdanova *et al.*, 2013). Therefore, FST results obtained from the DIO model have to be handled with cautious. However, when body weight was partialled out, the increased inactive time in ppDIO mice still persisted, suggesting that body weight cannot be the only factor producing the differences in time spent immobile.

Several studies reported an association between obesity and depression (de Wit *et al.*, 2010; Luppino *et al.*, 2010). Mechanisms that underlie the comorbidity of obesity with depression may involve decreased serotonergic tone (Owens & Nemeroff, 1994), elevated basal cortisol levels (in a sub-type of depression, Holsboer, 2000) and dysregulation of leptin signaling (Yamada *et al.*, 2011; Kloiber *et al.*, 2013). Research on mechanisms linking obesity with depression is urgently needed, because antidepressant treatment efficacy is impaired in obese patients with depression (Kloiber *et al.*, 2013). A depression-like and anxiety-related phenotype was also observed in DIO mice and rats (Gehlert *et al.*, 2009; Sharma & Fulton, 2013). Therefore, DIO rodent models may serve as a translational model to screen the

efficacy of antidepressant treatments in the obese. In this respect, it was shown that odansetron, a 5-HT$_3$ receptor antagonist, improved performance of DIO mice in an anxiety-related and depression-like behavioral test battery (Kurhe & Mahesh, 2015). Furthermore, a MCHR1 antagonist and a CB$_1$ receptor antagonist (rimonabant) improved depression-like and/or anxiety-related behaviors in DIO rodents (Gehlert *et al.*, 2009; Verty *et al.*, 2013). The efficacy of these antidepressant drugs still awaits testing in depressed obese patients.

In conclusion, a decrease in serotonergic tone may link obesity with sleep disturbances and mood disorders. Targeting these systems pharmacologically may improve behavioral aspects of depression in the obese. Thereby, the use of SSRIs could be more advantageous rather than the other antidepressants with a different mode of action.

1.4 Involvement of HPA axis activity in behavioral changes in ppDIO mice

In addition to serotonergic mechanisms that associate obesity with mood and sleep disorders, enhanced cortisol levels may provide an additional link between these disorders. In our study, basal serum corticosterone levels were elevated in 52 w old mice with a history of ppDIO. This elevation in basal corticosterone levels was paralleled by those in 10 w old ppDIO mice (at ZT8).

Previous studies reported alterations in HPA axis activity in obesity (Tannenbaum *et al.*, 1997; McNeilly *et al.*, 2015; Swierczynska *et al.*, 2015). In obese people, glucocorticoid production is increased, thus creating a vicious cycle, because cortisol promotes visceral fat accumulation (Pasquali & Vicennati, 2000), which further elevates cortisol levels due to its abundant expression of 11βHSD1, an enzyme that converts cortisone to the active steroid cortisol (Bujalska *et al.*, 1997). Similarly to obese humans, rodent obesity models display increased basal corticosterone levels (Tannenbaum *et al.*, 1997; McNeilly *et al.*, 2015; Swierczynska *et al.*, 2015), even though some contradicting results were demonstrated by other studies (Boukouvalas *et al.*, 2008; Boukouvalas *et al.*, 2010; Patchev *et al.*, 2014; Kohsaka *et al.*, 2007). Even an acute HFD feeding for seven days was enough to cause an exaggerated

corticosterone response after an acute stress in rats, suggesting that the diet itself (without the development of obesity) alters HPA axis function (Soulis et al., 2007). In our study, ppDIO mice showed elevated basal corticosterone levels at 10 w of age, when mice were still exposed to HFD, and at 52 w of age, when HFD had been removed for more than nine months.

During the peripubertal period, the HPA axis is still developing and a shift in hormonal stress reactivity occurs (McCormick et al., 2010; Romeo, 2010). For instance, in pre-pubertal rats the recovery of the HPA axis from an acute stress takes longer as compared to adult rats (McCormick et al., 2010; Romeo, 2010). Therefore, the peripubertal period might be a sensitive time window for aversive events that would modify HPA axis function.

Several studies have examined whether HPA axis activity undergoes developmental programming during the peripubertal period by various stimuli such as stress. Indeed, when applying a stressor during the peripubertal period, depending on the type and duration of a stressor, an exaggerated corticosterone response to stress was observed during adulthood (McCormick et al., 2010). In some studies, peripubertal stress paradigms yielded elevated concentrations even of basal corticosterone in adulthood (Lepsch et al., 2005; Schmidt et al., 2007; McCormick et al., 2010). However, literature on the relationship between peripubertal HFD feeding and HPA axis function is scarce. A recent study showed that maternal high-fat diet feeding impacts on the offspring HPA axis activity, an effect that manifests itself during adulthood (Sasaki et al., 2013). In addition, post-weaning HFD exposure increased basal corticosterone levels in females, but not in males, at puberty onset (Boukouvalas et al., 2008; Boukouvalas et al., 2010). In this thesis, we show for the first time that a history of ppDIO potentiates the long-term programming of basal corticosterone levels. Alarmingly, chronic elevations of glucocorticoids have been associated with the development of a variety of disorders such as diabetes, hypertension, impaired learning, depression etc. (Yau & Seckl, 2012). Further, glucocorticoids facilitate the differentiation of pre-adipocytes to adipocytes and thus promote the accumulation of visceral fat, as described above (Chapman et al., 1985). Hence, chronically elevated glucocorticoid levels promote the development of visceral obesity.

Enhanced HPA axis activity impairs sleep quality, as observed by lower SWA and frequent awakenings after administration of CRH, ACTH or methylprednisolone, a synthetic glucocorticoid receptor agonist (Holsboer *et al.*, 1988; Antonijevic & Steiger, 2003; Steiger & Kimura, 2010). These sleep changes also characteristically appear in patients with major depression (reviewed in Steiger & Kimura, 2010). In addition, a disinhibition of REMS is typically seen in depressed patients and in mouse models of depression. An overexpression of CRH appears to underlie increased REMS in the latter (Steiger & Kimura, 2010). However, a disinhibition of REMS was not observed in our ppDIO mouse model, suggesting a different contribution of enhanced HPA activity to sleep-wake alterations in our study. Nevertheless, an enhanced corticosterone secretion prior to dark onset may be partly responsible for decreased SWA in ppDIO mice, because the wake-promoting actions of the HPA axis may interfere with the maintenance of deep sleep. Hence, enhanced corticosterone may provide a second link between obesity, mood disorders and sleep disturbances. However, elevated corticosterone at dark onset would promote wakefulness rather than sleep. In contrast, ppDIO mice display elevated nocturnal sleep time, creating a mismatch to elevated corticosterone levels. Therefore, enhanced corticosterone levels cannot be a reason for the sleep-wake alterations in the case of ppDIO mice (Romanowski *et al.*, 2010), even though they may contribute to increased body weights and depression-like behaviors in our mouse model. On the other hand, based on our data, we emphasize that a decreased serotonergic tone provides a more likely link between obesity, mood disorders in sleep disturbances.

2 Strategies aimed at reducing elevated nocturnal sleep time in ppDIO mice

Based on our finding that a history of ppDIO increased nocturnal sleep time at 10 and 52 w of age, we aimed to examine whether pharmacotherapy or other weight loss strategies may reverse these sleep changes. To this end, we tested the administration of PYY_{3-36}, a peripheral satiety hormone, and a 24-hour fasting challenge and analyzed sleep-wake and food intake behaviors thereafter.

2.1 Injections of PYY_{3-36} decrease elevated NREMS time in ppDIO mice

Akanmu and colleagues have reported that PYY_{3-36} injections after dark onset increase in NREMS time in healthy male rats (Akanmu *et al.*, 2006). In this thesis, we reproduced this finding in normal C57BL/6N male mice. However, PYY_{3-36} elicits opposite effects on NREMS in ppDIO mice; instead of promoting NREMS, PYY_{3-36} reduced NREMS time in 10 and 52 w old mice with a history of ppDIO. Thereby, PYY_{3-36} abolished the initial differences in nocturnal NREMS time between ppDIO and control mice.

PYY_{3-36} was recently suggested as a pharmacotherapy option for weight loss strategies in obesity. PYY_{3-36} potently inhibits food intake through central mechanisms (Batterham *et al.*, 2002). PYY_{3-36} crosses the blood-brain barrier (Nonaka *et al.*, 2003) and binds to the NPY Y2 receptor with high affinity (Walther *et al.*, 2011), inhibiting the action of NPY neurons, thus indirectly stimulating POMC neurons (Batterham *et al.*, 2002; Challis *et al.*, 2003). PYY originally consists of 36 amino acids (PYY_{1-36}), binds to all known NPY receptor subtypes and rather promotes food intake (Keire *et al.*, 2002). But the major proportion of circulating PYY is represented by the cleaved form PYY_{3-36} (Grandt *et al.*, 1994). In obesity, PYY levels are generally decreased (Batterham *et al.*, 2003; Karra *et al.*, 2009), whereas its signaling efficacy is as high as in lean subjects (R. D. Reidelberger *et al.*, 2008). Therefore, supplementation of PYY_{3-36} in obese subjects may improve brain-periphery communication in obesity and lead to weight loss. Indeed, an intermittent

treatment with PYY_{3-36} achieved significant weight loss in DIO rats, even during *ad libitum* HFD exposure (R. D. Reidelberger *et al.*, 2008).

In our study, a bolus injection of PYY_{3-36} improved sleep-wake behaviors in ppDIO mice. However, the effect was only observed for the first post-injection hour. Previous reports have shown that the PYY_{3-36} effect on sleep persists for four hours in control animals (Akanmu *et al.*, 2006). Our animals were left undisturbed for one hour after the injections and were then briefly disturbed for the monitoring of food intake. This brief disturbance might have interfered with further effects of PYY_{3-36} on sleep. Moreover, the development of PYY_{3-36} analogues with longer-lasting actions could be beneficial for the treatment of obesity and obesity-associated disorders.

Intriguingly, the administration of PYY_{3-36} induced opposite effects in ppDIO to those observed in control mice with respect to sleep-wake behaviors, even though feeding was similarly suppressed. This suggests that a) different pathways were recruited by PYY_{3-36} in ppDIO and control mice, or b) initial differences in neuronal signaling between ppDIO and control mice were assimilated by PYY_{3-36}. In line with our results showing a down-regulation of the serotonergic tone in both 10 w and 52 w old ppDIO mice as compared to controls, we would expect that the alteration in serotonin signaling contributes to the modifications of sleep-wake behaviors by PYY_{3-36}. The most prominent target of PYY_{3-36} is ARC NPY neurons (Batterham *et al.*, 2002; Challis *et al.*, 2003). NPY and serotonin have opposing actions in the regulation of food intake, thus the decrease of either one may lead to an increase of the other. Therefore, a further reduction of NPY expression in ppDIO mice by PYY_{3-36} may have beneficial effects on serotonergic neurotransmission. Thus, PYY_{3-36} would have possibly restored serotonergic signaling, which may help improving sleep-wake behaviors in ppDIO mice. However, direct evidence on the effects of PYY_{3-36} on serotonin is still missing in literature.

Alternatively, PYY_{3-36} activates mesolimbic and nigrostriatal dopaminergic pathways (Batterham *et al.*, 2007). In rat striatal slices, PYY_{3-36} increased the synthesis and the release of dopamine (Adewale *et al.*, 2005, 2007). When PYY_{3-36} was given peripherally in mice, the locomotor response to subsequent amphetamine

administration was potentiated (Stadlbauer *et al.*, 2014). Hence, PYY$_{3\text{-}36}$ may have increased the dopaminergic tone in ppDIO mice, which would have taken part in promoting wakefulness (Saper *et al.*, 2010).

2.2 Differences in fasting-induced wakefulness between ppDIO and control mice at 52 w of age

Fasting has beneficial effects on many medical conditions such as diabetes, hypertension and arthritis due to its positive effects on insulin sensitivity (increasing), inflammation (decreasing), oxidative stress (reducing) etc. (Longo & Mattson, 2014). Fasting is also beneficial to lose weight, but is not known whether comorbid symptoms of obesity are cured by fasting as well. On the other hand, it is well known that reduced food availability increases wakefulness to facilitate food seeking behaviors (Borbely, 1977; Danguir & Nicolaidis, 1979). To test the possibility of reducing NREMS, we applied a 24-hour period of fasting in 52 w old ppDIO and control mice. Both diet groups showed a suppression of nocturnal NREMS time, but the suppression was significantly less pronounced in ppDIO mice. However, re-feeding induced similar alterations in sleep-wake behaviors in ppDIO and control animals. Food intake during re-feeding and body weight loss during fasting was also not different between the diet groups.

During the period of fasting, the peripheral and central satiety signaling machinery is attenuated, whereas the hunger machinery is activated. For example, peripheral satiety signals such as leptin decrease during fasting, whereas peripheral ghrelin concentrations rise, resulting in a central activation of NPY and orexin neurons, which stimulate feeding (Schwartz *et al.*, 2000). However, so far, little is known about the mechanism how fasting increases wakefulness per se. It was shown that hypothalamic orexin neurons are involved in the mediation of fasting-induced wakefulness (Yamanaka *et al.*, 2003). Therefore, we assumed that in our study, fasting might activate orexin neurons to promote wakefulness during food seeking behaviors. However, 52 w old ppDIO mice orexin activation would have occurred less than in control mice. The reduced orexin activation might have led to a lower suppression of nocturnal NREMS time as compared to controls. This idea is in line

with a previous study, in which fasting-induced hypothalamic activation and the hyperphagic response after fasting was attenuated in DIO mice (Briggs *et al.*, 2011). In addition, fasting usually elicits neuronal activation (as validated by c-Fos expression) in the PVN and the ARC in control mice, but failed to do so in mice that develop late-onset obesity (Briggs *et al.*, 2011; Becskei *et al.*, 2009).

Further, fasting leads to a lower reduction of leptin concentrations in late-onset obese mice than in lean controls (Becskei *et al.*, 2009). The increased adipose tissue in late-onset obese mice leads to elevated basal leptin concentrations (Considine *et al.*, 1996). Therefore, fasting elicited a lower leptin suppression in late-onset obese mice as compared to lean controls. Nevertheless, ghrelin release is similarly promoted in DIO and control mice after fasting (Briggs *et al.*, 2011). This suggests that leptin and ghrelin compete centrally to induce satiety and hunger during fasting, respectively. In our study, ppDIO mice had greater body weights than control mice, which may be caused by increased fat depots, which in turn elevated peripheral leptin concentrations. During fasting, leptin levels may have been higher in 52 w old ppDIO mice as compared to controls, which would interfere with ghrelin-mediated activation of central appetite-promoting pathways. This may have led to a blunted induction of orexin signaling and thereby to a reduced promotion of wakefulness. Indeed, administration of satiety factors, such as leptin or PYY_{3-36}, lead to an increase in NREMS (Sinton *et al.*, 1999; Akanmu *et al.*, 2006). Therefore, similarly to late-onset obesity mice, fasting may have elicited a decreased activation of hunger centers in our 52 w old ppDIO mice as compared to control mice, which resulted in a decreased promotion of wakefulness. Nevertheless, the suppression of nocturnal NREMS time was substantial in ppDIO mice, suggesting that fasting might be a beneficial treatment option in obesity by resetting homeostatic food regulatory centers.

3. High-fat diet withdrawal elicits increased wakefulness and alterations in brain reward centers

3.1 High-fat diet availability modulates nocturnal sleep-wake activity

As mentioned in previous sections, ppDIO mice exhibited increased nocturnal NREMS time during chronic HFD exposure at 10 w and again at 52 w of age under NC-feeding. However, when HFD was acutely replaced by NC, nocturnal NREMS time was dramatically reduced in 10 w old ppDIO mice, reaching levels even below control animals.

A recent study demonstrated that replacement of HFD by NC in adult DIO mice reduced elevated sleep time, in which sleep time in DIO mice was not distinguishable from control mice after four weeks on NC (Guan *et al.*, 2008). In contrast to our study, the animals in Guan's study were adult at the onset of HFD feeding. Therefore, in our study the dramatic reduction in NREMS time during HFD withdrawal could be an effect specific to DIO during the peripubertal period as compared to DIO during adulthood. Further, in Guan's study sleep-wake behaviors in DIO mice were recorded four weeks after the HFD was withdrawn. However, we took a closer look at NREMS reduction during the acute HFD withdrawal phase (within the first two weeks). Therefore, our earlier timing of sleep-wake observations directly after HFD withdrawal revealed the effect that could not be found by previous studies. Later on, in 24 w old ppDIO mice, this difference was not evident, further underlining that the dramatic increase in wakefulness is specific to HFD withdrawal in ppDIO mice. Hence, based on previous and our own observations, we assume that also adult DIO mice may show elevated awake time in the acute phase of HFD withdrawal.

Further, our results raise the question whether HFD withdrawal or body weight loss causes the dramatic reduction in NREMS time in ppDIO mice. It was previously suggested that weight loss also helps reducing upregulated nocturnal sleep in DIO mice. However, in our study, changes in NREMS time preceded the occurrence of significant weight loss; NREMS time is reduced already in the first six hours after

HFD is withdrawn. Furthermore, nocturnal NREMS time returned to initial levels in ppDIO mice when HFD was returned suggesting that nocturnal NREMS time depends on HFD availability in 10 w old ppDIO mice. Another study also showed that increased wakefulness is the result of the absence of HFD rather than weight loss per se (Perron *et al.*, 2015). The absence of HFD may trigger homeostatic responses in the brain and in the periphery that may elicit increased wakefulness to facilitate food seeking behaviors. Indeed, when HFD was replaced by NC, ppDIO mice underwent a voluntary hypophagia, which might have led to a negative energy balance and could activate homeostatic food intake regulators. Similarly, fasting in control mice produces a negative energy balance and reduces nocturnal NREMS time, as already reported previously (Borbely, 1977; Danguir & Nicolaidis, 1979). Therefore, we postulated that both HFD withdrawal in ppDIO mice and fasting in controls may recruit common mechanisms to promote wakefulness. One of these mechanisms may involve homeostatic regulators of feeding behaviors, as further elaborated on in the following section.

3.2 Homeostatic food intake regulation is activated by the HFD-NC switch

To investigate whether HFD withdrawal activates homeostatic brain mechanisms in the control of food intake, we compared NPY, MCH and orexin expression in ppDIO animals after *ad libitum* HFD exposure and after a 24 hour HFD-NC switch. *Ad libitum* fed controls and fasted controls were examined in parallel. Interestingly, NPY expression was up-regulated in the ARC of ppDIO mice following the HFD-NC switch as compared to uninterrupted HFD feeding. In addition, orexin concentrations in the LH were increased when HFD was withdrawn, although orexin mRNA expression showed no changes. On the other hand, fasted controls displayed both elevated NPY and orexin expression.

Orexin neurons are densely innervated by ARC NPY neurons (Broberger *et al.*, 1998; Elias *et al.*, 1998). NPY injections into the LH promote food intake (Campbell *et al.*, 2003). Based on the postulate that ARC NPY and POMC neurons serve as first-order neurons in the regulation of food intake, we expected that NPY expression would be primarily altered by fasting, followed by orexin expression that lies downstream from

NPY and POMC (Elias *et al.*, 1998). Indeed, as mentioned above, both NPY expression and orexin peptide levels were increased during states of negative energy balance. This finding suggests that HFD withdrawal elicits a homeostatic response in food intake regulatory regions comparable to the fasting response in control animals.

Further, we postulate that the increase in orexin levels may be partly responsible for the suppression of NREMS time during HFD withdrawal. This is supported by a previous study showing that orexin signaling is responsible for fasting-induced elevation of wakefulness (Yamanaka *et al.*, 2003). Thus, an increase in orexinergic tone is a likely common determinant in elevating wakefulness during fasting in controls and during the HFD-NC switch in ppDIO mice.

In conclusion, HFD withdrawal activated hunger centers that probably produced an increased homeostatic drive to consume food in ppDIO mice. However, feeding was dramatically reduced during the HFD-NC switch, even though the animals had *ad libitum* access to NC. This suggests that a different mechanism acted to suppress intake of NC, even when the energy balance was negative. Therefore, we continued our analysis in regions that are responsible for hedonic signals during feeding behaviors.

3.3 Contribution of reduced activities in the reward and motivational system to hypophagia during the HFD-NC switch

Ingestion of palatable foods is known to elicit dopamine efflux in reward- and motivation-related brain areas such as the NAc and VTA (Bassareo & Di Chiara, 1997; Hernandez & Hoebel, 1988; Roitman *et al.*, 2008). In our study, we found that dopamine concentrations in the NAc were dramatically reduced during the HFD-NC switch in ppDIO mice as compared to *ad libitum* HFD feeding. This effect was not observed in fasted controls, suggesting a specific NAc dopamine response to HFD withdrawal. Interestingly, previous studies have shown that the ingestion of aversive substances such as quinine decreases NAc dopamine (Roitman *et al.*, 2008). Consequently, low levels of NAc dopamine might be a direct reflection of the aversive perception of NC in ppDIO mice. This may explain the hypophagia occurring during

the HFD-NC switch, despite the negative energy balance as seen by increased NPY and orexin signals and by the observed body weight loss.

Recently the role of dopamine in encoding rewarding stimuli has been revisited (Berridge *et al.*, 2010); many studies support the idea that dopamine mediates food "wanting", i.e. the motivation or the drive to eat, instead of food "liking", i.e. the rewarding properties of food or the pleasure that food creates. In terms of obesity, one study showed that obesity decreases motivation rather than creating a reward deficiency syndrome; obese mice are less motivated to work for a palatable milk reward than control animals in an operant conditioning task. Moreover, their preference for sweet solution is lower than in control mice, especially when their maintenance diet was available *ad libitum* (Harb & Almeida, 2014). Further, reduced dopaminergic transmission in the NAc is well known to reduce the motivation to work for food and drugs (Barbano & Cador, 2007; Berridge, 2009; Salamone & Correa, 2009). Therefore, reduced dopamine levels in the NAc of ppDIO mice after HFD withdrawal most likely reflect a lower motivation to consume NC rather than the reduced pleasure elicited by NC consumption. On the other hand, HFD re-feeding caused an up-regulation of NAc dopamine concentrations, which probably translates into increased motivation to re-feed on HFD.

Which other factors may then cause the possibly aversive response to NC? The taste and smell information about NC is most likely encoded by opioid and endocannabinoid signaling in reward hotspots, such as the NAc, ventral pallidum, parabrachial nucleus etc. (Berridge *et al.*, 2010). In our study, the replacement of HFD by the less palatable NC may have triggered a disliking response in ppDIO mice, which may have been induced by alterations in opioid and endocannabinoid signaling to reward hotspots. This may have led to reduced motivation to continue NC consumption, as displayed by lower dopamine levels in the NAc.

In conclusion, these results support the idea that the hedonic drives towards food can override homeostatic control. In our study, orexigenic signals such as NPY and orexin were up-regulated during HFD withdrawal and provided a signal about the negative energy balance similarly to fasting in controls. However, the ingestion of NC

instead of HFD may have caused an aversive reaction that reduced the motivation to continue ingesting NC. This line of events may have caused hypophagia and resulted in body weight loss in ppDIO mice. However, the hunger signals during the hypophagia probably prompted ppDIO mice to increase their nocturnal awake time during the HFD-NC switch.

Recently, the role of NAc in sleep-wake regulation has received attention. NAc lesions elevated wakefulness throughout 24 hours, while REMS was not affected, and increased the number of NREMS-WAKE transitions (Qiu et al., 2010). Furthermore, the arousing effect of caffeine is mediated via the adenosine A_{2A} receptor in the NAc shell region (Lazarus et al., 2011). It seems that the NAc generally inhibits arousal centers such as the LH, TMN, DR and LC through its GABAergic projections (Kelley et al., 2005; Lazarus et al., 2011). High concentrations of dopamine in the NAc would thus lead to an inhibition of medium spiny neurons (MSN) through post-synaptic D2-like receptors. This would result in a blockade of inhibitory projections from the NAc to arousal centers, thereby wakefulness is promoted. Conversely, when dopamine concentrations are low in the NAc, D1-like receptors are activated and lead to a depolarization of MSNs, thus, arousal areas are inhibited and sleep can occur. In our study, the decreased concentrations of dopamine in the NAc would lead to a disinhibition of MSNs. Thus, MSNs would increase their inhibitory tone onto the LH, thereby providing a signal to sleep. However, as seen in our study, orexin peptide concentrations were elevated and sleep was suppressed during HFD withdrawal. This suggests that another mechanism may interfere with the inhibition of orexin neurons by NAc projections. To evaluate this hypothesis, we took a closer look at dopamine and serotonin neurotransmission in arousal-associated areas including the LH, DR and VTA.

3.4 Involvement of dopaminergic and serotonergic signaling in the LH in the control of HFD withdrawal-induced NREMS suppression and hypophagia

HFD withdrawal increased serotonin concentrations in the LH, while serotonin turnover was decreased in ppDIO mice. In addition, LH dopamine turnover was up-regulated after HFD withdrawal. In control mice, LH dopamine and serotonin

transmission were not altered by fasting. These results suggest that fasting in controls and HFD withdrawal in ppDIO mice elicit different responses in dopaminergic and serotonergic signaling in the LH, which may exert different effects on the promotion of wakefulness and food intake. As described above, serotonin may elicit differential post-synaptic responses depending on receptor availability. Intriguingly, pharmacological studies have shown that 5-HT$_{2C}$ activation increases wakefulness and reduces food intake in the rat (Nonogaki, 2012). The abundant expression of 5-HT$_{2C}$ receptors in the LH may mediate these effects (Collin *et al.*, 2002; Park *et al.*, 1999; Tabuchi *et al.*, 2013).

However, it is still not fully delineated how serotonin may mediate the dissociation between sleep-feeding. Previous studies suggested a functional dichotomy of orexin neurons; orexin neurons located in the lateral parts of the LH may serve reward-associated functions including feeding, whereas more medial parts of the LH may be involved in the maintenance of wakefulness (Fig. 50, Aston-Jones *et al.*, 2010). Projections from medial and lateral LH parts also differ in their target areas; arousal-associated orexin projections innervate mostly regions that promote wakefulness including the LC, DR, TMN and VTA, while reward-associated orexin projections primarily innervate the mPFC, VTA and NAc (Aston-Jones *et al.*, 2010). In addition, reward-associated orexin signaling appears to inhibit dopaminergic VTA neurons, while arousal-mediating orexin neurons excite DR serotonin neurons and VTA dopamine neurons.
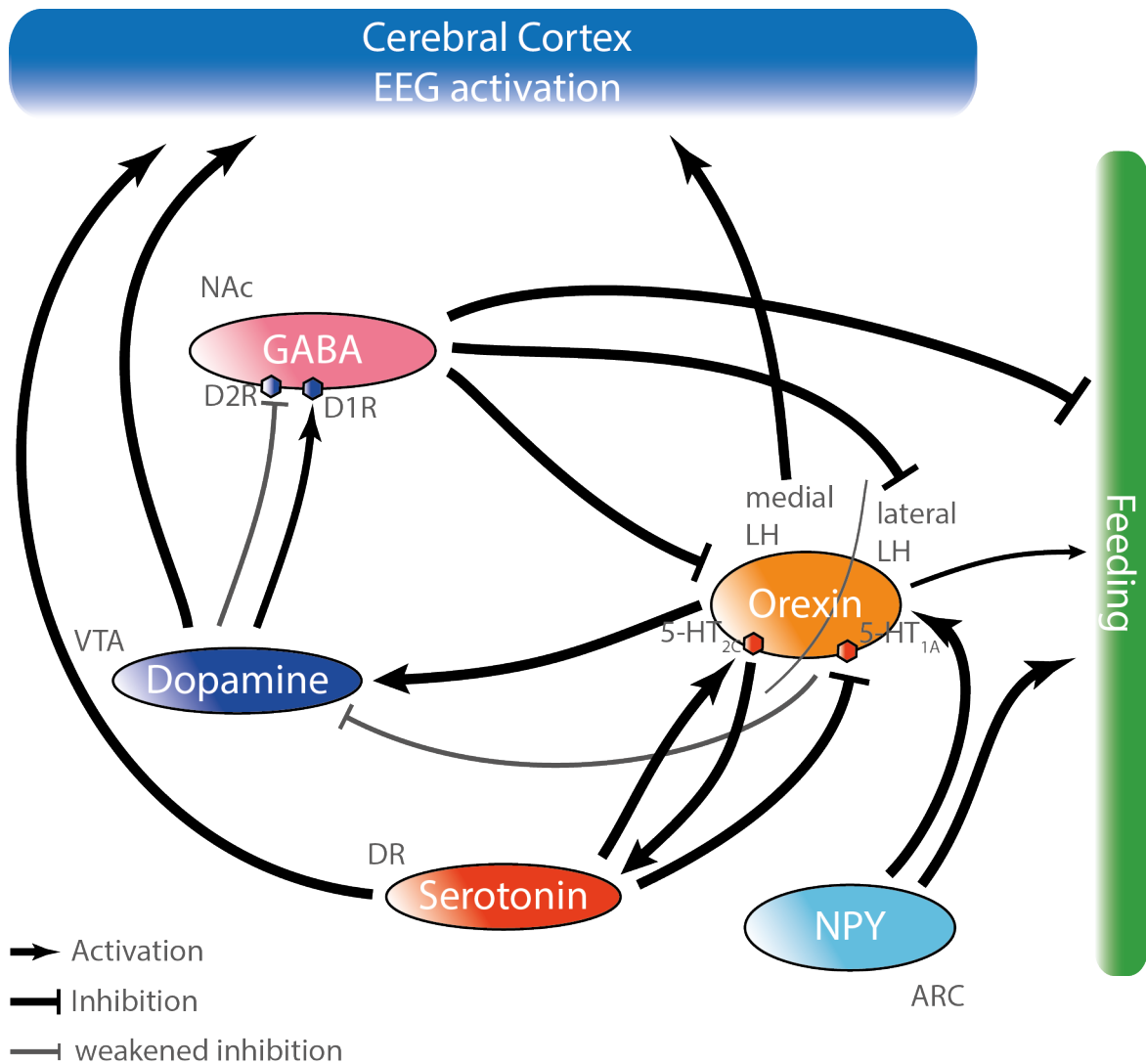
Figure 50: Schematic overview of interactions between arousal- and feeding-related nuclei during HFD withdrawal.

Wake-promoting areas such as the medial LH, VTA and DR are activated during HFD withdrawal and induce cortical activation. Homeostatic signals that facilitate food intake behaviors (Arc NPY neurons) are activated as well, but their function is overridden by hedonic inputs into the NAc. Thereby, the NAc inhibits lateral LH neurons resulting in hypophagia. In addition, serotonergic signaling is increased in the LH. We propose that serotonin acts on the medial LH via activation of 5-HT$_{2C}$ receptors to promote wakefulness and also on lateral LH, where it inhibits orexin neurons via 5-HT$_{1A}$ receptors to inhibit feeding.

Therefore, we propose that increased orexin-mediated excitation of arousal nuclei may have led to increased VTA dopamine and DR serotonin concentrations in ppDIO mice withdrawn from HFD, and thus this may have promoted the time spent awake. In turn, increased serotonin actions in the LH may have contributed to the hypophagia during HFD withdrawal by acting on lateral aspects of the LH. Serotonin may still activate orexin neurons through the 5-HT$_{2C}$ receptors, but due to different projection targets of orexin neurons arising from this area, may inhibit feeding on NC.

Alternatively, activation of 5-HT$_{1C}$ receptors may play a role in the mediation of the satiety effects of serotonin. These receptors are expressed in the LH as well and their activation potently inhibits orexin neurons (Muraki *et al.*, 2004). Different anatomical locations of serotonin receptor subtypes may further contribute to the dissociation of sleep and feeding behaviors in ppDIO mice (Fig. 50).

General conclusions and outlook

The present study demonstrates that a history of early-life obesity, here shown in the context of peripubertal development, programmed life-time sleep disturbances such as decreased sleep quality and elevated nocturnal sleep time. These sleep disturbances appeared during chronic HFD exposure and nine months after HFD was replaced by NC, suggesting that the peripubertal HFD experience permanently altered sleep-wake regulation. In addition, ppDIO mice exhibited elevated depression-like behaviors during aging. Hence, we explored the involvement of particular brain regions in the programming of sleep disturbances and of emotional behaviors in ppDIO mice. Our analysis disclosed a reduced serotonergic tone in the LH, suggesting its central role in the association between obesity, sleep disturbances and mood disorders. Further investigations should address the causality between lower serotonin, sleep disturbances and mood disorders. Thereby, a targeted pharmacological approach aimed at modulating serotonin signaling in the LH may reveal if lower LH serotonin levels produce increased nocturnal sleep time in ppDIO mice. Further, in vivo microdialysis may provide additional information about the chronological dynamics of serotonergic neurotransmission in the LH in obesity.

Furthermore, this study highlights new treatment options for ameliorating sleep disturbances in obesity. We have shown that both the administration of PYY_{3-36} and a 24-hour period of fasting potently reduced sleep time in ppDIO mice. Based on the results obtained in this and previous studies, dopaminergic and serotonergic actions in the brain are likely mediators of the PYY_{3-36} effect on sleep. The diverging effects of PYY_{3-36} on sleep time in control and ppDIO mice provide interesting contact points for further studies aimed at characterizing the underlying brain mechanism. Moreover, it would be worth investigating whether fasting and PYY_{3-36} exert positive effects on depression-like behaviors in ppDIO mice.

Interestingly, similarly to fasting in controls, the replacement of HFD by NC suppressed nocturnal sleep and increased LH orexin concentrations in ppDIO mice. When HFD was withdrawn in ppDIO mice, sleep time was even lower than in controls. Indeed, serotonin levels in the LH increased during HFD withdrawal, further emphasizing that serotonin and sleep time are causally related in ppDIO mice.

However, despite the increased feeding signals with HFD withdrawal, ppDIO mice underwent a voluntary hypophagia and did not consume the available NC. This behavioral response resulted in weight loss after acute HFD withdrawal. Due to the differences in palatability between the two diets, NC may have been less attractive to ppDIO mice, which in turn led to a decreased motivation to consume NC, as reflected by decreased dopamine levels in the NAc. Hence, during HFD withdrawal, hedonic properties of foods overwhelmed the homeostatic control that promotes feeding. This hypothesis should be further tested, for example by increasing dopaminergic neurotransmission in the NAc to induce motivated behaviors for feeding or by interfering with serotonergic inputs into the LH to reduce the arousal response to HFD withdrawal. Besides, an additional question we should address in the future is whether low dopamine levels in the NAc can be interpreted as reduced motivation for NC after HFD withdrawal. Furthermore, an anatomical dissection of the LH regarding the expression orexin and serotonin receptor subtypes may provide further insights into how serotonin regulates satiety and wakefulness. Finally, an exploration of a direct function of the NAc in the regulation of sleep-wake behaviors may add to better understanding of the association between obesity and sleep disturbances.

List of Abbreviations

5-HT	Serotonin
ACTH	adrenocorticotropic hormone
ad lib	ad libitum
AGRP	Agouti-related protein
AP	Area postrema
ARAS	ascending reticular activating system
ARC	Arcuate nucleus
BMI	Body mass index
CART	Cocaine- and amphetamine-related transcript
CCK	cholecystokinin
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
Ctrl	Control
D1R	Dopamine receptor 1
D2R	Dopamine receptor 2
DA	Dopamine
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamus
DR	Dorsal raphe nucleus
EDS	Excessive daytime sleepiness
EEG	Electroencephalography
EMG	Electromyography
FFT	Fast Fourier transformation
FST	Forced Swim Test
GLP-1	glucagon-like petide 1
HFD	High-fat/high-carbohydrate diet
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High pressure liquid chromatography
i.p.	Intraperitoneal
icv	intracerebroventricular
LC	Locus coeruleus
LDT	laterodorsal tegemental nucleus
LH	Lateral hypothalamus
LPT	Letral pontine tegmentum
MCH	Melanin concentrating hormone
MCHR1	MCH receptor 1
MnPO	median pre-optic nucleus
MPB	Medial parabrachial nucleus

mRNA	Messenger RNA
mRNA	Median raphe nucleus
NAc	Nucleus accumbens
NC	Standard laboratory food, normal chow
NPY	Neuropeptide Y
NREMS	Non-rapid eye movement sleep
NTS	Nucleus tractus solitarius
P	Postnatal day
PC	Precoeruleus region
POMC	Pro-opiomelanocortin
ppDIO	Peripubertal diet-induced obesity
PPT	pedunculopontine tegmental nucleus
PVN	Paraventricular nucleus
PYY	Peptide tyrosine-tyrosine
REMS	Rapid eye movement sleep
RIA	Radioimmunoassay
RT	Room temperature
s.c.	subcutaneously
SCN	suprachiasmatic nucleus
SEM	Standard error of the mean
SLD	sub-laterodorsal nucleus
SWA	Slow-wave activity
SWS	Slow wave sleep
TdT	terminal deoxynucleotidyl transferase
TH	Tyrosine hydroxylase
TMN	Tuberomammillary nucleus
vlPAG	ventrolateral periaqueductal gray
VLPO	Ventrolateral preoptic area
VMH	Ventromedial hypothalamus
VTA	Ventral tegmental area
ZT	Zeitgeber time

List of Figures and Tables

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List of buffers and solutions

Buffer and solutions used for RNA in situ hybridization

<u>TE buffer</u>

1 ml	1M Tris-HCl (pH 8.0)
0.2 ml	0.5M EDTA
100 ml	Water, fill up to 100 ml

<u>10x PBS</u>

90 g	NaCl
1.22 g	KH_2PO_4
8.15 g	Na_2PO_4
1 l	Distilled water, fill up to 1l
1 ml	DEPC (Sigma Aldrich)

10x PBS was mixed on a magnetic stirrer and autoclaved subsequently.

<u>DEPC-treated water</u>

5 ml	DEPC (Sigma Aldrich)
5 l	Distilled water

DEPC-treated water was mixed on a magnetic stirrer and autoclaved subsequently.

<u>20x SSC</u>

350.6 g	NaCl
176.4 g	Tri-Natriumcitrat-Dihydrat
2 l	Distilled water, fill up to 2l
2 ml	DEPC (Sigma Aldrich)

20x SSC was mixed on a magnetic stirrer and autoclaved subsequently.

<u>Hybridization buffer</u>

20 ml	Formamide (Emplura, Merck KGaA)
8 ml	20x SSC
2 ml	Salmon sperm DNA (Sigma Aldrich)
0.4 ml	Yest tRNA (25mg/ml, Sigma-Aldrich)
0.8 ml	50x Denhardt's solution (Sigma Aldrich)

0.8 ml   Sterile water

Hybridization buffer was mixed, aliquoted (2 ml aliquots) and stored in a deep freezer (-80°C).

0.1M TEA/HCl

13.3 ml   Tri-ethanolamide (TEA, Sigma Aldrich)

6 ml   6N HCl

980.7 ml   DEPC-treated water

TEA/HCl was mixed and adjusted to a pH of 8.0 with NaOH.

Acetic anhydride (Sigma Aldrich) was added *ex tempore* (for example, 1 ml acetic anhydride was added to 400 ml TEA/HCl).

4% Formaldehyde in 1x PBS

50 ml   37.6% Formaldehyde (Sigma Aldrich)

50 ml   10x PBS

500 ml   DEPC-treated water

Buffer and solutions used for homogenization of tissue samples

0.1M perchloric acid (kindly provided by E. Anderzhanova)

430 µl   70 % $HClO_4$

50 ml   HPLC grade water

Buffer and solutions used for Lowry Assay

Lowry Reagent

50 ml   Solution A (2% sodium carbonate in 0.1M NaOH)

500 µl   Solution B (2% sodium potassium tartrate)

500 µl   Solution C (1% cupric sulfate)

Distilled water was used to prepare solutions A-C.

Folin reagant

25 ml   Folin-Ciocalteus phenol reagent

25 ml   Distilled water

Acknowledgements

Curriculum Vitae

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Education

2011 – 2015	**Max Planck Institute of Psychiatry and Ludwig-Maximilian University, Munich, Germany**	
	PhD candidate in Neurosciences	
	Thesis	Sleep-wake behaviors resulting from early life obesity – Hedonic and homeostatic signal contributions
	Supervisor	Prof. Mayumi Kimura, PhD
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2009 – 2011	**University of Bonn, Bonn, Germany**	
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2006 – 2009	**University of Cologne, Cologne, Germany**	
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2004 – 2006	**Gymnasium Rodenkirchen, Cologne, Germany**	
	Secondary school, Abitur (German university entrance qualification, A-levels)	
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	Major	Biology, Mathematics

Research experience

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2011 – 2015	**Max Planck Institute of Psychiatry, Munich**	
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	Co-Supervisors	Prof. Osborne Almeida, PhD
		Dr. Alexandre Patchev

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	Laboratory Visit	Experimental sleep studies on cats, rats and mice
	Supervisor	Prof. Isabel de Andrés
Mar – Sep, 2011	**Institute for Reconstructive Neurobiology** **University of Bonn, Bonn**	
	Master thesis	The role of primary cilia in the development of dopaminergic neurons in the murine ventral midbrain
	Supervisor	Dr. Sandra Blaess
Jan – Feb, 2011	**Research center caesar, Bonn**	
	Internship	Department of Molecular Sensory Systems
	Supervisors	Dr. Timo Strünker & Dr. Dagmar Wachten
	Project	The electrophysiological properties of a receptor-type guanylate cyclase in sperm cells of Arbacia punctulata
Oct – Dec, 2010	**University of Bonn, Institute of Anatomy, Bonn**	
	Internship	Department of Anatomy & Cell Biology, Neurodevelopmental Lab
	Supervisor	Prof. Dr. Karl Schilling
	Project	Cell-type specific expression of Mtss1 splice variants in the developing and adult murine retina and cerebellum
Jun – Jul, 2010	**University of Bonn, Institute of Reconstructive Biology, Bonn**	
	Internship	Developmental Neurogenetics
	Supervisor	Dr. Sandra Blaess
	Project	The role of primary cilia in the development of dopaminergic neurons in the murine ventral midbrain
May – Dec, 2009	**Max Planck Institute for Neurological Research, Cologne**	
	Student assistant	RG Neurorehabilitation and –regeneration
	Supervisor	PD Dr. Christian Grefkes
	Project	Involvement in various projects (human studies) including transcranial magnetic stimulation, motor-evoked potentials and fMRI
Apr – Jun, 2009	**Max Planck Institute of Psychiatry, Munich**	
	Bachelor thesis	The Age-Dependent Effect of Ghrelin on the Secretion of Cortisol and Growth Hormone and on Sleep-EEG in Healthy Men
	Supervisor	Prof. Dr. Axel Steiger
Mar – Sep, 2008	**Max Planck Institute of Psychiatry, Munich**	
	Internship	RG Endocrinology of Sleep & RG Clinical Sleep Research
	Supervisors	Prof. Dr. Axel Steiger & PD Dr. Thomas Wetter
	Projects	1. Narcolepsy: An EEG and fMRI study 2. The effect of corticotropin releasing hormone on the sleep EEG of hypophysectomized patients

Academic achievements

Publications

Accepted/published

Gazea M, Tasouri E, Bosch V, Kabanova A, Gojak C, Kurtulmus B, Spatz J, Yoder BK, Pereira G, Brodski C, Tucker KL, Blaess S (**2016**), Primary cilia are critical for Sonic hedgehog-mediated dopaminergic neurogenesis in the embryonic midbrain. Developmental Biology 409(1):55-71.

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Gazea M, Patchev AV, Leidmaa E, Anderzhanova E, Flachskamm C, Almeida OFX, Kimura M (submitted to PNAS), Restoration of serotoninergic homeostasis in the lateral hypothalamus rescues sleep disturbances induced by early-life obesity.

Pawlowski M, **Gazea M**, Wollweber B, Dresler M, Holsboer F, Keck ME, Steiger A, Mikoteit T, Adamczyk M (submitted to Sleep) Cordance and heart rate variability derived from rapid eye-movement sleep as biomarkers of depression and treatment response.

Gazea M, Leidmaa E, Anderzhanova E, Pissioti A, Flachskamm C, Patchev AV, Almeida OFX, Kimura M (to be submitted to Front Neurosci), The central link between hypophagia and enhanced wakefulness following HFD withdrawal in early life obese mice.

Leidmaa E, Patchev AV, **Gazea M**, Pissioti A, Gassen N, Laszlo B, Kallo I, Liposits Z, Almeida OFX (in preparation). Hedonic food overrides with the efficacy of leptin effects on orexin neurons by interfering with galaninergic system.

Leidmaa E, **Gazea M**, Anderzhanova E, Pissioti A, Stoffel R, Patchev AV, Almeida OFX (in preparation). Dysregulation in serotoninergic neurotransmission upon acute exposure to palatable food.

Honors and Awards

2015   Travel Grant by PERC-FENS World Congress for the participation in the 9th World Congress of the International Brain Research Organization (IBRO), Rio de Janeiro, Brazil

2013   Travel grant by ESRS Network of Sleep Research Laboratories for the visit of the sleep laboratory of Prof. Dr. Isabel de Andrés

2011   Poster Award at Neurovisionen 7 meeting in Essen, Germany
"The role of primary cilia in the development of dopaminergic neurons in the murine ventral midbrain"

Voluntary work during PhD work

2015   Organizer of "Visions in Science 2015 – Branch and Connect" and 1st Max Planck Career Fair in Bonn, Germany
Website: http://www.visions-in-science.org/visions/

2014 – 2015   PhD representative at the Max-Planck-Institute of Psychiatry, Munich, Germany

2013   Organizer of the "Sleep Retreat 2013" in Starnberg, Germany (retreat for sleep researchers of the Max Planck Institute of Psychiatry)

Supervision of international summer students

Contributions to scientific meetings during PhD work

2015   **Poster presentation:** "Withdrawal from high-fat diet alters the activity of brain reward centers and homeostatic feeding regulators in peripubertal diet-induced obese mice." 9th World Congress of the International Brain Research Organization, 2015, Rio de Janeiro
Oral presentation: "Peripubertal diet-induced obesity: effects on sleep-wake behaviors and endocrine function during aging." Switchbox Final Meeting, 2015, Paris

2014   **Poster presentation:** "Peripubertal diet-induced obesity impairs sleep quality through changes in the dopaminergic system during aging in mice." 22nd Congress of the European Sleep Research Society, 2014, Tallinn
Poster presentation: "Peripubertal diet-induced obesity affects sleep quality in mice during aging." 9th FENS Forum of Neuroscience, Milan
Oral presentation: "My mouse eats and sleeps!" Switchbox Workshop "Brain, Behaviour and Ageing on the Danube", Budapest

2013   **Poster presentation:** "Primary cilia are crucial for Shh-mediated dopaminergic neurogenesis in the embryonic midbrain." Neuroscience 2013, SfN, San Diego, California
Poster presentation: "Peripubertal diet-induced obesity causes sleep disturbances in mice." Nutrition, Metabolism & the Brain Colloquium, Amsterdam
Poster presentation: "Peripubertal high-caloric nutrition causes sleep disturbances in mice." <interact> 6th Munich Life Science Symposium for Young Scientists, Munich

Oral presentation: "Peripubertal diet-induced obesity affects sleep and emotional behavior in mice". Max Planck Institute Symposium, Munich

Oral presentation: "Peripubertal diet-induced obesity programs sleep disturbances and overweight in mice." Sleep Retreat by Max-Planck-Institute of Psychiatry, Munich

2012   **Oral presentation:** "Malnutrition during puberty affects sleep and emotional behavior in mice." Sleep Retreat by Max-Planck-Institute of Psychiatry, Munich

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