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Research report

Effects of feeding time on daily rhythms of neuropeptide and clock gene expression in the rat hypothalamus

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ABSTRACT

Shiftworkers are exposed to several adverse health conditions, one being eating at night. Food consumption at an unnatural time-of-day is thought to be one of the main factors responsible for the increased risk of developing metabolic diseases, such as obesity and diabetes mellitus. The underlying mechanism is considered to include disruption of the circadian organization of physiology, leading to disruption of metabolism. When food is consumed at night, the hypothalamus, a brain region central to homeostasis, receives contradicting input from the central clock and the systemic circulation. This study investigated how timing of feeding affects hypothalamic function by studying, in different hypothalamic nuclei, expression of clock genes and key neuropeptide genes involved in energy metabolism, including orexin, melanin-concentrating hormone (MCH) and neuropeptide Y. Animals with food available *ad libitum* showed diurnal variation in the expression of clock genes *Per1* and *Per2* in the perifornical area and arcuate nucleus. Clock gene rhythms were lost in both nuclei when food was restricted to the light (i.e., sleep) period. Neuropeptide genes did not display significant daily variation in either feeding groups, except for orexin-receptor 1 in *ad libitum* animals. Analysis of genes involved in glutamatergic and GABAergic signaling did not reveal diurnal variation in expression, nor effects of feeding time. In conclusion, feeding at the 'wrong' time-of-day not only induces desynchronization between brain and body clocks but also within the hypothalamus, which may contribute further to the underlying pathology of metabolic dysregulation.

1. Introduction

Daily rhythms in physiology and behavior exist in many organisms, from cyanobacteria to humans. In mammals, these daily rhythms are driven by the suprachiasmatic nucleus (SCN) within the brain: the central and endogenous generator of approximately 24-h (i.e., circadian) rhythms. The molecular mechanism of the endogenous circadian pacemaker is composed of rhythmic transcription and translation of interlocking negative feedback loops, including *Bmal1*, *Clock*, and *Rorα* as positive and *Cry*, *Per* and *Rev-erbα* as negative elements (Bailey et al., 2014; Zarrinpar et al., 2016). In addition to the central clock in the SCN, this molecular clock mechanism is also present in most peripheral tissues, so-called peripheral clocks.

Synchronizing internal rhythmicity with environmental time is essential to optimally prepare the body for predictable daily challenges. Environmental light is the most dominant stimulus to synchronize the central clock in the SCN with environmental time

Abbreviations: BMAL1, brain and muscle Arnt-like protein 1; CLC3, voltage gated chloride channel 3; CLOCK, Circadian locomotor output cycles kaput; CRY, cryptochrome; DBP, D site of albumin promoter (albumin D-box) binding protein; GABA, gamma aminobutyric acid; PER, period; RORα, retinoic acid related-related orphan receptor; REV-ERBα, reverse viral erythroblastosis oncogene product α; vGat, vesicular GABA transporter; vGlut2, vesicular Glutamate transporter.

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(Nicolaides et al., 2014; Welsh et al., 2010). But besides the light/-dark cycle, mammals also use non-photic stimuli to entrain the circadian timing system (Zarrinpar et al., 2016). Non-photic stimuli such as feeding and locomotor activity mainly impact the peripheral clocks. Hypothetically, entrainment stimuli such as exercise or food consumption during the resting/fasting phase, deliver contradicting inputs to the circadian timing system. These conflicting stimuli may lead to abnormal daily rhythms of clock and metabolic functions, which eventually may contribute to development of metabolic diseases. Conflicting entrainment stimuli occur frequently in shiftworkers, and indeed epidemiological studies have shown an increased risk to develop metabolic health problems, such as obesity and diabetes type II, in shiftworkers (Di Lorenzo et al., 2003; Karlsson et al., 2001). Furthermore, experimental studies in rats reported that, for instance, 'working' and eating during the sleep phase induced a loss or reversal of daily rhythms in plasma glucose and triglyceride concentrations, which may be responsible for the increased adiposity observed in these animals (Salgado-Delgado et al., 2008). In addition, in mice, daytime feeding resulted in body weight gain due to an increased calorie intake, whereas locomotor activity remained the same (Arble et al., 2009). These metabolic disturbances were always accompanied by abnormal daily rhythms of clock gene expression in peripheral organs, such as liver, muscle and adipose tissue. Animal studies have shown that daytime feeding (i.e., during the inactive phase) fully reversed daily rhythms of clock and metabolic gene expression in the liver (Hatori et al., 2012; Opperhuizen et al., 2016; Reznick et al., 2013; Vollmers et al., 2009). Though, daily rhythms of peripheral clock gene expression were strongly influenced by the timing of feeding, hardly any alterations were found in the central SCN clock (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001), confirming the distinct pathways of entrainment for different elements of the circadian timing system.

Hypothalamic nuclei execute numerous functions important to maintaining energy homeostasis, including regulation of the feeding/fasting rhythm (Saper et al., 2002), sleep/wake cycle, energy balance, and hormone secretion (Kalsbeek et al., 2010). The highly heterogenic nature of hypothalamic structures includes the presence of numerous neuropeptides involved in energy homeostasis. For instance, orexin (ORX) (Sakurai et al., 1998), also known as hypocretin (de Lecea et al., 1998), and melanin-concentrating hormone (MCH), are neuropeptides produced in the perifornical area (PeF) of the lateral hypothalamus (LH) (Sakurai et al., 1998; Sakurai, 2014), albeit in separate neuron populations (Broberger et al., 1998; Elias et al., 1998; Sakurai, 2005). Both peptides are orexigenic (Kageyama et al., 2012; Qu et al., 1996) and are oppositely involved in the control of the mammalian sleep/wake cycle (Harris and Aston-Jones, 2006; Hassani et al., 2009; Lee et al., 2005) and glucose metabolism (Tsuneki et al., 2010). Another key nucleus involved in the regulation of feeding behavior is the arcuate nucleus (ARC) containing, amongst others, neuropeptide Y (NPY) producing neurons. NPY has a strong stimulatory effect on food intake (Edwards et al., 1999), influences the energy balance and glucose metabolism (Muroi and Ishii, 2016; Thorens, 2012; Yi et al., 2012) and acts on the ORX- and MCH-neurons in the PeF (Stanley and Thomas, 1993). A first attempt to study the hypothalamic reaction to conflicting input showed area-specific responses in animals forced to be active (Salgado-Delgado et al., 2010) or feed (Angeles-Castellanos et al., 2004; De Araujo et al., 2016; Minana-Solis et al., 2009) during their normal sleep phase.

Balancing the excitatory glutamatergic and inhibitory γ-aminobutyric acid (GABA)ergic neurotransmission in hypothalamic areas is an important SCN output mechanism (Kalsbeek et al., 2008). Abundant presence of both the vesicular glutamate (*vGlut2*) and GABA transporter (*vGat*) has been reported in the LH, including on ORX- and MCH-producing neurons (Backberg et al., 2004; Stuber and Wise, 2016). Moreover, disruption of GABA functioning in the LH has been shown to disrupt ORX-mediated glucose control (Yi et al., 2009) and sleep (Alam et al., 2005).

Until now, there have only been limited studies on how the molecular clocks within these SCN target areas respond to conflicting entraining signals such as eating at the 'wrong' time-of-day (i.e., during the sleep period). We hypothesized that such conflicting signals will disturb the molecular clocks in these hypothalamic nuclei and subsequently their homeostatic functions. Therefore, we investigated whether alterations in the timing of food intake, known to affect entrainment of peripheral clocks in liver and muscle, would affect the molecular clock and neuropeptide expression in SCN target areas. We studied the daily variation in the expression of clock (*Per1*, *Per2 and Bmal1*) and orexigenic (*Orx*, *Orx receptors*, *Mch and Npy*) genes in the PeF and ARC of male rats. Furthermore, we studied whether feeding time affects the expression of genes involved in synaptic glutamate/GABA neurotransmission (*vGat*, *vGlut2* and the chloride channel transporter *Clc3*) in the PeF.

2. Results

Metabolic data of the animals in this study have been published before (Reznick et al., 2013) and therefore are not reported here. In short, the authors observed that restricting food availability to the light phase induced a decrease in food intake without alterations in bodyweight or adiposity, a reversal of the daily RER rhythm and a disruption of the daily locomotor activity pattern and the oxygen consumption rhythm. Furthermore, clock and metabolic gene expression and protein content were differentially affected in liver and skeletal muscle tissue.

2.1. Effects of feeding time on clock gene expression in the perifornical area and arcuate nucleus of the hypothalamus

Control animals with food available *ad libitum* (ALF) demonstrated clear daily rhythms for the clock genes *Per1* and *Per2*, but not for *Bmal1*, in the PeF. The rhythmic expression of both *Per*-genes showed a peak at the beginning of the dark phase, at ZT15, and a trough at the beginning of the light phase (Fig. 1a, c, e). One-way ANOVA showed a significant effect of *Time* for *Per1* and *Per2* (both genes $p = 0.002$, Table 2), but not for *Bmal1* ($p = 0.507$) and JTK Cycle analysis indicated a significant 24 h rhythm of expression for both *Per* genes (*Per1:* $p < 0.001$; *Per2*: $p = 0.009$, Table 3), but not *Bmal1*. Animals with food available only during the day (DTF) showed pronounced alterations in the expression patterns of the clock genes with a loss of rhythmicity in both *Per* genes (Fig. 1a, c, e, Tables 2 and 3). Two-way ANOVA analysis to compare ALF and DTF animals showed significant effects of *Time*

Table 1
Primer sequences for RT-PCR.

Gene	Forward primer	Reverse primer
Bmal1	CCGATGACGAACTGAAACACCT	TGCAGTGTCCGAGGAAGATAGC
Per1	CGCACTTCGGGAGCTCAAACTTC	GTCCATGGCACAGGGCTCACC
Per2	CACCCTGAAAAGAAAGTGCGA	CAACGCCAAGGAGCTCAAGT
Orx	TCCTCACTCTGGGAAAGCG	CAGGGCAGGGATATGGCTCTA
OrxR1	AGAGAGCAGAGAGCGTTGTAAACC	TTCACAGGGACACATTGCTGC
OrxR2	TGTTCAAGAGCACAGCCAAACG	GCCAATACCATAAGACACAGGGG
Mch	CCACAAAGAACACAGGCTCC	GGTCCTTTCAGAGCGAGGTAAG
Npy	GACAATCCGGGCGAGGACGC	TCAAGCCTTGTTCTGGGGGCA
vGat	GGGTCACGACAAACCCAAGA	TAGGGTAGACCCAGCACGAA
vGlut2	ATCTGGTAAGGCTGGACACG	TAGCGGAGCCTTCTTCTCAG
Clc3	ACTGGGTGCGAGAAAAGTGT	AGCCATCCTGACCAAGCATC
Hprt	TTGGTCAAGCAGTACAGCCC	CTTGCCGCTGTCTTTTAGGC

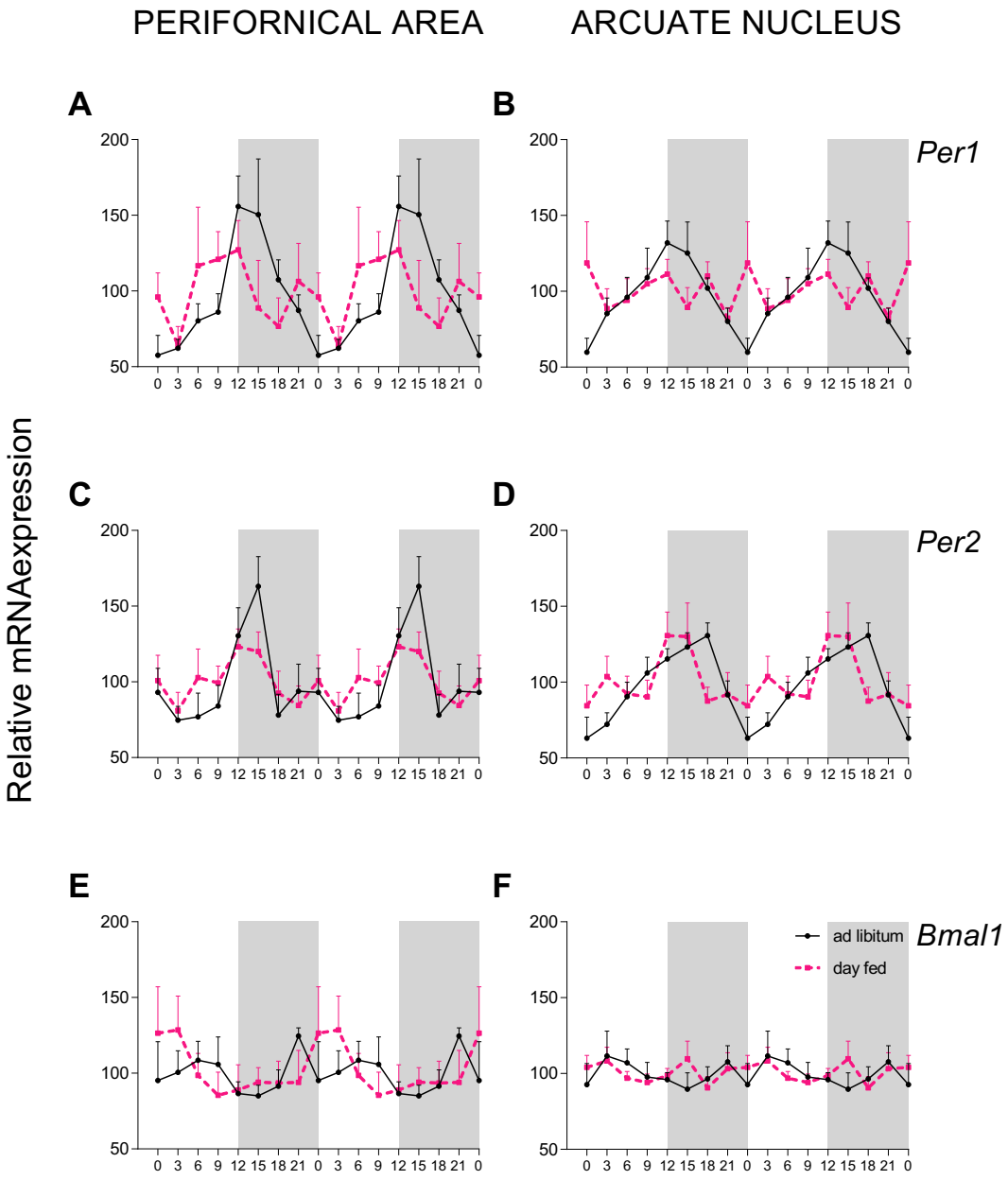

Fig. 1. Clock gene expression in hypothalamic nuclei. Double plotted expression of *Per1* (a, b), *Per2* (c, d), and *Bmal1* (e, f) in perifornical area (left graphs) and arcuate nucleus (right graphs) of *ad libitum* fed (solid lines) and daytime fed (dashed lines) animals. Data are expressed as mean ± SEM, n = 5–10 animals per data point.

Table 2
Results of one-way ANOVA analyses on gene expression.

Gene	Perifornical area		Gene	Arcuate nucleus	
	ALF	DTF		ALF	DTF
Per1	**0.002**	0.524	*Per1*	**0.022**	0.531
Per2	**0.002**	0.437	*Per2*	**<0.001**	0.123
Bmal1	0.507	0.622	*Bmal1*	0.802	0.624
Orx	0.303	0.435	*Npy*	0.067	0.852
OrxR1	0.084	0.709			
OrxR2	0.597	0.734			
Mch	0.059	0.417			
vGlut2	0.817	0.494			
Clc3	0.318	0.092			
vGat	0.702	0.811			

One-way ANOVA analysis was used to determine the effect of *Time* in individual feeding groups. *P* value is depicted for relative expression of each gene in PeF and ARC of *ad libitum* fed (ALF) and daytime fed (DTF) groups. Significant results are presented in bold, trends are underlined.

Table 3
Results of JTK Cycle analyses on rhythmicity of gene expression.

	P-Value		Amplitude		Acrophase	
	ALF	DTF	ALF	DTF	ALF	DTF
PeF						
Per1	<0.001	n.s	32.76	20.75	15	
Per2	0.009	n.s	22.51	12.99	15	
Bmal1	n.s	n.s	5.28	12.75		
Orx	n.s	n.s	4.96	2.68		
OrxR1	0.005	n.s	10.39	5.48	15	
OrxR2	n.s	n.s	9.83	9.91		
Mch	n.s	n.s	7.75	5.15		
vGlut2	n.s	n.s	2.57	7.33		
Clc3	n.s	n.s	7.01	12.94		
vGat	n.s	n.s	12.99	7.74		
ARC						
Per1	<0.001	n.s	20.48	9.44	13.5	
Per2	<0.001	n.s	26.85	7.85	15	
Bmal1	n.s	n.s	6.67	1.69		
Npy	n.s	n.s	6.35	4.11		

Parameters of fitted wave of JTK Cycle analysis for 24 h rhythm of gene expression in PeF and ARC of *ad libitum* fed (ALF) and daytime fed (DTF) groups. Converted values of gene expression into percentages are used. N.s indicates no significant diurnal variation. Acrophase is only provided if a gene was significantly rhythmic. *P*-value is provided when significant.

Table 4
Results of two-way ANOVA analyses on the effect of time of feeding on gene expression.

Gene	Perifornical area			Gene	Arcuate nucleus		
	Feeding	Time	Interaction		Feeding	Time	Interaction
Per1	0.914	**0.026**	0.192	*Per1*	0.864	*0.089*	*0.077*
Per2	0.872	**<0.001**	0.455	*Per2*	0.713	**<0.001**	0.105
Bmal1	0.867	0.651	0.601	*Bmal1*	0.848	0.703	0.763
Orx	0.992	0.378	0.125	*Npy*	0.878	0.228	0.682
OrxR1	0.982	0.208	**0.048**				
OrxR2	0.930	0.589	0.851				
Mch	0.973	0.105	**0.023**				
vGlut2	0.967	0.301	0.972				
Clc3	0.791	0.107	0.300				
vGat	0.996	0.923	0.434				

Two-way ANOVA was used to detect *Treatment, Time* and *Interaction* effects between *ad libitum* and daytime feeding groups. Converted values of gene expression into percentages are used. *P* values are depicted for the three terms of the ANOVA and significant results are presented in bold.

in *Per* genes, but no *Treatment* (i.e., feeding group) or *Interaction* effects were observed (Table 4).

In the arcuate nucleus, ALF animals also demonstrated clear daily variation in expression of *Per1* and *Per2*, but not *Bmal1,* with peak expression at ZT13.5 and ZT15, respectively (Fig. 1b, d, f, Table 3). Significant *Time* effects and JTK Cycle analysis were found for both *Per* genes (ANOVA *Per1*: $p = 0.022$; *Per2*: $p < 0.001$, Table 2; JTK Cycle $p < 0.001$ for both genes, Table 3). Again, alterations in the timing of feeding affected clock gene expression and induced a loss of significant rhythmic variation (Fig. 1b, d, f). Comparison between DTF and ALF animals revealed a significant *Time* effect ($p < 0.001$) for *Per2* expression and a trend for a *Time* ($p = 0.089$) and *Interaction* ($p = 0.077$) effect in *Per1* expression (Table 4). In ALF animals, the amplitude for the rhythm of *Per1* expression was larger in the PeF than in the ARC (32.8 vs. 20.5), whereas for *Per2* this was comparable (22.5 vs. 26.9, Table 3). Daytime fed animals showed a lower amplitude in *Bmal1* expression in the ARC compared to ALF animals, whereas in the PeF the reverse was observed

2.2. Effects of feeding time on expression of orexigenic genes in PeF and ARC of the hypothalamus

To study whether timing of feeding affects daily expression rhythms of neuropeptides involved in energy homeostasis, we measured expression of *Orx,* orexin receptors 1 and 2, *Mch* in the PeF and *Npy* in the ARC. Overall, the amplitude values of neuropeptide expression rhythms were lower than those for clock genes. *Orx* (Fig. 2a) and *Orx-receptor2* (Fig. 2d) did not show significant diurnal variation of expression in the ALF or DTF groups. *Mch* expression showed a trend ($p = 0.059$) for an effect of *Time* in the ALF group (Fig. 2b). *Orx-receptor1* showed daily variation in expression only in ALF animals with an acrophase at ZT15 (JTK Cycle $p = 0.005$, Table 3, ANOVA *Time* effect: $p = 0.08$, Table 2, Fig. 2c). Comparison between DTF and ALF showed a significant *Interaction* effect for *Mch* ($p = 0.023$) and *OrxR1* ($p = 0.048$) expression (Table 4).

None of the feeding groups demonstrated daily variation of *Npy* expression in the ARC, only a trend was found in one-way ANOVA analysis of the ALF animals ($p = 0.067$, Fig. 2e, Table 2 and 3).

2.3. Effects of feeding time on expression of genes involved with glutamate and GABA neurotransmission

In order to investigate the balance of glutamate and GABA neurotransmission, expression of *vGlut2*, *vGat* and *Clc3* in the PeF punches was studied for an effect of feeding time. Individual gene expression of *vGlut2*, *vGat* and *Clc3* did not display significant daily fluctuations in either ALF or DTF animals (Fig. 3a–c, Table 3). Only a trend was found for an effect of *Time* in daytime fed animals in *Clc3* expression ($p = 0.092$, Table 2). The ratio of *vGlut2*/*vGat* provides an indication of the GABAergic tone (Martisova et al., 2012). Average 24 levels of this ratio were not different between the feeding

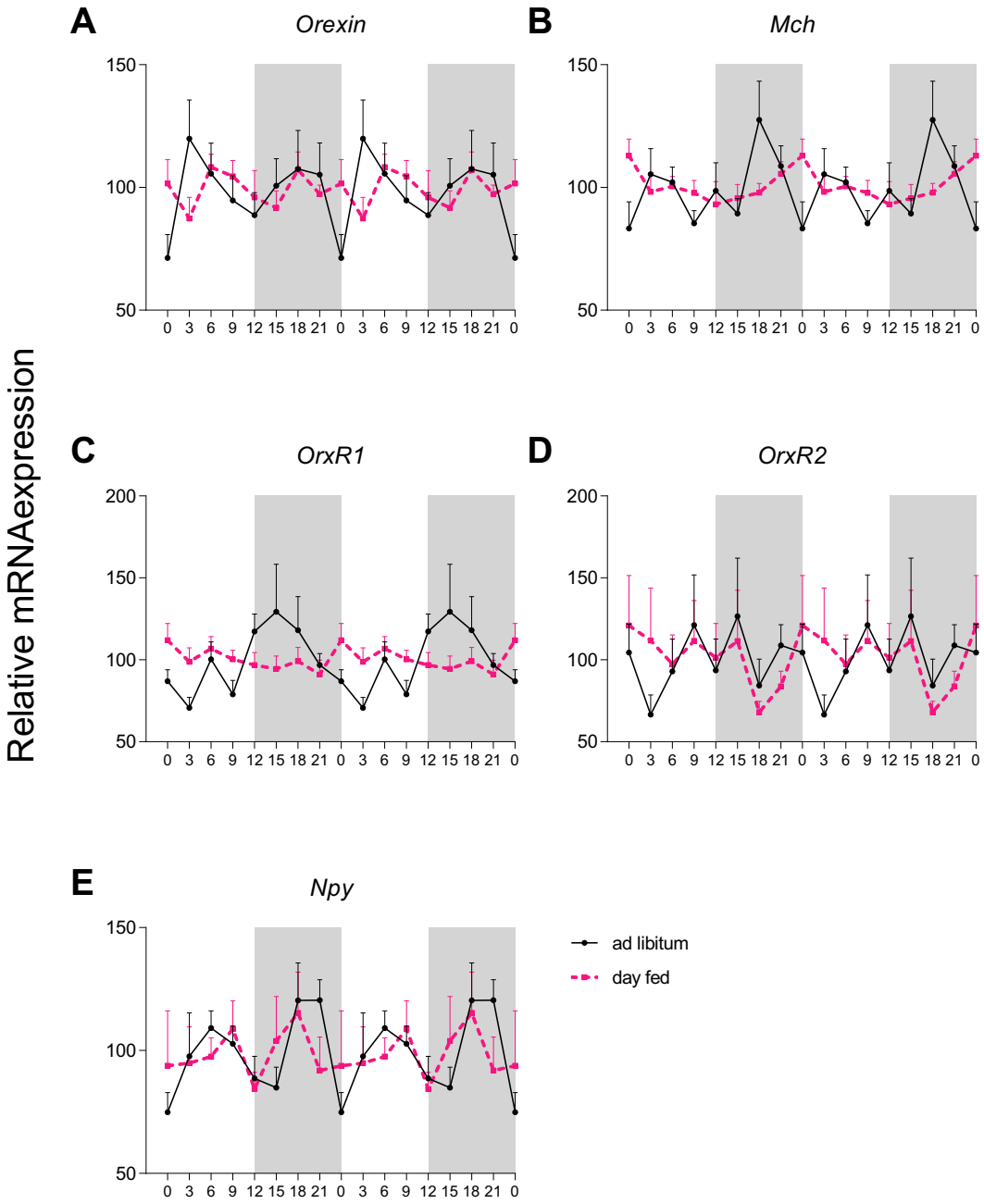

Fig. 2. Neuropeptide expression in hypothalamic nuclei. Double plotted expression of *Orexin* (a), *Mch* (b), *OrxR1* (c) and *OrxR2* (d) in perifornical area and *Npy* (e) in arcuate nucleus of *ad libitum* fed (solid lines) and daytime fed (dashed lines) animals. Data are expressed as mean ± SEM, n = 5–10 animals per data point.

groups (ALF 1.11 ± 0.05; DTF 1.06 ± 0.04). No significant effects of *Time* were found in DTF ($p = 0.568$) and ALF ($p = 0.259$) animals, nor did two-way ANOVA reveal effects of feeding time (*Time* $p = 0.345$, *Feeding* $p = 0.575$, *Interaction* $p = 0.401$, Fig. 3d) on the *vGlut2*/*vGat* ratio. Inhibition by GABA is partly facilitated by CLC3, and, indeed, a significant positive correlation between *vGat* and *Clc3* expression was observed in DTF animals, but not in ALF animals (Fig. 3e). Timing of food intake did not affect the negative correlation between GABA tonus (i.e., the *vGlut2/vGat* ratio) and *orexin* expression (Fig. 3f).

3. Discussion

In the present study, we show that restricting food intake to an unnatural time-of-day (i.e., the light phase for rats) induces a loss of all rhythms present in the perifornical area and arcuate nucleus during *ad libitum* feeding conditions, both those of clock genes and neuropeptides involved in the control of energy homeostasis. Thus contrary to the SCN itself, alterations in feeding time do affect the molecular clock mechanism and neuropeptide expression in the hypothalamic nuclei that are targeted by SCN output. The arhythmicity of clock gene expression in the SCN target areas is most likely a reflection of the contrasting inputs received by these brain areas: entrained signals from the SCN and partly or completely shifted signals from the periphery. Thus compared to the SCN and peripheral clocks, the response of the SCN target areas probably best reflects the internal desynchronization caused by the opposing entrainment stimuli of feeding during the sleeping phase.

Desynchronization of molecular clocks as a result of opposing entraining properties has been reported before and different kinds

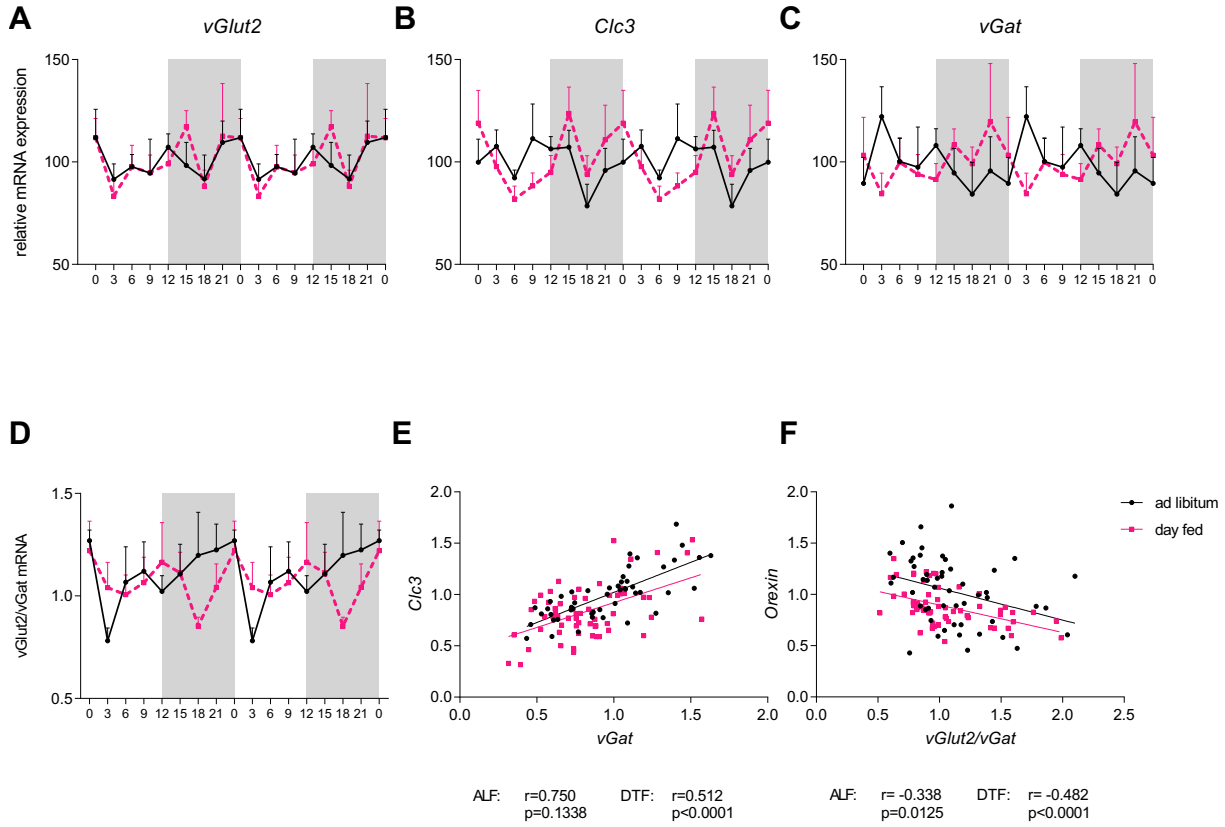

Fig. 3. GABA and glutamate neurotransmission. Double plotted expression of *vGlut2* (a), *Clc3* (b), *vGat* (c) and the *vGlut2/vGat* ratio (d) in the perifornical area of *ad libitum* fed (solid lines) and daytime fed (dashed lines) animals. A positive correlation between *vGat* and *Clc3* (e) was significant in DTF animals, but not in ALF animals. A negative correlation between the *vGlut2/vGat* ratio and *Orexin* expression (f) was found in both feeding groups. Data are expressed as mean ± SEM, n = 5–10 animals per data point.

of desynchronization have been described after changes in the timing of food intake. Peripheral desynchronization has been demonstrated to occur between the central and peripheral clocks (Damiola et al., 2000), as well as, between clocks of different peripheral organs (Opperhuizen et al., 2016; Reznick et al., 2013). In addition, internal desynchronization has been proposed to occur between clock genes and metabolic genes and even within the molecular clock mechanism, between different clock genes (Mukherji et al., 2015; Opperhuizen et al., 2015; Salgado-Delgado et al., 2013). The concept of hypothalamic desynchronization is relatively new and has only been studied occasionally (Opperhuizen et al., 2016; Ramirez-Plascencia et al., 2017; Salgado-Delgado et al., 2010). Moreover, the interaction between clock genes, desynchronization and neuropeptide expression and function is completely unknown.

The data in the current study were derived from brains of animals in a study comparing *ad libitum* feeding with daytime feeding, published previously (Reznick et al., 2013). Daytime fed animals in that study maintained equal body weight compared to animals eating *ad libitum*, suggesting alterations in nutrient absorption and/or oxidation. Daytime feeding affected concentrations of hormones, disturbed locomotor activity behavior and induced desynchronization between two essential metabolic organs, the liver and the skeletal muscle. Tissue specific proteins were differently affected in liver and muscle tissue by daytime feeding, demonstrating also functional changes besides changes in mRNA expression. These results were quite comparable to those of a previous study of our own using a longer period of daytime feeding (8 weeks versus 4 weeks) (Opperhuizen et al., 2016).

In addition to the SCN, other hypothalamic nuclei have also been reported to express components of the molecular clock. The phase, robustness and entraining properties, however, of these other hypothalamic clocks remain to be investigated. Localization of the lateral hypothalamus (LH) and arcuate nucleus (ARC) nearby the SCN, and the well-reported connectivity with the SCN, suggests strong influences of the SCN on these two brain regions. On the other hand, *in vitro* studies indicate these rhythms to be independent of the SCN (Abe et al., 2002; Guilding et al., 2009). Several studies have demonstrated that the SCN itself does not change its rhythmicity with alterations in the timing of food intake (Angeles-Castellanos et al., 2004; Damiola et al., 2000). On the other hand, the current and other studies (De Araujo et al., 2016) have shown that hypothalamic nuclei such as the LH (Opperhuizen et al., 2016) and ARC (Ramirez-Plascencia et al., 2017) are sensitive to changes in feeding time. For instance, daytime feeding was shown to reverse the daily rhythms of PER1 and PER2 protein expression in the dorso-medial hypothalamus (DMH) (Verwey et al., 2009; Verwey and Amir, 2011), and affect diurnal gene expression levels of *Per1, Per2* and *Bmal1* in the PVN (Girotti et al., 2009) and DMH (Moriya et al., 2009) in mice. Our data are in line with the latter observations, as daily variation in *Per1* and *Per2* gene expression was lost in both the LH and the ARC by daytime feeding.

The hypothalamus is recognized for its metabolic functions, strategically located near the third ventricle to sense fluctuations in energy levels immediately and respond accordingly by stimulating or inhibiting food intake, sleep and activity, glucose production and body temperature. Orexin and MCH, two of the neuropeptides contributing to this homeostasis and produced in the perifornical area of the LH, did not show a significant daily variation at the mRNA level in the current study. For orexin, this agrees with our previous report (Opperhuizen et al., 2016) and two previous

studies in mice (Akiyama et al., 2004; Stutz et al., 2007). However, these observations are not in line with another study showing daily oscillations in *prepro-orexin* (Taheri et al., 2000), as well as reports of rhythmic orexin peptide levels in the cerebrospinal fluid (Desarnaud et al., 2004; Zeitzer et al., 2003; Zhang et al., 2004) and the number of orexin-containing neurons (Martinez et al., 2002). Limited data are available on rhythmic expression of MCH peptide or mRNA, but in mice daily variation in hypothalamic *Mch* expression (Stutz et al., 2007) and peptide concentrations (Gerics et al., 2017) have been reported. Furthermore, direct projections from the SCN to MCH neurons have been shown (Abrahamson et al., 2001), suggesting them to be under circadian control. Changes in the timing of food intake did not induce major alterations in orexin and *Mch* expression. In our study, we did not measure peptide concentrations, and therefore it is possible that the orexin or *Mch* system is affected at the level of peptide production, release or function. Congruent with our previous study (Opperhuizen et al., 2016), *OrxR1* was significantly rhythmic only in control animals with an acrophase at ZT15. This rhythm was lost in the daytime fed group, whereas no changes were observed in *OrxR2* mRNA expression.

Previously it has been proposed that the daily rhythmicity of the orexin system is controlled by the GABAergic and glutamatergic output from the SCN (Yi et al., 2009), and low GABA activity, occurring naturally during the dark phase or induced by bicuculline infusion, increases orexin activity (Alam et al., 2005). The negative correlation between orexin mRNA and the ratio of glutamate transporters over GABA transporters we observed, indicates increased orexin mRNA expression with increasing GABAergic transmission. The unexpected direction of this correlation probably is explained by the fact that the peak activity of orexin mRNA expression is in opposite phase with the peak of peptide expression (Taheri et al., 2000), as well as with extracellular orexin concentrations (Zhang et al., 2004) and neuronal activity of orexin neurons (Estabrooke et al., 2001; Martinez et al., 2002). The latter three peak during the dark (i.e., active) phase in nocturnal animals. Thus the negative correlation between the *vGlut2/vGat* ratio and orexin mRNA expression indicates more orexin release with lower GABA transmission. Studying the daily variation of the ratio of *vGlut2/vGat* expression indeed shows a lower ratio, i.e., more orexin mRNA but less orexin release, during the light period. The data in Fig. 3d also suggest that the time of the highest GABA tone shifted from the early light phase to the mid dark phase by daytime feeding, but these changes were not significant. Moreover, the relation between *vGlut2/vGat* and orexin mRNA was also not affected by feeding time (Fig. 3f).

NPY neurons in the arcuate nucleus have been studied extensively for their role in feeding behavior and are considered to act in part via the MCH- and orexin neurons in the LH (Horvath et al., 1999; Kageyama et al., 2012). Several studies have shown (a trend towards) significant daily variation in *Npy* mRNA expression in *ad libitum* fed animals (Akabayashi et al., 1994; Kohsaka et al., 2007; McKibbin et al., 1991; Stutz et al., 2007; Xu et al., 1999). Also in the current study we only found a trend towards a significant effect of time-of-day.

The hypothalamic neuropeptides *Orexin*, *Mch* and *Npy* studied in this experiment did not show statistical significant daily variation of mRNA expression levels. Rhythmicity of the neuropeptides has been reported in CSF (Desarnaud et al., 2004; Djeridane et al., 2004; Pelluru et al., 2013; Zeitzer et al., 2003; Zhang et al., 2004), neuronal activity (Martinez et al., 2002; Ramirez-Plascencia et al., 2017) or in tissue (Jhanwar-Uniyal et al., 1990), but likely it is more difficult to detect similar changes in mRNA expression. This might be due to post-transcriptional processes, to the heterogeneity of the tissue punches or to the method of estimation. Absence of rhythmicity in *ad libitum* fed animals, for either methodological or biological reasons, increased the challenge to observe effects of timing of food intake. Future experiments including other techniques should be done before discarding the idea that food timing affects these peptidergic systems.

Thus contrary to the SCN, restricting feeding time to the light phase induces significant changes in clock gene expression rhythms in SCN target areas, with all genes losing their rhythmicity. Daily rhythms in neuropeptide expression in SCN target areas were non-significant, but comparable reductions in amplitude were observed. Literature data support interactions between the molecular clock and hypothalamic neuropeptide expression. For instance, absolute expression and diurnal variation in, amongst others, orexin expression was affected in the hypothalamus of *Clock* mutant mice (Turek et al., 2005).

4. Conclusion

This study highlights that time-dependent food intake strongly influences the molecular clock in hypothalamic SCN target areas. Thus although the molecular clock of the SCN itself seems to be insensitive for feeding at the wrong time-of-day, the hypothalamic clock in SCN target areas is clearly affected. 'Wrong' timing of food intake occurs frequently in large populations around the world, and literature is increasing on which levels of physiology are influenced by food timing, and possibly contribute to the increased risk of metabolic health problems. With this study, we emphasize the importance of including the hypothalamus, the epicenter of homeostatic regulation, in the hypothesis that circadian desynchronization underlies metabolic disruption.

5. Experimental procedure

5.1. Animals

The material for the current publication is derived from the previously published study by Reznick et al. (Reznick et al., 2013). For the current study, only brains were included from animals fed with regular chow (Rat maintenance Diet, including 8% fat, 21% protein and 71% carbohydrate plus fiber, vitamins and minerals; Gordon's Specialty Feeds, Sydney Australia) from two groups of adult male Wistar rats (Animal Resources Centre, Perth, Australia):

1) ***Ad libitum* (ALF)**; animals fed *ad libitum*, these animals consumed ~90% of their food during the dark phase;

2) **Daytime fed** (**DTF**); animals fed during daytime with food available only when lights were on (ZT0) to lights off (ZT12).

Water was available *ad libitum* for both groups. All experimental procedures performed were approved by the Animal Ethics Committee (Garvan Institute of Medical Research/St Vincent's Hospital, Darlinghurst, Australia) and were in accordance with the National Health and Medical Research Council of Australia's guidelines on animal experimentation.

5.2. Brain collection and brain areas separation

After four weeks on the feeding regime, rats were assigned to groups to be sacrificed at 3 h intervals over the 24 h period. Rats were sacrificed at ZT0, 3, 6, 9, 12, 15, 18 and 21, brains were immediately removed, placed on an aluminum plate cooled on dry ice, and stored at -80 °C until brain area dissection. Each time point comprises 5–10 animals per feeding group. The brains were sent to The Netherlands, and after arrival in Amsterdam, brains were sectioned into 250 μm slices at -20 °C with a cryostat (Leica CM 1950) and brain slices were soaked directly into RNA later (Ambion, Waltham, MA, USA). Bilateral punches from the perifornical area of the lateral hypothalamus (PeF) and arcuate nucleus

(ARC) were obtained using a 1mm diameter needle and collected into tubes with green beads (Roche, Basel, Switzerland). Approximately 8 bilateral PeF punches (bregma −2.40 to −3.84) and 4 bilateral ARC punches (bregma −1.72 to −3.12) were collected and homogenized by MagNA lyser Green beads (30 s, 6500 g, Roche, Basel, Switzerland) and stored in −80 °C until RNA isolation.

5.3. RNA isolation and cDNA synthesis

Total RNA was isolated from brain punches using RNA isolation kit (ALF animals: high pure RNA tissue kit, Roche, USA; DTF animals: RNA, DNA & Protein purification kit, Bioké, The Netherlands) according to manufacturer's instructions. RNA levels were detected by UV spectrophotometer (DS-11, DeNovix, USA). Equal amounts of total RNA in the same brain area among both groups were reverse transcribed using Transcriptor First cDNA Synthesis Kit (Roche, USA) with oligo-dT primers (30 min at 55 °C, 5 min at 85 °C). Additional reverse transcriptase minus (−RT) controls were randomly chosen and run to control for genomic DNA contamination.

5.4. Real-time quantitative PCR

The reaction system to measure gene expression using quantitative RT-PCR is that 2 μl cDNA was incubated in a final volume of 10 μl reaction containing 1 × SensiFAST SYBR NO-ROX Mix and 25 ng of each primer (forward and reverse). Quantitative RT-PCR (qRT-PCR) was performed in Lightcycler®480 (Roche), primer information is presented in Table 1. The qPCR data were first corrected by dividing the absolute expression by the mean expression of the whole plate (to correct of inter-plate differences). The relative amount of each gene was normalized against the expression of *Hprt* (Hypoxantine-guanine phosphoribosyl transferase) as a housekeeping gene for both brain areas. *Orx*, *OrxR1*, *OrxR2*, *Mch*, *vGlut2*, *Clc3* and *vGat* were measured in the PeF, *Npy* was measured in the ARC. The clock genes *Per1*, *Per2* and *Bmal1* were measured in both brain areas.

5.5. Statistical analysis

All analyses were conducted using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla, CA, USA) and JTK Cycle software (Hughes et al., 2010). For technical reasons RNA was prepared with a different protocol in the ALF animals than in the DTF animals. In order to compare amplitude and acrophase between the feeding groups, relative expression levels were converted to a percentage. This was done by setting the average 24 h expression of each gene per feeding group at 100%, and subsequently converting the relative expression of each animal into a percentage. After conversion, the effect of *Time* in the single treatment groups was analyzed using one-way ANOVA (Table 2). Furthermore, feeding groups were analyzed by two-way ANOVA analysis, for the effect of feeding group (*Treatment*), ZT time (*Time*) and *Interaction* (Table 4).

In addition, gene expression in the single treatment groups was analyzed using JTK Cycle software to test for rhythmicity (Table 3). Acrophase and *p*-value were obtained by fitting the data on a curve with a fixed 24 h period and are only reported for rhythmically expressed genes. *P* values reported are the result of the F-test, and the 24 h rhythm was confirmed if $p < 0.05$. JTK Cycle analysis produced equal *P*-value and acrophase estimation for converted and non-converted data (Table 3: converted data; Supplementary Table 1: non-converted data). Correlation analyses were done by calculation of Pearson's correlation coefficient r in GraphPad. Results were considered statistically significant if $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainres.2017.07.006.

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