Region-specific effects of restricted feeding on daily rhythms of the clock proteins, Period1 and Period2, in the forebrain of the male Wistar rat

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Abstract

Region-specific effects of restricted feeding on daily rhythms of the clock proteins, Period1 and Period2, in the forebrain of the male Wistar rat

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Circadian rhythms are driven by clocks and oscillators, which are distributed throughout the brain and body, and rely on daily rhythms in the expression of canonical clock genes such as Period1 (PER1) and Period2 (PER2). In rodents, restricted feeding (RF) schedules limit food-access to a single meal each day and entrain circadian rhythms in food-anticipatory activity. The dorsomedial hypothalamic nucleus (DMH) is one brain area where daily rhythms in PER1 and PER2 are adjusted in response to RF, and this shift is potentially involved in generating food-entrained rhythms in behavior. The limbic forebrain, which is important in the regulation of motivation and emotion, also exhibits daily rhythms of PER2 expression that are adjusted by RF. The elucidation of RF-induced changes in the daily rhythm of PER1 and PER2 expression not only helps to identify brain areas that could be involved in the generation of food-entrained circadian rhythms, but also helps to define the nature and importance of these region-specific circadian rhythms. The experiments in the present thesis use male Wistar rats in order to study RF-induced changes of daily rhythms of PER1 and PER2 protein expression in the DMH, limbic forebrain and the “master” light-

entrained clock, the suprachiasmatic nucleus (SCN). These studies demonstrate a nucleus-specific response to RF, which is associated with to the start-time, predictability, and duration of the daily RF meal. In the DMH, RF-induced rhythms of PER1 and PER2 expression were not strictly food-entrained, nor were they associated with the generation of circadian rhythms in food-anticipatory running-wheel activity. In the limbic forebrain, daily rhythms of PER2 expression in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and the central nucleus of the amygdala (CEA) appeared to become food-entrained. Also in the limbic forebrain, daily rhythms of PER2 expression in the basolateral amygdala (BLA) and dentate gyrus (DG) were adjusted in response to daytime food-access, which is a novel feeding time for these nocturnal rodents. Finally, daily rhythms of PER1 and PER2 expression in the SCN failed to respond to RF and, instead, remained in synchrony with the environmental light-dark cycle. In summary, these studies show that RF has widespread effects on the daily rhythms of PER1 and PER2 expression in brain areas that are not traditionally linked to the regulation of circadian rhythms, *per se,* but are instead associated with the regulation of motivation, arousal, emotion, and learning.

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Table of Contents

List of Figures xii

List of Tables xxi

List of Abbreviations xxii

Introduction 1

The circadian autoregulatory feedback loop 4

Daily rhythms in clock gene expression and their consequences 8

Restricted feeding and food-anticipatory activity 13

The suprachiasmatic nucleus 17

The dorsomedial hypothalamic nucleus 19

The limbic forebrain and dorsal striatum 22

The present thesis 25

Chapter 1: Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats 29

Contribution of authors 30

Abstract 31

Introduction 33

Method 35

Results 38

Discussion.. 49

Acknowledgments.. 55

Chapter 2: Region-specific modulation of PER2 expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats.. 56

Contribution of authors.. 57

Abstract.. 58

Study.. 60

Acknowledgements.. 72

Chapter 3: Circadian rhythms of PERIOD1 expression in the dorsomedial hypothalamic nucleus in the absence of entrained food-anticipatory activity rhythms in rats.. 73

Contribution of authors.. 74

Abstract.. 75

Introduction.. 76

Method.. 78

Results.. 81

Discussion.. 87

Acknowledgements.. 93

Chapter 4: Variable restricted feeding disrupts the daily oscillations of PERIOD2 expression in the limbic forebrain and dorsal striatum in rats.. 94

Contribution of authors.. 95

Abstract 96

Introduction 97

Method 99

Results 103

Discussion 110

Acknowledgements 115

Chapter 5: Nucleus-specific effects of meal duration on daily profiles of Period1 and Period2 protein expression in rats housed under restricted feeding. 116

Contribution of authors 117

Abstract 118

Introduction 120

Method 122

Results 126

Discussion 138

Acknowledgements 143

General Discussion 144

The DMH 146

The SCN 153

The limbic forebrain 154

Potential mechanisms 156

Final thoughts 158

References 161

Appendix A: Food-entrainable circadian oscillators in the brain........ 197

Contribution of authors........ 198

Abstract........ 199

Introduction 200

Circadian clock gene expression in the brain........ 203

Brain areas sensitive to restricted feeding 204

The circadian basis of food-anticipatory activities 213

The DMH and food-anticipatory activity 215

Potential mechanisms........ 217

Acknowledgements........ 220

Appendix B: REPRINT (CHAPTER 1) - Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats…….221

Appendix C: REPRINT (CHAPTER 2) - Region-specific modulation of PER2 expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats……….231

Appendix D: REPRINT (CHAPTER 3) - Circadian rhythms of PERIOD1 expression in the dorsomedial hypothalamic nucleus in the absence of entrained food-anticipatory activity rhythms in rats……………………………………………………237

Appendix E: REPRINT (CHAPTER 4) - Variable restricted feeding disrupts the daily oscillations of PERIOD2 expression in the limbic forebrain and dorsal striatum in rats……………………………………………………………………………..………..244

Appendix F: REPRINT (CHAPTER 5) - Nucleus-specific effects of meal duration on daily profiles of Period1 and Period2 protein expression in rats housed under restricted feeding…………………………………………………………………..252

Appendix G: REPRINT (APPENDIX A) - Food-entrainable circadian oscillators in the brain……………………………………………………………………….…261

List of Figures

Chapter 1

Figure 1.1 39

Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food-deprived (RF, n=24) and free-fed (RT, n=24) rats. Ensure was presented for 2h each day from ZT4-6 (4-6h after lights-on).

Figure 1.2 40

Representative single-plotted actograms of wheel-running activity in AL control rats, as well as food-deprived (RF) and free-fed (RT) rats that received a daily presentation of chocolate Ensure from ZT4-6 (4-6h after lights on; illustrated by gray rectangles). All RF rats showed 'anticipatory' wheel running preceding the daily meal, while only 37% of RT rats showed a similar behavioral pattern (bottom panel, on left). All rats were housed under a 12h:12h LD cycle. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10min. Successive days are plotted from top to bottom.

Figure 1.3 42

RF, but not RT synchronizes PER2 expression. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of PER2 immunoreactivity. (b) Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL) rats killed at ZT1 or 13 (scale bar=200μm). (c) Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group).

Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation.

Figure 1.4 45

After 10 days of repeated exposure, presentation of Ensure increases Fos expression in all regions (except the SCN) in RF rats, but only the CEA, BLA and DMH in RT rats. **(a)** Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos immunoreactivity. **(b)** Examples of Fos expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL), RF and RT rats killed at ZT5 (scale bar=200μm). (c) Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

Figure 1.5 48

Ensure presentation for the first time does not affect PER2 expression but it increases Fos expression in all areas (except the SCN) in food-deprived (24h; RF) and free-fed (RT) rats. **(a)** Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos and PER2 immunoreactivity. **(b)** Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in control (AL), RF and RT

rats (n=4/group). **(c)** Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=4/group). Dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

Chapter 2

Figure 2.1 64

Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food deprived (RF, n=24) and free-fed (RT, n=24) rats. Ensure was presented for 2h each night from ZT16-18 (4-6h after lights-off) in each group.

Figure 2.2 65

Double-plotted actograms of wheel-running activity from representative rats from the free-fed, *ad libitum* (AL) control group, the restricted feeding group (RF) and the restricted treat group (RT). The nightly presentation of Ensure occurred from ZT16-18 (4-6h after lights-off; illustrated by rectangles). All rats were housed under a 12:12h LD cycle which is illustrated by the bars at the top of each actogram. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10min. Successive days are plotted from top to bottom. The graph in the lower right shows mean (±S.E.M.) total daily number of wheel-revolutions per group (n=24) starting 4 days before and throughout the 10 days of restricted feeding.

Figure 2.3 66

PER2 expression in control (AL) and in food-deprived (RF) and free-fed (RT) rats under nighttime restricted access to Ensure. Left panel, brain maps showing location of regions under study. The shaded square in each map indicates the area scanned for quantification of PER2 immunoreactivity. Middle panel, examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in AL rats killed at ZT1 or 13. Right panel, graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=4/group). Vertical rectangles inside the graphs indicate the time of Ensure presentation.

Chapter 3

Figure 3.1 82

Wheel running activity records for a single representative rat from the ad libitum (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups. Below each actogram, bar graphs show the average wheel running activity in the day and night for Days 5-9 (inclusive) for all rats in each group (n=15-16/group). Actograms illustrate the 12h:12h LD cycle at the top of each record and sequential days are plotted from top to bottom. Vertical marks indicate periods of activity of at least 5 wheel-revolutions/10min. Semi-transparent rectangles indicate time of Ensure availability.

Figure 3.2 83

(A) Ensure consumption for each group across the 10-day variable restricted feeding (VRF) schedules, **(B)** total number of wheel running revolutions per 24h for each day of the experiment, and **(C)** percentage of total wheel running activity that took place during the 12h of light. Symbols and vertical lines indicate mean ± S.E.M. (n=15-16/group). Arrows indicate the start of restricted feeding schedules.

Figure 3.3 85

Photomicrographs showing examples of PER1-immunostaining in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) across the day in rats from the ad libitum (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups.

Figure 3.4 86

Graphs showing mean (± SEM) number of PER1-immunoreactive (PER1-IR) nuclei in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) as a function of ZT for the ad libitum (AL), **(A)** daytime variable restricted feeding (D-VRF), **(B)** nighttime variable restricted feeding (N-VRF) and **(C)** anytime variable restricted feeding (ANY-VRF) groups (n = 3–4/group).

Chapter 4

Figure 4.1 104

Daily patterns of PER2 expression under daytime VRF (D-VRF; left column), nighttime VRF (N-VRF; middle column), and anytime VRF (ANY-VRF; right

column), compared to AL controls. Each brain area is graphed separately and is listed on the far left of each row. Graphs illustrate the mean (± S.E.M.) PER2-immunoreactivity (PER2-IR) in each brain area, according to the zeitgeber time (ZT) when the rats were killed. Asterisks (*) represent a statistically significant difference ($p<0.05$) between groups at a single time point.

Figure 4.2 108

Daily patterns of corticosterone under daytime VRF (D-VRF; left), nighttime VRF (N-VRF; middle), and anytime VRF (ANY-VRF; right), compared to AL controls. Graphs illustrate the mean (± S.E.M.) corticosterone concentration in blood plasma (µg/dl) according to the zeitgeber time (ZT) when the blood plasma samples were collected. Asterisks (*) represent a statistically significant difference ($p<0.05$) between groups at a single time point.

Chapter 5

Figure 5.1 127

Food consumption (top graph) and body weight (bottom graph) for each group over the course of the experiment (daily mean ± S.E.M.; n=24/group). RF schedules lasted from day 6-15 and the vertical gray lines mark the day food was restricted in the 2hRF and 6hRF groups. Day 15 has been omitted from the top graph because some rats were perfused before the end of the 10th meal.

Figure 5.2 128

Single-plotted actograms illustrating the daily pattern of running-wheel activity for a representative rat from each group. Each horizontal line graphs 24h of running-wheel activity (data is binned every 10min), while the white and black

rectangles along the top of each record illustrate the 12h:12h light-dark cycle. The rat in the AL group had free access to food throughout the entire record. In contrast, the rats in the 2hRF and 6hRF groups also had free access to food for the first 6 days of each record, and then the RF mealtimes are illustrated by the shaded rectangles within each plot for the last 9 days of each record.

Figure 5.3 129

Mean running-wheel activity for each group (n=24/group; 10-min bins), from ZT0 on day 14 until ZT4 on day 15. The light-dark cycle is illustrated along the bottom of each graph, along with ZT. The 9th daily meal is illustrated by horizontal rectangles within the top graph (filled = 2hRF, open = 6hRF) and the graph ends immediately before the 10th and last meal. In contrast, the AL group in the bottom graph had free access to food at all times.

Figure 5.4 130

Mean (± S.E.M.) running-wheel revolutions for each group (n=24/group) for each day of the experiment (top graph) and during the food-anticipatory period (ZT1-4, middle graph). The proportion of the total activity that occurred during the food-anticipatory period is also shown (bottom graph). All groups had unrestricted food-access from day 1-5, but food was restricted in the 2hRF and 6hRF group from day 6-14. The onset of restricted food-access is marked by the vertical grey lines in each graph. Day 15 has been omitted from all graphs, because it was the last day of the experiment and incomplete data was available.

Figure 5.5 132

Schematics of the location of the SCN, BNSTov, CEA, BLA and DG (left column) and the mean PER2-immunoreactivity (PER2-IR ± S.E.M; n=4/group/ZT) in each structure across the 24h light-dark cycle.

Figure 5.6 136

Schematic of the location of the DMH (left) and mean PER1-immunoreactivity (PER1-IR ± S.E.M.; n=4/group/ZT) in this structure across the 24h light-dark cycle (right). Asterisks (*) denote a statistically significant difference between two groups (p<0.05).

Appendix A

Figure 6.1 202

Actograms illustrating the daily pattern of running-wheel activity for two representative rats that each received ad libitum food access (AL; first 9 days of the record) followed by restricted feeding (RF; last 10 days of the record). Each horizontal line plots 24h and sequential days are arranged from top to bottom. Rats were housed in a 12h:12h light-dark schedule, illustrated by the empty (light phase) and shaded (dark phase) areas in each actogram. Numbers above the actograms indicate zeitgeber time (ZT). Under RF, rats received 2h of access to food each day (ZT4-6), illustrated by the shaded rectangle. As expected, RF produced characteristic food-anticipatory running-wheel activity in the few hours leading up to the predictable mealtime.

Figure 6.2 207

Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of *ad libitum*-fed rats. Each line illustrates the estimated mean of PER2 immunoreactivity in a particular structure, plotted according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The 12h:12h light-dark cycle is also illustrated by the shaded and unshaded areas of the graph. With the exception of the DMH, all structures exhibited robust daily rhythms in PER2 expression. Peak PER2 expression was observed in the SCN, BNSTov and CEA at ZT13 whereas peak PER2 expression was observed in the BLA and DG at ZT1 (redrawn from data published in Verwey et al., 2007).

Figure 6.3 209

Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of rats under RF and ad libitum (AL) feeding conditions. Mean PER2 immunoreactivity is plotted for each structure, according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The daily meal of Ensure was provided either in the middle of the day (RF-day; ZT4-6; open rectangle) or middle of the night (RF-night; ZT16-18; shaded rectangle). The daily pattern of PER2 expression in the BNSTov, CEA and DMH was affected by both RF schedules. In contrast, the daily pattern of PER2 expression in the BLA and DG was only shifted under RF-day conditions. Finally, PER2 expression in the SCN was unaffected by either RF schedule (redrawn from data published in Verwey et al., 2007 and 2008).

List of Tables

Table 1.1 43

Results from ANOVAs carried out to assess the effect of feeding schedule and time of day on PER2 expression in each brain area under study

Table 2.1 68

Results from ANOVAs carried out to assess the effect of feeding schedule (AL, NF, NT) and time of day on PER2 expression in each brain area under study

Table 4.1 105

Daily patterns of PER2 expression in each brain area, under each feeding condition

Table 4.2 107

Feeding conditions and the time of day (ZT) both modulate PER2 expression, compared to AL controls

Table 4.3 109

Daily patterns in the amount of corticosterone under each condition, as well as how the corticosterone levels under each VRF schedule compared to AL controls

Table 5.1 133

ANOVA of PER2 expression in the SCN, limbic forebrain and DMH

Table 5.2 137

Two-way ANOVA of PER1 expression in the DMH

List of Abbreviations

AL	*Ad libitum*
ANOVA	Analysis of variance
Any-VRF	Anytime variable restricted feeding
BLA	Basolateral nucleus of the amygdala
Bmal1	Aryl hydrocarbon receptor nuclear translocator-like gene
BMAL1	*Bmal1* protein
BNST	Bed nucleus of the stria terminalis
BNSTov	Oval nucleus of the bed nucleus of the stria terminalis
CEA	Central nucleus of the amygdala
Clock	Circadian locomotor output cycles kaput gene
CLOCK	*Clock* protein
Cry1	Cryptochrome 1 gene
CRY1	Cryptochrome 2 protein
Cry2	Cryptochrome 2 gene
CRY2	Cryptochrome 2 protein
DAB	3,3-Diaminobenzidine
DF	Daytime restricted feeding
DG	Dentate gyrus of the hippocampus
DMH	Dorsomedial hypothalamic nucleus
DT	Daytime restricted treat
D-VRF	Daytime variable restricted feeding
Ensure	Chocolate Ensure Plus

FOS	FBJ osteosarcoma oncogene protein
IR	Immunoreactive
NF	Nighttime restricted feeding
NPAS2	Neuronal PAS domain protein 2
ns	Not significant
NT	Nighttime restricted treat
N-VRF	Nighttime variable restricted feeding
Per1	Period homolog 1 gene
PER1	Period homolog 1 protein
Per2	Period homolog 2 gene
PER2	Period homolog 2 protein
Per3	Period homolog 3 gene
PER3	Period homolog 3 protein
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1α
PPARɣ	Peroxisome proliferator-activated receptor gamma
Rev-erbα	*Nr1d1:* nuclear receptor subfamily 1, group D, member 1 gene
REVERB*α*	*Rev-erbα* protein
RF	Restricted feeding
RT	Restricted treat
SCN	Suprachiasmatic nucleus
S.E.M.	Standard error of the mean
Sirt1	Sirtuin1 gene

SIRT1	Sirt1 protein
VRF	Variable restricted feeding
ZT	Zeitgeber time

Introduction

Circadian rhythms are predictable fluctuations in behavior and physiology that have a period of approximately 24h and are commonly driven by biological clocks and oscillators that are distributed throughout the brain and body (Guilding & Piggins, 2007; Pittendrigh, 1976a; Reppert & Weaver, 2002). In mammals, the "master" circadian clock is located in the brain, in the suprachiasmatic nucleus (SCN), and is normally in synchrony with the environmental day-night cycle (Mohawk & Takahashi, 2011; Takahashi & Zatz, 1982). There are also subordinate circadian oscillators that are located in many other brain areas and peripheral tissues (Abe et al., 2002; Guilding & Piggins, 2007; Yamazaki et al., 2000; Yoo et al., 2004). Many of these oscillators are synchronized by rhythmic signals from the SCN, but can also be influenced by rhythms in core body temperature, hormone release, neurotransmitter release, feeding, and metabolism (Buhr, Yoo, & Takahashi, 2010; Challet, Caldelas, Graff, & Pevet, 2003; Yamamoto et al., 2005; Yujnovsky, Hirayama, Doi, Borrelli, & Sassone-Corsi, 2006). The focus of the present thesis is to examine the influence and importance of feeding in the adjustment of circadian clocks and oscillators in the brain. This work not only helps to elucidate the nature and function of these putative circadian oscillators, but also aids in our understanding of the diverse brain regions that can be affected by feeding and fasting (Verwey & Amir, 2009; Appendix A). Collectively, these experiments demonstrate that feeding schedules can shift daily rhythms in many brain regions that, instead of being

involved in the regulation of circadian rhythms, are more traditionally linked with the regulation of motivation, arousal, learning, and emotion.

Restricted feeding (RF) schedules limit food-access to a single meal, provided at the same time each day, and can produce fundamental changes in many circadian rhythms (Mistlberger, 2011; Richter, 1922; Stephan, 2002). Mice and rats are usually nocturnal under *ad libitum* (AL) feeding conditions, active and eating during the night and largely inactive and sleeping during the day. In contrast, RF schedules that provide a daytime meal will induce a novel bout of daytime activity, 2-3h before the predictable mealtime. This novel bout of activity is commonly referred to as food-anticipatory activity and, because it is synchronized with the mealtime, is also said to be food-entrained. Although food-entrained anticipatory activities are expressed with many of the characteristics and constraints of a *bona fide* circadian rhythm, they do not rely on the SCN. The site of a “master” food-entrained oscillator, however, has remained elusive (Davidson, 2006; Mistlberger, 2011; Silver, Balsam, Butler, & Lesauter, 2011; Stephan, Swann, & Sisk, 1979a).

Circadian clocks and oscillators rely on the expression of several canonical clock genes and proteins (Reppert & Weaver, 2002). Daily rhythms in clock gene expression are observed in many brain areas and peripheral tissues, and these rhythms suggest the presence of locally-regulated circadian clocks and oscillators (Abe et al., 2002; Yamazaki et al., 2000; Yoo et al., 2004). In the SCN, daily rhythms of clock gene expression are adjusted by light and have been linked with the regulation and timing of rhythms in locomotor activity and core

body temperature (Fuller, Lu, & Saper, 2008; Gavrila et al., 2008). In contrast, daily rhythms of clock gene expression in other brain areas and peripheral tissues are sensitive to hormones, neurotransmitters, and body temperature, and have been implicated in the modulation of local physiological processes (Buhr et al., 2010; Challet et al., 2003; Yamamoto et al., 2005; Yujnovsky et al., 2006). Thus, while SCN-based rhythms will normally remain in synchrony with the light-dark (LD) cycle, extra-SCN rhythms will often respond to many of the physiological changes associated with RF. In the brain, the limbic forebrain, dorsal striatum, and dorsomedial hypothalamic nucleus (DMH) are some key areas that are involved in the regulation of motivation, emotion, sleep, and arousal. These forebrain areas also express clock genes, and their daily patterns of expression are adjusted in response to RF (Lamont, Diaz, Barry-Shaw, Stewart, & Amir, 2005; Mieda, Williams, Richardson, Tanaka, & Yanagisawa, 2006; Waddington Lamont et al., 2007). In particular, the DMH has been implicated in the generation and timing of food-anticipatory activity (Fuller et al., 2008; Gooley, Schomer, & Saper, 2006), but this hypothesis has been widely debated (Landry, Simon, Webb, & Mistlberger, 2006; Mistlberger et al., 2009b; Moriya et al., 2009). Studies contained within the present thesis also study the DMH, and thus, make important contributions to this field (Verwey & Amir, 2009, 2011b, 2011a; Verwey, Khoja, Stewart, & Amir, 2007, 2008; Verwey, Lam, & Amir, 2009).

Each chapter of this thesis describes daily rhythms of clock gene expression in several forebrain areas, and demonstrates how they are adjusted

in response to RF. In order to introduce these studies, an expanded version of the canonical circadian feedback loop (The circadian autoregulatory feedback loop: Page 4) as well as the tissue-specific consequences of clock gene expression (Daily rhythms in clock gene expression and their consequences: Page 8) will be described. The rationale behind using RF to engage and entrain circadian rhythms will be introduced next (Restricted feeding and food-anticipatory activity: Page 13), and because these studies focus on gene expression in the SCN, DMH, limbic forebrain, and dorsal striatum, a short introduction to each of these brain areas is also warranted (The suprachiasmatic nucleus: Page 17; The dorsomedial hypothalamic nucleus: Page 19; The limbic forebrain and dorsal striatum: Page 22). Finally, the introduction ends with a synopsis of the issues that are addressed in each chapter (The present thesis: Page 25).

The circadian autoregulatory feedback loop

Daily rhythms in the expression of canonical clock genes contribute to the generation of circadian rhythms in behavior and physiology (Quintero, Kuhlman, & McMahon, 2003; Reppert & Weaver, 2002). In the SCN, daily rhythms in clock gene expression are entrained by the environmental light-dark (LD) cycle, and contribute to the generation of daily rhythms in locomotor activity and core body temperature in rodents (Fuller et al., 2008; Gavrila et al., 2008; Hamada, Antle, & Silver, 2004). In contrast, the factors that entrain daily rhythms of clock gene expression outside of the SCN and the consequences of these rhythms, vary in a

nucleus- and tissue-specific manner (Guilding & Piggins, 2007; Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007; Storch et al., 2007).

At the core of the mammalian circadian feedback loop, the *Circadian locomotor output cycles kaput* (CLOCK) and *Aryl hydrocarbon receptor nuclear translocator-like1* (BMAL1) genes are transcribed and translated into proteins that form heterodimers. CLOCK:BMAL1 dimers increase the expression of the *Period* (*1-3;* PER1, PER2, and PER3; (Bunger et al., 2000; Gekakis et al., 1998; Honma et al., 1998), *Cryptochrome* (*1-2;* CRY1 and CRY2; (Kume et al., 1999), and *Rev-erbα* (REVERBα; (Zheng et al., 1999) genes. Over the course of several hours, PER and CRY proteins accumulate and exert negative feedback, which interferes with the transcriptional activity of CLOCK:BMAL1 (Miyazaki, Mesaki, & Ishida, 2001). Concurrently, REVERB*α* protein, which is a nuclear receptor that is important in the regulation of *Bmal1* expression, also accumulates and provides additional negative feedback (Preitner et al., 2002). Importantly, the expression, accumulation, and degradation of many of these clock proteins, and in particular PER1 and PER2, is highly rhythmic and exhibits a robust 24h rhythm (Abe et al., 2002; Yoo et al., 2004).

In addition to multiple PERs and CRYs, there are also potential substitutes for both CLOCK and BMAL1 (Asher & Schibler, 2006; DeBruyne, Weaver, & Reppert, 2007; Reick, Garcia, Dudley, & McKnight, 2001; Shi et al., 2010). In particular, *Neuronal PAS domain protein 2* (NPAS2) can effectively substitute for CLOCK (DeBruyne et al., 2007; Reick et al., 2001). However, CLOCK mutant mice (Vitaterna et al., 1994) possess a dominant-negative mutation that can still

disrupt circadian rhythms (Asher & Schibler, 2006). In this case, mutated CLOCK protein still binds BMAL1, but these dimers are ineffective transcriptional regulators. By sequestering BMAL1 protein, the potential NPAS2 substitution is blocked by the mutated CLOCK protein. In contrast, when the *Clock* gene is knocked out entirely, NPAS2 can dimerize with BMAL1 and maintain relatively normal circadian rhythms (DeBruyne et al., 2007). Next, although BMAL1 has been considered the only single gene deletion that disables the circadian clock, BMAL2 can act as a functional substitute for BMAL1 (Shi et al., 2010). However, BMAL2 expression is regulated by BMAL1, and thus, both are disrupted in BMAL1 knockout mice. But circadian rhythms are restored when BMAL2 is driven constitutively throughout the brain and body, even if BMAL1 is not expressed (Shi et al., 2010). Thus, the circadian feedback loop has been described extensively in the past (Reppert & Weaver, 2002), but new studies continue to expand our understanding of the genes, proteins, kinases, receptors and signalling pathways that are involved.

Many of the circadian clock proteins, and in particular PER2, also have effects on animal behavior, cellular physiology, and metabolism. In addition to disrupted circadian rhythms in locomotor activity and body temperature (Albrecht, Zheng, Larkin, Sun, & Lee, 2001; Bae et al., 2001; Zheng et al., 2001; Zheng et al., 1999), mice with a mutation of the *Per2* gene exhibit an increased propensity to consume alcohol, a hyper-sensitized response to cocaine, and decreased glutamate reuptake (Abarca, Albrecht, & Spanagel, 2002; Fu, Pelicano, Liu, Huang, & Lee, 2002; Gu et al., 2011; Spanagel et al., 2005). Changes in the cell

cycle, increased apoptosis, increased cancer development, and deficient bone development are also observed in PER2 mutant mice (Arjona & Sarkar, 2006; Fu et al., 2002; Hua et al., 2006; Maronde et al., 2010). In addition to these diverse behavioral and cellular effects, PER2 is also able to influence the expression of metabolically-relevant genes such as *Hnf1α* and *Glucose-6-phosphatase* through an interaction with nuclear receptors such as peroxisome proliferator-activated receptor gamma (PPARɣ) and REVERBα (Grimaldi et al., 2010; Schmutz, Ripperger, Baeriswyl-Aebischer, & Albrecht, 2010). Nucleus-specific rhythms in PER2 expression continue to hold an unknown importance in many brain areas and peripheral tissues. Thus, the influence of PER2 on genes that regulate metabolism could represent one important consequence of these localized rhythms in clock gene expression.

PER2 not only contributes to the regulation of metabolism, but also responds to metabolic and physiological challenges (Arble, Ramsey, Bass, & Turek, 2010; Asher et al., 2008; Bass & Takahashi, 2010; Buhr et al., 2010; Green, Takahashi, & Bass, 2008; Reinke et al., 2008). For instance, when BMAL1 expression is selectively disrupted in the liver, daily rhythms of PER2 expression are still observed in this organ (Kornmann et al., 2007). It is still unclear what is driving these oscillations, but naturally occurring rhythms in body temperature and feeding could both be involved (Damiola et al., 2000; Reinke et al., 2008). Daily temperature-cycles reset and entrain PER2 rhythms in the periphery, in part, through the induction of heat shock proteins (Buhr et al., 2010). In addition, the expression of sirtuin1 (SIRT1) in the liver responds to

feeding and fasting, exhibits a daily oscillation, and deacetylates and degrades PER2 (Asher et al., 2008; Belden & Dunlap, 2008; Nakahata et al., 2008; Rodgers, Lerin, Gerhart-Hines, & Puigserver, 2008). SIRT1 also interacts with the PPARɣ transcriptional co-activator-1 (PGC-1α), the expression of which in the brain, is also influenced by fasting (Liu, Li, Liu, Borjigin, & Lin, 2007; Nemoto, Fergusson, & Finkel, 2005; Ramadori et al., 2008). While the importance of these temperature- and metabolism-related factors seems to vary from tissue-to-tissue and from nucleus-to-nucleus, they are clear examples that PER2 is regulated by several factors that lie outside of the canonical circadian clockwork (Guo, Brewer, Champhekar, Harris, & Bittman, 2005).

Daily rhythms in clock gene expression and their consequences

In order to study circadian rhythms within a single tissue or brain region, many studies focus on the expression of a single clock gene across the 24h period (Abraham, Prior, Granados-Fuentes, Piwnica-Worms, & Herzog, 2005; Amir, Lamont, Robinson, & Stewart, 2004; Chen et al., 2005; Granados-Fuentes, Prolo, Abraham, & Herzog, 2004; Lamont, Robinson, Stewart, & Amir, 2005; Yoo et al., 2004). In particular, the use of PER1 or PER2 as circadian markers, allows several different tissue-specific and nucleus-specific rhythms to be studied concurrently. PER1 and PER2 expression is influenced by diverse physiological and metabolic factors, and can modulate a battery of physiological processes (Asher et al., 2008; Buhr et al., 2010; Hampp et al., 2008; Schmutz et al., 2010; Yamamoto et al., 2005). Thus, in addition to the importance of PER1 and PER2

within the canonical circadian feedback loop (Bae et al., 2001), the expression of these proteins could also mediate important changes in cellular physiology and metabolism directly.

Daily oscillations in the expression of *Per1* and *Per2* can be studied through several measures of gene and/or protein expression. For instance, *in situ* hybridization can be used to study transcript levels while immunohistochemistry can be used to study protein expression. These approaches, which stain post-mortem tissue, produce a daily profile of gene expression that is a composite of many rodents killed at several different times of day. This between-subject profile assumes that the clock gene of interest is expressed with a similar rhythm in all of the individuals within a group. These approaches have the distinct advantage that they provide an *in vivo* measure of gene expression. In contrast, there are also approaches that record daily oscillations in cultured neurons, brain slices, or tissues held *in vitro*. Specifically, expression of the bioluminescent enzyme luciferase can be driven by a *Per* promoter or fused to a PER protein (Abe et al., 2002; Yoo et al., 2004). Thus, the amount of light that is emitted can be used to indicate either transcript levels if luciferase expression is driven by the promoter or protein expression if this bioluminescent enzyme is fused to the protein. Fusion proteins have the added advantage that the bioluminescence will also reflect some of the post-transcriptional regulation and post-translational modifications that also influence PER expression (Dardente & Cermakian, 2007).

Some of the first experiments that examined clock gene expression throughout the brain and body focused on PER1 expression. In particular, in a line of transgenic rats, the bioluminescent luciferase enzyme was driven by the *Per1* promoter (Abe et al., 2002; Yamazaki et al., 2000). These studies demonstrated circadian rhythms in several brain areas and tissues such as the pineal gland, pituitary, and arcuate nucleus, as well as the liver, lung and skeletal muscle (Abe et al., 2002; Yamazaki et al., 2000). However, these extra-SCN rhythms typically dampened after 2-3 days (Yamazaki et al., 2000). Later, similar studies were performed using a line of mice that expressed a PER2-luciferase fusion protein (Yoo et al., 2004). In contrast to the earlier studies that used the *Per1* promoter to drive luciferase expression, rhythms in the brain and the periphery of tissues explanted from mice expressing the PER2-luciferase fusion protein were self-sustained for several days or weeks, even without the SCN (Yoo et al., 2004). Thus, endogenous circadian rhythms in PER2 expression have been observed in a surprising number of peripheral tissues and brain nuclei.

The SCN is the “master” circadian clock and manipulations that restore or disrupt clock gene expression selectively within the SCN, have widespread effects on behavior and physiology. For instance, mice with a global disruption of the *Bmal1* gene express disrupted and unstable daily patterns of running-wheel activity that become arrhythmic when the mice are housed in constant darkness (Bunger et al., 2000). However, if *Bmal1* expression is specifically restored in the SCN of mice that are deficient in BMAL1, circadian rhythms in behavior and body

temperature are rescued (Fuller et al., 2008). Conversely, Per2-interfering RNA injected into the SCN of wildtype rats, has also been reported to disrupt the daily rhythm of running-wheel activity (Gavrila et al., 2008). Thus, a strong connection has been made between clock gene expression in the SCN with rhythms in locomotor activity and core body temperature. In contrast, the specific aspects of behavior and physiology that might depend on daily rhythms of clock gene expression in other brain areas remain unclear (Guilding & Piggins, 2007).

In order to elucidate the importance of circadian clock gene expression outside of the SCN, researchers have typically sought to disrupt these rhythms in a tissue-dependent manner. For instance, a retina-specific disruption of the *Bmal1* gene disrupts circadian rhythms in the retinal response to light (Storch et al., 2007). The adrenals also express circadian clock genes, and their expression in this endocrine gland could also be important in the modulation of local physiological responses (Kalsbeek et al., 2011). Mice with mutations of canonical circadian clock genes exhibit disrupted circadian rhythms of corticosterone release (Oster et al., 2006). Moreover, if the adrenals are transplanted from these circadian clock mutants into an adrenalectomized control mouse with otherwise normal rhythms, the daily profile of corticosterone release from the transplanted adrenals is still arrhythmic, which suggests that clock gene expression within the adrenals influences corticosterone release from this endocrine gland (Oster et al., 2006). Finally, the expression of circadian clock genes in the pancreas also modulates hormone release. When BMAL1 expression is specifically disrupted within the pancreas, mice exhibit a diabetes-

like phenotype due to the changes that are observed in insulin release (Marcheva et al., 2010). Thus, clock gene expression has been linked to certain physiological and biological functions, but this research area is forced to move forward in a sequential manner, with individual studies focusing on individual tissues. It is also relatively simple to study extra-SCN rhythms in tissues that have highly specialized functions. In the brain however, a high degree of interconnectedness makes it considerably more difficult to ascertain the specific roles of daily rhythms of clock gene expression in discrete brain regions.

One approach to studying daily rhythms of clock gene expression in the brain has been to concurrently study FOS expression. FOS is a transcription factor and an immediate early gene, the expression of which is often interpreted as cellular activation. The basal amount and the increase in FOS expression can also vary according to the time-of-day. For example, olfactory stimulation will induce FOS in the olfactory bulbs, and the magnitude of this induction exhibits a circadian rhythm (Amir, Cain, Sullivan, Robinson, & Stewart, 1999). The olfactory bulbs are also unique because they exhibit circadian rhythms in clock gene expression, even after SCN lesions (Granados-Fuentes et al., 2004). Thus, the observation that daily rhythms in FOS-induction persist, even after SCN lesions, suggests that this rhythm could be driven by local rhythms of clock gene expression in the olfactory bulb (Granados-Fuentes, Tseng, & Herzog, 2006). Therefore, these results support the position that daily rhythms in clock gene expression can have important effects on locally-mediated phenomena.

Restricted feeding and food-anticipatory activity

RF schedules limit food-access to a few hours at the same time each day and, in many rodents, lead to the induction and entrainment of circadian rhythms in food-anticipatory activity (Caba & Gonzalez-Mariscal, 2009; Fuller et al., 2008; Richter, 1922; Rusak, Mistlberger, Losier, & Jones, 1988; Stephan, 1981; Stephan, 1983; Stephan, Swann, & Sisk, 1979b). Anticipatory activities have also been reported in AL-fed rats given a similarly restricted access to a daily chocolate treat (Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, & Escobar, 2008; Hsu, Patton, Mistlberger, & Steele, 2010; Mistlberger & Rusak, 1987). Food-anticipatory activities begin approximately 3h before the food arrives and are food-entrained to the predictable mealtime. During the food-anticipatory period there is an increase in body temperature, circulating stress hormones, and gut peptides (Boulos & Terman, 1980; Nelson, Scheving, & Halberg, 1975). There are also food-anticipatory increases in food-bin approaches, general locomotor activity, and running-wheel activity (Balsam, Sanchez-Castillo, Taylor, Van Volkinburg, & Ward, 2009). Although there is a concert of changes during the food-anticipatory period, many studies focus on a single aspect of behavior or physiology. In the present thesis, food-anticipatory activity is measured by the amount of running-wheel activity in the hours before the predictable mealtime.

The regulation and timing of food-anticipatory activities appear to rely on a putative food-entrained circadian oscillator (Mistlberger, 1994, 2011; Stephan, 1992b). In particular, food-anticipatory activities are expressed with several of

the properties and constraints of light-entrained circadian rhythms (Boulos & Terman, 1980; Mistlberger, 1994, 2009; Stephan, 2002). For instance, rats continue to express food-anticipatory activity before the predictable mealtime, even during a 2-3 day fast (Mistlberger, 2011). The daily expression of food-anticipatory activity could be reliant on a simple biological "timer" that counts forward 24h since the last meal, rather than a circadian oscillator. However, the expression of food-anticipatory activity on the second or third day of fasting is inconsistent with a simple timer-based model and suggests a circadian mechanism (Mistlberger, 2011). Moreover, limits of entrainment have also been observed in both food-entrained and light-entrained rhythms. In particular, food-anticipatory activities will deviate from the RF schedule if the meals are presented outside of the circadian range, for instance with a period of less than about 21-22h or longer than around 30-31h (Stephan, 1981). This limited circadian range also indicates that the expression of food-anticipation could rely on a food-entrained circadian oscillator. Finally, if the RF meal is phase-shifted to a different time-of-day, the bout of food-anticipatory activity shifts to the new mealtime, gradually, over several days (Stephan, 1992a, 1992b). This finding also supports the hypothesis that anticipatory activities rely on a circadian oscillator. If, however, it was based on a simple hourglass-mechanism, it would have reset immediately after the first meal at the new time-of-day. Strikingly, food-entrainment seems to occur independently from the SCN (Damiola et al., 2000; Stephan, 1983) but the location of a "master" food-entrained oscillator has remained elusive (Davidson, 2009; Mistlberger, 2009).

As food-anticipatory activities are regulated in a circadian manner, several groups have hypothesized that circadian clock genes could be involved, but data supporting this hypothesis have been mixed. When essential clock genes are disrupted through global gene mutations or deletions, some reports show that food-entrained rhythms persist while others find that food-entrainment is impaired (Challet, Mendoza, Dardente, & Pevet, 2009; Feillet et al., 2006; Fuller et al., 2008; Pendergast et al., 2009; Storch & Weitz, 2009). For instance, according to one report, mice with a targeted disruption of the BMAL1 gene lack both light- and food-entrained circadian rhythms (Fuller et al., 2008). In other studies, however, similar mice showed relatively normal rhythms in food-anticipatory running-wheel activity, even though light-entrained rhythms were still disrupted (Pendergast et al., 2009; Storch & Weitz, 2009). Moreover, targeted mutation of the *Per2* gene also disrupts light-entrained (Bae et al., 2001) and food-entrained circadian rhythms (Feillet et al., 2006). However, food-entrained rhythms have also been reported in PER1/PER2 double-mutant mice (Storch & Weitz, 2009). Finally, the CLOCK-paralog NPAS2 could also be involved in the behavioral adaptation to RF. Under RF schedules, NPAS2-deficient mice eat less, lose more weight, and exhibit more RF-related sickness than wildtype controls (Dudley et al., 2003). Thus, the importance and involvement of the canonical circadian clock in the expression of food-anticipatory activities continues to unfold. However, these experiments mutated and knocked out genes throughout the brain and body. Thus, it is still unclear whether the expression of these

genes in a particular brain nucleus and/or peripheral tissue might be especially important in these effects.

Daily rhythms in clock gene expression may not be required for the expression of circadian rhythms in food-anticipatory activity, *per se*, but RF does modulate the daily rhythms of clock gene expression in many brain nuclei and peripheral tissues (Damiola et al., 2000; Hara et al., 2001; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001; Wakamatsu et al., 2001). In particular, clock gene expression in the liver has received considerable attention. Specifically, it was reported that daily rhythms of PER1 and PER2 expression in the liver are shifted in response to RF (Damiola et al., 2000), and that this response is independent of the SCN (Hara et al., 2001). These findings support the hypothesis that periodic feeding is involved in the entrainment of clock gene expression in this organ. Moreover, RF also modulates the daily rhythms of clock gene expression in many areas of the brain. For example, daily rhythms in the hippocampus, nucleus accumbens, lateral suptum, cortex, striatum, and paraventricular nucleus are all affected by RF (Angeles-Castellanos, Mendoza, & Escobar, 2007; Wakamatsu et al., 2001). In addition, and particularly relevant to the present thesis, the daily rhythms of *Per* expression in the limbic forebrain and DMH are also modulated by RF (Mieda et al., 2006; Waddington Lamont et al., 2007). Thus, RF can be used as a tool to both uncover the neural and genetic elements that could be involved in food-anticipatory activity, and to help define the properties and characteristics of nucleus-specific rhythms in clock gene expression.

The suprachiasmatic nucleus

The circadian clock, which resides in the SCN, has been studied for many years through the careful examination of circadian rhythms in behavior and physiology (Pittendrigh, 1976a, 1976b, 1976c, 1976d, 1976e). The first evidence to suggest that the SCN was involved in the regulation of circadian rhythms was the observation that rodents became arrhythmic when the SCN was lesioned (Moore & Eichler, 1972; Stephan & Zucker, 1972). The importance of the SCN was further substantiated by the finding that circadian rhythms are restored in SCN-lesioned rodents, when SCN-tissue is replaced by neural transplantation (Lehman et al., 1987). However, these experiments demonstrated that the SCN is necessary and sufficient for the expression of the circadian rhythms, but did not show that the SCN is the site of rhythm-generation.

The evidence that firmly established the SCN as the "master" circadian clock came from a transplant study that used hamsters with an abnormal circadian period (Ralph, Foster, Davis, & Menaker, 1990). Specifically, *tau* mutant hamsters express a relatively short free-running period in running-wheel activity (homozygotes = ~20h period; heterozygotes = ~22h period) as compared to wildtype controls (~24h period). Because of this fundamental difference in period, researchers transplanted SCN-tissue from *tau* mutants into arrhythmic SCN-lesioned wildtype hamsters. SCN-tissue was also transplanted from wildtype hamsters into arrhythmic SCN-lesioned *tau* mutant hamsters. Remarkably, it was the rhythm of the SCN-donor that was expressed in the behavior of the transplant-recipients (Ralph et al., 1990). Arrhythmic wildtype

hamsters that received an SCN-transplant from a *tau* mutant hamster, exhibited short circadian rhythms that were consistent with the phenotype of the *tau* mutants. In contrast, *tau* mutants exhibited a wildtype-like period if they received an SCN-transplant from a wildtype hamster. Thus, the SCN was shown to generate *de novo* circadian rhythms in behavior.

Most circadian rhythms are entrained by the environmental LD cycles (Pittendrigh, 1976d). The SCN receives innervation from melanopsin-expressing retinal ganglion cells, which are independent from visual photoreceptors (Beaule, Robinson, Lamont, & Amir, 2003; Lucas et al., 2003). These melanopsin-containing cells project to the SCN via the retinohypothalamic tract (RHT) and when stimulated by light, can produce phase shifts in the circadian clock (Benarroch, 2011; Hatori & Panda, 2010; Pickard & Sollars, 2010). These light-induced phase shifts depend on two main factors, the time-of-day when the light pulse occurs and the amount of light (Pittendrigh, 1976b). During the subjective day when light-exposure would be expected, light does not result in a phase shift of the circadian clock. In contrast, during the subjective night when light-exposure is not expected, light pulses will phase-shift the clock in a time-dependent manner (Pittendrigh, 1976b). The brightness and the duration of the light pulse, also have significant effects on the magnitude of these phase shifts (Pittendrigh, 1976b). Higher-intensity light or longer duration light-pulses produce larger phase shifts of the circadian clock. In this way, circadian rhythms in the SCN are strongly light-entrained, but depending on the conditions, may respond to other stimuli.

Under constant light or constant darkness, the SCN and the behaviors it controls can become food-entrained (Challet et al., 2003; Challet, Malan, & Pevet, 1996; Mendoza, Pevet, & Challet, 2007). Constant light in particular, disrupts circadian rhythms of clock gene expression in the SCN (Ohta, Yamazaki, & McMahon, 2005) and produces arrhythmic behavior in freely-fed rats. Under these constant-light conditions, RF schedules can restore and entrain daily rhythms of running-wheel activity and PER2 expression in the SCN (Lamont, Diaz, et al., 2005). Under constant darkness, food-entrained rhythms also exhibit an interaction with light-entrained rhythms (Acosta-Galvan et al., 2011; Mendoza et al., 2007; Stephan, 1986; Stephan & Becker, 1989). In contrast, under a LD cycle, similar RF schedules do not affect the daily rhythms of PER2 expression in the SCN (Waddington Lamont et al., 2007), although, some studies demonstrate changes in the rhythm of PER1 expression (Mendoza et al., 2007). Thus, daily rhythms of clock gene expression in the SCN are typically light-entrained if a LD cycle is present, but RF schedules have some influence on these SCN-based rhythms under constant light or constant darkness.

The dorsomedial hypothalamic nucleus

The DMH is an important transmission point in the regulation of many circadian rhythms in behavior, hormone release, and arousal. The SCN sends projections to the DMH through the ventral subparaventricular zone, and the DMH in turn, sends inhibitory outputs to brain areas that promote sleep, such as the

ventrolateral preoptic area. There are also excitatory outputs to wake-promoting brain areas, such as orexin-expressing neurons in the lateral hypothalamus (Chou et al., 2003; Saper, Lu, Chou, & Gooley, 2005). The DMH is sensitive to leptin, and neuropeptide Y expression in this nucleus is involved in the regulation of body weight (Chao, Yang, Aja, Moran, & Bi, 2011; Elmquist, Ahima, Elias, Flier, & Saper, 1998; Elmquist, Elias, & Saper, 1999; Yang et al., 2009). The DMH also sends projections to the paraventricular nucleus, which is important in the regulation of glucocorticoid release (Elmquist et al., 1998). Therefore, wake, sleep, arousal, feeding, and hormone release are all influenced by the DMH. Many of these factors are also influenced by RF, which raises the possibility that the DMH could mediate some of these food-anticipatory activities.

In the search for a “master” food-entrained oscillator, many researchers have concentrated their efforts on studies that lesion a discrete brain area (Davidson, 2009; Davidson, Cappendijk, & Stephan, 2000; Gooley et al., 2006). In particular, DMH-lesions reduce the amount of food-anticipatory activity, which suggests that this brain nucleus could be necessary for some aspect of this response (Gooley et al., 2006). However, DMH-lesions do not eliminate food-entrainment altogether, which suggests that it could be involved but not required for these rhythms (Landry et al., 2006). One major limitation of DMH-lesions is that they alter many other circadian rhythms, even under AL conditions (Gooley et al., 2006). In particular, under AL feeding, the overall amount of locomotor activity and the amount of food that is consumed each day are both decreased after DMH-lesions (Gooley et al., 2006). Therefore, even though food-

anticipatory locomotor activity is decreased by DMH-lesions, because of the concurrent decrease in overall activity, the proportion of the total activity that occurs during the food-anticipatory period can remain quite similar between DMH-lesioned and DMH-intact rodents (Moriya et al., 2009). Thus, the study and characterization of daily rhythms of clock gene expression in the DMH in response to RF, is crucial to the understanding of how this nucleus might be involved in the production and timing of food-anticipatory activity.

The DMH exhibits a low-amplitude daily rhythm in clock gene expression under AL conditions, but demonstrates a relatively large-amplitude rhythm under RF (Mieda et al., 2006). The daily pattern of FOS expression, an immediate early gene that suggests local cellular activity, is also strongly rhythmic in the DMH under RF (Angeles-Castellanos, Aguilar-Roblero, & Escobar, 2004; Gooley et al., 2006). Thus, daily rhythms of clock gene expression and immediate early gene expression both suggest that the DMH is sensitive to RF. However, the importance of clock gene expression in the DMH in the production of food-anticipatory activities has remained controversial (Fuller et al., 2008; Mistlberger et al., 2009a; Mistlberger et al., 2008). Fuller et al. (2008) reported that *Bmal1* -/- mice do not exhibit food-anticipatory activity, yet when a viral vector was used to restore *Bmal1* expression in the DMH, circadian rhythms in food-anticipatory activity were rescued. These findings seemed to demonstrate a strong link between canonical circadian clock genes that are expressed in the DMH and circadian rhythms in food-anticipatory activity, however, this study used a short daily meal. Instead, if food-access is restricted more gradually, starting with a

long meal and then shortening the meal each day, Bmal1 -/- mice can show robust food-anticipatory activity (Pendergast et al., 2009; Storch & Weitz, 2009), which calls into question the "rescue" demonstrated by Fuller et al. (2008). Thus, the careful study of the daily rhythm of clock gene expression in the DMH could help to resolve these disparate findings. However, rhythms in several other areas of the forebrain are also sensitive to RF, and could also be involved in this food-anticipatory response.

The limbic forebrain and dorsal striatum

The limbic forebrain is involved in the regulation of motivational and emotional state, and includes the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG). Not only do these brain areas modulate the physiological and behavioral responses to stress and drugs of abuse (Erb, Salmaso, Rodaros, & Stewart, 2001; Gray, 1993; Loewy, 1991; Nijsen, Croiset, Diamant, De Wied, & Wiegant, 2001), they also affect anxiety, learning, reproduction, and feeding (Casada & Dafny, 1991; Figueiredo, Bodie, Tauchi, Dolgas, & Herman, 2003; Stefanova & Ovtscharoff, 2000; Van de Kar & Blair, 1999; Walker, Toufexis, & Burlet, 2001; Walker, Toufexis, & Davis, 2003). For example, the BNSTov and CEA have been implicated in stress-induced reinstatement of drug-seeking in cocaine self-administering rats (Erb et al., 2001). These brain areas also contribute to unique aspects of learning and memory, whereas the amygdala is implicated in conditioning and affect, the

hippocampus is utilized in spatial navigation (McDonald & White, 1993). In addition, the dorsal striatum is involved in the regulation of motor control and habit learning (Robbins, Cador, Taylor, & Everitt, 1989; McDonald & White, 1993; Stice, Spoor, Ng, & Zald, 2009). When dopamine production is disrupted in dopamine neurons, mice do not eat enough to survive and are hypoactive. But when dopamine production is selectively restored in the dorsal striatum of these dopamine-deficient mice, this manipulation facilitates feeding, reward-based learning, and locomotion (Palmiter, 2008). Importantly, many of these processes also vary according to the time-of-day and exhibit a robust circadian rhythm. Daily rhythms are observed in the release of stress and sex hormones, as well as in feeding, conditioned place preference learning, and mood (Ahlers, Smajda, & Ahlersova, 1980; Boivin et al., 1997; Cain, Chou, & Ralph, 2004; Keefe & Turek, 1985). However, whether these circadian rhythms are mediated by local circadian oscillators within the limbic forebrain and dorsal striatum, or if they only require the master clock in the SCN, remains an open question.

There are robust daily rhythms in the expression of the circadian clock protein, PER2, in the BNSTov, CEA, BLA, DG and dorsal striatum (Amir et al., 2004; Hood et al., 2010; Lamont, Robinson, et al., 2005). When rats are housed under a 12h:12h LD cycle, daily rhythms of PER2 expression in the BNSTov and CEA peak around the time the environmental lights turn off (dusk) and trough around the time the environmental lights turn on (dawn), a daily pattern that is approximately in synchrony with the SCN (Amir et al., 2004; Lamont, Robinson, et al., 2005). In contrast, the daily rhythms of PER2 expression in the BLA, DG,

and dorsal striatum peak around dawn and trough around dusk (Amir et al., 2004; Hood et al., 2010; Lamont, Robinson, et al., 2005), in anti-phase with the SCN, BNSTov and CEA. These differences in the phase of the PER2 rhythms seem to suggest that the signal(s) that entrain the BNSTov and CEA are probably different from those that entrain the BLA, DG, and dorsal striatum.

Daily rhythms of PER2 expression in certain areas of the limbic forebrain are sensitive to daily rhythms in hormone release. Specifically, in the rat, adrenal stress hormones, gonadal hormones, and thyroid hormones, all affect the daily rhythms of PER2 expression in the BNSTov and CEA but not rhythms in the SCN, BLA or DG (Amir et al., 2004; Amir & Robinson, 2006; Lamont, Robinson, et al., 2005; Perrin, Segall, Harbour, Woodside, & Amir, 2006). In particular, the daily rhythm of the adrenal stress hormone, corticosterone, is critical for daily rhythms in PER2 expression in the BNSTov and CEA (Amir et al., 2004; Lamont, Robinson, et al., 2005; Segall, Milet, Tronche, & Amir, 2009; Segall, Perrin, Walker, Stewart, & Amir, 2006). However, daily rhythms in PER2 expression can be induced by RF, even when the adrenal gland is removed, suggesting that RF and corticosterone may act in parallel to modulate daily rhythms of PER2 expression in these areas (Segall, Verwey, & Amir, 2008). In addition, the neurotransmitter dopamine is important for daily rhythms of PER2 expression in the dorsal striatum, which are blunted after neurotoxic lesions that target the dopamine system (Hood et al., 2010). In contrast to all of these factors that influence the daily rhythms of PER2 expression in the BNSTov, CEA, and dorsal striatum, the specific factors that modulate PER2 rhythms in the BLA and DG

have remained largely unclear. To date, the main experiments that have successfully shifted daily rhythms of PER2 expression in all of these limbic areas, including the BLA and DG, have used RF (Lamont, Diaz, et al., 2005; Waddington Lamont et al., 2007).

The present thesis

Food-anticipatory activities are induced by RF schedules and characterized by novel daily rhythms in behavior, metabolism, and hormone release. Moreover, RF schedules are also associated with shifts in the daily rhythms of clock gene expression in many areas of the brain and body. In particular, daily rhythms of clock gene expression in the DMH have been implicated in the generation and timing of food-anticipatory activity. In order to test this hypothesis, the present thesis describes the daily patterns of clock gene expression in the DMH in response to several different RF schedules. While the daily pattern of clock gene expression in the DMH did respond to RF, these data do not support a causal role of clock gene expression in the DMH in the generation of food-entrained anticipatory activity. In addition, the effects of RF on daily rhythms of clock gene expression in the limbic forebrain are also described. These brain areas are more typically linked to the regulation of motivation and emotion instead of circadian rhythms. Nevertheless, daily rhythms in PER2 expression are observed in the BNSTov, CEA, BLA, DG, and dorsal striatum, and these rhythms are sensitive to RF. By using RF to probe these daily rhythms in clock gene expression, the importance of these nucleus-specific rhythms can be elucidated. If the daily rhythm of PER1 or PER2 expression in a given brain area is always

shifted with respect to the mealtime, it could be considered food-entrained. In contrast, if the daily rhythm of PER1 or PER2 expression is adjusted differentially depending on the time-of-day when the meal is provided, it could be sensitive feeding or fasting at a specific time-of-day. If the shift in PER1 or PER2 expression varies according to the time-of-day when food is presented, this would indicate an interaction between light-entrained and food-entrained signals. Finally, if the daily rhythms in PER1 or PER2 fail to shift in response to RF, this would suggest that another zeitgeber (e.g. the light-dark cycle) is able to over-power the influence of feeding, fasting, or food-anticipatory activity. The body of the present thesis is composed of 5 peer-reviewed papers (Verwey & Amir, 2011a, 2011b; Verwey et al., 2007, 2008; Verwey et al., 2009), which appear as Chapters 1-5. Reprints of these publications can also be found in Appendices B-F.

In Chapters 1 and 2 (Verwey et al., 2007, 2008), daily profiles of PER2 expression were determined under RF schedules that provided a single meal at the same time each day. In Chapter 1, the RF group was given a daytime meal (daytime RF; DF), while in Chapter 2, the RF group received a nighttime meal (nighttime RF; NF). Each meal consisted of a complete meal replacement (Ensure), which was available for 2h/day. Two additional restricted “treat” (RT) groups were also described that had uninterrupted access to AL chow, and received a similar access to Ensure in the form of a daytime-treat (DT; Chapter 1) or a nighttime-treat (NT; Chapter 2). RT schedules induce some food-anticipatory activities, but without the periodic fasting that is associated with RF.

By contrasting these groups, the importance of the time-of-day the rats eat and the potential importance of fasting were both evaluated. Preliminary results for Chapters 1 and 2 were presented previously (Verwey, 2006), but the results pertaining to the BLA, DG, DMH and the publication of these data all occurred during my tenure within the doctoral program.

In Chapters 3 and 4 (Verwey & Amir, 2011a; Verwey et al., 2009), the daily profiles of clock gene expression were determined under RF schedules that provided an unpredictable meal, at a different time each day. These variable restricted feeding (VRF) schedules varied the 2h mealtime either within the 12h of light (Daytime VRF), within the 12h of dark (nighttime VRF) or across the entire 24h LD cycle (Anytime VRF). Under these conditions, it is impossible to accurately predict the arrival of the next meal, and thus, circadian rhythms in food-anticipatory activity fail to develop. By contrasting these groups, we were able to test the impact of 10-day RF schedules, without food-entrainment, *per se*. Similar to the previous chapters, these experiments also tested the importance of the time-of-day when rats ate. These experiments also evaluated whether or not, even in the absence of food-entrainment, the negative energy balance brought about by a 2h meal/day was sufficient to engage these DMH-based rhythms.

In Chapter 5 (Verwey & Amir, 2011b), the importance of the severity of the RF schedule was tested. Specifically, these experiments varied the meal duration, with one schedule providing a 2h-meal of standard rat chow (2hRF) and the other schedule providing a 6h-meal (6hRF) each day. Meal duration affects the amount of food that rats are able to consume, the amount of body weight

they lose, as well as the magnitude of food-anticipatory activity (Stephan & Becker, 1989). However, because the daily meals arrived at the same time in both groups, they also exhibited approximately synchronous food-anticipatory activity. Thus, these conditions helped to elucidate whether it was the RF, the amount of food-anticipatory activity, or the time of food-arrival/food-removal that could be mediating some of these effects on daily rhythms of PER1 and PER2 expression outside of the SCN.

In summary, the present thesis is an attempt to describe interactions between feeding, motivational state, and clock gene expression. This thesis demonstrates that, above and beyond the homeostatic consequences of feeding, meals also affect brain areas that are more traditionally linked to the regulation of motivation, arousal, emotion, and learning. Even though these brain areas are not typically associated with the regulation of circadian rhythms or feeding, *per se*, the expression of circadian clockwork within each brain area responds to RF. These findings not only illustrate a novel consequence of RF, but they also help to characterize the regulation of circadian rhythms in the limbic forebrain, dorsal striatum, and especially, the DMH.

Chapter 1: Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats

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Contribution of authors

Michael Verwey designed the experiments together with Dr. Amir and oversaw all aspects of this project, including restricted feeding, perfusions, brain slicing, immunohistochemistry, microscopy, imaging, cell counting, and statistical analyses. The publication was written by Michael Verwey and edited by Dr. Jane Stewart and Dr. Shimon Amir.

Zehra Khoja was an undergraduate student that helped with the animal care, restricted feeding, and some of the immunohistochemistry in these studies.

Dr. Jane Stewart is a professor in the Department of Psychology. She contributed to the preparation of the manuscript.

Dr. Shimon Amir is the principle investigator of the lab where this experiment took place and Michael Verwey's thesis supervisor. He participated in the design of the experiments, microscopy, cell counting, and editing of the manuscript.

Abstract

Circadian clock genes are rhythmically expressed in many areas of the brain and body and are thought to underlie most endogenous circadian behaviors and physiological processes. Daily rhythms of clock gene expression throughout the brain and body are normally coordinated by the suprachiasmatic nucleus (SCN), but they are also strongly influenced by daily temporal restrictions of food availability. Here, we studied the effects of a daily restricted presentation of highly palatable complete meal replacement, chocolate Ensure Plus (Ensure) in food-deprived (restricted feeding, RF) and free-fed (restricted treat, RT) rats, on the expression of the clock protein, Period2 (PER2) in regions of the brain involved in motivational and emotional regulation; these include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), the dentate gyrus (DG) and the dorsomedial hypothalamus (DMH). RF and RT rats consumed similar amounts of Ensure, but changes in the pattern of PER2 expression were seen only in the RF condition, suggesting that changes in PER2 expression in these regions are triggered by the daily alleviation of a negative metabolic state associated with RF and are independent of the positive incentive properties of the consumed substance, *per se*. In contrast, the expression of the immediate early gene, *Fos*, was increased in these regions by both RF and RT schedules, showing that signals concerning the incentive value of the consumed food reach these regions. No changes in either PER2 or Fos expression were observed in the SCN of RF or RT rats. These findings demonstrate that mechanisms leading

to changes in the expression of PER2 and those affecting the induction of Fos under RF and RT are, at least in part, dissociable.

Introduction

Circadian rhythms in the expression of clock genes in peripheral tissues and brain are orchestrated by a master pacemaker located in the suprachiasmatic nucleus (Guo et al., 2005; Guo, Brewer, Lehman, & Bittman, 2006; Lowrey & Takahashi, 2004; Yoo et al., 2004). These extra-SCN rhythms are also strongly influenced by feeding schedules that restrict food-access to the same time each day (Angeles-Castellanos et al., 2007; Challet et al., 2003; Damiola et al., 2000; Hara et al., 2001; Mieda et al., 2006; Stokkan et al., 2001; Waddington Lamont et al., 2007; Wakamatsu et al., 2001; Zvonic et al., 2006). Such schedules lead to novel daily fluctuations in energy balance as well as changes in the patterns of arousal, circulating corticosterone and daily activity rhythms (Davidson, Tataroglu, & Menaker, 2005; Mendoza, 2007; Mistlberger & Marchant, 1995; Stephan, 2002), suggesting that expression of clock genes in the brain and periphery is not only sensitive to signals from the SCN but also responds to changes in nutritional and/or motivational state independently of the SCN (Hara et al., 2001).

We have shown recently that scheduled restricted feeding (RF) in rats strongly affects the rhythm of expression of the clock protein Period2 (PER2) in limbic forebrain areas involved in the control of motivation and emotion, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and dentate gyrus (DG; Lamont, Diaz, et al., 2005; Waddington Lamont et al., 2007). Interestingly we also found that in the absence of food deprivation, daily limited access to highly

palatable substances such as sucrose or saccharine, had no effect on the rhythm of PER2 expression in these areas in spite of the fact that these substances were consumed in large quantities. Based on these findings, we hypothesized that the expression of PER2 in the limbic forebrain is sensitive to homeostatic signals arising from the daily alleviation of a negative metabolic state associated with scheduled feeding and is independent of the positive incentive properties of the consumed substance, *per se* (Waddington Lamont et al., 2007).

To study this issue further, we assessed the effect of daily limited access to a highly palatable complete meal replacement, chocolate Ensure Plus (Ensure; Abbott Laboratories, Abbott Park, IL, USA), on PER2 expression in the BNSTov, CEA, BLA, and DG in both food-deprived and free-fed rats. In addition, we assessed the effect of daily limited access to Ensure on neuronal activation in these brain regions in food-deprived and free-fed rats using the transcription factor, Fos, as a marker (Angeles-Castellanos et al., 2004; Angeles-Castellanos et al., 2007). Also, we assessed the effect of limited access to Ensure in food-deprived and free-fed rats on PER2 and Fos expression in the dorsomedial hypothalamic nucleus (DMH), an area shown recently to play a role in the interface between RF, PER2 expression and certain food anticipatory rhythms (Gooley et al., 2006; Landry et al., 2006; Mieda et al., 2006). Finally, in order to differentiate the circadian effects of serial food presentations from the potential acute effects of a single food presentation, we also studied rats on the first day of our restricted Ensure schedules. Preliminary results have been presented in an abstract form (Verwey, Khoja, & Amir, 2005).

Method

Animals and housing

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. All efforts were made to minimize the number of animals used and their suffering. A total of 96 male Wistar rats were used (225-250g; Charles River Laboratories, St. Constant, QC, Canada). The rats were housed individually in cages equipped with running wheels, under a 12h:12h light/dark (LD) schedule (300 lux at cage level) and had free access to Purina rat chow and water for at least 10 days before the start of each experiment. Running wheel activity was recorded by computer (Vitalview, Minimitter, OR, USA) and analyzed with Circadia software.

RF and restricted treats (RT)

Rats were assigned randomly to one of three groups: RF group, RT group or *ad libitum* (AL) group. During experimental stages, rats in the RF group were fed exclusively with unlimited Ensure for 2h each day, during the middle of the day, from zeitgeber time (ZT) 4-6 (ZT0 denotes time of lights on in a 12h:12h LD schedule). Rats in the RT group had continued access to Purina rat chow and in addition received an identical restricted access to Ensure during the day (ZT4-6). Rats in the AL control group had free access to Purina rat chow only. These schedules lasted for 10-13 days. This relatively short RT schedule was deliberately chosen so the RT group could be compared with the RF group.

Other experiments have generally used longer (4-6 week) palatable meal entrainment protocols and have focused more on treat-anticipatory behavior (Mendoza, Angeles-Castellanos, & Escobar, 2005a, 2005c; Mistlberger & Rusak, 1987). Our experiments did not set out to study the anticipation of a daily treat, *per se*, instead we focused on the metabolic and motivational consequences of the daily consumption of Ensure between fasted and free-fed rats.

Tissue preparation and immunocytochemistry

On the last day of the scheduled feeding, rats were deeply anesthetized with sodium pentobarbital (Somnotol, ~100 mg/kg) at one of six ZTs (ZT1, 5, 9, 13, 17, 21) and perfused intracardially with 300ml of cold saline (0.9% NaCl) followed by 300ml of cold, 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.3). Following perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4°C overnight. Serial coronal brain sections (50μm) containing regions of interest were collected from each animal using a vibratome and stored in Watson's cryoprotectant solution until use (Watson, Wiegand, Clough, & Hoffman, 1986). Immunocytochemistry for PER2 was performed on one set of brain sections as previously described (Amir et al., 2004) using an affinity-purified rabbit polyclonal antibody raised against PER2 (1:800, Alpha Diagnostics International, San Antonio, TX, USA). Immunocytochemistry for Fos was performed on a second set of brain sections collected from each rat as previously described (Beaule, Arvanitogiannis, & Amir, 2001) using a polyclonal *cFos* antibody, raised in rabbit (1:100,000, Oncogene Sciences, Boston, MA, USA).

Image analysis and statistics

PER2- and Fos-stained brain sections were mounted on gelatin-coated glass slides, cover-slipped, and examined under a light microscope. Images of brain areas containing the SCN, BNSTov, CEA, BLA, DG and DMH were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA), and Image SXM software (v1.6, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 or Fos were counted on captured images using a 400X400 μm (SCN, BNSTov, CEA, BLA, DMH) or a 200X400 μm (DG) frame. The mean number of PER2 or Fos immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analysis of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

Results

Ensure consumption and locomotor activity rhythms under RF and RT schedules

Daily rhythms of wheel running activity were assessed in AL rats housed under a 12h:12h LD schedule and in similarly housed rats that were placed on either a RF or RT schedule in which Ensure was given for 2h each day, from ZT4-6. Figure 1.1 shows the amounts of Ensure consumed each day during the 2h access period by rats from the RF and RT groups. It can be seen that with the exception of the first day of limited access, rats from the two groups consumed similar amounts of Ensure throughout the experiment. Notwithstanding, RT rats continued to eat regular chow and thus consumed more calories each day than RF rats (data not shown). As expected, rats from the RF group showed consistent changes in running wheel patterns and developed an anticipatory running wheel bout which began 2-3 h before daily food presentation (Figure 1.2).

In contrast, only 9 of the 24 rats from the RT group showed anticipatory running wheel activity (Figure 1.2). The remaining rats from the RT group did not develop anticipatory running and their circadian running patterns resembled those of AL rats that had free access to normal rat chow but not to Ensure. When the consumption of Ensure was re-examined, treat-anticipating RT rats tended to eat moderately more Ensure than non-anticipating RT rats, but this effect was not significant (P=0.075).

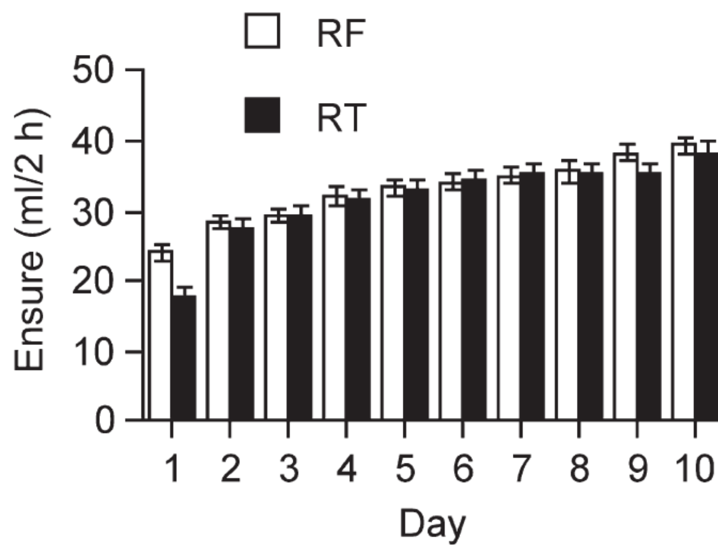

Figure 1.1
Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food-deprived (RF, n=24) and free-fed (RT, n=24) rats. Ensure was presented for 2h each day from ZT4-6 (4-6h after lights-on).

Figure 1.2
Representative single-plotted actograms of wheel-running activity in AL control rats, as well as food-deprived (RF) and free-fed (RT) rats that received a daily presentation of chocolate Ensure from ZT4-6 (4-6h after lights on; illustrated by gray rectangles). All RF rats showed 'anticipatory' wheel running preceding the daily meal, while only 37% of RT rats showed a similar behavioral pattern (bottom panel, on left). All rats were housed under a 12h:12h LD cycle. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10min. Successive days are plotted from top to bottom.

RF but not RT modifies the daily pattern of PER2 expression

Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of AL rats killed at ZT1 or ZT13 and graphs showing daily patterns of PER2 expression in AL, RF and RT groups are shown in Figure 1.3 and results from two-way ANOVA carried out for each brain region to assess group differences and changes across time are shown in Table 1.1. In AL rats the expression of PER2 in the SCN, BNSTov, CEA, BLA and DG was rhythmic, whereas expression in the DMH was arrhythmic, as previously reported (Amir et al., 2004; Lamont, Robinson, et al., 2005; Mieda et al., 2006). Specifically, in the SCN, BNSTov and CEA maximal nuclear staining for PER2 was seen in the evening, at ZT 13, whereas in BLA and DG PER2 expression was maximal in the morning, at ZT1 (see Figure 1.3). In food-deprived rats, restricted access to Ensure for 10 days had no effect on PER2 expression in the SCN. The rhythm of PER2 expression in the SCN in RF rats was similar to that seen in the SCN of AL rats, peaking at ZT13. In contrast, peak PER2 expression in the BNSTov, CEA, BLA and DG of rats from the RF group shifted to ZT17, 12h after the daily Ensure presentation (Figure 1.3). RF induced a strong PER2 rhythm in the DMH which peaked around the time of food presentation, consistent with a previous report in mice (Mieda et al., 2006).

Finally, robust rhythms of PER2 expression were seen in the SCN, BNSTov, CEA, BLA and DG of rats from the RT group (Figure 1.3). However, contrary to the effect of daily RF, daily RT had no effect on the rhythm of PER2

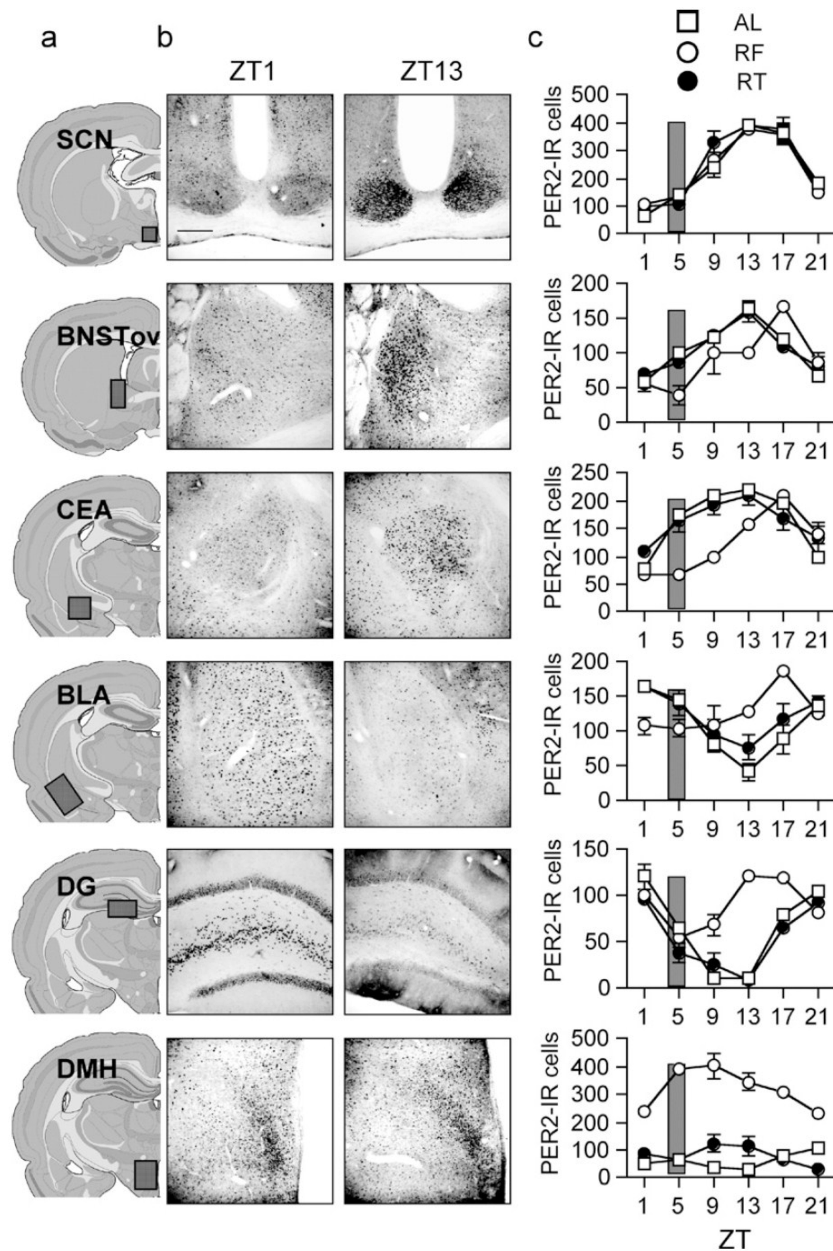

Figure 1.3
RF, but not RT synchronizes PER2 expression. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of PER2 immunoreactivity. (b) Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL) rats killed at ZT1 or 13 (scale bar=200μm). (c) Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation.

Brain Area	Group	Time of Day	Group x time
SCN	F(2, 54)=0.68, P=.5	F(5, 54)=91.40, P<0001	F(10, 54)=1.18, P=.3
BNSTov	F(2, 54)=3.65, P=.03	F(5, 54)=30.68, P<0001	F(10, 54)=5.85, P<.0001
CEA	F(2, 54)=17.31, P<0001	F(5, 54)=27.70, P<0001	F(10, 54)=7.59, P<.0001
BLA	F(2, 54)=2.02, P=.14	F(5, 54)=8.24, P<0001	F(10, 54)=5.00, P<.0001
DG	F(2, 54)=34.71, P<0001	F(5, 54)=34.52, P<0001	F(10, 54)=11.53, P<.0001
DMH	F(2, 54)=248.61, P<0001	F(5, 54)=4.18, P=.002	F(10, 54)=5.36, P<.0001

Table 1.1
Results from ANOVAs carried out to assess the effect of feeding schedule and time of day on PER2 expression in each brain area under study

expression in these areas. In these rats, PER2 expression in all regions resembled that of AL rats, consistent with our previous observation that consumption of a highly palatable substance in the absence of food deprivation is insufficient to bring about a change in the rhythm of PER2 expression (Waddington Lamont et al., 2007).

Fos expression under scheduled daily limited access to Ensure

Daily RF, but not RT, shifted the phase of PER2 expression in the limbic forebrain and induced rhythms in PER2 expression in the DMH, suggesting that the two feeding schedules might exert quantitatively or qualitatively different effects on neural activity within these brain regions. To investigate this possibility we assessed the expression of the cellular activity marker, Fos, before, during and after Ensure presentation in a second set of brain sections obtained from AL, RF and RT rats.

Representative photomicrographs of Fos immunoreactivity from AL, RF and RT rats killed 1h after Ensure presentation (ZT5) and graphs showing the levels of Fos expression from these groups 3h before (ZT1) and 1 and 5 h after (ZT5, ZT9) Ensure presentation (ZT4-6) are shown in Figure 1.4. In the SCN, the expression of Fos varied as a function of Time peaking at ZT1, 1h after lights on in all three groups ($F_{2,27}$=6.86; $P<0.001$). Likewise, Fos expression in the BNSTov varied as a function of Time ($F_{2,27}$=10.90; $P=0.0003$). Furthermore, in the BNSTov there was also a significant TimeXGroup interaction ($F_{4,27}$=5.31; $P=0.003$); expression of Fos at ZT5, 1h after Ensure presentation, was

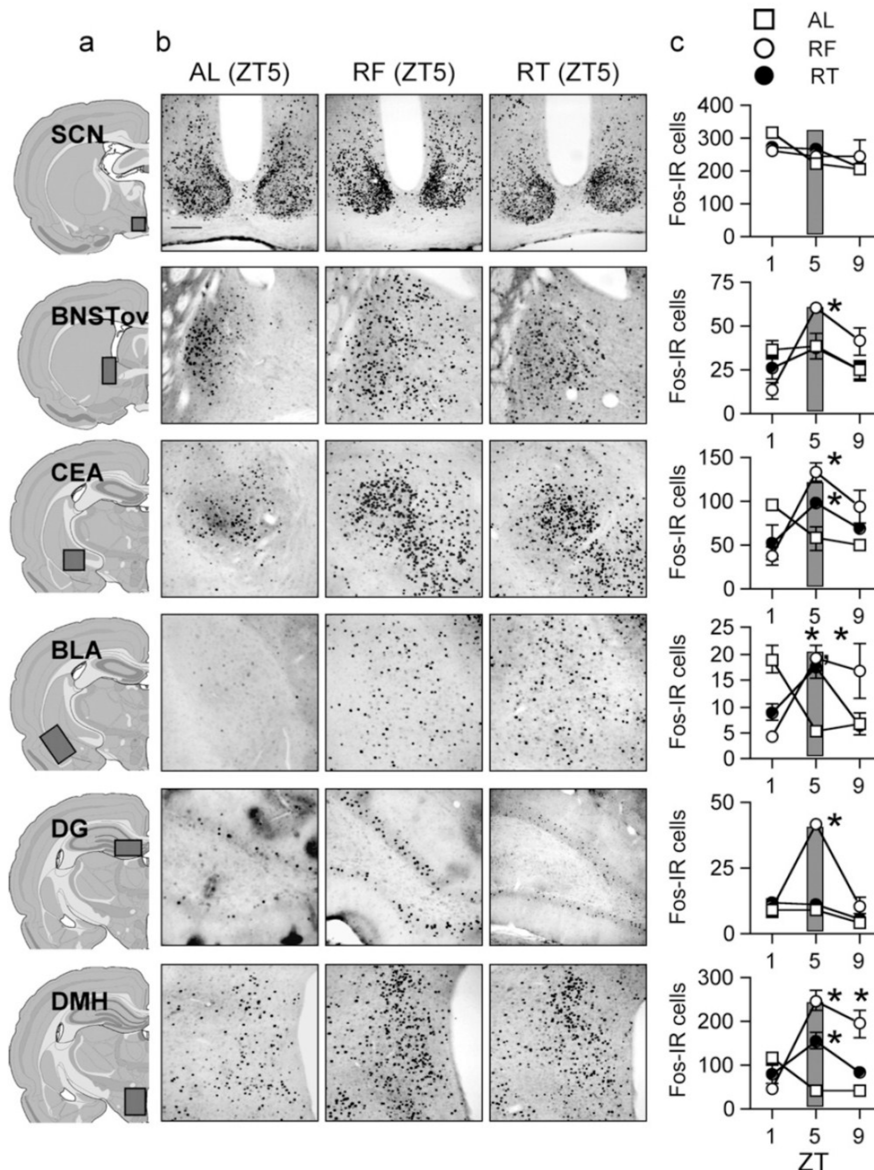

Figure 1.4
After 10 days of repeated exposure, presentation of Ensure increases Fos expression in all regions (except the SCN) in RF rats, but only the CEA, BLA and DMH in RT rats. **(a)** Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos immunoreactivity. **(b)** Examples of Fos expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL), RF and RT rats killed at ZT5 (scale bar=200μm). (c) Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3-4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

significantly higher in the RF group than in the AL group (Figure 1.4), but there was no difference between the AL the RT group.

In the CEA, Fos expression varied as a function of Time ($F_{2,27}$=6.29; P=0.006), and there was a significant TimeXGroup interaction ($F_{4,27}$=8.25; P=0.002). In this case, however, the level of Fos expression at ZT5 and ZT9, 1h and 5h after Ensure presentation was greater in both the RF and RT groups compared with the AL group (Figure 1.4). In the BLA, the RF and RT groups showed a similar pattern of Fos expression at ZT1 and ZT5 and in each case levels were significantly different from those seen in the AL group (TimeXGroup: $F_{4,27}$=11.18; P<0.001). In the DG only the RF group showed elevated Fos expression at ZT5 (TimeXGroup: $F_{4,27}$=23.36; P<0.001). Finally, in the DMH Fos expression in both RF and RT groups was significantly higher than in AL rats at ZT5, 1h after ensure presentation (TimeXGroup: $F_{4,27}$=19.03; P<0.001).

Taken together, the results show that after 10 days of repeated exposure, presentation of Ensure to RF rats increases Fos expression in all the regions studied, except the SCN. In contrast, in RT rats, exposure to Ensure increases Fos expression only in the CEA, BLA and DMH relative to AL rats. Furthermore, at the time of food presentation the increases in Fos expression were usually greater in RF than in RT rats. Thus, although the presentation of Ensure activates neural elements in most regions in both RF and RT groups after repeated Ensure presentation, the magnitude of this activation appears to be modulated by the metabolic and motivational consequences of food deprivation.

PER2 and Fos expression following acute Ensure feeding

The results from the scheduled feeding experiment suggest that the mechanism regulating the expression of PER2 in the limbic forebrain and DMH is sensitive to signals associated with the repeated mitigation of a negative metabolic state. Daily limited access to Ensure in the absence of food deprivation did not alter PER2 expression, suggesting that incentive signals associated with daily presentation of Ensure are not effective. However, both RF and RT induced Fos expression in these same regions, albeit somewhat differentially, suggesting that Fos expression is related to the incentive or nutritive aspects of Ensure.

To further test this idea, in a final experiment, we assessed Fos and PER2 expression before, during and after an acute presentation of Ensure in 24h food-deprived and free-fed rats. Groups of free-fed and fasted (24h) rats were killed 3h before (ZT1), and 1h (ZT5) and 5h (ZT9) after a single presentation of Ensure (ZT4-6). As shown in Figure 1.5, the first presentation of Ensure induced equally strong Fos expression in both groups in all regions, with the exception of the SCN where Fos expression was similar in all groups. PER2 expression was unaffected in all regions.

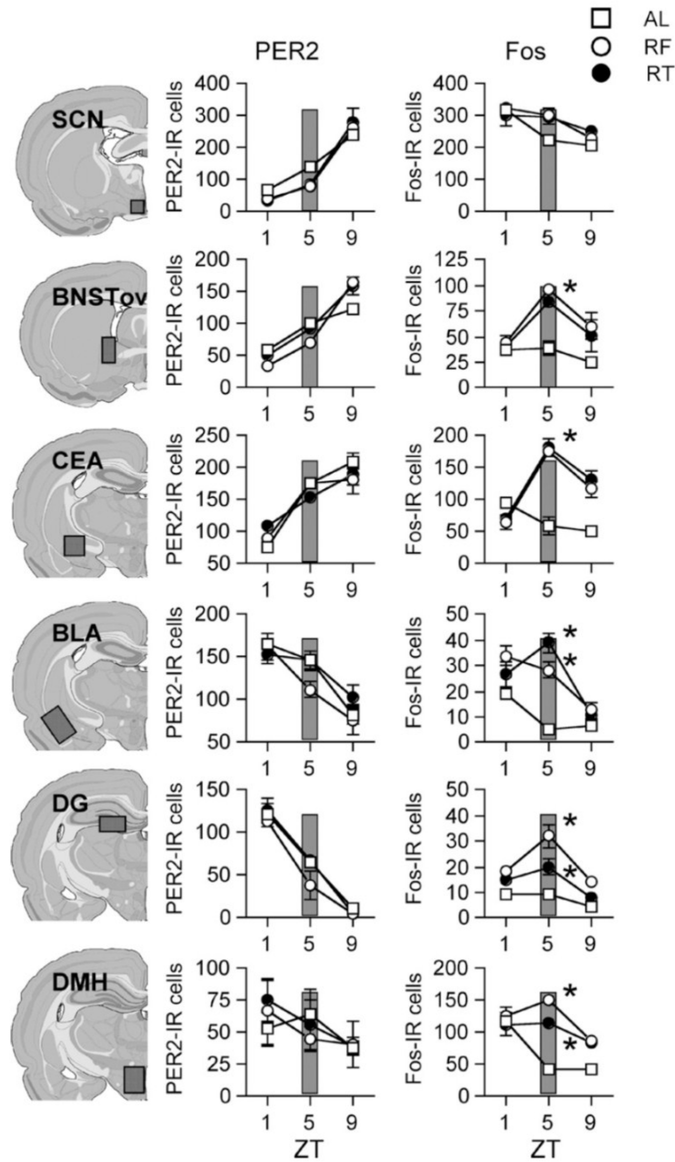

Figure 1.5
Ensure presentation for the first time does not affect PER2 expression but it increases Fos expression in all areas (except the SCN) in food-deprived (24h; RF) and free-fed (RT) rats. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos and PER2 immunoreactivity. (b) Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in control (AL), RF and RT rats (n=4/group). (c) Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=4/group). Dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4-6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

Discussion

The results of the present experiments show that in food-deprived rats daily restricted access to a highly palatable food, chocolate Ensure, synchronizes the rhythms of PER2 expression in limbic forebrain regions involved in motivational and emotional regulation and uncouples them from the rhythm in the SCN. These results are consistent with previous findings on the synchronizing effect of daily RF on the expression of PER2 and other clock genes in the brain and periphery in rodents (Angeles-Castellanos et al., 2007; Challet et al., 2003; Damiola et al., 2000; Hara et al., 2001; Mieda et al., 2006; Stokkan et al., 2001; Waddington Lamont et al., 2007; Wakamatsu et al., 2001; Zvonic et al., 2006). We also found that in the absence of food deprivation daily restricted access to Ensure had no effect on PER2 rhythms in the limbic forebrain in spite of the fact that consumption equalled that in food-deprived rats.

These results confirm and extend our previous observations in free-fed rats given restricted daily access to sucrose or saccharine solution (Waddington Lamont et al., 2007) by showing that even a highly palatable complete meal replacement has no effect on PER2 expression, suggesting that it is not the intake of nutrients, *per se*, that results in altered PER2 expression patterns. Furthermore, this supports the hypothesis that the effect on PER2 rhythms in these brain areas arises from signals associated with the daily alleviation of a negative metabolic state, and not from those associated with the incentive properties of the food. To the best of our knowledge there is one other study of the effect of daily RT on the expression of a clock gene in the brain in rats

(Mendoza, Angeles-Castellanos, et al., 2005a). In this study it was found that presentation of a daily palatable meal changed the pattern of PER1 expression in the SCN and the paraventricular thalamic nucleus, an area known to be highly sensitive to a range of arousing and rewarding stimuli. These results are at odds with the present finding of a lack of effect of RT on PER2 expression in the SCN.

These differences could be attributed to methodological differences, such as the number of days of limited access (6 weeks in Mendoza, Angeles-Castellanos, et al., 2005a, compared with ~2 weeks in the present study), the nature of the treat (chocolate bar in the Mendoza, Angeles-Castellanos, et al., 2005a study), type of limitation (limiting the amount of chocolate in the Mendoza, Angeles-Castellanos, et al., 2005a study instead of limiting the time treat was available in the current study), lighting conditions (constant darkness in the Mendoza, Angeles-Castellanos, et al., 2005a study instead of a light/dark cycle in the present study), and the type of clock protein measured (PER1 in the Mendoza, Angeles-Castellanos, et al., 2005a study instead of PER2 in the present study). Furthermore, differences in patterns of expression of PER1 and PER2 have also been observed in rats under RF schedules (Angeles-Castellanos et al., 2007; Lamont, Diaz, et al., 2005; Waddington Lamont et al., 2007) suggesting that the mechanism(s) that control the expression of different clock genes in the brain could be differentially sensitive to signals associated with feeding.

Our findings indicate that the rhythms of PER2 expression in the limbic forebrain are modulated by nutritional status and are insensitive to the incentive

properties of food, *per se*. To study whether the signals associated with the eating of Ensure in free-fed and food-deprived rats gained access to the regions under study we measured Fos induction before, during and after chronic Ensure presentation. In addition, we assessed Fos expression in food-deprived or free-fed rats receiving an acute presentation of Ensure. In rats that received daily Ensure presentations, we found that with the exception of the BNSTov and DG, restricted daily access to Ensure induced a significant increase in the expression of Fos in the limbic forebrain in both food-deprived and free-fed rats, indicating that the expression of Fos in these regions is modulated primarily by signals associated with the incentive or metabolic properties of the consumed food. This conclusion is further supported by the results from rats that received Ensure for the first time, where Fos expression increased equally in all regions (except the DG and SCN) regardless of nutritional state. However, a role for nutritional status in the modulation of Fos expression is suggested by the finding that, in rats given daily restricted access to Ensure, the increase in Fos expression in the different regions was greater in food-deprived than free-fed rats, (except the BLA and SCN). Together, the results from the rats given daily restricted access to Ensure are consistent with previous findings on the expression of Fos in multiple limbic areas in rats under RF and RT (Angeles-Castellanos et al., 2004; Angeles-Castellanos, Mendoza, Diaz-Munoz, & Escobar, 2005; Angeles-Castellanos et al., 2007; Gooley et al., 2006; Mendoza, Angeles-Castellanos, et al., 2005a; Mendoza, Angeles-Castellanos, & Escobar, 2005b; Mendoza, Angeles-Castellanos, et al., 2005c). Furthermore, they suggest that the neural signals and

cellular events leading to Fos induction in the BNSTov, CEA, BLA and DG under scheduled RF and RT and those responsible for the changes in PER2 expression these limbic forebrain regions are, at least in part, functionally dissociable.

In addition to the differences in PER2 and Fos expression discussed above, we observed differences in the prevalence of food anticipatory wheel-running. Previous studies have shown that daily limited access to a palatable meal can entrain food anticipatory rhythms in free-fed rats (Mendoza, Angeles-Castellanos, et al., 2005a; Mistlberger & Rusak, 1987); however, the aim of our study was to compare the metabolic and motivational consequences of the daily consumption of Ensure in food-deprived and free-fed rats and we used a protocol too short to reliably observe treat-anticipation. In the present study all food-deprived rats showed robust anticipatory wheel-running, whereas only 37% of free-fed rats that received daily limited access to Ensure did. To assess whether these behavioral differences, rather than differences in nutritional state, could account for the effects on PER2 expression, we compared rhythms of PER2 between free-fed rats that showed anticipatory wheel-running (n=9) and those that did not (n=15). We found that the circadian patterns of PER2 expression in these two sub-groups were indistinguishable and not different from those in AL control rats (data not shown). This finding suggests that anticipatory wheel-running, as such, does not account for the differences in the rhythms of PER2 expression.

We also studied PER2 and Fos expression in the DMH, a region of the hypothalamus that receives a major innervation from the SCN (Thompson & Swanson, 1998) and that has been implicated both in SCN-driven circadian rhythms (Chou et al., 2003) and in the control of circadian food anticipatory wheel-running activity and temperature rhythms (Gooley et al., 2006). Consistent with a previous study in mice (Mieda et al., 2006), we found that in free-fed rats the expression of PER2 in the DMH is arrhythmic and that in food-deprived rats, daily limited access to Ensure induces a robust rhythm that peaks around the time of food presentation. Importantly, we found that as in the case of the limbic forebrain, daily limited access to Ensure in free-fed rats had no effect on PER2 expression in the DMH whereas Fos was expressed equally in the DMH in both food-deprived and free-fed rats. These findings further demonstrate the dissociation between the effects of Ensure presentation on neuronal activation and on changes in rhythms of PER2 expression.

The findings concerning PER2 expression in the DMH are at odds with the hypothesis that the induction of a rhythm of PER2 in this region is critical for the expression of food anticipatory behavior (Mieda et al., 2006). At least 37% of free-fed rats given daily access to Ensure showed anticipatory wheel-running yet no changes in PER2 expression were observed in the DMH. In fact, it is possible that an even greater proportion of RT rats may have shown other forms of anticipatory behavior such as increased activity around the food receptacle before mealtime (Landry et al., 2006), but these alternate anticipatory behaviors were not tested. Thus, although our study confirms the previous finding that daily

restricted access to food induces a robust rhythm of PER2 expression in the DMH, we do not have evidence that this rhythm plays a critical role in the expression of food-anticipatory behaviors.

Taken together the present experiments show that daily restricted access to a highly palatable meal produces marked changes in PER2 rhythms in BNSTov, CEA, BLA, DG, and DMH in food-deprived rats, but does not affect the rhythms in these structures in free-fed rats. The evidence from the studies of Fos induction, shows that this lack of effect is not due to differences in the ability of food stimuli to gain access to these various brain regions. Thus, we conclude on the basis of these data and those from our previous studies that the effects on PER2 rhythms are due to the daily alleviation of a negative metabolic state and not to signals arising from the incentive or metabolic properties of the food, *per se*. We cannot, however, at this time relate these changes to any particular behavioral outcome of scheduled feeding. Although there is evidence from studies in mutant mice that global functional disruption of PER2 does affect the expression food anticipatory locomotor activity and temperature rhythms (Feillet et al., 2006), the particular brain regions important for these changes remain to be identified.

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Chapter 2: Region-specific modulation of PER2 expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats

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Contribution of authors

Michael Verwey designed the experiments together with Dr. Amir and oversaw all aspects of this project, including restricted feeding, perfusions, brain slicing, immunohistochemistry, microscopy, imaging, cell counting, and statistical analyses. The publication was written by Michael Verwey and edited by Dr. Jane Stewart and Dr. Shimon Amir.

Zehra Khoja was an undergraduate student that helped with the animal care, restricted feeding, and some of the immunohistochemistry in these studies.

Dr. Jane Stewart is a professor in the Department of Psychology. She contributed to the preparation of the manuscript.

Dr. Shimon Amir is the principle investigator of the lab where this experiment took place and Michael Verwey's thesis supervisor. He participated in the design of the experiments, microscopy, cell counting, and editing of the manuscript.

Abstract

Feeding schedules that restrict food access to a predictable daytime meal induce in rodents food-anticipatory behaviors, changes in physiological rhythms, and shifts in the rhythm of clock gene expression in the brain and periphery. However, little is known about the effects of nighttime restricted feeding. Previously, we showed that daytime restricted access to a highly palatable complete meal replacement, Ensure Plus (Ensure), shifts the rhythm of expression of the clock protein PER2 in limbic forebrain areas including the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG), and induces a rhythm in the dorsomedial hypothalamic nucleus (DMH) in food deprived (restricted feeding), but not free-fed rats (restricted treat). In the present study we investigated the effects of nighttime restricted feeding (Ensure only, 2h/night) and nighttime restricted treats (Ensure 2h/night + free access to chow) in order to determine whether these effects were dependent on the time of day the meal was provided. We found that nighttime restricted feeding, like daytime restricted feeding, shifted the rhythm of PER2 expression in the BNSTov and CEA and peak expression was observed ~12h after the mealtime. Also consistent with previous work, nighttime restricted feeding induced a rhythm of PER2 expression in the DMH and these effects occurred without affecting the rhythm in the suprachiasmatic nucleus (SCN). In contrast to previous work with daytime restricted feeding, nighttime restricted feeding had no effect on PER2 rhythms in the BLA and DG. Finally, nighttime restricted treats, as was the case

for daytime restricted treats, had no effect on PER2 expression in any of the brain areas studied. The present results together with our previous findings show that the effect of restricted feeding on PER2 rhythms in the limbic forebrain and hypothalamus depend on a negative energy balance and vary as a function of time of day in a brain region-specific manner.

Study

Feeding schedules that restrict food-availability to the middle of the day in rodents induce characteristic food-anticipatory rhythms in locomotor activity, body temperature and corticosterone release that are independent of the primary circadian clock in the suprachiasmatic nucleus (SCN; (Mistlberger, 1994; Stephan, 2002; Stephan et al., 1979b). Furthermore, such restricted feeding schedules shift the rhythms of expression of clock genes and clock proteins in the brain and periphery without affecting the rhythms in the SCN (Angeles-Castellanos et al., 2007; Challet et al., 2003; Damiola et al., 2000; Hara et al., 2001; Kawamoto et al., 2006; Kobayashi, Oishi, Hanai, & Ishida, 2004; Mendoza, 2007; Stokkan et al., 2001; Wakamatsu et al., 2001; Zvonic et al., 2006). It is unclear, however, whether the behavioral and molecular changes induced by daytime restricted feeding are due to the daily cycle of food deprivation and re-feeding, as such, or whether they are due to the fact that food is presented at a time of day when nocturnal rodents are relatively inactive and do not normally eat.

We have shown previously that restricted feeding with a predictable daytime access to the complete meal replacement Ensure Plus (Ensure) or with standard laboratory chow, shifts the rhythms of expression of the clock protein PER2 in limbic forebrain structures involved in motivational and emotional regulation, including the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG; (Amir et al., 2004; Lamont, Robinson, et al.,

2005; Verwey et al., 2007; Waddington Lamont et al., 2007). Moreover, daytime restricted feeding also induces a rhythm of PER2 expression in the dorsomedial hypothalamic nucleus (DMH), an area implicated in the control of food-anticipatory rhythms (Gooley et al., 2006; Verwey et al., 2007). In contrast to restricted feeding, when a similar daytime access to Ensure was delivered to free-fed rats (restricted treat) it had no effect on PER2 rhythms in any of these brain regions, emphasizing the importance of a negative energy balance in these effects (Verwey et al., 2007; Waddington Lamont et al., 2007). To investigate the importance of time of day, the present study placed food-deprived and free-fed rats on nighttime restricted access to Ensure and assessed the effect on rhythms of running-wheel activity and on rhythms of PER2 expression in the SCN, BNSTov, CEA, BLA, DG, and DMH. Preliminary results have been presented in an abstract form (Verwey et al., 2005).

All experimental procedures in this study followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Every effort was made to reduce the number of animals used and to minimize potential suffering. Male Wistar rats (225-250g; Charles River Laboratories, St. Constant, QC, Canada) were individually housed in cages equipped with running-wheels, under a 12h:12h light-dark (LD) schedule (~300 lux at cage level) and had free access to Purina rat chow and water. Running-wheel activity was continuously monitored using VitalView software (Mini Mitter Co. Inc., Sunriver, OR) and analyzed with Circadia software. Following acclimation to the housing environment, one group of rats (group RF;

restricted feeding) was food deprived and placed on a nighttime restricted feeding schedule in which Ensure Plus (Ensure; Abbott Laboratories, Abbott Park, IL, USA) was made available for 2h each night for 10 nights, from zeitgeber time (ZT) 16–18 (ZT12 denotes time of lights off in a 12:12 LD schedule). A second group was given the same nighttime restricted access to Ensure, but had free access to lab chow at all times (group RT, restricted treat). A third group of rats was given *ad libitum* access to normal rat chow only (group AL).

On the day following the last scheduled presentation of Ensure, rats were deeply anesthetized with an overdose of sodium pentobarbital (~100mg/kg) at one of six ZTs (ZT1, 5, 9, 13, 17, 21) and perfused intracardially with 300ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.3). Serial coronal brain sections (50μm) were taken using a vibratome. Immunocytochemistry for PER2 was performed as previously described (Verwey et al., 2007) using an affinity purified rabbit polyclonal antibody raised against PER2 (1:800, ADI, San Antonio, TX). PER2-stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 were counted using the captured images. For analysis, the mean number of PER2-immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analyses of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

Figure 2.1 shows the amount of Ensure consumed each night during the 2h access period by rats from the RF and RT groups. It can be seen that with the exception of the first night of limited access, rats from the RT group consumed more Ensure than rats from the RF group throughout the experiment ($P < 0.001$). In addition, rats in the RT group continued to eat chow and continued to gain weight, whereas the RF group lost weight. This points to a fundamental difference in energy balance between these groups. Figure 2.2 shows representative double-plotted actograms of wheel-running activity for one rat from each of the three groups (AL, RF and RT). All rats from the RF group showed clear changes in running-wheel patterns, developing a period of reduced running-wheel use during the Ensure presentation, but an overall increase in running-wheel activity over the 24h day. Rats from the RT group showed reduced running-wheel use during the 2h Ensure presentation, but no overall increase in running (Figure 2.3). ANOVA shows a significant effect of group ($F_{2,897} = 5.81$, $P < 0.004$) and a groupXday interaction ($F_{26,897} = 12.95$, $P < 0.0001$).

The daily patterns of PER2 expression in AL, RF and RT groups are shown in Figure 2.3. In both the AL and RT groups PER2 expression was similar and exhibited a circadian rhythm in the SCN, BNSTov and CEA which peaked at ZT13, whereas in the BLA and DG the rhythm peaked at ZT1, as previously described (Verwey et al., 2007). In the DMH, the expression of PER2 in AL and RT groups differed little as a function of time of day. In contrast, the RF group exhibited rhythms of PER2 expression in the BNSTov and CEA that were shifted

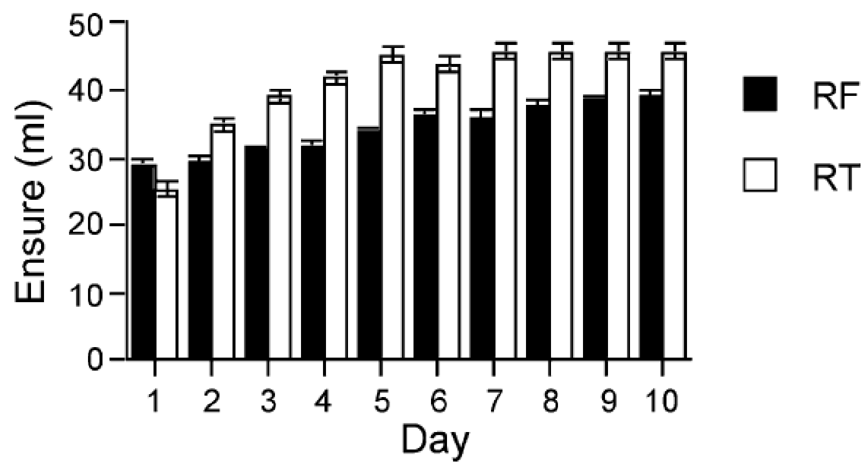

Figure 2.1
Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food deprived (RF, *n* = 24) and free-fed (RT, n=24) rats. Ensure was presented for 2h each night from ZT16-18 (4-6h after lights-off) in each group.

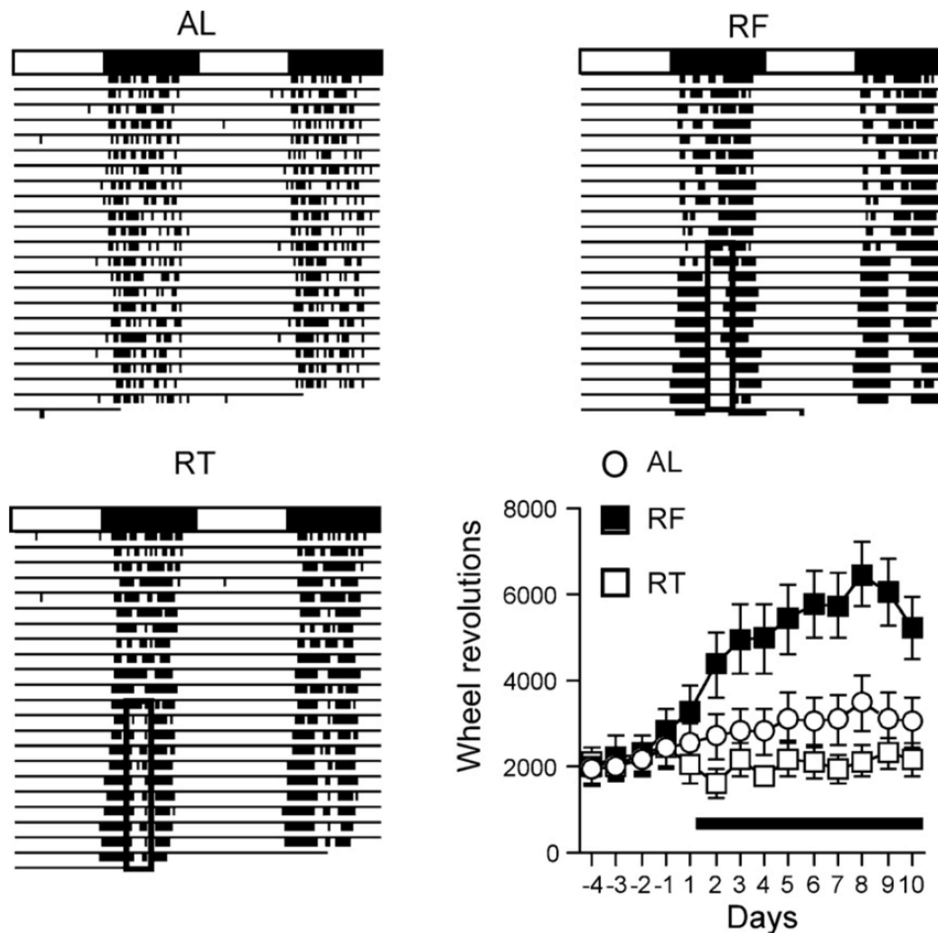

Figure 2.2
Double-plotted actograms of wheel-running activity from representative rats from the free-fed, *ad libitum* (AL) control group, the restricted feeding group (RF) and the restricted treat group (RT). The nightly presentation of Ensure occurred from ZT16-18 (4-6h after lights-off; illustrated by rectangles). All rats were housed under a 12:12h LD cycle which is illustrated by the bars at the top of each actogram. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10min. Successive days are plotted from top to bottom. The graph in the lower right shows mean (±S.E.M.) total daily number of wheel-revolutions per group (n=24) starting 4 days before and throughout the 10 days of restricted feeding.

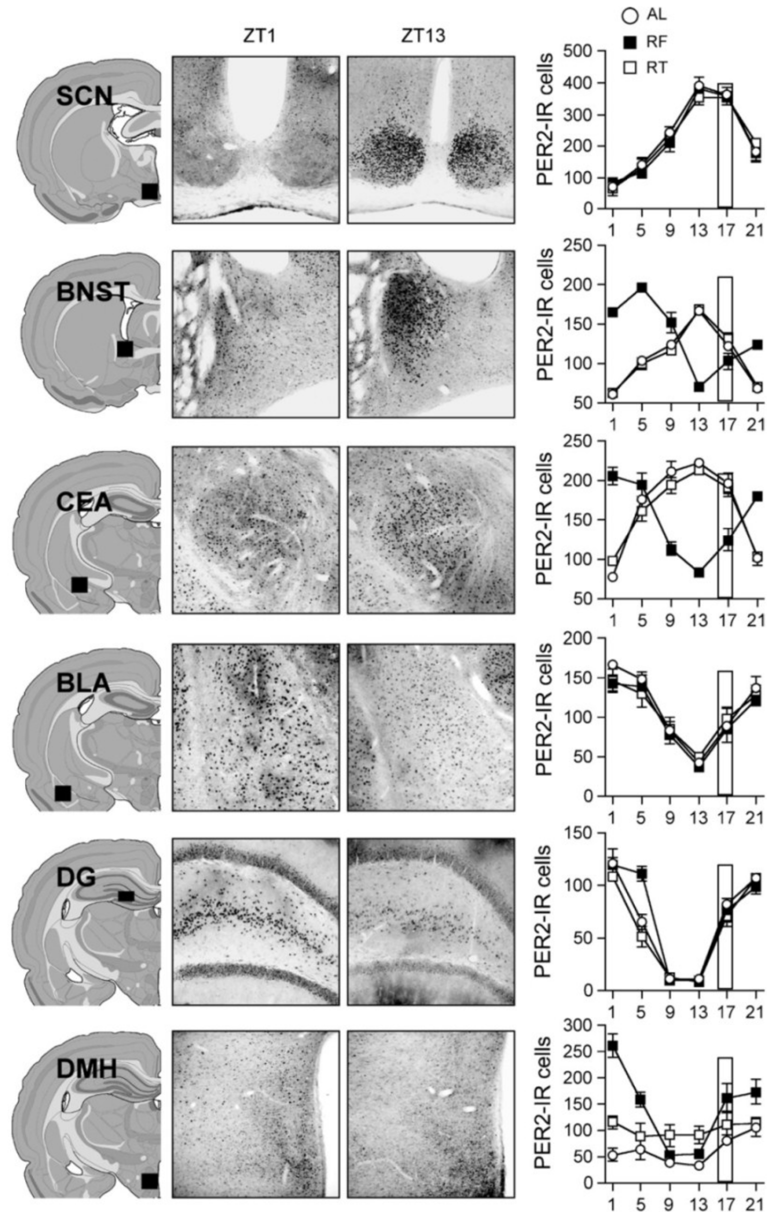

Figure 2.3
PER2 expression in control (AL) and in food-deprived (RF) and free-fed (RT) rats under nighttime restricted access to Ensure. Left panel, brain maps showing location of regions under study. The shaded square in each map indicates the area scanned for quantification of PER2 immunoreactivity. Middle panel, examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in AL rats killed at ZT1 or 13. Right panel, graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n = 4/group). Vertical rectangles inside the graphs indicate the time of Ensure presentation.

and peaked around ZT1-5. The rhythms of PER2 expression in BLA and DG were not shifted in the RF group and resembled those in the AL and RT groups. Finally, nighttime RF induced a robust rhythm of PER2 expression in the DMH. The results from the group x time ANOVAs for each brain area are shown in Table 1.1.

Restricted feeding is a powerful synchronizer of behavioral and physiological circadian rhythms and of rhythms of expression of clock genes in the brain and periphery in rodents (Challet et al., 2003; Mendoza, 2007; Mistlberger, 1994; Stephan, 2002). However, most studies on the circadian effects of such feeding schedules restrict food-availability to a daytime meal. The results of the present study in nighttime fed rats show that, indeed, the time of day meals are presented can play a significant role in the effects of restricted feeding on PER2 rhythms in the limbic forebrain and hypothalamus. We found that contrary to daytime restricted feeding, when rhythms of PER2 expression were shifted in all structures studied, nighttime restricted feeding had no effect on PER2 rhythms in the BLA and DG. This finding indicates that the effect of restricted feeding on PER2 expression in the BLA and DG seen in our previous study on daytime restricted feeding did not result from a negative energy balance, as such, but was dependent on some aspect unique to daytime restricted feeding.

Consistent with our earlier study on daytime restricted feeding, nighttime restricted feeding shifts the rhythm of PER2 expression in the BNSTov and CEA. In both cases PER2 expression peaks ~12h after the meal. We also found that,

Brain area	Group	Time of Day	Group x Time
SCN	F(2,54)=0.319, n.s.	F(5,54)=98.9, P<0.001	F(10,54)=0.498, n.s.
BNSTov	F(2,54)=34.3, P<0.001	F(5,54)=29.6, P<0.001	F(10,54)=45.0, P<0.001
CEA	F(2,54)=3.35, P=0.42	F(5,54)=18.6, P<0.001	F(10,54)=37.7, P<0.001
BLA	F(2,54)=1.13, n.s.	F(5,54)=34.1, P<0.001	F(10,54)=0.314, n.s.
DG	F(2,54)=4.17, P=0.021	F(5,54)=136, P<0.001	F(10,54)=3.71, P=0.001
DMH	F(2,54)=33.8, P<0.001	F(5,54)=13.3, P<0.001	F(10,54)=6.00, P<0.001

Table 2.1
Results from ANOVAs carried out to assess the effect of feeding schedule (AL, NF, NT) and time of day on PER2 expression in each brain area under study

as was the case with daytime restricted feeding, nighttime restricted feeding induced a rhythm of PER2 expression in the DMH (Mieda et al., 2006; Verwey et al., 2007). These results support the conclusion that unlike the BLA and DG, the effect of restricted feeding on PER2 rhythms in the BNSTov, CEA and DMH is strongly linked to a negative energy balance and is independent of the time of day when food is presented. The finding that restricted feeding induces a rhythm of PER2 expression in the DMH is particularly interesting in view of recent evidence implicating both Per2 and the DMH in the expression of certain food-anticipatory rhythms (Feillet et al., 2006; Gooley et al., 2006; Mieda et al., 2006). Finally, we found that in the absence of food deprivation nighttime restricted access to Ensure had no effect on PER2 expression in any of the brain regions under study. These results add support for the conclusion that the effects of scheduled access to Ensure on PER2 rhythms are linked primarily to its nutritional value and are relatively independent of its incentive properties, *per se* (Verwey et al., 2007).

The anticipatory behavioral and physiological circadian rhythms associated with restricted feeding are known to be independent of the SCN (Stephan et al., 1979b). However, restricted feeding can affect clock gene expression in the SCN under some circumstances (Castillo, Hochstetler, Tavernier, Greene, & Bult-Ito, 2004; Challet et al., 2003; Lamont, Diaz, et al., 2005). Our present findings suggest that some SCN-driven signal could be modulating the daily sensitivity of clock gene expression in the BLA and DG to metabolic cues associated with restricted feeding. Indeed, the finding that

restricted feeding shifts PER2 expression in the BLA and DG after daytime but not nighttime restricted feeding is reminiscent of the phase dependency of other synchronizing stimuli, photic as well as non-photic, whose effectiveness is temporally modulated by the SCN clock (Mistlberger & Skene, 2004; Mrosovsky, 1995; Rosenwasser & Dwyer, 2001).

The rhythms of PER2 expression in the BNSTov and CEA are distinct from those in the BLA and DG in several ways, including phase of peak expression and sensitivity to glucocorticoid, thyroid and gonadal hormones (Amir et al., 2004; Amir & Robinson, 2006; Lamont, Robinson, et al., 2005; Perrin et al., 2006; Segall et al., 2006). The present findings show that the rhythms in these structures are also distinct in their sensitivity to restricted feeding. Specifically, contrary to what was observed in the BLA and DG, PER2 rhythms in BNSTov and CEA were equally affected by daytime and nighttime restricted feeding, suggesting that the sensitivity of the BNSTov and CEA to feeding cues is not gated temporally across the day. Albeit, we are currently unable to explain the nature of the unwavering sensitivity of PER2 rhythms in the BNSTov and CEA to restricted feeding.

In summary, the present findings concerning the effect of nighttime restricted feeding on PER2 rhythms in the limbic forebrain, taken together with our previous work on daytime restricted feeding, point to a complex brain region-dependent interaction between feeding cues and the time of day food is presented. In the BLA and DG the effect of restricted feeding depends on a negative energy balance, but is gated by the time of day. In BNSTov, CEA and

DMH the effect of feeding on PER2 expression appears to depend solely on a negative energy balance. The basis and functional consequences of these region-specific differences remain to be determined.

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Chapter 3: Circadian rhythms of PERIOD1 expression in the dorsomedial hypothalamic nucleus in the absence of entrained food-anticipatory activity rhythms in rats

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Contribution of authors

Michael Verwey designed the experiments together with Dr. Amir and oversaw all aspects of this project, including restricted feeding, perfusions, brain slicing, immunohistochemistry, microscopy, imaging, cell counting, and statistical analyses. The publication was written by Michael Verwey and edited by Dr. Shimon Amir.

Germain Y. Lam was an undergraduate student that helped with the animal care and restricted feeding in this study.

Dr. Shimon Amir is the principle investigator of the lab where this experiment took place and Michael Verwey's thesis supervisor. He participated in the design of the experiments, microscopy, cell counting, and editing of the manuscript.

Abstract

When food availability is restricted to a single time of day, circadian rhythms of behavior and physiology in rodents shift to anticipate the predictable time of food arrival. It has been hypothesized that certain food-anticipatory rhythms are linked to the induction and entrainment of rhythms in clock gene expression in the dorsomedial hypothalamic nucleus (DMH), a putative food-entrained circadian oscillator. To study this concept further, we made food availability unpredictable by presenting the meal at a random time each day (variable restricted feeding, VRF), either during the day, night or throughout the 24h cycle. Wheel running activity and the expression of the clock protein, Period1 (PER1), in the DMH and the suprachiasmatic nucleus (SCN) were assessed. Rats exhibited increased levels of activity during the portion of the day when food was randomly presented but, as expected, failed to entrain anticipatory wheel running activity to a single time of day. PER1 expression in the SCN was unchanged by VRF schedules. In the DMH, PER1 expression became rhythmic, peaking at opposite times of day in rats fed only during the day or during the night. In rats fed randomly throughout the entire 24h cycle, PER1 expression in the DMH remained arrhythmic, but was elevated. These results demonstrate that VRF schedules confined to the day or night can induce circadian rhythms of clock gene expression in the DMH. Such feeding schedules cannot entrain behavioral rhythms, thereby showing that food-entrainment of behavior and circadian rhythms of clock gene expression in the DMH are dissociable.

Introduction

Feeding schedules that restrict food availability to a predictable time each day induce characteristic food-anticipatory circadian rhythms in behavior and physiology in rodents (Mistlberger, 2006; Richter, 1922; Stephan, 2002). Food-anticipatory rhythms appear to rely on endogenous food-entrained circadian oscillators that are independent from the master light-entrained circadian clock in the suprachiasmatic nucleus (SCN; Marchant & Mistlberger, 1997; Mistlberger, de Groot, Bossert, & Marchant, 1996; Stephan, 1983). The dorsomedial hypothalamic nucleus (DMH), a brain area implicated in the mediation of SCN-driven circadian rhythms (Chou et al., 2003), has recently been proposed to harbor a food-entrainable circadian oscillator that is both necessary and sufficient for the expression of some food-anticipatory rhythms (Fuller et al., 2008; Gooley et al., 2006). This proposal is based on the finding that DMH lesions can block some food-anticipatory rhythms and is supported by the evidence that restricted feeding schedules induce rhythmic expression of clock genes in the DMH (Fuller et al., 2008; Gooley et al., 2006; Mieda et al., 2006; Verwey et al., 2007, 2008). Although there is evidence suggesting that some clock genes play an essential role in food anticipation, evidence linking clock gene rhythms in the DMH, *per se*, and the expression of food-entrained behavioral rhythms is equivocal (Feillet et al., 2006; Fuller et al., 2008; Mistlberger et al., 2008; Storch & Weitz, 2009).

Predictable restricted feeding schedules reliably promote distinct food-anticipatory circadian rhythms in behavior and physiology, and strongly induce the rhythmic expression of clock proteins, such as period1 (PER1) and period2

(PER2), in the DMH (Fuller et al., 2008; Mieda et al., 2006; Saper & Fuller, 2007; Verwey et al., 2007, 2008). Here we describe the rhythmic expression of PER1 in the DMH and wheel-running activity rhythms in rats exposed to unpredictable feeding schedules where food was restricted to a different time each day. Such variable restricted feeding (VRF) schedules reproduce the daily fluctuations in energy balance normally associated with restricted feeding but, due to their unpredictable nature, preclude the emergence and entrainment of precise food-anticipatory circadian rhythms (Escobar, Martinez-Merlos, Angeles-Castellanos, del Carmen Minana, & Buijs, 2007). In the present experiment, food was presented at variable and unpredictable times during the 12h day (daytime VRF), the 12h night (nighttime VRF) or anytime across the entire 24h day-night cycle (anytime VRF) for a period of 10 days.

Method

Animals and housing

All experimental procedures followed the guidelines set out by the Canadian Council on Animal Care (http://www.ccac.ca/) and were approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada). All efforts were made to minimize the number of rats used; a total of 63 male Wistar rats (Charles River Laboratories, St Constant, QC, Canada) weighing 225–250 g at the beginning of the study were used. All rats were housed individually in cages equipped with running wheels under a 12h:12h light-dark cycle (LD cycle; 300 lux at cage level) and had free access to standard rodent diet (#5075; Charles River Laboratories) and water for at least 2 weeks before each experiment. Wheel running activity was recorded continuously by computer (Vitalview; Minimitter, Bend, OR, USA).

VRF

Rats were randomly assigned to one of four groups: daytime VRF; nighttime VRF; anytime VRF; or ad libitum (AL) chow. In accordance with the Animal Care and Use Committee at Concordia University, all meal replacement, chocolate Ensure Plus (Ensure, 1.5 Cal/mL; see complete nutritional facts at http://ensure.com/) for 2h each day; experimental feeding schedules lasted 10 days. The daytime VRF group received access to Ensure starting at zeitgeber time (ZT; ZT0 = when environmental lights turn on) 6, 3, 10, 4, 1, 7, 10, 2, 5 and 0, for Days 1-10, respectively. The nighttime VRF group received access to

Ensure starting at ZT (ZT12 = environmental lights turn off) 18, 15, 22, 16, 13, 19, 22, 14, 17 and 12, for Days 1-10, respectively. Rats in the anytime VRF group received access to Ensure starting at ZT4, 13, 21, 7, 14, 10, 19, 6, 15 and 0, for Days 1-10, respectively.

Tissue preparation and immunohistochemistry

On the last day of the experiment, rats were deeply anesthetized with sodium pentobarbital (Somnotol, 100 mg/kg) at one of four ZTs (ZT 1, 7, 13 or 19). Rats were perfused transcardially with 300 mL of cold saline (4ºC; 0.9% NaCl in distilled water) followed by 300 mL of cold paraformaldehyde solution (4ºC; 4% paraformaldehyde in 0.1M phosphate buffer), and brains were post-fixed for 24h in cold 4% paraformaldehyde solution. Serial coronal sections (50µm) containing the regions of interest were collected using a vibratome and stored in Watson's cryoprotectant at -20°C until processing (Watson et al., 1986).

Immunohistochemistry was performed using established protocols (Amir et al., 2004). Briefly, brain sections containing the SCN and DMH were incubated (40h, 4ºC) in a primary solution with polyclonal rabbit antibodies for PER1 (1:24 000; generous gift from Dr S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) and 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada) in 5% milk buffer in a Triton Trizma-buffered saline solution (0.3% Triton, 50 mM Trizma buffer, 0.9% saline). Free-floating sections were then incubated in a secondary antibody solution with biotinylated anti-rabbit IgG made in goat (1:200; Vector

Laboratories), followed by an incubation in an Avidin-Biotin-Peroxidase solution (Vectastain Elite ABC Kit; Vector Laboratories). Sections were then rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution, and immunoreactive (IR) cells were finally stained with a solution containing 0.5% DAB with 0.01% H_2O_2 and 8% $NiCl_2$. Blocking experiments performed by adding the PER1 peptide (1 mg/mL in phosphate-buffered saline) to the primary incubation solution prevented PER1 staining. Brain sections were mounted on gelatin-coated slides, dehydrated with alcohols, cleared with Citrisolv and glass coverslips were fixed in place with permount.

Microscopy and data analysis

PER1-stained sections were examined under a light microscope, and images of the SCN and DMH were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA) and image SXM software (v1.6, S D Barrett; http://www.ImageSXM.org.uk). IR-cells were counted for each image using a 400x400μm template, and means were calculated for each brain area based on the six unilateral images with the highest number of IR cells. Differences between groups were determined with analysis of variance (ANOVA), where the alpha level was set at 0.05.

Results

Food intake and wheel running activity

Wheel running activity records for representative rats from the AL, daytime VRF, nighttime VRF and anytime VRF groups as well as bar graphs showing the average day/night activity for each group for the last 5 days of the VRF schedule are shown in Figure 3.1. Graphs showing daily Ensure consumption as well as the daily totals and daily distributions of wheel running activity are shown in Figure 3.2. With the exception of the first few days of the experiment, Ensure consumption was virtually the same in all VRF groups (Figure 3.2A). However, throughout the course of the entire 10-day protocol, differences in weight loss were observed between groups. Specifically, whereas the daytime VRF group lost 85±5g (mean ± SEM) or 24% of their starting weight, the nighttime and anytime VRF groups lost 58±3g (~17%) and 61±4g (~17%) of their starting weight, respectively. VRF schedules increased the total amount of daily wheel running activity by two- to three-fold relative to daily running activity in AL controls (Figure 3.2B). Moreover, VRF schedules increased daytime running (Figure 3.2C) as compared with the AL control group. Daytime VRF was associated with the largest increase in daytime running. Nighttime VRF also resulted in a small increase in the percentage of daytime activity, while anytime VRF exhibited an intermediate increase in daytime running. Significantly, none of the VRF schedules led to the emergence of characteristic food-entrained circadian rhythms in anticipatory wheel running activity.

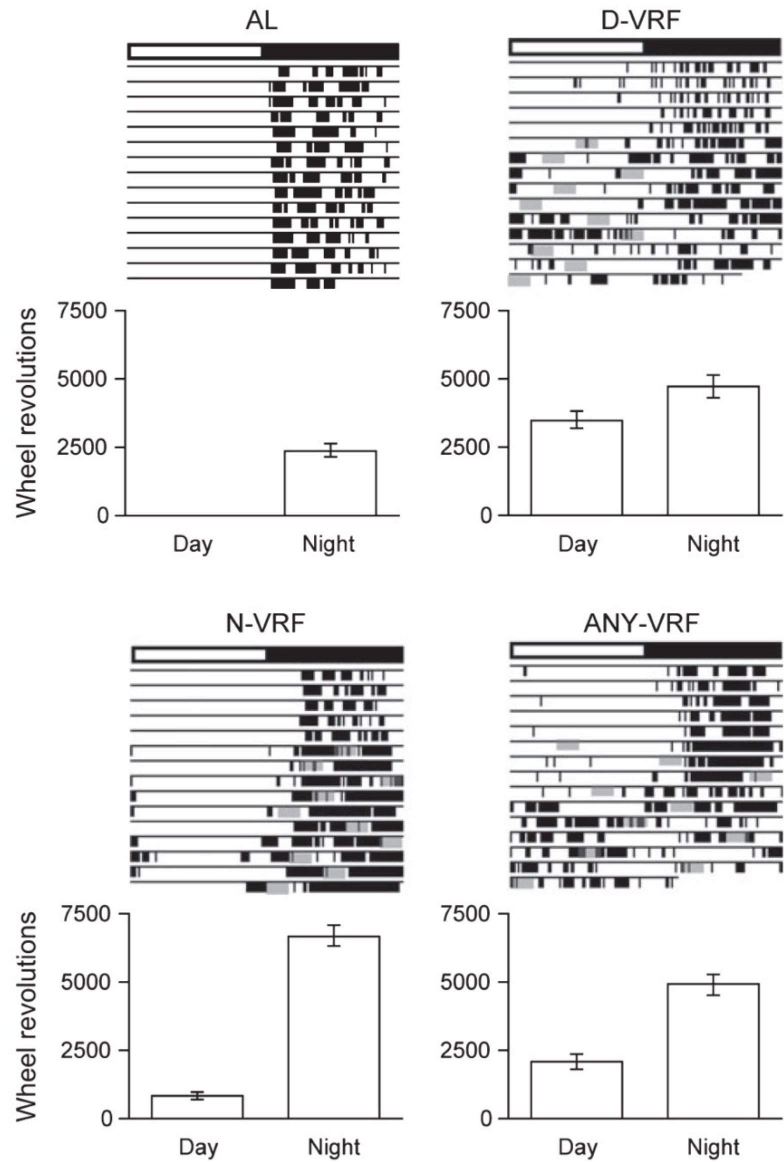

Figure 3.1
Wheel running activity records for a single representative rat from the ad libitum (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups. Below each actogram, bar graphs show the average wheel running activity in the day and night for Days 5–9 (inclusive) for all rats in each group (n=15-16/group). Actograms illustrate the 12:12h LD cycle at the top of each record and sequential days are plotted from top to bottom. Vertical marks indicate periods of activity of at least 5 wheel-revolutions/10min. Semi-transparent rectangles indicate time of Ensure availability.

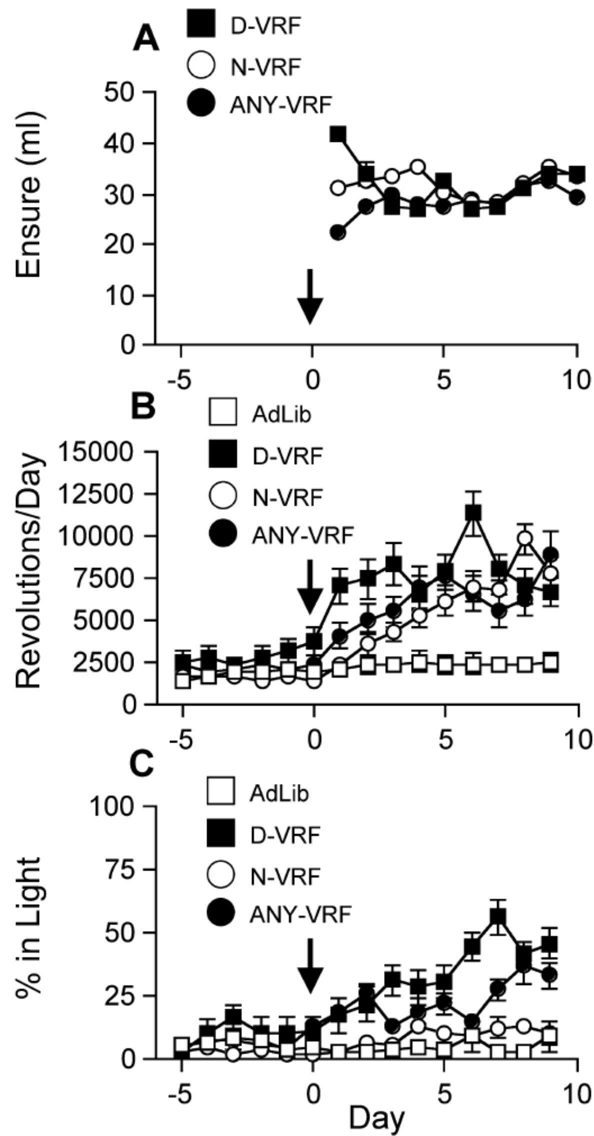

Figure 3.2
(A) Ensure consumption for each group across the 10-day variable restricted feeding (VRF) schedules, (B) total number of wheel running revolutions per 24h for each day of the experiment, and (C) percentage of total wheel running activity that took place during the 12h of light. Symbols and vertical lines indicate mean±S.E.M. (n=15-16/group). Arrows indicate the start of restricted feeding schedules.

PER1 expression

Photomicrographs showing examples of PER1 expression in the SCN and DMH of rats from the AL, daytime VRF, nighttime VRF and anytime VRF groups are shown in Figure 3.3, and graphs showing mean PER1 expression as a function of ZT for all VRF groups relative to AL values are shown in Figure 3.4. In all groups, PER1 expression in the SCN was rhythmic ($ANOVA_{TIME}$; AL: $F_{3,12} = 17.6$, $P < 0.001$; daytime VRF: $F_{3,11} = 18.9$, $P < 0.001$; nighttime VRF: $F_{3,12} = 28.8$, $P < 0.001$; anytime VRF: $F_{3,12} = 30.6$, $P < 0.001$), peaking at about ZT13 (see Figure 3.4). In contrast, in the DMH, PER1 expression varied as a function of feeding schedule (see Figure 3.4). The expression of PER1 in the DMH in AL fed rats was arrhythmic ($ANOVA_{TIME}$, $F_{3,12} = 1.76$, $P = 0.21$). In contrast, daytime VRF induced a circadian rhythm of PER1 expression in the DMH that peaked at about ZT13 ($ANOVA_{TIME}$, $F_{3,11} = 37.9$, $P < 0.0001$). Under nighttime VRF, PER1 expression in the DMH was also rhythmic but, contrary to daytime VRF, the peak expression occurred at about ZT1 ($ANOVA_{TIME}$, $F_{3,12} = 8.68$, $P < 0.002$). When VRF occurred anytime across the day and night, PER1 expression in the DMH was arrhythmic ($ANOVA_{TIME}$, $F_{3,12} = 1.48$, $P = 0.26$), although overall levels were higher than those seen in the AL group ($ANOVA_{GROUP}$, $F_{1,24} = 5.6$; $P < 0.05$).

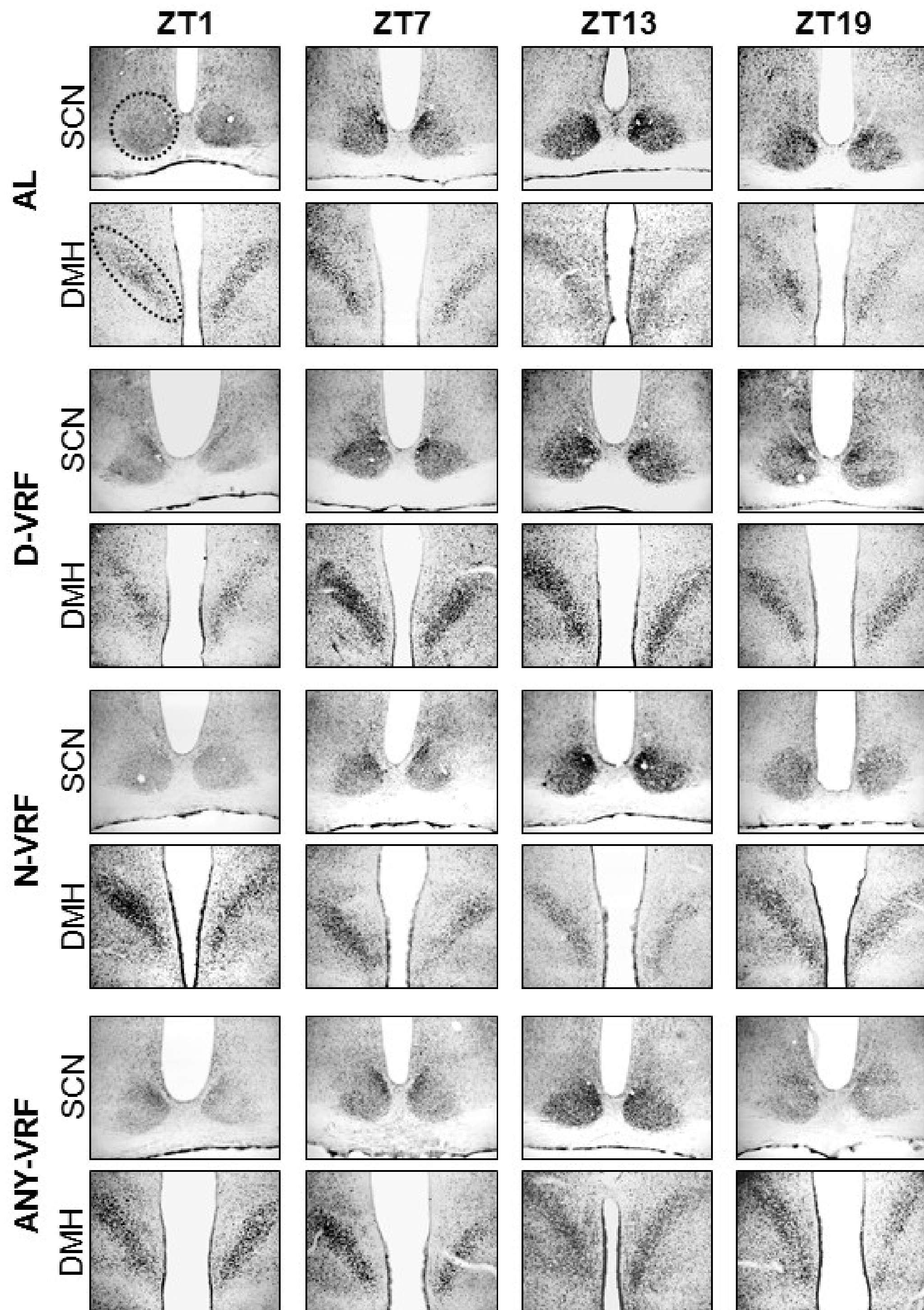

Figure 3.3
Photomicrographs showing examples of PER1-immunostaining in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) across the day in rats from the ad libitum (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups.

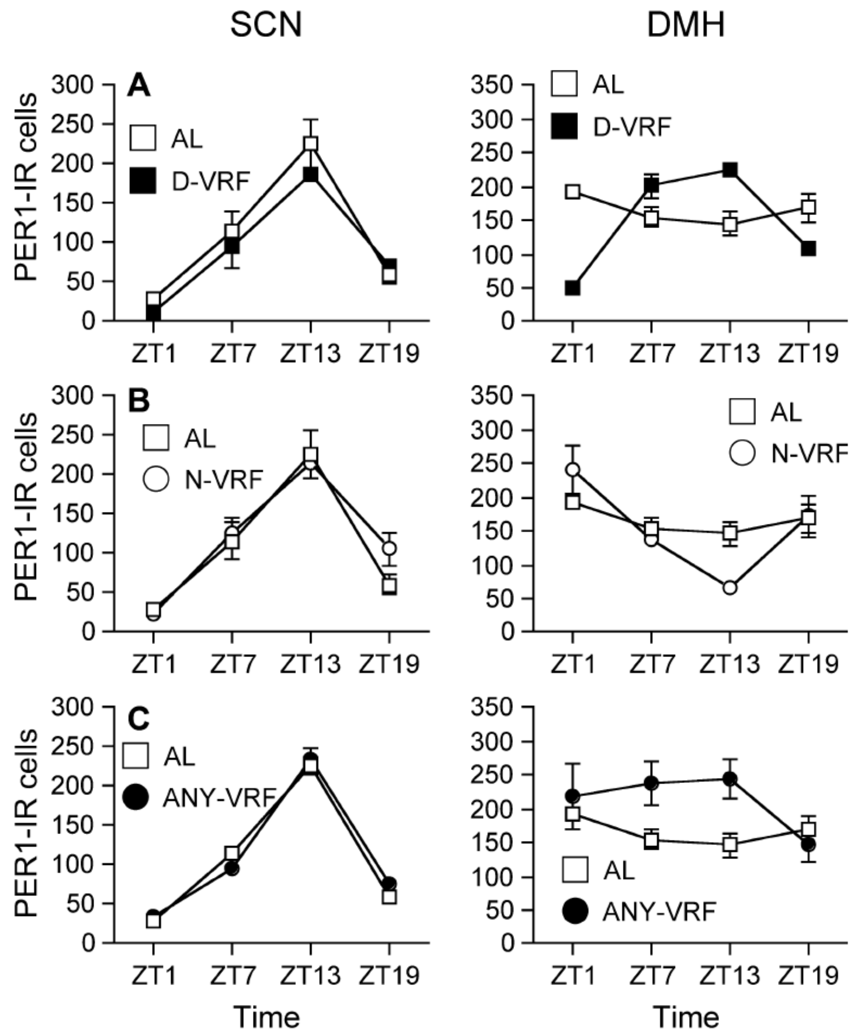

Figure 3.4
(A–C) Graphs showing mean (± SEM) number of PER1-immunoreactive (PER1-IR) nuclei in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) as a function of ZT for the ad libitum (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups (n = 3–4/group).

Discussion

Predictable restricted feeding schedules, in which food is presented at the same time each day, lead to the emergence of entrained food-anticipatory rhythms in behavior. Furthermore, such predictable schedules induce and entrain circadian rhythms of PER1 and PER2 expression in the DMH (Fuller et al., 2008; Mieda et al., 2006; Verwey et al., 2007). The DMH appears to contribute to the expression and control of some food-anticipatory rhythms, such as the rhythm in body temperature (Fuller et al., 2008; Gooley et al., 2006). Consequently, it has been proposed that the induction of a circadian rhythm in PER expression in this region is intimately linked to the emergence and entrainment of food-anticipatory rhythms under restricted feeding. In the present study we used unpredictable feeding schedules in which food was presented at a different time each day. Such schedules lead to gradual loss of body weight of between 17 and 24%, roughly the same magnitude of weight loss seen in rats subjected to 10 days of predictable feeding schedule with Ensure (2h/day). The levels and distribution of daily wheel running activity were differentially affected by these VRF schedules but, because of the unpredictability of the time of feeding, distinct food-entrained anticipatory rhythms in wheel running activity did not emerge. However, despite the unpredictability of the feeding times, the daytime VRF group showed the largest increase in wheel running activity during the day, the nighttime VRF group showed the majority of wheel running activity during the night, and the anytime VRF group demonstrated an intermediate distribution (Figure 3.2C). Circadian rhythms of PER1 expression in the SCN were not affected by any of the VRF

feeding schedules, consistent with results showing lack of effect of predictable restricted feeding schedules with Ensure on PER2 expression in this region (Verwey et al., 2007, 2008). In contrast, PER1 expression in the DMH, which was arrhythmic in freely fed rats, became rhythmic under daytime and nighttime VRF schedules. When the daily access to food was limited to the daytime, peak PER1 expression in the DMH was observed between ZT7 and ZT13, and an opposite rhythm was observed when food was restricted to the nighttime. However, PER1 expression in the DMH remained arrhythmic when food was presented at random times throughout the 24h day. These results demonstrate that in addition to predictable restricted feeding schedules (Fuller et al., 2008; Mieda et al., 2006; Verwey et al., 2007, 2008), VRF can also induce circadian rhythms of PER1 expression in the DMH. Furthermore, they show that the pattern and phase of the rhythm of PER1 expression induced in the DMH depends on whether food is given during the daytime or the nighttime, as previously shown for PER2 under predictable restricted feeding schedules (Verwey et al., 2007, 2008). Together, these results show that the induction of a circadian rhythm in PER1 expression in the DMH can be dissociated from the emergence of food-entrained behavioral rhythms in rats.

An important question that emerges from these findings concerns the critical factor involved in the induction of the different patterns and phase of the circadian rhythms of PER1 expression in the DMH. One possibility is that the observed rhythm in PER1 expression is dependent on the time of the last meal before the rats were killed. This is unlikely, however, because in the present

study the daytime and anytime VRF groups were both presented with their last meal at ZT0, but resulted in decidedly different daily profiles of PER1 expression in the DMH. Whereas the daytime VRF group exhibited rhythmic PER1 expression in the DMH that peaked during the day, in the group that received food randomly throughout the 24h day (anytime VRF) PER1 expression in the DMH was arrhythmic, albeit elevated. These data, together with our previous observation that exposure to a single episode of food deprivation and refeeding has no effect on PER2 expression in the DMH (Verwey et al., 2007, 2008), suggest that it is the entire 10-day VRF schedule and the portion of the day when food becomes available (daytime, nighttime or anytime) that determines subsequent circadian rhythms in PER1 expression and not simply the time the last meal was presented.

A second issue to be considered is the relation between the induction of PER rhythms in the DMH and the emergence of food-anticipatory rhythms. We found that daily restricted feeding can induce a rhythm in PER1 expression in the DMH, even when the feeding time is unpredictable and animals cannot accurately anticipate the precise time of the daily meal. These results show that the induction of PER1 rhythms in the DMH and the food-entrainment of activity rhythms are dissociable. Another example of such a dissociation comes from previous studies using restricted ‘treats’. Restricted treat schedules provide a daily highly palatable treat to a freely fed rat and induce treat-anticipatory activity (Angeles-Castellanos et al., 2008; Mendoza, Angeles-Castellanos, et al., 2005a, 2005c; Mistlberger & Rusak, 1987), but fail to induce a circadian rhythm of PER2

expression in the DMH (Verwey et al., 2007, 2008). Similarly it has been shown that food-entrained behavioral rhythms and the rhythm of expression of Per1 in digestive organs in rats are also dissociable (Davidson, Poole, Yamazaki, & Menaker, 2003).

The present results add in an important way to the current debate about the role of the DMH as a food-entrained circadian oscillator driving food-anticipatory rhythms. The mammalian circadian clock is based on daily oscillations in the expression of several clock genes (Reppert & Weaver, 2002). Among them, the *Bmal1* gene controls the expression of *Per* genes and plays an essential role in the generation of circadian rhythms. Bmal1 and Per2 mutant mice each exhibit pronounced deficits in the circadian modulation of behavior, and either mutation has the capacity to disrupt circadian clocks at the molecular level (Bae et al., 2001; Bunger et al., 2000; Shearman et al., 2000). Both Bmal1 and Per2 mutants have been reported to exhibit deficient or disrupted food-anticipatory rhythms under daily scheduled restricted feeding (Feillet et al., 2006; Fuller et al., 2008). Furthermore, restoring Bmal1 in the DMH was found to rescue food anticipatory rhythms of body temperature in Bmal1 mutant mice (Fuller et al., 2008). Although subsequent PER expression in the DMH of mutant mice was not assessed, it was suggested that a full complement of clock genes in the DMH is important for the expression of food-anticipatory changes in the rhythm of core body temperature. Contrary to these findings, other studies have shown that both Bmal1 and Per2 mutant mice exhibit clear food-anticipatory activity (Mistlberger et al., 2008; Storch & Weitz, 2009), suggesting that the food-

entrainment of behavior may be independent of certain clock genes. Future studies will need to focus on the self-sustainability of the restricted feeding-induced rhythms in clock gene expression in the DMH, as well as the extent to which the full complement of canonical clock genes are being expressed within this structure. The present results show that although the expression of *Per* genes in the DMH is sensitive to restricted feeding schedules, the induced rhythm does not depend on food being presented at a particular, predictable time each day. Rather, a rhythm of PER1 expression in the DMH can be induced when food is restricted to a certain portion of the day (i.e. daytime or nighttime). Together, these results indicate that the induction of a rhythm of PER expression in the DMH can be dissociated from and can exist in the absence of stable, food-entrained rhythms in behavior.

In summary, the present study shows that the induction of circadian PER1 expression in the DMH and circadian food anticipatory rhythms in wheel running activity are dissociable. Furthermore, they confirm previous evidence indicating that the daily pattern of PER expression in the DMH induced by restricted feeding varies as a function of circadian time of food presentation. Functional consequences of the circadian clock gene expression in the DMH remain unclear, but we would suggest that perhaps rhythms in the DMH are particularly relevant to the control of core body temperature (Dimicco & Zaretsky, 2007). The DMH is clearly affected by restricted feeding and appears to mediate at least some food-anticipatory circadian rhythms (Fuller et al., 2008; Gooley et al., 2006). However, VRF schedules were sufficient to induce a circadian rhythm of

PER1 expression in the DMH, suggesting that food-entrainment, *per se*, is not a requirement for circadian rhythms of clock gene expression in the DMH (Landry et al., 2006; Landry, Yamakawa, Webb, Mear, & Mistlberger, 2007; Verwey et al., 2007, 2008).

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Chapter 4: Variable restricted feeding disrupts the daily oscillations of PERIOD2 expression in the limbic forebrain and dorsal striatum in rats

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Contribution of authors

Michael Verwey designed the experiments together with Dr. Amir and oversaw all aspects of this project, including restricted feeding, perfusions, brain slicing, immunohistochemistry, microscopy, imaging, cell counting, and statistical analyses. The publication was written by Michael Verwey and edited by Dr. Shimon Amir.

Dr. Shimon Amir is the principle investigator of the lab where this experiment took place and Michael Verwey's thesis supervisor. He participated in the design of the experiments, and editing of the manuscript.

Abstract

Predictable restricted feeding schedules limit food-availability to a single meal at the same time each day, lead to the induction and entrainment of circadian rhythms in food-anticipatory activity, and shift daily rhythms of clock gene expression in areas of the brain that are important in the regulation of motivational and emotional state. In contrast, when food is delivered under a variable restricted feeding (VRF) schedule, at a new and unpredictable mealtime each day, circadian rhythms in food-anticipatory activity fail to develop. Here, we study the effects of VRF on the daily rhythm of plasma corticosterone and of clock gene expression in the limbic forebrain and dorsal striatum, of rats provided a 2h-access to a complete meal replacement (Ensure Plus) at an unpredictable time each day. VRF schedules varied the mealtimes within the 12h of light (daytime VRF), the 12h of dark (nighttime VRF), or across the 24h light-dark cycle (anytime VRF). Our results show that contrary to the synchronizing effects of predictable restricted feeding, VRF blunts the daily corticosterone rhythm and disrupts daily rhythms of PER2 expression in a region-specific and mealtime-dependent manner.

Introduction

Circadian rhythms can be entrained by restricted feeding cycles that provide a single meal at the same time each day (Mistlberger, 1994; Stephan et al., 1979b). In rodents, these predictable cycles of feeding and fasting will induce and entrain characteristic food-anticipatory rhythms in behavior and physiology (Richter, 1922), and synchronize the rhythms of clock gene expression in the periphery and brain (Angeles-Castellanos et al., 2007; Damiola et al., 2000; Davidson et al., 2003; Davidson, Stokkan, Yamazaki, & Menaker, 2002; Hara et al., 2001; Le Minh, Damiola, Tronche, Schutz, & Schibler, 2001; Verwey & Amir, 2009; Wakamatsu et al., 2001). In contrast, little is known about the circadian effects of variable restricted feeding (VRF) cycles, which provide a single meal at a new and unpredictable time each day and thus cannot entrain rhythms of food anticipatory behavior. There is some evidence that VRF cycles disrupt daily behavioral rhythms as well as daily rhythms in metabolite and hormone levels (Escobar et al., 2007; Valle, 1981; Verwey et al., 2009), but the effect of VRF on daily rhythms of clock gene expression has not been systematically examined.

In the present study, we assessed the effects of VRF on circulating levels of corticosterone and daily rhythms of the clock protein, Period2 (PER2). Specifically, PER2 expression was measured in the suprachiasmatic nucleus (SCN), the master circadian clock, and in several forebrain nuclei important in stress, motivation and emotional regulation. The dorsal striatum, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus

(DG) have all been shown to exhibit daily rhythms in PER2 expression in rats (Amir et al., 2004; Hood et al., 2010; Lamont, Robinson, et al., 2005). Moreover, predictable restricted feeding cycles have been shown to entrain the daily rhythms of PER2 expression in the limbic forebrain, without shifting the daily rhythm of PER2 expression in the SCN (Verwey et al., 2007, 2008). In the present study we show that, contrary to the synchronizing effects of predictable feeding schedules, VRF schedules disrupt the daily rhythms of PER2 expression in a region-specific and mealtime-dependent manner.

Method

Animals and housing

All experimental procedures were approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada) and adhered to the rules and recommendations set out by the Canadian Council on Animal Care (http://www.ccac.ca/). Male Wistar rats weighing between 225-250g at the start of the experiment (n=63; Charles River Laboratories, St. Constant, QC, Canada) were individually housed in cages equipped with running wheels, in a 12h-12h environmental light-dark (LD) cycle. Rats had free access to standard rat chow (Rodent diet #5075; Charles River Laboratories, St. Constant, QC, Canada) and water during the initial 2-week entrainment period. The ad libitum (AL) control group continued with free access to rat chow, but food-availability was restricted to a 2h meal/day for rats assigned to the VRF groups (see next section). Running wheel records and complete descriptions of the behavioral patterns under each VRF condition were reported previously (Verwey et al., 2009).

Variable restricted feeding

Variable restricted feeding schedules varied the time of food arrival within the 12h light phase (daytime VRF), the 12h dark phase (nighttime VRF) or across the entire 24h LD cycle (anytime VRF). Under all VRF conditions, the meals consisted of a 2h-access to the complete meal replacement chocolate Ensure Plus (Ensure, http://ensure.com/), each day for 10 days. The daytime VRF group

received a meal starting at zeitgeber time (ZT; ZT0 is when environmental lights turn on) 6, 3, 10, 4, 1, 7, 10, 2, 5, and 0, for days 1-10, respectively. The nighttime VRF group received a meal starting at ZT (ZT12 = Environmental lights turn off) 18, 15, 22, 16, 13, 19, 22, 14, 17, and 12, for days 1-10, respectively. Rats in the anytime VRF group received a meal starting at ZT 4, 13, 21, 7, 14, 10, 19, 6, 15, and 0, for days 1-10, respectively.

Perfusions and immunocytochemistry

Perfusions were carried out at ZT1, ZT7, ZT13, or ZT19. At each time, rats from each group were injected with sodium pentobarbital (Somnotol, 100 mg/kg) and perfused transcardially with 300mL of cold saline (4°C; 0.9% NaCl in distilled water) followed by 300mL of cold paraformaldehyde solution (4°C; 4% paraformaldehyde in 0.1M phosphate buffer). Brains were removed from the skull, and post-fixed for 24h (4% paraformaldehyde solution, 4°C). Alternate coronal brain sections (50 μm) containing the brain areas of interest were sliced on a vibratome and stored in cold Watson's cryoprotectant (-20°C) until staining (Watson et al., 1986). One set of brain sections were used to analyze PER1 expression in the SCN and DMH, and the data were published separately (Verwey et al., 2009). The present experiment used the second set of brain sections to examine PER2 expression in the SCN, limbic forebrain, and dorsal striatum.

Immunohistochemistry for PER2 was carried out as previously described (Amir et al., 2004). Briefly, sections were incubated (40h, 4°C) in a primary

antibody solution containing polyclonal rabbit antibodies for PER2 (1:1000; Alpha Diagnostics International, San Antonio, TX, USA) and 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada) in triton (0.3%) trizma-buffered saline (50mM trizma buffer with 0.9% NaCl). After rinsing the free-floating tissue in trizma-buffered saline, sections were incubated in a secondary antibody solution with biotinylated anti-rabbit IgG made in goat (1h, 4°C; 1:200; Vector Laboratories), rinsed again, then incubated in an avidin-biotin solution (2h, 4°C; Vectastain Elite ABC Kit; Vector Laboratories). The tissue was then bathed in a 0.5% 3,3-diaminobenzidine solution and immunoreactive cells were stained with a solution containing 0.5% 3,3-diaminobenzidine, 0.01% H_2O_2 and 8% $NiCl_2$. Brain sections were mounted on gelatin-coated slides, dehydrated in a series of alcohols, cleared with Citrisolv, and glass coverslips were fixed in place with permount glue (Fisher Scientific, Ottawa, ON, Canada).

Plasma sampling and corticosterone assay

Blood samples were collected prior to each perfusion, within 90s of first contact with the home cage. Plasma was isolated by centrifugation (10000rpm, 10min, 4°C) and stored at -80°C until the corticosterone assay was performed. Enzyme-linked immunosorbent assays were carried out according to the instructions provided (Corticosterone EIA Kit 900-097; Assay Designs Inc., Ann Arbor, MI, USA). Each sample was assayed in duplicate and values were averaged between wells containing the same sample (intra-assay variability <5%).

Microscopy and data analysis

Using a light microscope, brain sections were studied and images of the SCN, BNSTov, CEA, BLA, DG and dorsal striatum were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA) and image SXM software (v1.6, S D Barrett, http://www.ImageSXM.org.uk). PER2-immunoreactive (PER2-IR) cells were counted for each brain area using either a 400x400μm (SCN, BNSTov, CEA, BLA, dorsal striatum) or 400x200μm (DG) template, and means were calculated for each brain area based on the 6 unilateral images with the highest immunoreactivity. Differences between groups were determined with analysis of variance (ANOVA) where the alpha level was set at 0.05.

Results

On average, over the course of the 10-day experiment, each VRF group consumed a similar amount of Ensure (Daytime VRF = 47.8 ± 0.8 kCal/day; Nighttime VRF 46.9 ± 0.7 kCal/day; Anytime VRF = 43.2 ± 0.6 kCal/day). In response to these hypocaloric diets, the daily pattern of PER2 expression was modulated in several brain areas, and the daily profiles of PER2-IR are shown in Figure 4.1, where each VRF group is graphed with respect to AL controls. A robust daily rhythm in PER2 expression was observed in the SCN of all groups (Table 4.1), where peak PER2 expression was seen around ZT13 and trough PER2 expression around ZT1, and this SCN-rhythm was unaffected by VRF (Figure 4.1; Table 4.2). Consistent with previous findings (Amir et al., 2004; Hood et al., 2010; Lamont, Robinson, et al., 2005), rats that were freely fed with standard rat chow, also exhibited robust daily oscillations of PER2 expression in the BNSTov, CEA, BLA, DG, and dorsal striatum (Table 4.1). Specifically, in the BNSTov and CEA, PER2 expression peaked around ZT13 and troughed around ZT1 (Figure 4.1). In contrast, the BLA, DG, and dorsal striatum, each exhibited a daily oscillation of PER2 expression that peaked around ZT1 and troughed around ZT13 (Figure 4.1). It was these daily rhythms of PER2 expression, outside of the SCN, which were strongly affected by VRF.

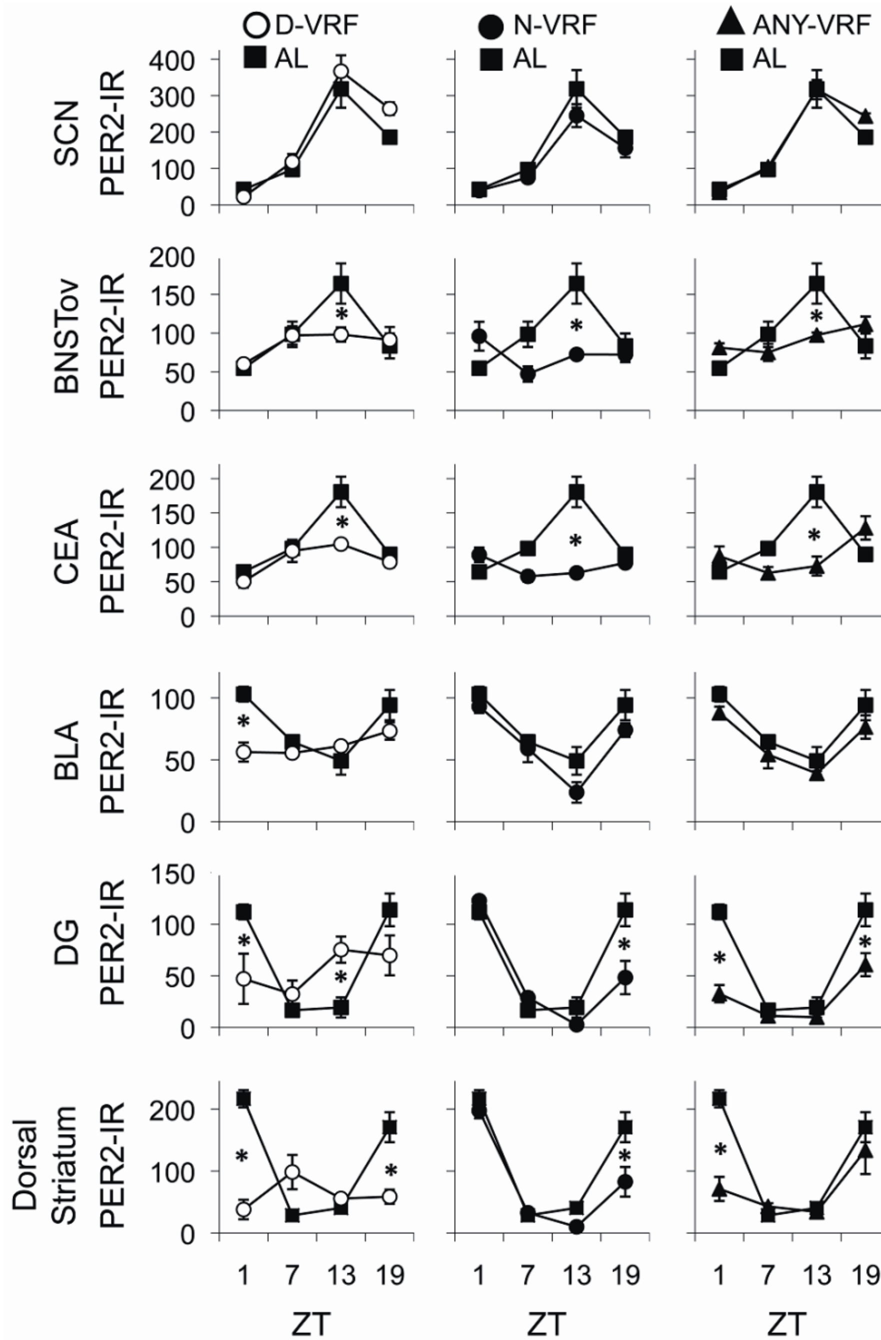

Figure 4.1
Daily patterns of PER2 expression under daytime VRF (D-VRF; left column), nighttime VRF (N-VRF; middle column), and anytime VRF (ANY-VRF; right column), compared to AL controls. Each brain area is graphed separately and is listed on the far left of each row. Graphs illustrate the mean (± sem) PER2-immunoreactivity (PER2-IR) in each brain area, according to the zeitgeber time (ZT) when the rats were killed. Asterisks (*) represent a statistically significant difference ($p<0.05$) between groups at a single time point.

Condition/Structure	$ANOVA_{ZT}$
AL Controls	
SCN	F(3,12)= 63.6, p<0.001
BNSTov	F(3,12)= 16.8, p<0.001
CEA	F(3,12)= 60.0, p<0.001
BLA	F(3,12)= 22.3, p<0.001
DG	F(3,12)= 82.2, p<0.001
Dorsal Striatum	F(3,12)= 41.1, p<0.001
D-VRF	
SCN	F(3,11)=28.2, p<0.001
BNSTov	F(3,11)=1.9, ns
CEA	F(3,11)=4.5, p<0.05
BLA	F(3,11)=2.6, ns
DG	F(3,11)=1.4, ns
Dorsal Striatum	F(3,11)=2.2, ns
N-VRF	
SCN	F(3,12)=19.1, p<0.001
BNSTov	F(3,12)=2.8, ns
CEA	F(3,12)=4.3, p<0.05
BLA	F(3,12)=13.9, p<0.001
DG	F(3,12)=56.1, p<0.001
Dorsal Striatum	F(3,12)=35.2, p<0.001
ANY-VRF	
SCN	F(3,12)=69.1, p<0.001
BNSTov	F(3,12)=4.1, p<0.05
CEA	F(3,12)=4.4, p<0.05
BLA	F(3,12)=8.0, p<0.01
DG	F(3,12)=11.4, p<0.01
Dorsal Striatum	F(3,12)=4.2, p<0.05

Table 4.1
Daily patterns of PER2 expression in each brain area, under each feeding condition

The effect of VRF on PER2 expression in the limbic forebrain and dorsal striatum is shown in Figure 4.1 and statistical analyses are shown in Table 4.2. In the daytime-VRF group, the daily rhythm of PER2 expression was dampened in all of the extra-SCN areas and the CEA was the only structure that continued to exhibit a daily oscillation in PER2 expression (Table 4.1). In the nighttime-VRF group, the circadian rhythm of PER2 expression in the BNSTov and CEA was dampened as compared to AL controls (Figure 4.1; Table 4.2), although PER2 expression in the CEA continued to show a significant daily oscillation (Table 4.1). The BLA, DG and dorsal striatum continued to exhibit robust circadian rhythms in the nighttime-VRF group (Table 4.1), comparable to AL controls. In the anytime-VRF group, all extra-SCN structures continued to show a daily oscillation in PER2 expression (Table 4.1). However, under anytime-VRF, the daily patterns of PER2 expression in the BNSTov, CEA, DG and dorsal striatum, were each blunted compared to AL controls (Figure 4.1; Table 4.2).

Daily patterns of plasma corticosterone are shown in Figure 4.2. The AL controls were the only group that showed a statistically significant daily oscillation (Table 4.3). The daily pattern of corticosterone in the daytime-VRF group was significantly different from the AL controls (Table 4.3). The nighttime-VRF group exhibited corticosterone levels that were generally higher than the AL controls (Table 4.3). Finally, while the anytime-VRF group was not statistically different from AL controls overall, it failed to show a significant daily oscillation (Table 4.3).

Condition/ Structure	$ANOVA_{Feeding}$	$ANOVA_{ZT}$	$ANOVA_{Feeding \times ZT}$
D-VRF v. AL			
SCN	F(1,23)=4.1, ns	F(3,23)=74.0, p<0.001	F(3,23)=1.7, ns
BNSTov	F(1,23)=2.6, ns	F(3,23)=13.0, p<0.001	F(3,23)=4.6, p<0.05
CEA	F(1,23)=18.1, p<0.01	F(3,23)=33.1, p<0.001	F(3,23)=7.7, p<0.001
BLA	F(1,23)=18.8, p<0.01	F(3,23)=14.6, p<0.001	F(3,23)=10.4, p<0.001
DG	F(1,23)=1.1, ns	F(3,23)=12.3, p<0.001	F(3,23)=9.7, p<0.001
Dorsal Striatum	F(1,23)=20.7, p<0.01	F(3,23)=11.6, p<0.001	F(3,23)=25.4, p<0.001
N-VRF v. AL			
SCN	F(1,24)=6.2, p<0.05	F(3,24)=67.4, p<0.001	F(3,24)=1.3, ns
BNSTov	F(1,24)=11.9, p<0.01	F(3,24)=6.9, p<0.01	F(3,24)=12.1, p<0.001
CEA	F(1,24)=60.0, p<0.01	F(3,24)=20.5, p<0.001	F(3,24)=41.1, p<0.001
BLA	F(1,24)=10.3, p<0.01	F(3,24)=32.2, p<0.001	F(3,24)=0.9, ns
DG	F(1,24)=10.1, p<0.01	F(3,24)=118, p<0.001	F(3,24)=14.3, p<0.001
Dorsal Striatum	F(1,24)=10.8, p<0.01	F(3,24)=72.8, p<0.001	F(3,24)=3.7, p<0.05
ANY-VRF v. AL			
SCN	F(1,24)=1.6, ns	F(3,24)=130, p<0.001	F(3,24)=1.9, ns
BNSTov	F(1,24)=1.6, ns	F(3,24)=14.3, p<0.001	F(3,24)=10.6, p<0.001
CEA	F(1,24)=7.3, ns	F(3,24)=10.0, p<0.001	F(3,24)=19.0, p<0.001
BLA	F(1,24)=8.0, p<0.01	F(3,24)=25.1, p<0.001	F(3,24)=0.2, ns
DG	F(1,24)=66.5, p<0.01	F(3,24)=71.0, p<0.001	F(3,24)=16.0, p<0.001
Dorsal Striatum	F(1,24)=11.0, p<0.01	F(3,24)=23.9, p<0.001	F(3,24)=7.3, p<0.001

Table 4.2
Feeding conditions and the time of day (ZT) both modulate PER2 expression, compared to AL controls

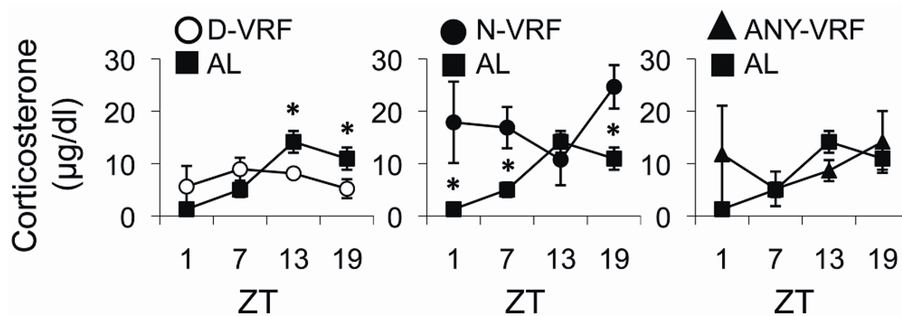

Figure 4.2
Daily patterns of corticosterone under daytime VRF (D-VRF; left), nighttime VRF (N-VRF; middle), and anytime VRF (ANY-VRF; right), compared to AL controls. Graphs illustrate the mean (± S.E.M.) corticosterone concentration in blood plasma (µg/dl) according to the zeitgeber time (ZT) when the blood plasma samples were collected. Asterisks (*) represent a statistically significant difference ($p<0.05$) between groups at a single time point.

Feeding Condition	$ANOVA_{ZT}$	$ANOVA_{Feeding}$	$ANOVA_{FeedingXZT}$
AL	$F(3,11)=12.7$, $p<0.001$		
D-VRF	$F(3,11)=0.7$, ns		
N-VRF	$F(3,12)=1.1$, ns		
ANY-VRF	$F(3,9)=0.5$, ns		
D-VRF v. AL	$F(3,22)= 5.0$, $p<0.01$	$F(1,22)= 0.4$, ns	$F(3,22)= 4.5$, $p<0.05$
N-VRF v. AL	$F(3,23)= 1.6$, ns	$F(1,23)= 11.0$, $p<0.01$	$F(3,23)= 2.2$, ns
ANY-VRF v. AL	$F(3,20)= 1.9$, ns	$F(1,20)= 0.6$, ns	$F(3,20)= 1.6$, ns

Table 4.3
Daily patterns in the amount of corticosterone under each condition, as well as how the corticosterone levels under each VRF schedule compared to AL controls

Discussion

The present results show that VRF schedules, which provide an unpredictable meal each day, can disrupt daily rhythms of PER2 expression in the dorsal striatum and certain regions of the limbic forebrain. While the daily rhythm of PER2 expression in the SCN failed to respond to the VRF schedules, and remained in synchrony with the LD cycle, the effects of VRF on PER2 expression outside of the SCN varied according to the brain area and the phase of the LD cycle when meals were given. The daily rhythms of PER2 expression in the dorsal striatum and limbic forebrain were blunted under daytime VRF (Figure 4.1). In contrast, nighttime VRF blunted the daily patterns of PER2 expression in the BNSTov and CEA, but daily rhythms were relatively undisturbed in the BLA, DG, and dorsal striatum (Figure 4.1). Daily patterns of PER2 expression under anytime VRF were similarly blunted in the BNSTov and CEA, but also showed some disruption in the DG and dorsal striatum (Figure 4.1), perhaps due to the daytime meals provided under this schedule (on day 1, 4, 6, 9, and 10 of the 10-day anytime VRF schedule). In addition, the daily rhythm of corticosterone release typically becomes food-entrained under predictable restricted feeding schedules, which provide the daily meal at the same time each day (Ahlers et al., 1980). It is therefore remarkable that VRF, which does not produce food-entrainment *per se*, also exhibited a strong effect on corticosterone release (Figure 4.1).

Phase-dependent effects of feeding, on daily rhythms of clock gene expression, have also been observed under other feeding schedules and in other

brain areas. Predictable restricted feeding schedules that provide a daytime meal will shift the daily rhythm of PER2 expression in the BLA and DG, but such a shift is not observed if a predictable nighttime meal is provided (Verwey et al., 2007, 2008). Thus, clock gene expression in the BLA and DG appears to respond differentially to daytime and nighttime meals, regardless of whether they are given according to a predictable or unpredictable schedule. Moreover, VRF induces yet another pattern of responding in the dorsomedial hypothalamic nucleus (DMH), a nucleus where clock gene expression is potentially involved in food-entrainment (Fuller et al., 2008; Mistlberger et al., 2008). Instead of blunting the daily profile of clock gene expression, as was observed in the BNSTov and CEA, daytime and nighttime VRF were shown to induce a daily rhythm of Period1 (PER1) expression in the DMH (Verwey et al., 2009). These differential effects are probably intrinsic to the brain areas, rather than specific to PER1 or PER2 expression, because both clock genes were similarly unaffected in the SCN. Nucleus-specific effects of feeding, on daily rhythms of PER1/PER2 expression, reaffirm that circadian clock genes are regulated in a decidedly nucleus-specific manner.

When rats are freely fed, the daily rhythm of corticosterone is important in sustaining daily rhythms of PER2 expression in the BNSTov and CEA (Perrin et al., 2006; Segall et al., 2009). In contrast, under predictable RF, the daily rhythm of corticosterone becomes food-entrained and will exhibit a food-anticipatory release before the daily mealtime (Ahlers et al., 1980; Honma, Honma, & Hiroshige, 1983). But even after adrenalectomy, restricted feeding can still

entrain daily rhythms of PER2 expression in the BNSTov and CEA (Segall et al., 2008). Such results demonstrate that glucocorticoids are one (Le Minh et al., 2001; Yamamoto et al., 2005; Yoshida et al., 2006), but not the only, link between feeding and circadian rhythms. Instead, feeding-related and fasting-related hormones or other metabolic factors might also be involved in the modulation of PER2 expression, corticosterone release, and behavior. For instance, the orexigenic hormone ghrelin, which typically increases with hunger and decreases in response to feeding, can influence corticosterone release as well as behavioral anxiety (Asakawa et al., 2001).

Feeding-responsive hormones, such as ghrelin, exert an important influence on the daily rhythms of corticosterone and behavior. It is interesting to note, however, that while ghrelin can influence light-induced FOS expression in the SCN (Yi et al., 2008), this was the only brain area where PER2 rhythms failed to respond to VRF. Alternatively, factors that are directly involved in metabolism, such as peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and sirtuin1 (SIRT1), have been shown to influence clock gene expression (Asher et al., 2008; Canaple et al., 2006; Liu et al., 2007; Nakahata et al., 2008). Thus, the nucleus-specific effects we observe on PER2 expression, may rely on the metabolic factors that could be specific to each of these brain areas. In addition, the effects that we observe on the daily rhythm of corticosterone release, probably rely on hormonal changes that influence the brain circuitry underlying stress.

If the link between restricted feeding and daily rhythms in clock gene expression were simply due to metabolic factors, however, the effects of a meal should not depend on the time-of-day when it is provided. Our previous studies with predictable restricted feeding and our current study with unpredictable VRF, both provide evidence that this pattern of response is not always true. Restricted feeding entrains PER2 rhythms in the BNSTov and CEA, so they peak approximately 12h after the predictable meal, regardless of whether meals are provided mid-day or mid-night (Verwey & Amir, 2009; Verwey et al., 2007, 2008). Moreover, the daily pattern of PER2 expression in the BNSTov and CEA was similarly blunted by both daytime VRF and nighttime VRF in the present study. Because the effects of restricted feeding seem to be independent from the time of day when food is given, we would propose that metabolic factors, like PGC-1α and SIRT1 (Asher et al., 2008; Canaple et al., 2006; Liu et al., 2007; Nakahata et al., 2008), could play an important role in mediating the effects of restricted feeding on clock gene expression in the BSNTov and CEA. In contrast, changes in the daily rhythm of PER2 expression in the BLA, DG, and dorsal striatum, depend on the time of day when meals are provided. Daytime feeding, whether it is predictable or unpredictable, seems to have strong effects on the daily rhythm of PER2 expression in the BLA, DG, and dorsal striatum, while nighttime meals leave these rhythms largely intact. Thus, we would also propose that metabolic factors, like PGC-1α and SIRT1, might be less important in the BLA, DG, and dorsal striatum. Collectively, these findings would seem to emphasize the

importance of studying metabolic factors and circadian clock gene expression concurrently.

The present study supports the view that irregular meals not only affect behavioral and physiological rhythms, as previously shown (Escobar et al., 2007; Verwey et al., 2009), but also bring about additional disruptions of daily rhythms in clock gene expression in the brain as well as daily rhythms in corticosterone release. Circadian disruptions and unstable entrainment have diverse consequences that, in various experiments, disrupt neural architecture and cognitive flexibility, as well as lead to cardiac and renal diseases (Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011; Martino et al., 2008). A growing body of literature suggests a fundamental difference between daytime and nighttime food consumption (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Fonken et al., 2010). Light-exposure during the night disrupts the circadian system of mice, and not only leads to an increase in daytime food-consumption, but also an increase in body mass and a decrease in glucose tolerance (Fonken et al., 2010). If food is provided only during the 12h of light, nocturnal mice will gain more weight than if food is provided instead during the 12h of dark (Arble et al., 2009). Causal links between clock gene expression, hormone release, and behavior remain to be clearly demonstrated, but these findings continue to support an important link between metabolism and the circadian system at behavioral, hormonal, and molecular levels (Asher et al., 2008; Canaple et al., 2006; Liu et al., 2007; Nakahata et al., 2008; Turek et al., 2005).

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Chapter 5: Nucleus-specific effects of meal duration on daily profiles of Period1 and Period2 protein expression in rats housed under restricted feeding.

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Contribution of authors

Michael Verwey designed the experiments together with Dr. Amir and oversaw all aspects of this project, including restricted feeding, perfusions, brain slicing, immunohistochemistry, microscopy, imaging, cell counting, and statistical analyses. The publication was written by Michael Verwey and edited by Dr. Shimon Amir.

Dr. Shimon Amir is the principle investigator of the lab where this experiment took place and Michael Verwey's thesis supervisor. He participated in the design of the experiments, and editing of the manuscript.

Abstract

Restricted feeding (RF) schedules provide a cycle of fasting and feeding each day, and induce circadian rhythms in food-anticipatory activity. In addition, daily rhythms in the expression of circadian clock genes, such as rhythms in Period1 (PER1) or Period2 (PER2), are also shifted in many brain areas that are important for the regulation of motivation and emotion. In order to differentiate brain areas that respond to the time of food presentation from areas that are sensitive to the degree of restriction, the present study compared RF schedules that provided rats with either a 2h-meal (2hRF) or a 6h-meal (6hRF) each day. As expected, 2hRF was associated with less food-consumption, more weight-loss, and more food-anticipatory running-wheel activity than 6hRF. In association with these metabolic and behavioral differences, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), which has been proposed to be integral to the generation and/or maintenance of food-anticipatory activities, peaked earlier in the 2hRF group and later in the 6hRF group. Because both RF groups exhibited approximately synchronous food-anticipatory activity, but phase shifted rhythms of PER1 and PER2 expression in the DMH, it suggests that the phase of food-anticipatory activity is not directly regulated by this brain area. Next, daily rhythms of PER2 expression in the limbic forebrain responded to each RF schedule in a nucleus-specific manner. In some brain areas, the amplitude of the PER2 rhythm was differentially adjusted in response to 2hRF and 6hRF, while other areas, responded similarly to both RF schedules. These findings demonstrate that daily

rhythms of clock gene expression can be modulated by the motivational state of the animal, as influenced by meal duration, weight loss and food-consumption.

Introduction

Restricted feeding (RF) schedules that provide a single meal at the same time each day induce robust circadian rhythms in behavior and physiology (Mistlberger, 2011; Richter, 1922). In rodents, these rhythms in food-anticipatory activity are associated with alterations in the daily expression rhythms of circadian clock genes and proteins, such as Period1 (PER1) and Period2 (PER2), in many peripheral tissues and brain nuclei (Angeles-Castellanos et al., 2007; Hara et al., 2001; Kudo et al., 2004; Mieda et al., 2006; Minami, Horikawa, Akiyama, & Shibata, 2002; Waddington Lamont et al., 2007; Wakamatsu et al., 2001). In recent years there has been considerable interest in the role that these tissue- and nucleus-specific rhythms might play in the generation of food-anticipatory activities as well as how they might interact with local metabolic processes (Asher et al., 2008; Belden & Dunlap, 2008; Challet et al., 2009; Escobar, Cailotto, Angeles-Castellanos, Delgado, & Buijs, 2009; Feillet et al., 2006; Fuller et al., 2008; Karatsoreos et al., 2011; Nakahata et al., 2008; Pendergast et al., 2009; Shirai, Oishi, Kudo, Shibata, & Ishida, 2007; Sonoda, Mehl, Chong, Nofsinger, & Evans, 2007; Storch & Weitz, 2009). However, the factors that influence the effect of RF on clock gene expression in the periphery and brain have not been fully explored. For example, we have shown that the effect of RF on the daily rhythm of PER2 expression in the rat forebrain varies as a function of whether food is presented during the light phase or dark phase of the LD cycle (Verwey et al., 2007, 2008) and whether it is given at the same or different time each day (Verwey & Amir, 2011a; Verwey et al., 2009). The

duration of the daily meal influences food-consumption, weight-loss, hunger, arousal, and food-anticipatory activity (Honma et al., 1983; Stephan & Becker, 1989), but whether or not it is important in the modulation of the daily pattern of clock gene expression has not been determined.

Daily PER2 rhythms, which are responsive to RF, have been reported in regions of the limbic forebrain that are important in the regulation of motivational and emotional state (Amir et al., 2004; Amir & Stewart, 2009; Lamont, Diaz, et al., 2005; Lamont, Robinson, et al., 2005; Waddington Lamont et al., 2007). These regions include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus (DG). Furthermore, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), an area that has been linked to the regulation of feeding and arousal, and to food-anticipatory activity in some studies, is also affected by RF (Fuller et al., 2008; Mieda et al., 2006; Verwey et al., 2007, 2008). Therefore, to study the impact of meal duration on clock gene expression in these brain regions, we provided rats with either a 2h-meal (2hRF) or a 6h-meal (6hRF) at the same time each day. The results show that meal duration plays a key role in the regulation of food-anticipatory activity and circadian rhythms of clock gene expression in the forebrain, and underscore the importance of motivational factors in the entrainment of behavioral and molecular circadian rhythms by RF in rats. Preliminary results have been presented in abstract form (Verwey & Amir, 2010).

Method

Animals, housing and restricted feeding

All experimental procedures were approved by the Animal Care Committee at Concordia University (Montréal, Québec, Canada) and followed the guidelines set out by the Canadian Council on Animal Care. Male Wistar rats (72 rats; 250-275g at the start of each experiment) were individually housed in cages equipped with running wheels, and kept in light-proof and sound-attenuated chambers. Running wheel activity was continuously recorded by computer (Vitalview, Minimitter, OR, USA) and inspected with circadia software. All rats were kept in a regular 12h:12h light-dark cycle (~300 lux at cage level when lights were on), and had free access to food (Rodent diet #5075, Charles River Laboratories, St. Constant, Québec, Canada) and water. After a two-week acclimation period, rats in the restricted feeding groups received either a single 2h meal/day (2hRF) or a 6h meal/day (6hRF) for 10 days. In both groups the meals began at zeitgeber time 4 (ZT4), 4h after the environmental lights turned on (ZT0 denotes time of light on). Accordingly, the 2hRF group had access to food from ZT4-6, while the 6hRF group had access to the food from ZT4-10. The ad libitum (AL) fed group had free access to food throughout the experiment.

Immunohistochemistry

At the end of the restricted feeding schedules, rats were injected with an overdose of euthanyl (~150mg/kg, CDMV, St. Hyacinthe, Québec, Canada) and perfused transcardially around the clock (ZT 1, 5, 9, 13, 17, 21; n=4/timepoint)

with 300mL of cold saline (4°C; 0.9% NaCl in distilled water) followed by 300mL of cold paraformaldehyde solution (4°C; 4% paraformaldehyde in 0.1M phosphate buffer). Brains were removed and post-fixed for ~24h in paraformaldehyde solution. Coronal brain sections (50 µm) were sliced on a vibratome, and brain sections containing the regions of interest were collected and stored at -20°C, in Watson's cryoprotectant, until staining (Watson et al., 1986). Sections containing the suprachiasmatic nucleus (SCN), the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), dentate gyrus (DG) and the dorsomedial hypothalamic nucleus (DMH) were immunostained for PER2 protein. Whereas, a second set of brain sections that contained only the DMH was stained for the PER1 protein.

Immunohistochemistry was performed as previously described (Amir et al., 2004; Verwey et al., 2009). Briefly, polyclonal antibodies for either PER1 (1:24000; made in rabbit; generous gift from Dr S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) or PER2 (1:800; made in goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Both primary antibody solutions were made with trizma-buffered saline (TBS; 50mM trizma buffer with 0.9% NaCl) and contained triton (0.3%). The PER1 antibody solution was also milk-buffered and contained 2% normal goat serum (Vector Laboratories, Burlington, Ontario, Canada), whereas the PER2-containing solution was not milk-buffered and contained 2% normal horse serum (Vector Laboratories, Burlington, Ontario, Canada). After rinsing the free-floating tissue in

fresh TBS, brain sections were placed into a secondary incubation solution containing either biotinylated anti-rabbit IgG made in goat (in the case of PER1-staining; 1h, 4°C; 1:200; Vector Laboratories) or biotinylated anti-goat IgG made in horse (in the case of PER2-staining; 1h, 4°C; 1:400; Vector Laboratories). Brain sections were rinsed in fresh TBS, and then incubated in an avidin-biotin solution for 2h (4°C; Vectastain Elite ABC Kit; Vector Laboratories). Next, to visualize the immunoreactive cells, sections were rinsed in a 0.5% 3,3-diaminobenzidine solution (10min), followed by a solution containing 0.5% 3,3-diaminobenzidine, 0.01% H_2O_2 and 8% $NiCl_2$ (10min). All sections were then mounted on gelatin-coated slides, underwent serial alcohol dehydration and cleared with citrisolv. Glass coverslips were then glued in place with permount (Fisher Scientific, Ottawa, Ontario, Canada).

Microscopy and data analysis

Slides were examined under a light microscope (Leitz Laborlux S) using a 20x objective. 400x400μm (SCN, BNSTov, CEA, BLA, DMH) or 400x200μm (DG) images were captured and analyzed using a Sony XC-77 camera (Sony, Tokyo, Japan) a Scion LG-3 Frame Grabber (Scion Corporation, Frederick, MD, USA) and Image SXM software (v.1.6, SD Barrett, http://www.imagesxm.org.uk). The mean number of stained nuclei in each brain region was determined by computing the average count from the 6 sections containing the greatest number of stained nuclei out of all the images taken of a given structure. Differences in clock gene expression between groups were determined with an analysis of

variance (ANOVA) where the alpha level was set at 0.05. Running-wheel activity, food consumption and body weight were analyzed with two-way repeated measures ANOVAs, and followed with post-hoc Tukey's tests.

Results

Food consumption, body weight and running wheel activity

When all rats were freely fed (day 1-5), there was no significant difference in food consumption between the groups. As expected, during RF (days 6-14), the 2hRF and 6hRF groups ate less food than the AL group ($p<0.01$; Figure 5.1, top graph). Moreover, during this period of RF, the 2hRF group ate less food than the 6hRF group ($p<0.01$). For example, on the 9th day of the RF schedule (day 14), the 2hRF group ate only 49% of their baseline food consumption, while the 6hRF group ate 71% on the same day. As a consequence of these differences in food-consumption, at the end of the 10-day schedules, the 2hRF group had lost 21% of their initial body weight while the 6hRF group had only lost 10% (Figure 5.1, Bottom graph). In contrast, the AL group did not change their daily food consumption and, from day 6 to day 15, gained an additional 10% in body weight (Figure 5.1).

Representative actograms, which illustrate the daily patterns of running-wheel activity before and after the initiation of the RF schedules for an individual in each group, can be seen in Figure 5.2. Based on all of the running-wheel activity records, both RF groups developed food-anticipatory activity, at a time-of-day when AL controls were normally inactive (Figure 5.3). The total daily activity was increased in both RF groups, as compared to the AL group ($p<0.01$; Figure 5.4, top graph), and the 2hRF group ran more than the 6hRF group ($p<0.01$). The 2hRF group also exhibited more food-anticipatory running-wheel activity,

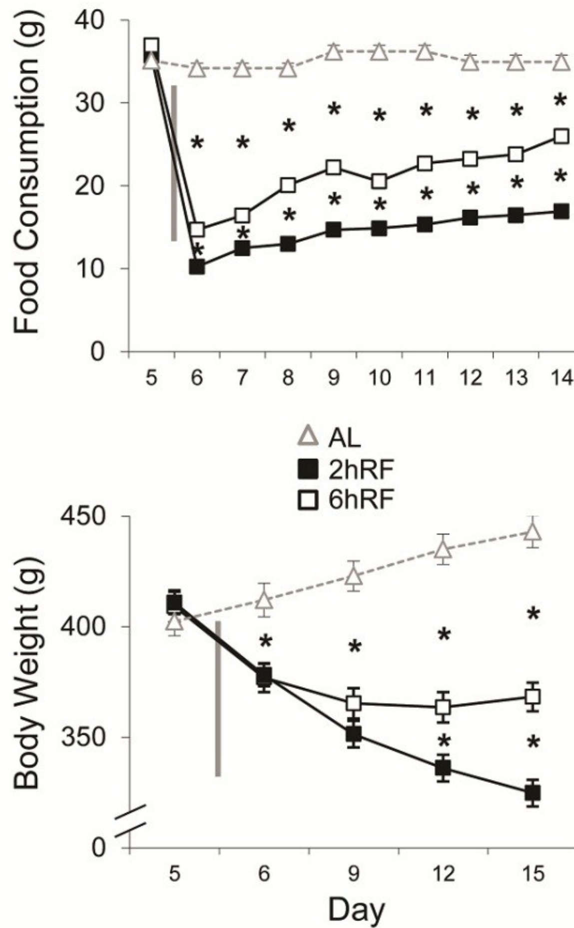

Figure 5.1
Food consumption (top graph) and body weight (bottom graph) for each group over the course of the experiment (daily mean ± S.E.M.; n=24/group). RF schedules lasted from day 6-15 and the vertical gray lines mark the day food was restricted in the 2hRF and 6hRF groups. Day 15 has been omitted from the top graph because some rats were perfused before the end of the 10th meal.

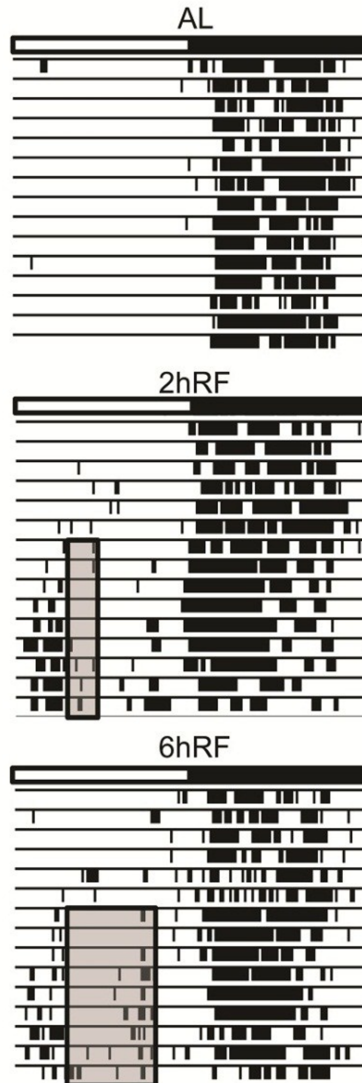

Figure 5.2
Single-plotted actograms illustrating the daily pattern of running-wheel activity for a representative rat from each group. Each horizontal line graphs 24h of running-wheel activity (data is binned every 10min), while the white and black rectangles along the top of each record illustrate the 12h:12h light-dark cycle. The rat in the AL group had free access to food throughout the entire record. In contrast, the rats in the 2hRF and 6hRF groups also had free access to food for the first 6 days of each record, and then the RF mealtimes are illustrated by the shaded rectangles within each plot for the last 9 days of each record.

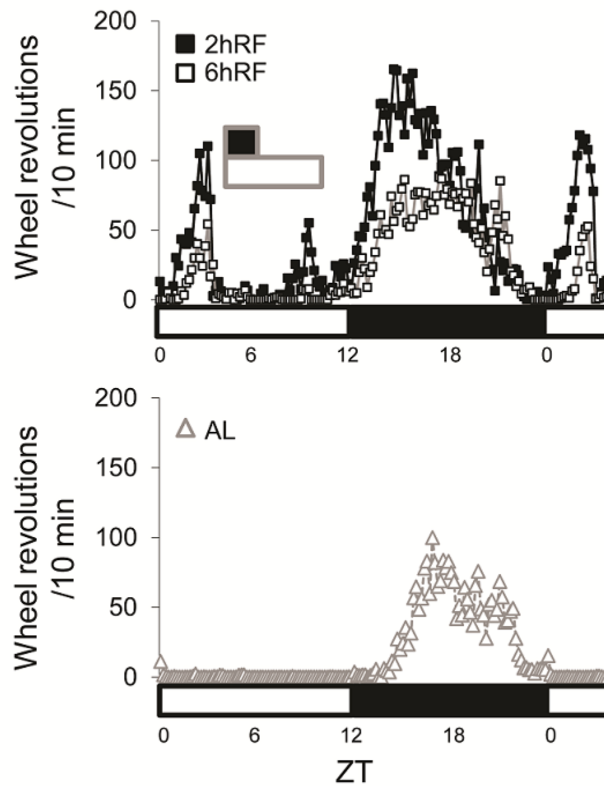

Figure 5.3
Mean running-wheel activity for each group (n=24/group; 10min bins), from ZT0 on day 14 until ZT4 on day 15. The light-dark cycle is illustrated along the bottom of each graph, along with ZT. The 9th daily meal is illustrated by horizontal rectangles within the top graph (filled = 2hRF, open = 6hRF) and the graph ends immediately before the 10th and last meal. In contrast, the AL group in the bottom graph had free access to food at all times.

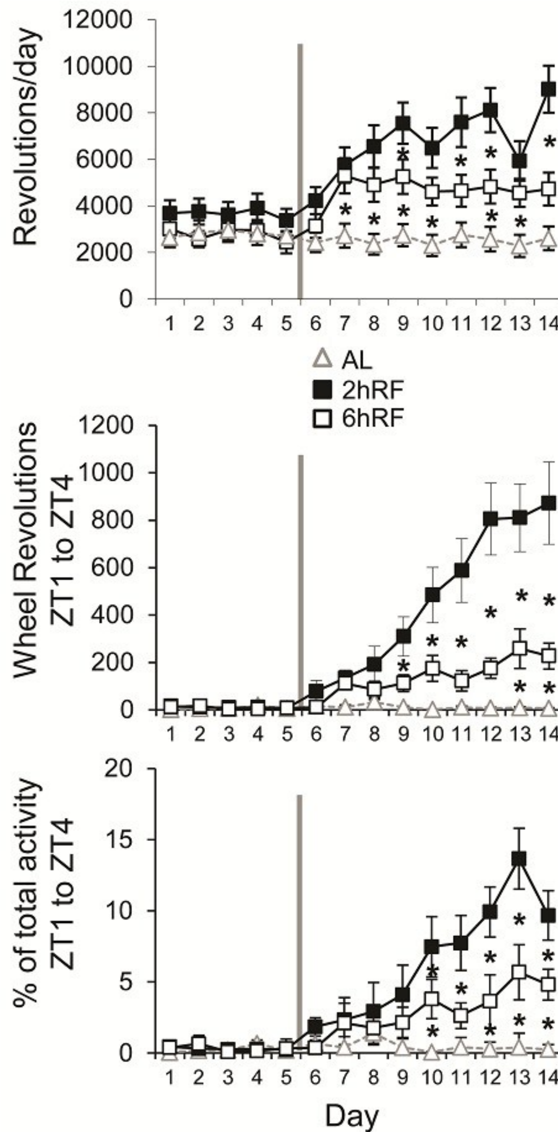

Figure 5.4
Mean (± S.E.M.) running-wheel revolutions for each group (n=24/group) for each day of the experiment (top graph) and during the food-anticipatory period (ZT1-4, middle graph). The proportion of the total activity that occurred during the food-anticipatory period is also shown (bottom graph). All groups had unrestricted food-access from day 1-5, but food was restricted in the 2hRF and 6hRF group from day 6-14. The onset of restricted food-access is marked by the vertical grey lines in each graph. Day 15 has been omitted from all graphs, because it was the last day of the experiment and incomplete data was available.

from ZT1-4, than the 6hRF group ($p<0.01$; Figure 5.4, middle graph). Accounting for the differences in total activity (Figure 5.4, bottom graph), the 2hRF group still exhibited a higher proportion of activity during the food-anticipatory period than the 6hRF group (e.g. Day 14, 2hRF:12.9±0.1%, 6hRF:8.4±0.1%).

PER2 expression in the SCN, limbic forebrain and DMH

Robust daily oscillations of PER2 expression were observed in the SCN and limbic forebrain under AL conditions (Figure 5.5). In the AL group, PER2 expression in the SCN, BNSTov and CEA was highest around ZT13 and lowest around ZT1. Whereas, in the BLA and DG, anti-phase oscillations were observed that peaked around ZT1 and troughed around ZT13, which is consistent with previous reports (Amir et al., 2004; Lamont, Robinson, et al., 2005). The DMH also exhibited a low-amplitude rhythm under AL conditions, with a peak at ZT21 ($p<0.05$). RF had no effect on the rhythm of PER2 expression in the SCN, but daily rhythms were adjusted by RF in all other regions (Table 5.1; Figure 5.5).

In general, all areas continued to exhibit a daily rhythm of PER2-IR under most feeding conditions ($p<0.05$). The only exceptions were the BNSTov under 6hRF and the BLA under 2hRF, which failed to show a daily oscillation in PER2-IR ($p>0.05$). Specifically, we found that the rhythm of PER2 expression in the BNSTov (Figure 5.5) was modulated differentially by the two RF schedules (Table 5.1). In particular, PER2 expression in the BNSTov was elevated at ZT17

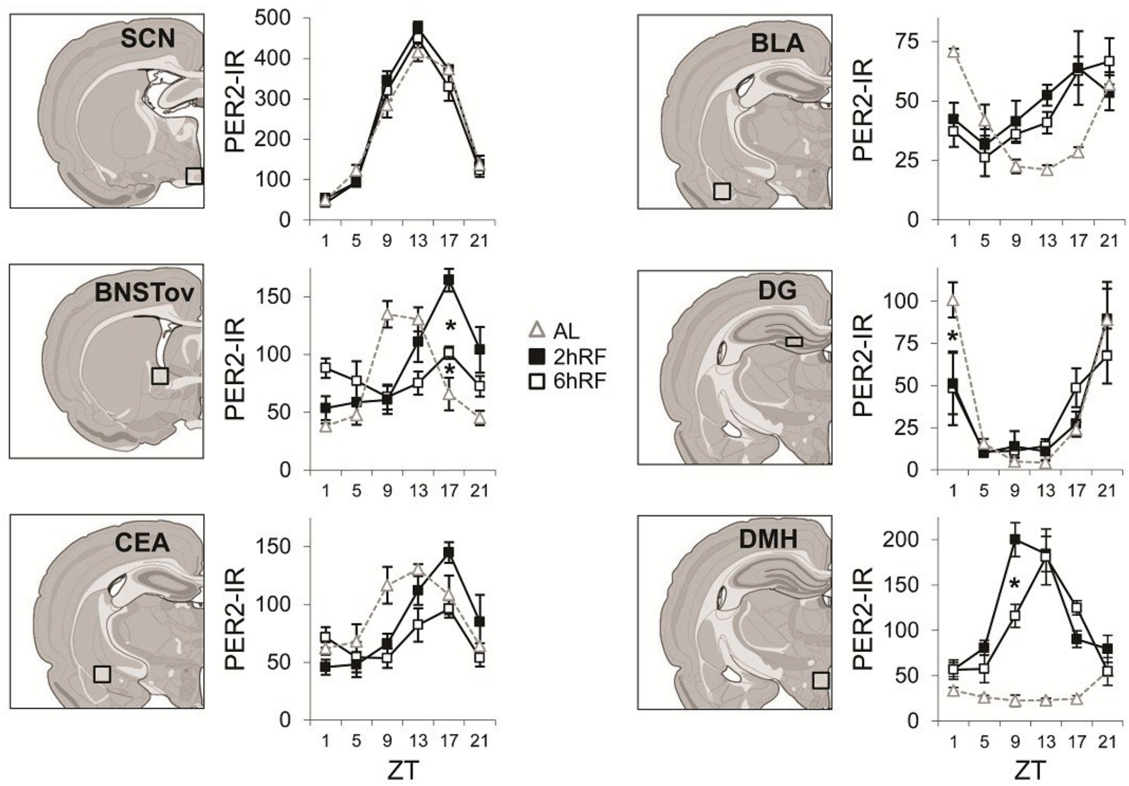

Figure 5.5
Schematics of the location of the SCN, BNSTov, CEA, BLA and DG (left column) and the mean PER2-immunoreactivity (PER2-IR ± S.E.M; n=4/group/ZT) in each structure across the 24h light-dark cycle.

Brain Region	Two-way ANOVA	Statistical Significance
SCN	**$ANOVA_{TIME}$**	$F(5,54)=232$, $p<0.01$
	$ANOVA_{GROUP}$	$F(2,54)=1.9$, ns
	$ANOVA_{TIMExGROUP}$	$F(10,54)=1.3$, ns
BNSTov	**$ANOVA_{TIME}$**	$F(5,54)=9.7$, $p<0.01$
	$ANOVA_{GROUP}$	$F(2,54)=2.6$, ns
	$ANOVA_{TIMExGROUP}$	$F(10,54)=8.7$, $p<0.01$
CEA	**$ANOVA_{TIME}$**	$F(5,54)=14.1$, $p<0.01$
	$ANOVA_{GROUP}$	$F(2,54)=5.9$, $p<0.01$
	$ANOVA_{TIMExGROUP}$	$F(10,54)=3.0$, $p<0.01$
BLA	**$ANOVA_{TIME}$**	$F(5,54)=22.7$, $p<0.01$
	$ANOVA_{GROUP}$	$F(2,54)=0.6$, ns
	$ANOVA_{TIMExGROUP}$	$F(10,54)=1.8$, ns
DG	**$ANOVA_{TIME}$**	$F(5,54)=7.6$, $p<0.01$
	$ANOVA_{GROUP}$	$F(2,54)=1.7$, ns
	$ANOVA_{TIMExGROUP}$	$F(10,54)=4.8$, $p<0.01$
DMH	**$ANOVA_{TIME}$**	$F(5,54)=19.3$, $p<0.001$
	$ANOVA_{GROUP}$	$F(2,54)=71.7$, $p<0.001$
	$ANOVA_{TIMExGROUP}$	$F(10,54)=9.7$, $p<0.001$

Table 5.1
ANOVA of PER2 expression in the SCN, limbic forebrain and DMH

in both RF groups compared to the AL group ($p<0.05$), but was lower in the 6hRF group compared to the 2hRF group ($p<0.05$). Moreover, while a robust daily rhythm in PER2 expression was observed in the BNSTov of 2hRF rats, the daily pattern was arrhythmic in the 6hRF group. In the CEA, peak PER2 expression was also shifted to ZT17 under 2hRF and 6hRF (Figure 5.5, but both daily profiles remained rhythmic. At ZT17, however, the 2hRF exhibited higher PER2 expression in the CEA than the 6hRF group ($p<0.05$), which is consistent with a higher-amplitude rhythm under 2hRF (Table 5.1). Finally, the RF-induced rhythm of PER2 expression in the DMH also depended on the length of the daily meal. Specifically, less PER2-IR was observed at ZT9 in the 6hRF than the 2hRF group, which could suggest a phase delay of the DMH rhythm.

In contrast to the differential effects of 2hRF and 6hRF that we observed in the BNSTov and CEA, both RF groups lead to a similar shift in the rhythm of PER2 expression in the BLA. The daily rhythm of PER2 expression in the BLA peaked around ZT17-21 in the 6hRF group instead of around ZT1 in AL controls. In the DG, the main effect of 2hRF and 6hRF on the daily rhythm of PER2 expression was a reduction in the number of cells expressing PER2 at ZT1 as compared to the AL group at the same time point ($p<0.01$). Thus, 2hRF and 6hRF had differential effects on the amplitude of daily oscillations of PER2 expression in the BNSTov, CEA, and DMH, but similar effects on the daily rhythms of PER2 expression in the BLA and DG (Table 5.1).

PER1 expression in the DMH

Even though the PER1 and PER2 genes are homologous, there are also many differences in their regulation and consequences. For these reasons, we also evaluated PER1 expression in the DMH in the same rats. Consistent with earlier studies, PER1 expression in the DMH exhibited a low-amplitude rhythm under AL conditions ($p<0.05$; Figure 5.6). In contrast, robust daily rhythms in PER1 expression were observed under both RF schedules ($p<0.01$; Figure 5.6). Importantly, RF increased the amplitude and shifted the daily rhythm of PER1 expression in the DMH, so that there was a statistically significant interaction between time and feeding group (Table 5.2). PER1-IR was significantly higher at ZT13 in both RF groups compared to controls ($p<0.01$). Moreover, at ZT9, the 2hRF group exhibited higher PER1-IR than the 6hRF group ($p<0.01$), which is consistent with a phase delay of this rhythm in the 6hRF group with respect to the 2hRF group.

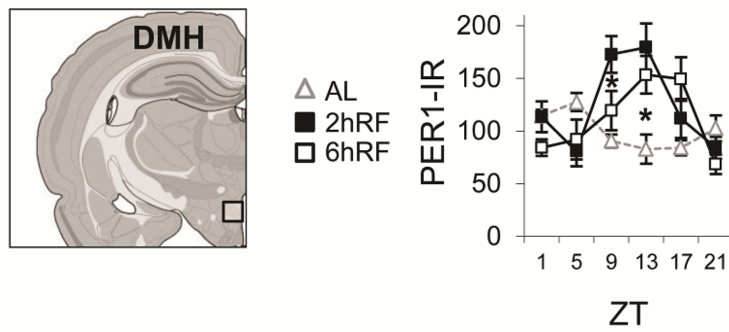

Figure 5.6
Schematic of the location of the DMH (left) and mean PER1-immunoreactivity (PER1-IR ± S.E.M; n=4/group/ZT) in this structure across the 24h light-dark cycle (right). Asterisks (*) denote a statistically significant difference between two groups ($p<0.05$).

Two-way ANOVA	Statistical Significance
$ANOVA_{TIME}$	$F(5,54)=5.5$, $p<0.01$
$ANOVA_{GROUP}$	$F(2,54)=6.0$, $p<0.01$
$ANOVA_{TIMExGROUP}$	$F(10,54)=5.3$, $p<0.01$

Table 5.2
Two-way ANOVA of PER1 expression in the DMH

Discussion

RF schedules modulate daily rhythms of clock gene expression in the brain and periphery (Angeles-Castellanos et al., 2007; Hara et al., 2001; Kudo et al., 2004; Mieda et al., 2006; Minami et al., 2002; Waddington Lamont et al., 2007; Wakamatsu et al., 2001). By contrasting 2hRF and 6hRF the present experiment has demonstrated that, depending on the brain area, these effects can be dependent or independent of the meal duration. Meal duration influences the amount of food rats are able to consume each day, and therefore also influences body weight (Honma et al., 1983; Stephan & Becker, 1989). These differences in body weight, arguably, influence the motivation for food, which could be reflected in the finding that the 2hRF group exhibited significantly more food-anticipatory running-wheel activity than the 6hRF group. However, these RF schedules also had general effects that were not specifically linked to the mealtime. For instance, the overall daily activity was highest in the 2hRF group, intermediate in the 6hRF group, and lowest in the AL group. In parallel with this finding, nocturnal onset of activity also appeared to be earliest in the 2hRF group, intermediate in the 6hRF group and relatively late in the AL group. Because the daily rhythm of PER2 expression was unchanged in the SCN, we suggest that this effect on the phase angle of entrainment is not related to the entrainment of the master clock, *per se*, and could instead be related to the non-specific changes in the amount of running-wheel activity. Finally and most importantly, where the daily rhythms of PER1 and PER2 expression in the DMH as well as PER2 expression in the BNSTov and CEA were each differentially responsive to

2hRF and 6hRF, PER2 rhythms in the BLA and DG responded similarly to both RF schedules. These findings reflect an ever-growing complexity of tissue-specific effects of feeding on daily rhythms of PER1 and PER2 protein expression in the rat forebrain and hypothalamus.

One factor that could mediate some of this tissue-specificity could be the adrenal stress hormone corticosterone. When rats are freely-fed, the daily rhythm of corticosterone release is fundamentally important to sustain daily rhythms of PER2 expression in the BNSTov and CEA but not in the BLA and DG (Amir et al., 2004; Lamont, Robinson, et al., 2005; Segall et al., 2009; Segall et al., 2006). In contrast, the importance of this stress hormone in modulating clock gene expression in the DMH is not well understood. In addition, RF schedules that provide a short daily meal produce a robust food-anticipatory release of corticosterone, whereas RF schedules that provide a long daily meal do not produce this food-anticipatory release (Honma et al., 1983). Thus, these differential patterns of corticosterone release, coupled with region-specific effects on the daily patterns of PER2 expression, could be important for the changes in PER2 rhythms that we observe in response to 2hRF and 6hRF. However, RF has also been shown to modulate the daily rhythms of clock gene expression in the brains of adrenalectomized rats (Segall et al., 2008), suggesting that this hormone is not the only factor influencing these rhythms.

Core metabolic factors could also be modulating daily rhythms of clock gene expression. Metabolic factors like peroxisome proliferative activated receptor gamma coactivator-1α (Ppargc1a) and sirtuin1 (Sirt1), have both been

shown to influence clock gene expression (Asher et al., 2008; Belden & Dunlap, 2008; Liu et al., 2007; Rodgers et al., 2008; Rodgers et al., 2005). In particular, Sirt1 expression has been reported in the BNST and DMH (Ramadori et al., 2008), and provides a metabolic influence on PER2 expression (Asher et al., 2008). However, RF-induced changes of Sirt1 expression have also been observed in the SCN (Satoh et al., 2010), a brain area where in the present study PER2 expression was unaffected by RF. We should note that Asher et al. (2008) demonstrated an influence of SIRT1 on PER2 expression in the liver and in cell culture. Thus, we propose that the importance of SIRT1 may vary in a nucleus-specific manner and that, in the SCN of freely moving rats, the environmental LD cycle could supersede the influence of SIRT1 on the daily rhythm of PER2 expression. Consistent with these nucleus-specific responses, while Sirt1 expression is increased in the hypothalamus by fasting, this modulation has not been observed in the forebrain (Ramadori et al., 2008). In contrast, PGC-1α is also expressed in several key brain areas that are involved in energy homeostasis (Sarruf et al., 2009), many of which interact and influence the brain nuclei in the present study. However, the nucleus-specific circadian rhythms in the expression of these factors, and the way this daily pattern might change under conditions of RF, remain topics for future study.

The DMH has been an area of great interest in the regulation of circadian rhythms in food-anticipatory activity (Fuller et al., 2008; Gooley et al., 2006; Landry et al., 2006; Mieda et al., 2006; Mistlberger et al., 2009a; Mistlberger et al., 2008). Instead of being necessary or sufficient for rhythms in food-

anticipatory activity, it was recently suggested that the DMH might be involved in modulating the amount of food-anticipatory activity (Mistlberger, 2011). In the present study, the rats in the 2hRF and 6hRF groups both exhibited different amounts of synchronous food-anticipatory activity. In contrast, the daily rhythms of PER1 and PER2 expression, under each RF schedule, were out of synchrony between the two groups. Because the DMH rhythm under 6hRF appeared to be phase delayed compared the rhythm under 2hRF, this is another demonstration that daily rhythms of PER1 and PER2 expression in this brain area are dissociable from the timing of food-anticipatory activity. Consistent with this dissociation, variable RF schedules that provide an unpredictable meal each day, fail to induce circadian rhythms in food-anticipatory activity, but robust daily rhythms of PER1 expression in the DMH are still observed (Verwey et al., 2009). Nor does this observed dissociation, between rhythms in clock gene expression and rhythms in food-anticipatory activity, appear to be limited only to PER1. We have previously reported that daily rhythms in anticipatory running-wheel activity, induced by highly palatable treat-access, do not induce daily rhythms of PER2 expression in the DMH (Verwey et al., 2007, 2008). Thus, even though some reports have suggested that daily rhythms in food-anticipatory activity rely on an intact canonical circadian feedback loop (Feillet et al., 2006; Fuller et al., 2008), other studies suggest that we must start to look beyond this prototypical molecular clock (Pendergast et al., 2009; Storch & Weitz, 2009).

The present study has demonstrated that the amount of food that is consumed is able to modulate, in a nucleus-specific manner, the daily rhythms of

clock gene expression in the limbic forebrain and hypothalamus. Because a daily treat will induce some food-anticipatory activities but fail to adjust daily rhythms in PER2 expression (Verwey et al., 2007, 2008), we have known for some time that a negative energy balance is important. However, this study demonstrates that the degree of food-restriction is also important in determining the daily rhythms of clock gene expression in the brain. This intimate link, between circadian rhythms and metabolism, has been emerging for several years (Asher et al., 2008; Damiola et al., 2000; Escobar et al., 2009; Karatsoreos et al., 2011; Lamia, Storch, & Weitz, 2008; Liu et al., 2007; Wakamatsu et al., 2001), but it is only more recently that we have started to appreciate the remarkable tissue-specificity of these effects.

Acknowledgements

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General Discussion[1]

RF schedules induce circadian rhythms in food-anticipatory activity and these rhythms are unique because they do not rely on the SCN (Stephan et al., 1979a). However, the putative food-entrained oscillator(s) that underlie these circadian rhythms have remained elusive (Balsam et al., 2009; Caba & Gonzalez-Mariscal, 2009; Challet et al., 2009; Davidson, 2009; Dietrich & Horvath, 2009; Escobar et al., 2009; Honma & Honma, 2009; Mistlberger, 2009; Webb, Baltazar, Lehman, & Coolen, 2009). Thus, the discovery of food-entrained oscillators can indicate brain regions that respond to RF, are involved in the generation and timing of food-anticipation, or both (Antle & Silver, 2009). Moreover, this field of study also helps to elucidate the nature and regulation of those nucleus-specific rhythms (Verwey & Amir, 2009). RF-responsive rhythms in PER1 and PER2 expression, which could influence local neural activity and processing, have been observed in several nuclei of the limbic forebrain as well as in the DMH (Mieda et al., 2006; Waddington Lamont et al., 2007). In particular, the DMH has been implicated in the expression of circadian rhythms in food-anticipatory activity (Acosta-Galvan et al., 2011; Fuller et al., 2008; Gooley et al., 2006). Although daily rhythms of

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Verwey, M., & Amir, S. (2009). Food-entrainable circadian oscillators in the brain. European Journal of Neuroscience. 30, 1650-1657.

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PER1 expression in the DMH are affected by RF (Fuller et al., 2008; Mieda et al., 2006), whether or not these rhythms are food-entrained and their link with food-anticipatory activity has not been determined.

In order to examine the potential correlates of RF and food-anticipatory activity, the experiments in the present thesis focused on daily rhythms of PER1 and PER2 expression in the DMH as well as PER2 expression in the limbic forebrain. In particular, in Chapters 1 and 2, RF schedules provided a daily meal at a predictable time during the day (Chapter 1; Verwey et al., 2007) or during the night (Chapter 2; Verwey et al., 2008). In Chapters 3 and 4, RF schedules provided a daily meal at an unpredictable time during the day or during the night (Chapters 3 and 4; Verwey & Amir, 2011a; Verwey et al., 2009). Finally in Chapter 5, the daily meal arrived at the same time each day but the duration of the meal was either short or long (Chapter 5; Verwey & Amir, 2011b). These schedules were used to test the potential link between food-entrainment, anticipatory running-wheel activity and daily rhythms of PER1 and PER2 expression in the forebrain. Our findings suggest that daily rhythms of clock gene expression in the DMH are dissociable from daily rhythms in food-anticipatory activity. In part, to test whether this effect was specific to the DMH or a general property of areas that exhibit daily rhythms in clock gene expression, daily rhythms in the limbic forebrain and SCN were also studied. While the SCN remained light-entrained during all of these RF schedules, the daily rhythms of PER2 expression in the limbic forebrain exhibited a nucleus-specific response. These findings offer new insight into the regulation of daily rhythms in PER1 and

PER2 expression in these brain areas and demonstrate that cycles of fasting and feeding can affect circadian rhythms in brain areas that are more typically linked to the regulation of motivation, emotion, arousal, and learning.

The DMH

The present studies have contributed a number of key findings to the discussion and understanding of RF-induced rhythms in the DMH. In a previous study, Fuller et al. (2008) injected a viral vector that restored BMAL1 expression in the DMH of BMAL1 -/- mice. In this study, daily rhythms in food-anticipatory activity were deficient in BMAL1 -/- mice. However, when a viral vector was injected in the DMH, which allowed the expression of BMAL1, it rescued the deficits in the production and timing of food-anticipatory activity in the BMAL -/- mice. While this manipulation caused BMAL1 to be expressed in the DMH, and this facilitated Per1 expression at one time-point, the authors did not describe the local rhythm(s) of clock gene expression after this manipulation (Fuller et al., 2008). Mieda et al. (2006) and Fuller et al. (2008) have both shown that a novel daily rhythm of PER1 expression is induced by RF in wildtype mice, but this observation suggests that this region is food-sensitive, and does not constitute food-entrainment or a causal link with food-anticipatory activity. Our studies have focused on daily rhythms of PER1 and PER2 expression in the DMH of rats in response to novel RF schedules and found converging evidence that these DMH-rhythms are only partially food-entrained and are dissociable from circadian rhythms in food-anticipatory running-wheel activity.

Partial food-entrainment of the DMH. Theoretically, food-entrainment should synchronize the daily rhythm of gene expression with the time of the daily meal. One implication of food-entrainment is if a brain region becomes exclusively food-entrained, the daily rhythms in that brain area should exhibit an equal and constant shift with respect to the mealtime. Thus, contrasting the daily rhythms of PER2 expression that result from a RF schedule that delivers a daytime meal in Chapter 1 (Verwey et al., 2007), with the daily rhythms of PER2 expression that result from a RF schedule that delivers a nighttime meal in Chapter 2 (Verwey et al., 2008) should provide one test of food-entrainment (Verwey & Amir, 2009). Making this comparison, we find that a daytime meal produces a rhythm of PER2 expression in the DMH with peak expression 1-5h after food-arrival, while a nighttime meal produces a peak approximately 9h after food-arrival (Appendix A; Figure 6.3, bottom right). Because of these different phase angles of entrainment, between the time of food-arrival and the time of peak PER2 expression, the DMH-rhythm in PER2 expression does not appear to be exclusively food-entrained.

Results from Chapter 3, which used variable restricted feeding (VRF), also demonstrate that daily rhythms of PER1 expression in the DMH do not become food-entrained (Verwey et al., 2009). VRF schedules provided an unpredictable meal at a new time each day, which did not entrain food-anticipatory running-wheel activity (Escobar et al., 2007). Despite this lack of behavioral food-entrainment, robust daily rhythms of PER1 expression in the DMH were observed after VRF schedules that varied the meals within the 12h of light

(daytime VRF) or within the 12h of dark (nighttime VRF; (Verwey et al., 2009). However, if meals were provided randomly around the 24h LD-cycle (anytime VRF), an arrhythmic daily pattern of PER1 expression was observed. Thus, daytime-VRF and nighttime-VRF demonstrate that while the daily rhythm of PER1 expression does relate to the time-of-day when food is available, food-entrainment is not required for the induction of daily rhythms in this brain nucleus. Finally, in Chapter 5, meal duration was also shown to influence the daily rhythms of PER1 and PER2 expression in the DMH, even though both meals began at the same time-of-day (Verwey & Amir, 2011b). Specifically, the daily rhythms of PER1 and PER2 expression in the DMH of rats that received a 2h meal each day peaked earlier than a group that received a 6h meal each day. Thus, PER1 and PER2 expression in the DMH also appears to be sensitive to either the time the daily meal ends, or the severity of the RF schedule.

Even if it is not exclusively food-entrained, the daily rhythm of PER1 and PER2 expression in the DMH does appear to be related to the time-of-day when food is made available. In most cases, the peak in PER1/PER2 expression occurred within several hours of the final meal. One exception was seen in the anytime-VRF group, which received a random meal anytime during the day or night, and exhibited an arrhythmic daily profile of PER1 expression. This finding is important because the final meal in the anytime-VRF group was given at the same time as the final meal in the daytime-VRF group, but these groups exhibited decidedly different profiles of PER1 expression. Re-feeding after an acute fast is sufficient to induce *Per1* and *Per2* gene expression in the DMH

(Mieda et al., 2006; Moriya et al., 2009), but it appears to be the entire 10-day schedule that determines the daily pattern of gene expression in these experiments. Thus, the daily profiles that we observe after several days of RF do not qualify as food-entrainment, but seem to be the result of a gradual adaptive process that relates to the time-of-day when food is provided.

Food-anticipatory activity and the DMH. Rhythms of PER1 or PER2 expression in the DMH were not linked to daily rhythms of food-anticipatory running-wheel activity. In Chapter 1, several rats in the restricted treat (RT) condition exhibited treat-anticipatory running-wheel activity, but PER2 expression in the DMH was similar to the non-anticipating RT rats and the AL controls (Verwey et al., 2007). In Chapter 2, it was impossible to identify food-anticipatory activity in the night-fed rats because it was obscured by the normally-occurring nocturnal running-wheel activity. However, the nightly pattern of running-wheel activity was similar between the RT and RF groups, while the daily profiles of PER2 expression were decidedly different (Verwey et al., 2008). Thus, in Chapters 1 and 2, we demonstrated that daily rhythms in treat-anticipatory activity were not associated with daily rhythms of PER2 expression in the DMH. However, it is still not clear if treat-anticipation is homologous with the food-anticipation observed under RF.

In Chapter 3, variable RF (VRF) schedules provided a 2h meal to fasted rats, at a pseudo-random time each day (Verwey et al., 2009). These VRF schedules make accurate food-entrained anticipatory activity impossible. Nevertheless, daytime-VRF and nighttime-VRF schedules, which varied the

meals within the 12h of light and the 12h of dark respectively, both induced robust daily rhythms of PER1 expression in the DMH. This is another demonstration of daily rhythms in the DMH in the absence of daily rhythms in anticipatory activity. Finally, in Chapter 5, we compared 2hRF and 6hRF schedules, which both provided the daily meal at ZT4 (Verwey & Amir, 2011b). Although the total amount of food-anticipatory running-wheel activity in the 2hRF group was greater than in the 6hRF group, the food-anticipatory activity was synchronous. In contrast, the rhythms of PER1 and PER2 expression in the DMH were delayed in the 6hRF group as compared to the 2hRF group. Thus, these findings collectively support the hypothesis that daily rhythms of PER1 or PER2 expression in the DMH are not causally linked to circadian rhythms in food-anticipatory activity.

DMH – Future directions. The DMH responds to RF (Acosta-Galvan et al., 2011; Angeles-Castellanos et al., 2004; Caba et al., 2008; Gooley et al., 2006; Mieda et al., 2006; Moriya et al., 2009; Verwey & Amir, 2010, 2011b; Verwey et al., 2007, 2008; Verwey et al., 2009), and RF-induced changes in PER1 and PER2 expression in the DMH remain an important and interesting phenomenon. Although these studies have not linked DMH-rhythms with daily rhythms in food-anticipatory running-wheel activity, there could still be some functional consequence to these changes. In particular, because the DMH is connected to brain areas that regulate sleep and arousal (Chou et al., 2003), there could be a link between DMH-rhythms in PER1 or PER2 expression and the time-of-day when rats sleep, the sleep stage they experience, or the waking

times. In humans, the time-of-day when rapid eye movement (REM) sleep occurs is strongly influenced by the circadian clock (Dijk, Duffy, Riel, Shanahan, & Czeisler, 1999). In contrast, non-REM (NREM) sleep appears to be driven by homeostatic mechanisms, which promote NREM sleep based on the amount of time an individual has been awake. Thus, REM sleep in particular could be useful to monitor in future experiments that focus on these DMH-based rhythms.

One emerging hypothesis has been that the DMH and SCN interact (Acosta-Galvan et al., 2011), both through neuronal connections and through their respective influences on behavior. To determine the role of SCN-rhythms in the induction and modulation of DMH-rhythms, SCN-lesions (Acosta-Galvan et al., 2011) or constant light housing that disrupts daily rhythms in the SCN of rats (Ohta et al., 2005), could be used to disrupt the SCN-influence on the DMH. The daily rhythms of PER1 and PER2 expression in the DMH were not exclusively food-entrained in our studies, but this could have been because of the influence of an intact SCN, which remained in synchrony with the LD-cycle. SCN-lesions would have an effect on the DMH, not only through the disruption of SCN-DMH connections, but also through the behavioral and physiological arrhythmicity that results from ablating the "master" circadian clock. Constant light would also result in a similar behavioral arrhythmicity with the structure of the SCN remaining intact, but SCN-rhythms would likely be restored and food-entrained under RF (Lamont, Diaz, et al., 2005). Without light-entrained signals from the SCN to the DMH, the DMH may then be free to become food-entrained.

Another topic for future study could be the hormonal influences on clock gene expression in the DMH. Adrenal hormones, such as corticosterone, have been extensively studied in the context of RF schedules and food-anticipatory activity. In addition to the normal nocturnal release of corticosterone, this glucocorticoid is also released during the food-anticipatory period under RF (Ahlers et al., 1980; Honma et al., 1983). These daily rhythms in corticosterone also influence daily rhythms of clock gene expression in several brain areas and in the periphery (Le Minh et al., 2001; Stokkan et al., 2001; Yamamoto et al., 2005; Yoshida et al., 2006). Segall et al. (2006) demonstrated that it was the daily rhythms in corticosterone that sustained daily rhythms of PER2 expression in the BNSTov and CEA. However, the importance of corticosterone in the regulation of clock gene expression in the DMH is presently unknown. Food-anticipatory corticosterone release is observed in association with short daily meals, but not long daily meals (Honma et al., 1983). This implied difference in corticosterone release could have been one of the factors driving the differences we observed in PER1 and PER2 expression in the DMH in Chapter 5 (Verwey & Amir, 2011b). Thus, surgical removal of the adrenal glands could help to elucidate the importance of this hormone in RF-induced changes in the rhythms of PER1 and PER2 expression (Segall et al., 2008).

The importance of DMH-rhythms could also be addressed through suppression of PER2 expression, specifically within this brain area. Gavrila et al. (2008) have previously accomplished this type of suppression in the SCN of wildtype rats, through the injection of double-stranded PER2-interfering RNA.

Stereotaxic surgeries that microinjected this interfering RNA into the SCN, produced a transient suppression of PER2 expression, which lasted 3-6 days. This suppression also disrupts SCN-driven circadian rhythms in locomotor activity (Gavrila et al., 2008). However, it takes several days to induce and entrain food-anticipatory running-wheel activity, and the surgery alone will often produce weight loss and changes in the amount of running-wheel activity. Therefore, a modified approach would be required in order to target food-entrained rhythms. Specifically, if the DMH were cannulated, rats could be food-entrained for 1-2 weeks and then PER2-interfering RNA or a control sequence of RNA could be injected into the DMH without anesthesia or additional surgery.

The SCN

Certain studies have demonstrated that RF can have an effect on light-entrained circadian rhythms (Challet et al., 1996; Challet, Solberg, & Turek, 1998; Mendoza, Angeles-Castellanos, et al., 2005c; Ohta et al., 2008). However, the SCN remained light-entrained in our experiments and this finding is consistent with many other studies (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Waddington Lamont et al., 2007; Wakamatsu et al., 2001), but it remains unclear which aspect of these experiments might account for these fundamentally different results. For instance, many of the experiments that find the SCN remains light-entrained used rats, while many that find an effect of RF on SCN-rhythms have used mice (Challet et al., 2003; Challet et al., 1998). Effects of RF on the SCN are rarely observed in a 12h:12h LD cycle, and are

observed more often under constant light or constant darkness (Lamont, Diaz, et al., 2005). Many of the studies that have found an effect of RF on the SCN, have studied PER1 expression (Mendoza, Graff, Dardente, Pevet, & Challet, 2005; Mendoza et al., 2007), and most of the present studies have focused on PER2 expression. There has also been some speculation that the SCN could be particularly sensitive to caloric restriction (Mendoza, 2007). Instead of providing an AL amount of food for a short meal each day, calorie restrictions deliver 50-70% of the daily food intake and the rats can consume at their leisure. However, depending on the meal duration, rats will consume a similar proportion of their AL food-consumption under RF. Thus, it is impossible to attribute these different findings to any one variable in particular. However, the light-entrainment of the SCN has served as a powerful internal control for our experiments. With so many different groups of animals killed at different times-of-day, having a brain area that does not respond to RF has helped to emphasize and validate the differences that are observed in other brain regions.

The limbic forebrain

BNSTov and CEA. Daily rhythms of PER2 expression in the BNSTov and CEA become food-entrained under RF (Verwey & Amir, 2009). Specifically, daytime RF and nighttime RF schedules, in Chapters 1 and 2, both produced peak PER2 expression in the BNSTov and CEA approximately 12h after the meal (Appendix A; Figure 6.3). Because there is a similar phase angle of entrainment, between mealtime and peak PER2 expression, this finding is

consistent with food-entrainment. In addition, VRF schedules in Chapter 4, which did not produce food-entrained anticipatory activity, were associated with blunted or arrhythmic daily profiles of PER2 expression in these brain areas (Verwey & Amir, 2011a). Finally, in Chapter 5, although long and short daily meals produced differential rhythm amplitudes, of PER2 expression in these brain nuclei, the phase of the rhythm was similar between the two groups, which also exhibited synchronous food-anticipatory activity (Verwey & Amir, 2011b). Daily rhythms of food-anticipatory activity persist, even after extensive lesions of the limbic forebrain (Mistlberger & Mumby, 1992). Therefore, the effects of RF on daily rhythms of PER2 expression in the limbic forebrain are probably a response to these behavioral and metabolic changes that are induced by RF, rather than a rhythm generator for the timing of anticipation.

BLA and DG. Daily rhythms of PER2 expression in the BLA and DG did not become food-entrained. In particular, RF schedules that provided nighttime-access to food (e.g. NF in Chapter 2 and nighttime VRF in Chapter 4) had negligible effects on the daily rhythms in these brain areas. Therefore, it is probably something that is specific to daytime feeding that is adjusting these rhythms. Because the restricted treat (RT) rats also consume a large amount of food at the same time-of-day, it is probably not the daytime food-consumption, *per se*. It could also be some other disruption caused by the food-anticipatory activities that are induced by daytime RF. However, the rats in the 2hRF group and the 6hRF group, which exhibited very different amounts of food-anticipatory running-wheel activity, also exhibited similar daily rhythms in the BLA and DG

(Verwey & Amir, 2011b). This finding suggests that the changes in the BLA and DG are not proportional to the magnitude or amount of daytime activity. Another consequence, however, is that under daytime feeding schedules the rats are fasted overnight. Therefore, it could be this nighttime food-access, present in the nighttime RF schedules and absent in the daytime RF schedules, which could be important for maintaining normal BLA- and DG-based rhythms.

Potential mechanisms

Nucleus-specific effects of RF on daily rhythms of PER1 and PER2 expression suggest that different mechanisms could be relevant in different brain areas. For instance, in the SCN, neural inputs from photosensitive retinal ganglia could have blocked the potential effects of RF by keeping this brain nucleus strongly light-entrained. Hormone release, particularly daily rhythms in corticosterone release, has already been shown to be important for daily rhythms of PER2 expression in the BNSTov and CEA (Segall et al., 2009; Segall et al., 2006). Although RF can influence daily rhythms of PER2 expression in the BNSTov and CEA, even after adrenalectomy, the observed rhythms are not identical between the adrenal-intact and adrenalectomized groups under RF (Segall et al., 2008). This finding suggests that corticosterone could still have a role, influencing PER2 expression in the BNSTov and CEA, even under RF. The release of thyroid hormones and sex hormones have also been shown to influence daily rhythms of PER2 expression in the limbic forebrain (Amir & Robinson, 2006; Perrin et al., 2006), but their importance in RF-induced changes remains to be determined.

Body temperature has also been shown to have a robust influence on the daily rhythms of clock gene expression (Buhr et al., 2010). The daily rhythm of core body temperature is altered after RF, and an increase in body temperature is typically observed during the food-anticipatory period (Gooley et al., 2006). However, more research is needed to elucidate and document the specific temperature cycles that are experienced within the brain. Circadian clocks are typically temperature-compensated, and hence, the circadian feedback loop will generate 24h periods at most biologically-relevant temperatures. However, even with temperature compensation, heat shock proteins still modulate the expression of circadian clock genes (Buhr et al., 2010; Kornmann et al., 2007). This type of temperature-related signalling is an ideal mechanism to synchronize and coordinate a wide array of circadian clocks and oscillators in diverse tissues throughout the brain and body. However, the nucleus-specificity of the effects that we observe would seem to indicate more specific mechanism(s).

Core metabolic factors are another potential influence that could mediate the effects of RF on daily rhythms of PER1 and PER2 expression in the limbic forebrain and DMH. As the BNSTov and CEA become food-entrained, they could be responding directly to some correlate of fasting or feeding. For instance, SIRT1, PGC-1α, and PPARɣ are core metabolic factors that respond to fasting and feeding, which could be involved in modulating the daily rhythm of PER2 expression (Asher et al., 2008; Grimaldi et al., 2010; Liu et al., 2007). Reciprocally, daily rhythms in PER2 expression could also be influencing the activity or expression of these metabolic factors (Schmutz et al., 2010) and the

directionality of these effects still need to be elucidated. Additional studies should also focus on the nucleus-specific expression of these signals. SIRT1 is expressed in the SCN (Satoh et al., 2010), but daily rhythms of PER1 and PER2 expression in this brain area normally do not adjust in response to RF. Thus, the importance of metabolic factors may also vary in a nucleus- or tissue-specific manner.

Final thoughts

Over the course of evolutionary time, light was typically only present during the environmental day. In contrast, modern societies generate light artificially, at all times of day. This fundamental change has produced conditions whereby, instead of correcting and compensating to ensure synchrony between environmental cycles and biological rhythms, circadian clocks and oscillators are susceptible to the exacerbation and amplification of desynchrony with the environment. Such desynchrony is also observed in shift workers and is typically associated with adverse consequences that include an increased risk of cancer, decreased fertility, reduced sleep quality, impaired waking performance, and an increased risk for obesity (Dodson & Zee, 2010; Karlsson, Knutsson, & Lindahl, 2001; Knutsson, 1989; Lawson et al., 2011; Raslear, Hursh, & Van Dongen, 2011). In addition to this shift from environmentally-derived to technologically-produced light, there has also been a shift in the availability and caloric value of food.

In broad terms, two main factors contribute to energy balance, calories-consumed and calories-burned, and the net change will result in weight gain or weight loss. The production of high-calorie food and the concomitant decrease in physical activity, has led to an obesity "epidemic" (Millward & Spinney, 2011). Moreover, the cosmetic and health consequences of obesity have fostered a weight-loss culture and an industry around how to do it. Weight-loss plans will typically have two main components, increased physical activity and reduced caloric consumption, which are also observed when rats are housed under RF. RF schedules in these experiments were typically 10-days long, comparable to a short diet in humans. The 6hRF group in Chapter 5 lost about 10% of their body weight, which is also comparable to the weight loss of an effective diet. Thus, the behavioral and physiological parallels between RF and dieting, suggest that some of the changes that were observed in these experiments may also be occurring in humans.

Food consumption has diverse homeostatic, metabolic, rewarding, and satiating consequences. With the obesity "epidemic", the elucidation and understanding of these diverse consequences of feeding have become increasingly important. The conventional understanding of the limbic forebrain and DMH is that these brain areas are involved in the regulation of motivation, emotion, and arousal. However, these findings have expanded this understanding to include local circadian rhythms that demonstrate a nucleus-specific response to RF. Daily rhythms of clock gene expression in the DMH have been implicated in the generation and timing of food-anticipatory activities.

The present studies clearly support the finding that daily rhythms within the DMH respond to RF. However, they also suggest that these DMH-rhythms are not exclusively food-entrained and are dissociable from daily rhythms in food-anticipatory activity. Therefore, the DMH should not be considered the "master" food-entrained oscillator. However, the aim of this work was not only to confirm or deny this hypothesis, but it was also to elucidate the molecular consequences of fasting and feeding on circadian rhythms outside the SCN. These studies have described a rich profile of nucleus-specific effects of RF on daily rhythms of PER1 and PER2 expression, which expand our current understanding of the brain regions that respond to feeding and fasting. In summary, these studies highlight several specific aspects of RF, such as the time-of-day, predictability, and duration of the meal, which uniquely modulate the daily rhythms of PER1 and PER2 expression in the brain and help to describe the characteristics and constraints of these nucleus-specific circadian rhythms.

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Appendix A: Food-entrainable circadian oscillators in the brain

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Michael Verwey wrote this paper, which was edited by Dr. Shimon Amir.

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Abstract

Circadian rhythms in mammalian behaviour and physiology rely on daily oscillations in the expression of canonical clock genes. Circadian rhythms in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus but are also observed in many other brain regions that have diverse roles, including influences on motivational and emotional state, learning, hormone release and feeding. Increasingly, important links between circadian rhythms and metabolism are being uncovered. In particular, restricted feeding (RF) schedules which limit food availability to a single meal each day lead to the induction and entrainment of circadian rhythms in food-anticipatory activities in rodents. Food-anticipatory activities include increases in core body temperature, activity and hormone release in the hours leading up to the predictable mealtime. Crucially, RF schedules and the accompanying food-anticipatory activities are also associated with shifts in the daily oscillation of clock gene expression in diverse brain areas involved in feeding, energy balance, learning and memory, and motivation. Moreover, lesions of specific brain nuclei can affect the way rats will respond to RF, but have generally failed to eliminate all food-anticipatory activities. As a consequence, it is likely that a distributed neural system underlies the generation and regulation of food-anticipatory activities under RF. Thus, in the future, we would suggest that a more comprehensive approach should be taken, one that investigates the interactions between multiple circadian oscillators in the brain and body, and starts to report on potential neural systems rather than individual and discrete brain areas.

Introduction

Circadian rhythms are observed in many aspects of animal behaviour and physiology, and include daily rhythms in motivated behaviours, hormone release, body temperature, cognition and mood (Hastings, O'Neill, & Maywood, 2007; Lamont, James, Boivin, & Cermakian, 2007; Mendoza, 2007; Schibler, 2007; Wirz-Justice, 2008). At the molecular level, circadian clocks rely on daily oscillations in the transcription and translation of canonical clock genes (Reppert & Weaver, 2002). In mammals, circadian oscillations in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus (SCN), and are fundamentally linked to the generation and regulation of circadian rhythms within this structure (Fuller et al., 2008; Gavrila et al., 2008). Circadian rhythms in clock gene expression are also observed in many brain areas outside the SCN, areas with diverse roles in behaviour and physiology (Guilding & Piggins, 2007). Tissue-specific clock gene expression is thought to modulate many essential cellular, homeostatic and metabolic processes (Challet et al., 2003; Hastings et al., 2007; Pardini & Kaeffer, 2006; Rutter, Reick, & McKnight, 2002). In turn, it is becoming increasingly clear that metabolic signals influence clock gene expression (Belden & Dunlap, 2008; Grimaldi, Nakahata, Kaluzova, Masubuchi, & Sassone-Corsi, 2009; Liu et al., 2007; Nakahata et al., 2008; Rodgers et al., 2008). Thus, patterns of food intake not only affect satiety and hunger but also have the potential to influence clock gene expression throughout the brain and body.

To probe the interaction between metabolism and clock gene expression, in rodents, restricted feeding (RF) schedules have been used. RF schedules limit food availability to a single meal each day and, in response, animals gradually develop a number of food-anticipatory activities (see Figure 6.1). Specifically, increases in locomotor activity, body temperature and hormone release are observed before the predictable daily meal (Stephan, 2002). Most importantly, RF schedules also shift or entrain the daily rhythm of clock gene expression in many brain areas (Abe, Honma, & Honma, 2007; Angeles-Castellanos et al., 2007; Girotti, Weinberg, & Spencer, 2009; Mieda et al., 2006; Wakamatsu et al., 2001). Thus, RF provides a powerful tool with which to study the neural and molecular bases of food-anticipatory activities as well as the nature and function of daily oscillations of clock gene expression in the brain.

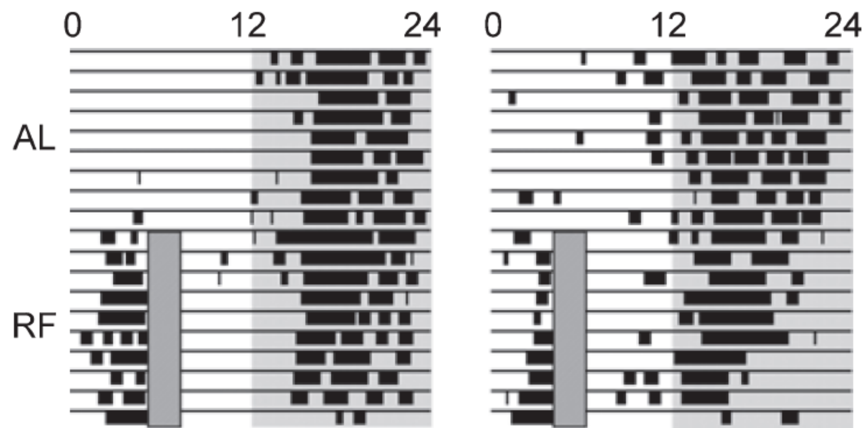

Figure 6.1
Actograms illustrating the daily pattern of running-wheel activity for two representative rats that each received ad libitum food access (AL; first 9 days of the record) followed by restricted feeding (RF; last 10 days of the record). Each horizontal line plots 24h and sequential days are arranged from top to bottom. Rats were housed in a 12h:12h light-dark schedule, illustrated by the empty (light phase) and shaded (dark phase) areas in each actogram. Numbers above the actograms indicate zeitgeber time (ZT). Under RF, rats received 2h of access to food each day (ZT4-6), illustrated by the shaded rectangle. As expected, RF produced characteristic food-anticipatory running-wheel activity in the few hours leading up to the predictable mealtime.

Circadian clock gene expression in the brain

The basis for self-sustained circadian rhythms is a highly conserved molecular autoregulatory feedback loop that oscillates with an ~24h period (Asher et al., 2008; Belden & Dunlap, 2008; Kornmann et al., 2007; Liu et al., 2007; Reppert & Weaver, 2002; Yoo et al., 2004). Briefly, at the core of this loop the genes *Clock* and *Bmal1* encode proteins that dimerize and act as a transcription factor. CLOCK: BMAL1 heterodimers then enhance the transcription of *Period* (*Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes (Gekakis et al., 1998; Hogenesch, Gu, Jain, & Bradfield, 1998). PER and CRY expression oscillate across the day and the proteins feed back into the nucleus, and interfere with the transcriptional activity of CLOCK:BMAL1 (Kume et al., 1999; Shearman et al., 2000; Vitaterna et al., 1999). In essence, these genes and their protein products are the gears and motors that allow circadian clocks to 'tick'. Thus, daily oscillations in clock gene expression (e.g. PER1 and PER2) allow for the identification of brain areas that express crucial circadian clockwork.

Circadian rhythms in clock gene expression have been found in many brain regions outside the SCN including the hippocampus, piriform cortex, cingulate cortex, prefrontal cortex, striatum, paraventricular hypothalamic nucleus, arcuate nucleus, olfactory bulb, nucleus accumbens and pituitary (Abe et al., 2002; Angeles-Castellanos et al., 2007; Gillespie, Chan, Roy, Cai, & Belsham, 2003; Granados-Fuentes et al., 2004; Kriegsfeld, Korets, & Silver, 2003; Shieh, 2003; Shieh, Yang, Lu, Akil, & Watson, 2005; Yamamoto et al., 2001). Furthermore, we recently identified robust rhythms of PER2 expression in

select regions of the limbic forebrain, areas that are important in the regulation of stress, motivation and emotion (Amir et al., 2004; Lamont, Robinson, et al., 2005). These limbic areas include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus of the hippocampus (DG). Although circadian oscillations in clock gene expression in most brain areas are either directly or indirectly under the control of the SCN, rhythms in brain regions outside the SCN are also sensitive to diverse hormonal and behavioural manipulations, including restricted feeding (Amir, Harbour, & Robinson, 2006; Angeles-Castellanos et al., 2007; Lamont, Diaz, et al., 2005; Perrin et al., 2006; Segall et al., 2006; Verwey et al., 2007, 2008; Verwey et al., 2009; Waddington Lamont et al., 2007; Wakamatsu et al., 2001).

Brain areas sensitive to restricted feeding

There are two reasons to study circadian rhythms of gene expression under RF. The first reason is to determine which brain areas are involved in the regulation and entrainment of food-anticipatory activities per se. The second reason is to study of the properties and functions of putative circadian oscillators outside the SCN. Our recent work has been concerned primarily with the latter issue and we have recently started to define some of the hormonal and behavioural influences that affect clock gene expression in the limbic forebrain (Amir et al., 2004; Amir & Stewart, 2009; Lamont, Robinson, et al., 2005). To carry out these studies, we and other have studied immediate-early gene expression (e.g. cFOS) and the

expression of clock genes (e.g. PER1, PER2) across the circadian cycle (Angeles-Castellanos et al., 2004; Angeles-Castellanos et al., 2007; Mendoza, Angeles-Castellanos, et al., 2005c; Verwey et al., 2007).

Immediate-early genes, such as cFOS, are readily expressed throughout the brain in response to diverse stimuli. Although cFOS expression might be elevated in a particular brain area, clock gene expression may be unaffected in the same region. Our own research has illustrated this point by contrasting rats on an RF schedule with rats on a restricted treat (RT) schedule (Verwey et al., 2007). Specifically, a restricted daily meal of highly palatable complete meal replacement, chocolate Ensure Plus (Ensure), was given to fasted (RF) and freely-fed (RT) rats. Although both groups consumed similar amounts of Ensure, only 37% of the RT group exhibited anticipatory running wheel activity in the hours leading up to the Ensure access while 100% of the RF group anticipated the daily mealtime. Of note, in response to Ensure, both groups exhibited increases in cFOS expression in the limbic forebrain and hypothalamus. In contrast, the daily pattern of PER2 expression in the same brain structures was only affected by RF, while the RT group and the ad libitum-fed control groups were indistinguishable. Thus, this dissociation between cFOS and PER2 expression in response to RF and RT emphasizes the importance of distinguishing conclusions based on immediate-early gene expression from conclusions based on clock gene expression. Moreover, the differential effects of RF and RT on PER2 expression suggest that clock gene expression in the limbic forebrain and hypothalamus is relatively insensitive to the incentive value

of food and, instead, requires the metabolic challenges associated with fasting under RF.

The limbic forebrain

The limbic forebrain contributes to the regulation of motivation and emotion, and includes brain areas such as the bed nucleus of the stria terminalis (BNST), amygdala and hippocampus. Collectively, these areas modulate neuroendocrine, autonomic and behavioural responses to different types of stress and to drugs of abuse (Erb et al., 2001; Gray, 1993; Loewy, 1991; Nijsen et al., 2001). Moreover, these brain areas also modulate fear and anxiety, learning and memory, reproductive and maternal behaviours, and ingestive behaviours (Casada & Dafny, 1991; Figueiredo et al., 2003; Stefanova & Ovtscharoff, 2000; Van de Kar & Blair, 1999; Walker et al., 2001; Walker et al., 2003). Under ad libitum feeding conditions, PER2 is expressed with a circadian rhythm in the BNSTov, CEA, BLA and DG (Amir et al., 2004; Lamont, Robinson, et al., 2005). On a 12h:12h light-dark schedule, daily oscillations of PER2 expression in the BNSTov and CEA peak around the time of transition from day to night (dusk), a daily expression profile which is in synchrony with PER2 expression in the SCN (Amir et al., 2004; Lamont, Robinson, et al., 2005). In contrast, peak PER2 expression in the BLA and DG is observed around the time of transition from night to day (dawn; (Lamont, Robinson, et al., 2005), opposite to the oscillation observed in the BNSTov, CEA and SCN (see Figure 6.2).

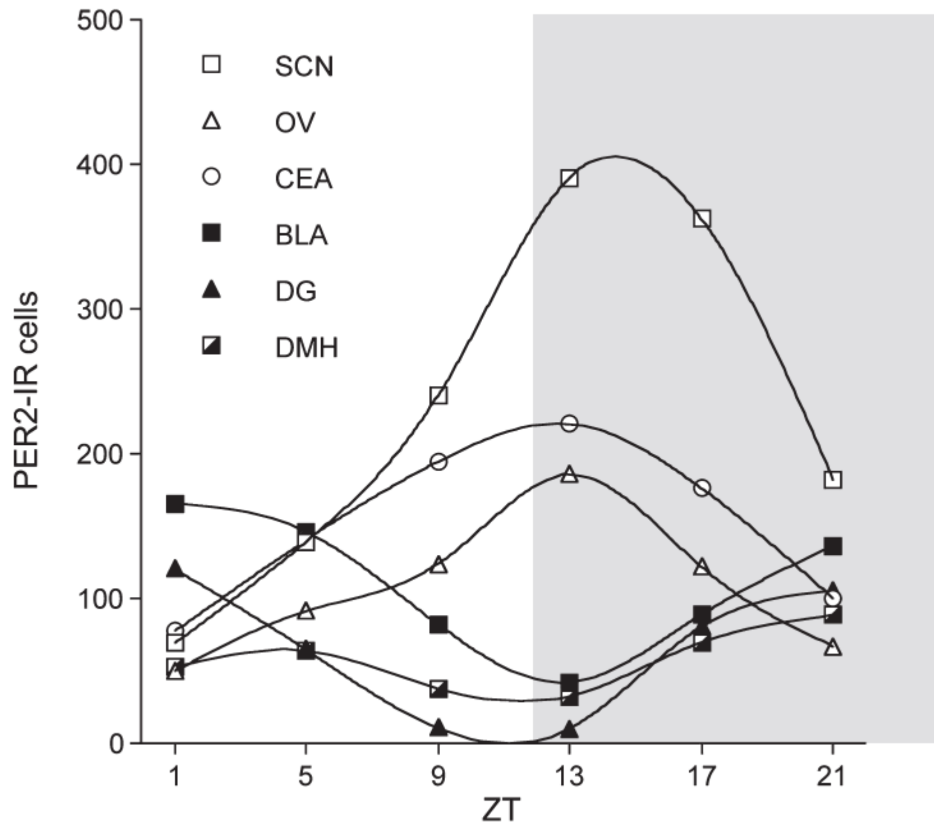

Figure 6.2
Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of ad libitum-fed rats. Each line illustrates the estimated mean of PER2 immunoreactivity in a particular structure, plotted according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The 12h:12h light-dark cycle is also illustrated by the shaded and unshaded areas of the graph. With the exception of the DMH, all structures exhibited robust daily rhythms in PER2 expression. Peak PER2 expression was observed in the SCN, BNSTov and CEA at ZT13 whereas peak PER2 expression was observed in the BLA and DG at ZT1 (redrawn from data published in Verwey et al., 2007).

Consistent with other extra- SCN circadian oscillators (Sakamoto et al., 1998), no circadian rhythm is observed in PER2 expression in the limbic forebrain after the SCN is lesioned (Amir et al., 2004; Lamont, Robinson, et al., 2005). Additionally, daily rhythms of PER2 expression in the BNSTov and CEA are also sensitive to hormonal signals. In the rat, adrenal stress hormones (corticosterone), gonadal hormones (Oestrogen, testosterone) and thyroid hormones all modulate PER2 expression in the BNSTov and CEA though, importantly, these hormones do not modulate PER2 expression in the SCN, BLA or DG (Amir et al., 2004; Amir & Robinson, 2006; Lamont, Robinson, et al., 2005; Perrin et al., 2006). In particular, the daily rhythm of corticosterone, the main adrenal glucocorticoid in rats, is critical for daily rhythms in PER2 expression in the BNSTov and CEA (Segall et al., 2009; Segall et al., 2006). Interestingly, in contrast to many other manipulations that fail to modulate PER2 expression in the BLA and DG, the daily rhythm of PER2 expression in these areas is affected by RF schedules.

In our studies on the effect of RF on PER2 expression in the limbic forebrain we found that, when food is restricted to the middle of the day when rats normally do not eat (daytime RF), the daily rhythms of PER2 expression in the BNSTov, CEA, BLA and DG shift such that peak PER2 expression in all of these structures is observed ~12h after the daytime meal (Verwey et al., 2007). In contrast, RF schedules that provide a nighttime meal (nighttime RF), a more appropriate mealtime for nocturnal rats, shift the daily rhythms of PER2 expression in the BNSTov and CEA but not in the BLA and DG (see Figure 6.3). As was the case for daytime RF, peak PER2 expression was observed in the

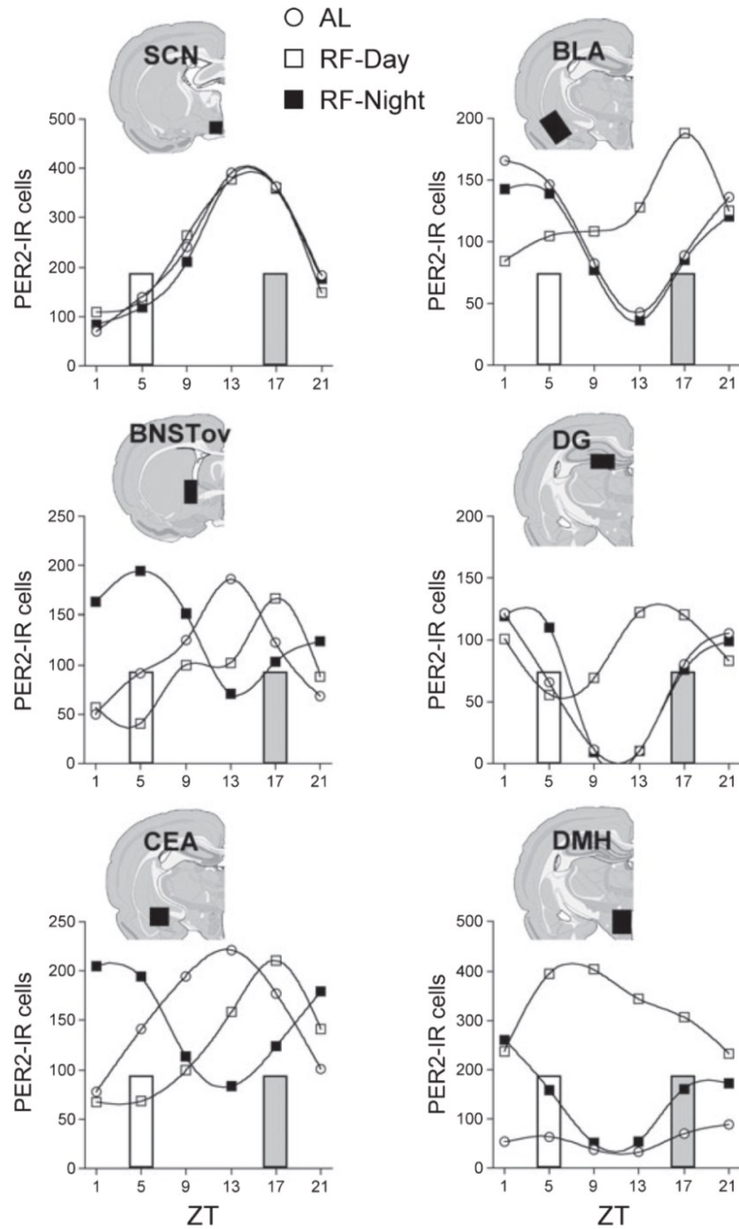

Figure 6.3
Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of rats under RF and ad libitum (AL) feeding conditions. Mean PER2 immunoreactivity is plotted for each structure, according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The daily meal of Ensure was provided either in the middle of the day (RF-day; ZT4-6; open rectangle) or middle of the night (RF-night; ZT16-18; shaded rectangle). The daily pattern of PER2 expression in the BNSTov, CEA and DMH was affected by both RF schedules. In contrast, the daily pattern of PER2 expression in the BLA and DG was only shifted under RF-day conditions. Finally, PER2 expression in the SCN was unaffected by either RF schedule (redrawn from data published in Verwey et al., 2007 and 2008).

BNSTov and CEA ~12h after the predictable mealtime; however, PER2 expression in the BLA and DG was unaffected by nighttime RF and similar to that in ad libitum-fed controls (Verwey et al., 2008). Thus clock gene expression in the BLA and DG is affected only by daytime RF whereas, PER2 expression in the BNSTov and CEA peaks ~12h after both daytime RF and nighttime RF. Thus clock gene expression in the BNSTov and CEA would appear to be food-entrained.

The changes in clock gene expression seen in response to RF vary as a function of the gene studied. Differences between PER1 and PER2 expression have been observed in the limbic forebrain. In ad libitum-fed rats, PER1 expression is reportedly rhythmic in the BNST and hippocampus but not rhythmic in the CEA or BLA (Angeles-Castellanos et al., 2007) whereas, as previously noted, PER2 expression is rhythmic in all of these brain areas under ad libitum feeding (Amir et al., 2004; Lamont, Robinson, et al., 2005). Under a daytime RF schedule, PER1 expression was reported to be unchanged in the CEA and BLA but altered in the BNST and hippocampus (Angeles-Castellanos et al., 2007). In contrast, under similar conditions we found robust shifts in PER2 expression in all these regions (Verwey et al., 2007, 2008; Waddington Lamont et al., 2007). These data suggest key differences in the regulation of PER1 and PER2 expression in the limbic forebrain by RF, but these two genes have not been studied concurrently in the same experiment so a note of caution is necessary. Indeed, whereas we quantified PER2 expression specifically in the BNSTov, Angeles-Castellanos et al. (2007) quantified PER1 immunoreactivity in the BNST

as a whole. Similarly, we quantified PER2 expression specifically in the DG whereas Angeles-Castellanos et al. (2007) examined PER1 expression throughout the hippocampus. Both the BNST and hippocampus are heterogeneous structures that contain many distinct subregions with different functions and these subregions should be analysed individually. Nevertheless, there is also evidence that PER1 and PER2 are differentially expressed in the limbic forebrain in the mouse (Feillet, Mendoza, Albrecht, Pevet, & Challet, 2008).

The SCN

The SCN also exhibits changes in the expression of clock genes in response to RF schedules but, compared to the large shifts observed in the limbic forebrain, these changes are usually subtle. For example, the daily rhythm of expression of Per1 mRNA in the SCN shifts in response to RF, but these changes are relatively small. The rhythms of Per1 in the SCN did not become entrained by the RF schedule (Mendoza, Angeles-Castellanos, et al., 2005a). In our studies we have not observed RF-induced changes the expression of PER2 in the SCN (Verwey et al., 2007, 2008). One exception to this generalization occurs when rats are housed in constant light. Under these conditions, circadian locomotor activity rhythms become disrupted and PER2 expression in the SCN does not exhibit a circadian rhythm. Remarkably, when placed on an RF schedule in these constant light conditions, both locomotor activity and PER2 rhythms are reinstated and are entrained to the time of feeding (Lamont, Diaz, et al., 2005).

Some of the most robust effects on clock gene expression in the SCN have been observed in response to caloric restriction (Andrade, Pereira, Silva, Sa, & Lukoyanov, 2004; Challet et al., 1996; Challet et al., 1998; Mendoza, Graff, et al., 2005). In mice, hypocaloric feeding schedules can entrain SCN-driven circadian rhythms and affect clock gene expression in this brain area. Importantly, this effect has been attributed to the hypocaloric feeding *per se* rather than the reorganization of circadian activities and the development of food-anticipatory activities. Specifically, delivering a single hypocaloric meal each day leads to the development of food-anticipatory activities. However, the delivery of six meals per day do not lead to the same development of coordinated food-anticipation and yet still affect clock gene expression in the SCN (Mendoza, Drevet, Pevet, & Challet, 2008). This work clearly demonstrates that certain changes in clock gene expression can be brought on simply by reduced caloric intake rather than food-entrainment per se.

The dorsomedial hypothalamic nucleus (DMH)

The DMH is an important relay in the transmission of signals from the SCN to the rest of the brain and body. This structure also appears to integrate metabolic signals with inputs it receives from the SCN (Elmquist et al., 1998) and provides important outputs to brain areas that regulate sleep, such as the ventrolateral preoptic nucleus, and arousal, such as the lateral hypothalamus (Chou et al., 2003). Under ad libitum feeding conditions the DMH does not exhibit robust daily oscillations in clock gene expression but, under RF, large-amplitude circadian

rhythms in Bmal1, PER1 and PER2 expression emerge (Fuller et al., 2008; Mieda et al., 2006; Moriya et al., 2009; Verwey et al., 2007, 2008). These daily oscillations in clock gene expression in the DMH depend on the time of day when meals are provided. Specifically, under a daytime RF schedule, peak PER2 expression is observed in the DMH soon after the mealtime. However, under a nighttime RF schedule, peak PER2 expression is observed in the DMH several hours after the mealtime (Verwey et al., 2007, 2008). This interaction between the time when food is given and the time of peak expression suggests that, although clock gene expression in the DMH is affected by RF schedules, it may not be exclusively food-entrained. To date, studies of clock gene expression in the DMH have been carried out in SCN-intact rats and mice and the DMH has strong connections to the SCN (Chou et al., 2003). An intriguing question, therefore, is whether clock gene expression in the DMH could be food-entrained if the SCN were lesioned.

The circadian basis of food-anticipatory activities

The characteristics of food-anticipatory activities are consistent with an underlying food-entrained circadian oscillator. For example, when rats are fasted for 2-4 days after being on an RF schedule, food-anticipatory activities continue to appear each day around the predicted mealtime (Boulos, Rosenwasser, & Terman, 1980). An hourglass model, whereby rats anticipate subsequent food arrival a fixed number of hours after the last meal, would not account for daily food anticipation under a 72h fast. Also consistent with a circadian mechanism,

food-anticipatory activities show limits of entrainment. Although rodents accurately predict 24h food intervals, 18h intervals do not lead to characteristic food-anticipatory activities (Stephan, 1981). If food-anticipatory behaviours were based on an hourglass mechanism, 18h and 24h schedules should represent similar challenges. Based on these findings, putative circadian oscillators throughout the brain and body are thought to contribute to the regulation and entrainment of food-anticipatory activities. Although some aspects of food anticipation persist when canonical circadian clock genes are mutated or knocked out, deficits are also observed (Feillet et al., 2006; Fuller et al., 2008; Storch & Weitz, 2009). Light-entrained and food-entrained circadian oscillators are viewed as separate and relatively independent systems (Mistlberger, 1994; Stephan, 2002). Consistent with this hypothesis, there are generally subtle effects of RF on clock gene expression in the SCN, and SCN lesions do not interfere with the development of food-anticipatory activities (Stephan et al., 1979a; Stephan et al., 1979b). However, because no single brain lesion eliminates all food-anticipatory activities, the specific areas and tissues of the brain and body that are involved in circadian food-entrained anticipatory activities remain unclear. Food-entrained and light-entrained oscillators affect the expression of overlapping canonical clock genes in many brain areas and so the focus of experiments should start to shift towards uncovering networks of RF-sensitive brain areas.

The DMH and food-anticipatory activity

It has been suggested that clock gene expression in the DMH is necessary for the expression of food-anticipatory activities (Fuller et al., 2008; Gooley et al., 2006). Specifically, mutant mice with a targeted disruption of the Bmal1 gene are reported to exhibit deficient circadian rhythms and disruptions in their ability to show food-anticipatory increases in core body temperature (Fuller et al., 2008). Moreover, food anticipation has been reported to be restored when exogenous Bmal1 is transfected to the DMH (Fuller et al., 2008). Lesions of the DMH have also been reported to interfere with the expression of food-anticipatory activities (Gooley et al., 2006). Clearly, these studies suggest that the DMH could be important in the expression of certain food-anticipatory rhythms. However, there are also a number of other studies that contradict this conclusion (Landry et al., 2006; Landry et al., 2007; Moriya et al., 2009; Storch & Weitz, 2009). For example, it has recently been reported that some food-anticipatory behaviours persist in Bmal1-mutant mice (Pendergast et al., 2009; Storch & Weitz, 2009). Furthermore, there are a number of reports of robust food-anticipatory activities in DMH-lesioned rats (Landry et al., 2006; Landry et al., 2007). These discrepancies have led to active debate in the literature (Fuller et al., 2008; Gooley et al., 2006; Landry et al., 2006; Mistlberger et al., 2008) and it is clear that a more comprehensive understanding of the control of food-anticipatory rhythms will depend on further experimentation and replication.

Accurate food-anticipatory activities are not required for robust circadian rhythms in clock gene expression in the DMH. Variable restricted feeding

schedules provide an unpredictable meal each day, and inherently prohibit accurate food-anticipation (Escobar et al., 2007; Verwey et al., 2009). When the mealtime is varied within either the 12h of light or the 12h of darkness, large-amplitude rhythms in PER1 expression are clearly observed in the DMH despite a lack of food-entrained running wheel activity (Verwey et al., 2009). In contrast, when food availability is varied throughout the entire 24h cycle, PER1 expression in the DMH is elevated across the day as compared to controls, though no daily rhythm is observed (Verwey et al., 2009). Thus, robust circadian rhythms in PER1 expression can be induced by RF schedules even without the entrainment of food-anticipatory activity. Conversely, food-anticipatory activities have also been observed in the absence of circadian rhythms in clock gene expression in the DMH. Food-anticipatory activities have been reported in rats receiving a daily chocolate treat (Mendoza, Angeles-Castellanos, et al., 2005a; Mistlberger & Rusak, 1987) but, in those rats anticipating the daily treat, PER2 expression in the DMH is similar to ad libitum-fed controls (Verwey et al., 2007, 2008). Collectively, these findings demonstrate that the food entrainment of running wheel activity and PER expression in the DMH are dissociable. However, the induction and entrainment by RF of daily rhythms in clock gene expression in the DMH certainly represents an interesting and important area of study, whether or not it is crucial to all food-anticipatory behaviours.

Potential mechanisms

In the anticipation of food, canonical clock proteins do not operate in isolation. Homeostatic signals, learning and as yet undiscovered circadian mechanisms all may contribute to the expression of food-anticipatory rhythms. Furthermore, in spite of the observation that certain food-anticipatory activities persist in mice with circadian gene mutations, extra-SCN clock gene expression remains an important area of study in the context of restricted feeding. Indeed, several lines of evidence, mostly from work in the periphery, have made it abundantly clear that clock gene expression has an important influence on tissue-specific metabolism and physiology (Chen-Goodspeed & Lee, 2007; Fu et al., 2002; Oster et al., 2006; Winter, Bosnoyan-Collins, Pinnaduwage, & Andrulis, 2007). Fundamentally, understanding the regulation and consequence of clock gene expression in different regions of the brain is not only important for the elucidation of mechanisms underlying RF but also essential to understanding the interaction between the circadian system and physiology. Several pathways influence circadian clock gene expression throughout the brain and body. Daily rhythms of clock gene expression in the limbic forebrain and hypothalamus are selectively sensitive to RF and not RT. Thus, rather than pathways associated with reward and feeding per se, metabolic pathways associated with fasting are probably involved in the regulation of clock gene expression in these areas. One metabolic pathway whereby RF schedules could influence circadian oscillators in the brain involves two interacting proteins that relate directly to cellular metabolism and clock gene expression, *Peroxisome proliferator-activated*

receptor gamma co-activator 1alpha (PGC-1α) and *Sirtuin1* (SIRT1; Asher et al., 2008; Belden & Dunlap, 2008; Liu et al., 2007; Rodgers et al., 2005). Specifically, the expression of PGC-1α in peripheral tissues such as liver and muscle follows a circadian rhythm (Liu et al., 2007). In turn, PGC-1α has been found to induce and regulate the rhythmic expression of clock genes in peripheral tissues and, significantly, to affect behavioural and physiological circadian rhythms (Canaple et al., 2006; Liu et al., 2007). In contrast, SIRT1 is directly sensitive to food availability and it deacetylates PER2, thus also affecting the circadian feedback loop (Asher et al., 2008; Belden & Dunlap, 2008). These mechanisms have been studied in the periphery (Asher et al., 2008; Liu et al., 2007; Nakahata et al., 2008) but additional research is needed to demonstrate their importance in the brain. PGC-1α mRNA is expressed in many brain areas, including the olfactory bulb, cerebral cortex, septal nucleus, striatum, hippocampus and substantia nigra (Cowell, Blake, & Russell, 2007; Tritos et al., 2003). Furthermore, the expression of PGC-1α in cortical cells has been shown to be regulated by neuronal activity (Meng, Liang, & Wong-Riley, 2007), raising the intriguing possibility that PGC-1α may mediate the effects of signals arising from daily restricted feeding and from other perturbations of physiology and behaviour on the expression of clock genes in the brain. A second pathway whereby RF schedules could influence clock gene expression in the limbic forebrain is through the action of glucocorticoids. Glucocorticoids modulate clock gene expression in the periphery, and daily rhythms in glucocorticoid release are needed to sustain at least certain brain oscillators (Segall et al., 2006). The daily

rhythm of glucocorticoid release is also changed by RF, and a food-anticipatory release of this stress hormone is well documented (Ahlers et al., 1980). Although glucocorticoids do not appear to influence PER2 expression in the limbic forebrain under RF (Segall et al., 2008), the RF modulation of glucocorticoid release could have a role in the modulation in the brain of clock gene expression, especially of PER1, which contains a glucocorticoid-responsive element in its promoter region (Yamamoto et al., 2005). A third pathway whereby RF schedules could influence clock gene expression is through body temperature. The daily rhythm of body temperature is entrained by RF and has been studied extensively in the context of the DMH. Circadian rhythms in cerebral temperature also occur (Boudreau et al., 2008), but their importance to clock gene expression remains unknown. In the periphery, circadian rhythms in body temperature and the associated induction of heat shock proteins appear to have some influence on clock gene expression (Kornmann et al., 2007). Through a combination of inputs, some of which have been discussed here, RF schedules are able to influence clock gene expression in a region-dependent manner. Elucidating the mechanisms whereby RF is able to alter clock gene expression in some brain areas and not others, and uncovering the consequences of these effects, should be a major focus of future research.

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Appendix B: REPRINT (CHAPTER 1) - Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats.

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DIFFERENTIAL REGULATION OF THE EXPRESSION OF Period2 PROTEIN IN THE LIMBIC FOREBRAIN AND DORSOMEDIAL HYPOTHALAMUS BY DAILY LIMITED ACCESS TO HIGHLY PALATABLE FOOD IN FOOD-DEPRIVED AND FREE-FED RATS

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Abstract—Circadian clock genes are rhythmically expressed in many areas of the brain and body and are thought to underlie most endogenous circadian behaviors and physiological processes. Daily rhythms of clock gene expression throughout the brain and body are normally coordinated by the suprachiasmatic nucleus (SCN), but they are also strongly influenced by daily temporal restrictions of food availability. Here, we studied the effects of a daily restricted presentation of highly palatable complete meal replacement, chocolate Ensure Plus (Ensure) in food-deprived (restricted feeding, RF) and free-fed (restricted treat, RT) rats, on the expression of the clock protein, Period2 (PER2) in regions of the brain involved in motivational and emotional regulation; these include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), the dentate gyrus (DG) and the dorsomedial hypothalamus (DMH). RF and RT rats consumed similar amounts of Ensure, but changes in the pattern of PER2 expression were seen only in the RF condition, suggesting that changes in PER2 expression in these regions are triggered by the daily alleviation of a negative metabolic state associated with RF and are independent of the positive incentive properties of the consumed substance, per se. In contrast, the expression of the immediate early gene, Fos, was increased in these regions by both RF and RT schedules, showing that signals concerning the incentive value of the consumed food reach these regions. No changes in either PER2 or Fos expression were observed in the SCN of RF or RT rats. These findings demonstrate that mechanisms leading to changes in the expression of PER2 and those affecting the induction of Fos under RF and RT are, at least in part, dissociable. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: restricted feeding, clock genes, bed nucleus of the stria terminalis, amygdala, hippocampus, food-anticipatory behavior.

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Abbreviations: AL, *ad libitum*; ANOVA, analysis of variance; BLA, basolateral amygdala; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DG, dentate gyrus; DMH, dorsomedial hypothalamic nucleus; Ensure, complete meal replacement, chocolate Ensure Plus; LD, light/dark; PER2, Period2; RF, restricted feeding; RT, restricted treat; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

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Circadian rhythms in the expression of clock genes in peripheral tissues and brain are orchestrated by a master pacemaker located in the suprachiasmatic nucleus (SCN) (Lowrey and Takahashi, 2004; Yoo et al., 2004; Guo et al., 2005, 2006). These extra-SCN rhythms are also strongly influenced by feeding schedules that restrict food access to the same time each day (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001; Challet et al., 2003; Mieda et al., 2006; Zvonic et al., 2006; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). Such schedules lead to novel daily fluctuations in energy balance as well as changes in the patterns of arousal, circulating corticosterone and daily activity rhythms (Mistlberger and Marchant, 1995; Stephan, 2002; Davidson et al., 2005; Mendoza, 2007), suggesting that expression of clock genes in the brain and periphery is not only sensitive to signals from the SCN but also responds to changes in nutritional and/or motivational state independently of the SCN (Hara et al., 2001).

We have shown recently that scheduled restricted feeding (RF) in rats strongly affects the rhythm of expression of the clock protein Period2 (PER2) in limbic forebrain areas involved in the control of motivation and emotion, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and dentate gyrus (DG) (Lamont et al., 2005a; Waddington Lamont et al., 2007). Interestingly we also found that in the absence of food deprivation, daily limited access to highly palatable substances such as sucrose or saccharine, had no effect on the rhythm of PER2 expression in these areas in spite of the fact that these substances were consumed in large quantities. Based on these findings, we hypothesized that the expression of PER2 in the limbic forebrain is sensitive to homeostatic signals arising from the daily alleviation of a negative metabolic state associated with scheduled feeding and is independent of the positive incentive properties of the consumed substance, per se (Waddington Lamont et al., 2007).

To study this issue further, we assessed the effect of daily limited access to a highly palatable complete meal replacement, chocolate Ensure Plus (Ensure; Abbott Laboratories, Abbott Park, IL, USA), on PER2 expression in the BNSTov, CEA, BLA, and DG in both food-deprived and free-fed rats. In addition, we assessed the effect of daily limited access to Ensure on neuronal activation in these brain regions in food-deprived and free-fed rats using the

transcription factor, Fos, as a marker (Angeles-Castellanos et al., 2004, 2007). Also, we assessed the effect of limited access to Ensure in food-deprived and free-fed rats on PER2 and Fos expression in the dorsomedial hypothalamic nucleus (DMH), an area shown recently to play a role in the interface between RF, PER2 expression and certain food anticipatory rhythms (Gooley et al., 2006; Landry et al., 2006; Mieda et al., 2006). Finally, in order to differentiate the circadian effects of serial food presentations from the potential acute effects of a single food presentation, we also studied rats on the first day of our restricted Ensure schedules. Preliminary results have been presented in an abstract form (Verwey et al., 2005).

EXPERIMENTAL PROCEDURES

Animals and housing

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. All efforts were made to minimize the number of animals used and their suffering. A total of 96 male Wistar rats were used (225–250 g; Charles River Laboratories, St. Constant, QC, Canada). The rats were housed individually in cages equipped with running wheels, under a 12-h light/dark (LD) schedule (300 lux at cage level) and had free access to Purina rat chow and water for at least 10 days before the start of each experiment. Running wheel activity was recorded by computer (Vitalview, Minimitter, OR, USA) and analyzed with Circadia software.

RF and restricted treats (RT)

Rats were assigned randomly to one of three groups: RF group, RT group or *ad libitum* (AL) group. During experimental stages, rats in the RF group were fed exclusively with unlimited Ensure for 2 h each day, during the middle of the day, from zeitgeber time (ZT) 4–6 (ZT0 denotes time of lights on in a 12-h LD schedule). Rats in the RT group had continued access to Purina rat chow and in addition received an identical restricted access to Ensure during the day (ZT4–6). Rats in the AL control group had free access to Purina rat chow only. These schedules lasted for 10–13 days. This relatively short RT schedule was deliberately chosen so the RT group could be compared with the RF group. Other experiments have generally used longer (4–6 week) palatable meal entrainment protocols and have focused more on treat-anticipatory behavior (Mistlberger and Rusak, 1987; Mendoza et al., 2005a,c). Our experiments did not set out to study the anticipation of a daily treat, per se, instead we focused on the metabolic and motivational consequences of the daily consumption of Ensure between fasted and free-fed rats.

Tissue preparation and immunocytochemistry

On the last day of the scheduled feeding, rats were deeply anesthetized with sodium pentobarbital (Somnotol, ~100 mg/kg) at one of six ZTs (ZT1, 5, 9, 13, 17, 21) and perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Following perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4 °C overnight. Serial coronal brain sections (50 μm) containing regions of interest were collected from each animal using a vibratome and stored in Watson's cryoprotectant solution until use (Watson et al., 1986). Immunocytochemistry for PER2 was performed on one set of brain sections as previously described (Amir et al., 2004) using an affinity-purified rabbit polyclonal antibody raised against PER2 (1:800, Alpha Diagnostics International, San Antonio, TX, USA). Immunocytochemistry for Fos was performed on a second set of brain sections collected from each rat as previously described (Beaule et al., 2001) using a polyclonal *cFos* antibody, raised in rabbit (1:100,000, Oncogene Sciences, Boston, MA, USA).

Image analysis and statistics

PER2- and Fos-stained brain sections were mounted on gelatin-coated glass slides, coverslipped and examined under a light microscope. Images of brain areas containing the SCN, BNSTov, CEA, BLA, DG and DMH were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA), and Image SXM software (v1.6, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 or Fos were counted on captured images using a 400×400 μm (SCN, BNSTov, CEA, BLA, DMH) or a 200×400 μm (DG) frame. The mean number of PER2 or Fos immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analysis of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

RESULTS

Ensure consumption and locomotor activity rhythms under RF and RT schedules

Daily rhythms of wheel running activity were assessed in AL rats housed under a 12-h LD schedule and in similarly housed rats that were placed on either a RF or RT schedule in which Ensure was given for 2 h each day, from ZT4–6. Fig. 1 shows the amounts of Ensure consumed each day during the 2-h access period by rats from the RF and RT groups. It can be seen that with the exception of the first day of limited access, rats from the two groups consumed similar amounts of Ensure throughout the experiment. Notwithstanding, RT rats continued to eat regular chow and thus consumed more calories each day than RF rats (data not shown). As expected, rats from the RF group showed consistent changes in running wheel patterns and developed an anticipatory running wheel bout which began 2–3 h before daily food presentation (Fig. 2).

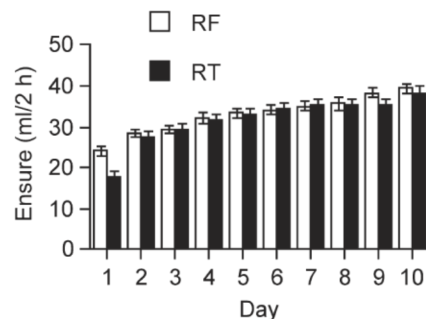

Fig. 1. Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food-deprived (RF, n=24) and free-fed (RT, n=24) rats. Ensure was presented for 2 h each day from ZT4–6 (4–6 h after lights-on).

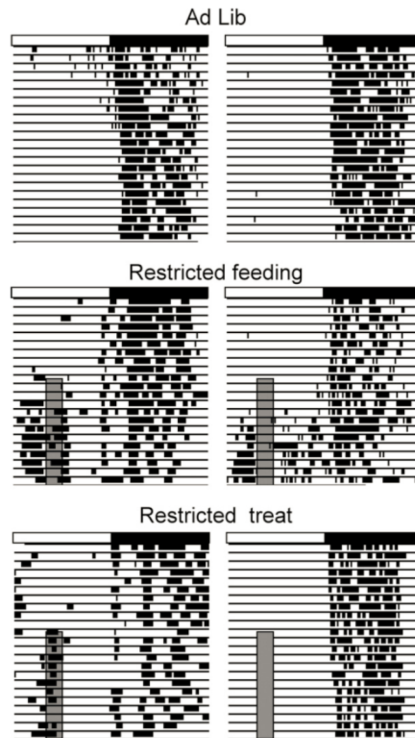

Fig. 2. Representative single-plotted actograms of wheel-running activity in AL control rats, as well as food-deprived (RF) and free-fed (RT) rats that received a daily presentation of chocolate Ensure from ZT4–6 (4–6 h after lights on; illustrated by gray rectangles). All RF rats showed 'anticipatory' wheel running preceding the daily meal, while only 37% of RT rats showed a similar behavioral pattern (bottom panel, on left). All rats were housed under a 12-h LD cycle. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10 min. Successive days are plotted from top to bottom.

In contrast, only 9 of the 24 rats from the RT group showed anticipatory running wheel activity (Fig. 2). The remaining rats from the RT group did not develop anticipatory running and their circadian running patterns resembled those of AL rats that had free access to normal rat chow but not to Ensure. When the consumption of Ensure was reexamined, treat-anticipating RT rats tended to eat moderately more Ensure than non-anticipating RT rats, but this effect was not significant ($P=0.075$).

RF but not RT modifies the daily pattern of PER2 expression

Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of AL rats killed at ZT1 or ZT13 and graphs showing daily patterns of PER2 expression in AL, RF and RT groups are shown in Fig. 3 and results from two-way ANOVA carried out for each brain region to assess group differences and changes across time are shown in Table 1. In AL rats the expression of PER2 in the SCN, BNSTov, CEA, BLA and DG was rhythmic, whereas expression in the DMH was arrhythmic, as previously reported (Amir et al., 2004; Lamont et al., 2005b; Mieda et al., 2006). Specifically, in the SCN, BNSTov and CEA maximal nuclear staining for PER2 was seen in the evening, at ZT 13, whereas in BLA and DG PER2 expression was maximal in the morning, at ZT1 (see Fig. 3).

In food-deprived rats, restricted access to Ensure for 10 days had no effect on PER2 expression in the SCN. The rhythm of PER2 expression in the SCN in RF rats was similar to that seen in the SCN of AL rats, peaking at ZT13. In contrast, peak PER2 expression in the BNSTov, CEA, BLA and DG of rats from the RF group shifted to ZT17, 12 h after the daily Ensure presentation (Fig. 3). RF induced a strong PER2 rhythm in the DMH which peaked around the time of food presentation, consistent with a previous report in mice (Mieda et al., 2006).

Finally, robust rhythms of PER2 expression were seen in the SCN, BNSTov, CEA, BLA and DG of rats from the RT group (Fig. 3). However, contrary to the effect of daily RF, daily RT had no effect on the rhythm of PER2 expression in these areas. In these rats, PER2 expression in all regions resembled that of AL rats, consistent with our previous observation that consumption of a highly palatable substance in the absence of food deprivation is insufficient to bring about a change in the rhythm of PER2 expression (Waddington Lamont et al., 2007).

Fos expression under scheduled daily limited access to Ensure

Daily RF, but not RT, shifted the phase of PER2 expression in the limbic forebrain and induced rhythms in PER2 expression in the DMH, suggesting that the two feeding schedules might exert quantitatively or qualitatively different effects on neural activity within these brain regions. To investigate this possibility we assessed the expression of the cellular activity marker, Fos, before, during and after Ensure presentation in a second set of brain sections obtained from AL, RF and RT rats.

Representative photomicrographs of Fos immunoreactivity from AL, RF and RT rats killed 1 h after Ensure presentation (ZT5) and graphs showing the levels of Fos expression from these groups 3 h before (ZT1) and 1 and 5 h after (ZT5, ZT9) Ensure presentation (ZT4–6) are shown in Fig. 4. In the SCN, the expression of Fos varied as a function of Time peaking at ZT1, 1 h after lights on in all three groups ($F[2,27]=6.86$; $P<0.001$). Likewise, Fos expression in the BNSTov varied as a function of Time ($F[2,27]=10.90$; $P<0.0003$). Furthermore, in the BNSTov there was also a significant Time×Group interaction ($F[4,27]=5.31$; $P<0.003$); expression of Fos at ZT5, 1 h after Ensure presentation, was significantly higher in the RF group than in the AL group (Fig. 4), but there was no difference between the AL the RT group.

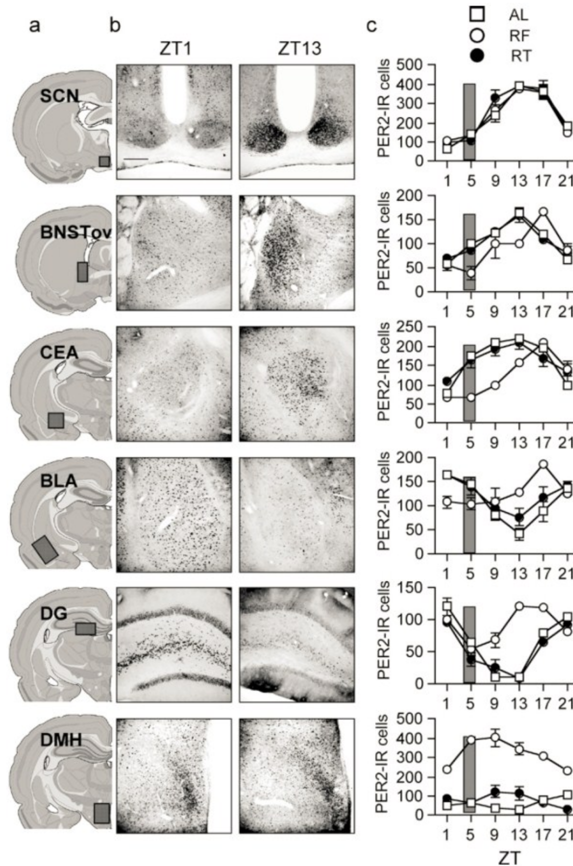

Fig. 3. RF, but not RT synchronizes PER2 expression. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of PER2 immunoreactivity. (b) Examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL) rats killed at ZT1 or 13 (scale bar=200 μm). (c) Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3–4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation.

In the CEA, Fos expression varied as a function of Time ($F[2,27]=6.29$; $P<0.006$), and there was a significant Time×Group interaction ($F[4,27]=8.25$; $P<0.002$). In this case, however, the level of Fos expression at ZT5 and ZT9, 1 and 5 h after Ensure presentation was greater in both the RF and RT groups compared with the AL group (Fig. 4). In the BLA, the RF and RT groups showed a similar patter of Fos expression at ZT1 and ZT5 and in each case levels were significantly different from those seen in the AL group (Time×Group: $F(4,27)=11.18$; $P<0.0001$). In the DG only the RF group showed elevated Fos expression at ZT5 (Time×Group: $F(4,27)=23.36$; $P<0.0001$). Finally, in the DMH Fos expression in both RF and RT groups was significantly higher than in AL rats at ZT5, 1 h after ensure presentation (Time×Group: $F(4, 27)=19.03$; $P<0.0001$).

Taken together, the results show that after 10 days of repeated exposure, presentation of Ensure to RF rats increases Fos expression in all the regions studied, except the SCN. In contrast, in RT rats, exposure to Ensure increases Fos expression only in the CEA, BLA and DMH relative to AL rats. Furthermore, at the time of food presentation the increases in Fos expression were usually greater in RF than in RT rats. Thus, although the presen-

Table 1. Results from ANOVAs carried out to assess the effect of feeding schedule and time of day on PER2 expression in each brain area under study

Brain area	Group	Time of day	Group×time
SCN	$F(2, 54)=0.68$, $P=0.5$	$F(5, 54)=91.40$, $P<0.0001$	$F(10, 54)=1.18$, $P=0.3$
BNSTov	$F(2, 54)=3.65$, $P=0.03$	$F(5, 54)=30.68$, $P<0.0001$	$F(10, 54)=5.85$, $P<0.0001$
CEA	$F(2, 54)=17.31$, $P<0.0001$	$F(5, 54)=27.70$, $P<0.0001$	$F(10, 54)=7.59$, $P<0.0001$
BLA	$F(2, 54)=2.02$, $P=0.14$	$F(5, 54)=8.24$, $P<0.0001$	$F(10, 54)=5.00$, $P<0.0001$
DG	$F(2, 54)=34.71$, $P<0.0001$	$F(5, 54)=34.52$, $P<0.0001$	$F(10, 54)=11.53$, $P<0.0001$
DMH	$F(2, 54)=248.61$, $P<0.0001$	$F(5, 54)=4.18$, $P=0.002$	$F(10, 54)=5.36$, $P<0.0001$

tation of Ensure activates neural elements in most regions in both RF and RT groups after repeated Ensure presentation, the magnitude of this activation appears to be modulated by the metabolic and motivational consequences of food deprivation.

PER2 and Fos expression following acute Ensure feeding

The results from the scheduled feeding experiment suggest that the mechanism regulating the expression of PER2 in the limbic forebrain and DMH is sensitive to signals associated with the repeated mitigation of a negative metabolic state. Daily limited access to Ensure in the absence of food deprivation did not alter PER2 expression, suggesting that incentive signals associated with daily presentation of Ensure are not effective. However, both RF and RT induced Fos expression in these same regions, albeit somewhat differentially, suggesting that Fos expression is related to the incentive or nutritive aspects of Ensure. To further test this idea, in a final experiment, we assessed Fos and PER2 expression before, during and after an acute presentation of Ensure in 24-h food-deprived and free-fed rats. Groups of free-fed and fasted (24 h) rats were killed 3 h before (ZT 1), and 1 (ZT5) and 5 h (ZT 9) after a single presentation of Ensure (ZT4–6).

As shown in Fig. 5, the first presentation of Ensure induced equally strong Fos expression in both groups in all regions, with the exception of the SCN where Fos expression was similar in all groups. PER2 expression was unaffected in all regions.

DISCUSSION

The results of the present experiments show that in food-deprived rats daily restricted access to a highly palatable food, chocolate Ensure, synchronizes the rhythms of PER2 expression in limbic forebrain regions involved in motivational and emotional regulation and uncouples them from the rhythm in the SCN. These results are consistent with previous findings on the synchronizing effect of daily RF on the expression of PER2 and other clock genes in the brain and periphery in rodents (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001; Challet et al., 2003; Mieda et al., 2006; Zvonic et al., 2006; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). We also found that in the absence of food deprivation daily restricted access to Ensure had no effect on PER2 rhythms in the limbic forebrain in spite of the fact that consumption equaled that in food-deprived rats. These results confirm and extend our previous observations in free-fed rats given restricted daily access to sucrose or saccharine solution (Waddington Lamont et al., 2007) by showing that even a highly palatable complete meal replacement has no effect on PER2 expression, suggesting that it is not the intake of nutrients, per se, that results in altered PER2 expression patterns. Furthermore, this supports the hypothesis that the effect on PER2 rhythms in these brain areas arises from signals associated with the daily alleviation of a negative metabolic state, and not from those associated with the incentive properties of the food. To the best of our knowledge there is one other study of the effect of daily RT on the expression of a clock gene in the brain in rats (Mendoza et al., 2005a). In this study it was found that presentation of a daily palatable meal changed the pattern of PER1 expression in the SCN and the paraventricular thalamic nucleus, an area known to be highly sensitive to a range of arousing and rewarding stimuli. These results are at odds with the present finding of a lack of effect of RT on PER2 expression in the SCN. These differences could be attributed to methodological differences, such as the number of days of limited access (6 weeks in the Mendoza et al. (2005a) study compared with <2 weeks in the present study), the nature of the treat (chocolate bar in the Mendoza et al. (2005a) study), type of limitation (limiting the amount of chocolate in the Mendoza et al. (2005a) study instead of limiting the time treat was available in the current study), lighting conditions (constant darkness in the Mendoza et al. (2005a) study instead of a light/dark cycle in the present study), and the type of clock protein measured (PER1 in the Mendoza et al. (2005a) study instead of PER2 in the present study). Furthermore, differences in patterns of expression of PER1 and PER2 have also been observed in rats under RF schedules (Lamont et al., 2005a; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007) suggesting that the mechanism(s) that control the expression of different clock genes in the brain could be differentially sensitive to signals associated with feeding.

Our findings indicate that the rhythms of PER2 expression in the limbic forebrain are modulated by nutritional status and are insensitive to the incentive properties of food, per se. To study whether the signals associated with the eating of Ensure in free-fed and food-deprived rats gained access to the regions under study we measured Fos induction before, during and after chronic Ensure presentation. In addition, we assessed Fos expression in

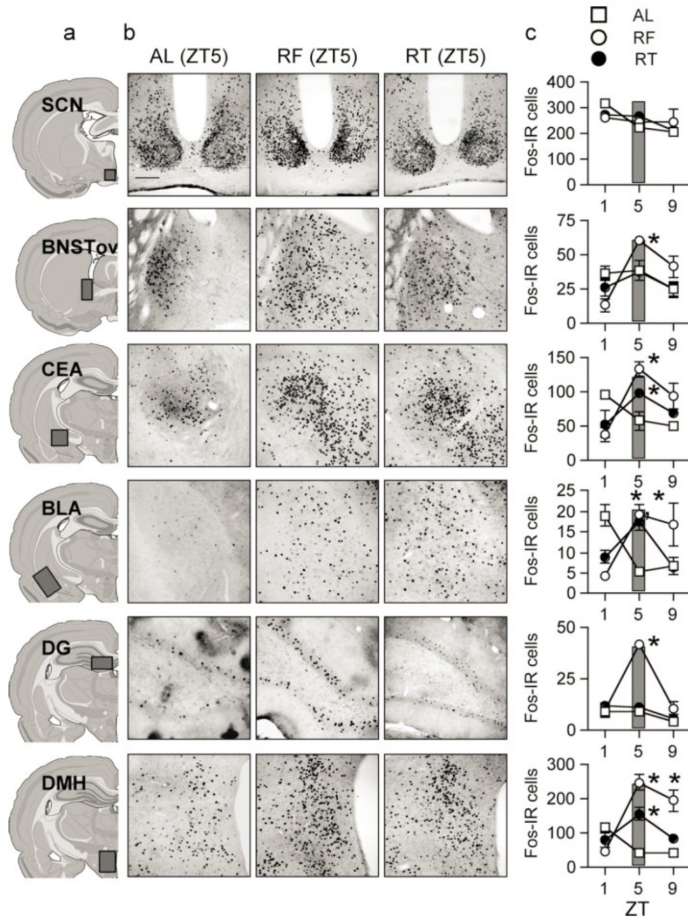

Fig. 4. After 10 days of repeated exposure, presentation of Ensure increases Fos expression in all regions (except the SCN) in RF rats, but only the CEA, BLA and DMH in RT rats. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos immunoreactivity. (b) Examples of Fos expression in the SCN, BNSTov, CEA, BLA, DG and DMH in control (AL), RF and RT rats killed at ZT5 (scale bar=200 μm). (c) Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=3–4/group). Vertical dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4–6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

food-deprived or free-fed rats receiving an acute presentation of Ensure. In rats that received daily Ensure presentations, we found that with the exception of the BNSTov and DG, restricted daily access to Ensure induced a significant increase in the expression of Fos in the limbic forebrain in both food-deprived and free-fed rats, indicating that the expression of Fos in these regions is modulated primarily by signals associated with the incentive or metabolic properties of the consumed food. This conclusion is further supported by the results from rats that received Ensure for the first time, where Fos expression increased equally in all regions (except the DG and SCN) regardless of nutritional state. However, a role for nutritional status in the modulation of Fos expression is suggested by the finding that, in rats given daily restricted access to Ensure, the increase in Fos expression in the different regions was greater in food-deprived than free-fed rats, (except the BLA and SCN). Together, the results from the rats given

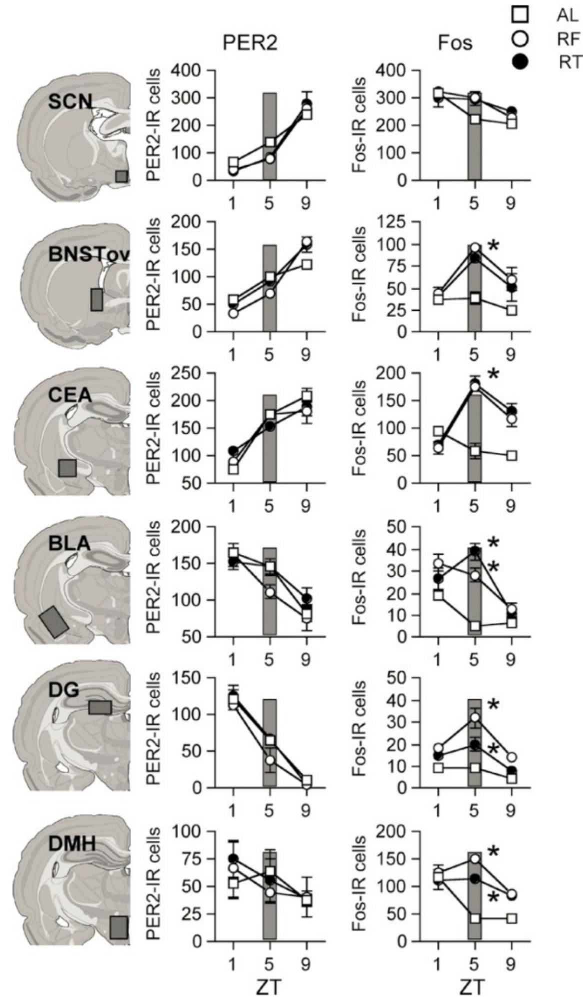

Fig. 5. Ensure presentation for the first time does not affect PER2 expression but it increases Fos expression in all areas (except the SCN) in food-deprived (24 h; RF) and free-fed (RT) rats. (a) Brain maps showing location of regions under study. The dotted square in each map indicates the area scanned for quantification of Fos and PER2 immunoreactivity. (b) Graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in control (AL), RF and RT rats (n=4/group). (c) Graphs showing mean (±S.E.M.) number of Fos-immunoreactive (Fos-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats (n=4/group). Dotted rectangles inside the graphs indicate the time of Ensure presentation (ZT4–6). Asterisks indicate significant difference from corresponding AL group (Student-Newman-Keuls, $P<0.05$).

daily restricted access to Ensure are consistent with previous findings on the expression of Fos in multiple limbic areas in rats under RF and RT (Angeles-Castellanos et al., 2004, 2005, 2007; Mendoza et al., 2005a,b,c; Gooley et al., 2006). Furthermore, they suggest that the neural signals and cellular events leading to Fos induction in the BNSTov, CEA, BLA and DG under scheduled RF and RT and those responsible for the changes in PER2 expression

these limbic forebrain regions are, at least in part, functionally dissociable.

In addition to the differences in PER2 and Fos expression discussed above, we observed differences in the prevalence of food anticipatory wheel-running. Previous studies have shown that daily limited access to a palatable meal can entrain food anticipatory rhythms in free-fed rats (Mistlberger and Rusak, 1987; Mendoza et al., 2005a); however, the aim of our study was to compare the metabolic and motivational consequences of the daily consumption of Ensure in food-deprived and free-fed rats and we used a protocol too short to reliably observe treat-anticipation. In the present study all food-deprived rats showed robust anticipatory wheel-running, whereas only 37% of free-fed rats that received daily limited access to Ensure did. To assess whether these behavioral differences, rather than differences in nutritional state, could account for the effects on PER2 expression, we compared rhythms of PER2 between free-fed rats that showed anticipatory wheel-running ($n=9$) and those that did not ($n=15$). We found that the circadian patterns of PER2 expression in these two sub-groups were indistinguishable and not different from those in AL control rats (data not shown). This finding suggests that anticipatory wheel-running, as such, does not account for the differences in the rhythms of PER2 expression.

We also studied PER2 and Fos expression in the DMH, a region of the hypothalamus that receives a major innervation from the SCN (Thompson and Swanson, 1998) and that has been implicated both in SCN-driven circadian rhythms (Chou et al., 2003) and in the control of circadian food anticipatory wheel-running activity and temperature rhythms (Gooley et al., 2006). Consistent with a previous study in mice (Mieda et al., 2006), we found that in free-fed rats the expression of PER2 in the DMH is arrhythmic and that in food-deprived rats, daily limited access to Ensure induces a robust rhythm that peaks around the time of food presentation. Importantly, we found that as in the case of the limbic forebrain, daily limited access to Ensure in free-fed rats had no effect on PER2 expression in the DMH whereas Fos was expressed equally in the DMH in both food-deprived and free-fed rats. These findings further demonstrate the dissociation between the effects of Ensure presentation on neuronal activation and on changes in rhythms of PER2 expression.

The findings concerning PER2 expression in the DMH are at odds with the hypothesis that the induction of a rhythm of PER2 in this region is critical for the expression of food anticipatory behavior (Mieda et al., 2006). At least 37% of free-fed rats given daily access to Ensure showed anticipatory wheel-running yet no changes in PER2 expression were observed in the DMH. In fact, it is possible that an even greater proportion of RT rats may have shown other forms of anticipatory behavior such as increased activity around the food receptacle before mealtime (Landry et al., 2006), but these alternate anticipatory behaviors were not tested. Thus, although our study confirms the previous finding that daily restricted access to food induces a robust rhythm of PER2 expression in the DMH, we do not have evidence that this rhythm plays a critical role in the expression of food-anticipatory behaviors.

Taken together the present experiments show that daily restricted access to a highly palatable meal produces marked changes in PER2 rhythms in BNSTov, CEA, BLA, DG, and DMH in food-deprived rats, but does not affect the rhythms in these structures in free-fed rats. The evidence from the studies of Fos induction, shows that this lack of effect is not due to differences in the ability of food stimuli to gain access to these various brain regions. Thus, we conclude on the basis of these data and those from our previous studies that the effects on PER2 rhythms are due to the daily alleviation of a negative metabolic state and not to signals arising from the incentive or metabolic properties of the food, per se. We cannot, however, at this time relate these changes to any particular behavioral outcome of scheduled feeding. Although there is evidence from studies in mutant mice that global functional disruption of PER2 does affect the expression food anticipatory locomotor activity and temperature rhythms (Feillet et al., 2006), the particular brain regions important for these changes remain to be identified.

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Appendix C: REPRINT (CHAPTER 2) - Region-specific modulation of PER2 expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats

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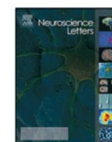

Region-specific modulation of PER2 expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats

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ABSTRACT

Feeding schedules that restrict food access to a predictable daytime meal induce in rodents food-anticipatory behaviors, changes in physiological rhythms and shifts in the rhythm of clock gene expression in the brain and periphery. However, little is known about the effects of nighttime restricted feeding. Previously, we showed that daytime restricted access to a highly palatable complete meal replacement, Ensure Plus (Ensure), shifts the rhythm of expression of the clock protein PER2 in limbic forebrain areas including the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG), and induces a rhythm in the dorsomedial hypothalamic nucleus (DMH) in food deprived (restricted feeding), but not free-fed rats (restricted treat). In the present study we investigated the effects of nighttime restricted feeding (Ensure only, 2 h/night) and nighttime restricted treats (Ensure 2 h/night + free access to chow) in order to determine whether these effects were dependent on the time of day the meal was provided. We found that nighttime restricted feeding, like daytime restricted feeding, shifted the rhythm of PER2 expression in the BNSTov and CEA and peak expression was observed ~12 h after the mealtime. Also consistent with previous work, nighttime restricted feeding induced a rhythm of PER2 expression in the DMH and these effects occurred without affecting the rhythm in the suprachiasmatic nucleus (SCN). In contrast to previous work with daytime restricted feeding, nighttime restricted feeding had no effect on PER2 rhythms in the BLA and DG. Finally, nighttime restricted treats, as was the case for daytime restricted treats, had no effect on PER2 expression in any of the brain areas studied. The present results together with our previous findings show that the effect of restricted feeding on PER2 rhythms in the limbic forebrain and hypothalamus depend on a negative energy balance and vary as a function of time of day in a brain region-specific manner.

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Feeding schedules that restrict food-availability to the middle of the day in rodents induce characteristic food-anticipatory rhythms in locomotor activity, body temperature and corticosterone release that are independent of the primary circadian clock in the suprachiasmatic nucleus (SCN) [16,22,23]. Furthermore, such restricted feeding schedules shift the rhythms of expression of clock genes and clock proteins in the brain and periphery without affecting the rhythms in the SCN [3,5,6,9–11,14,24,28,29]. It is unclear, however, whether the behavioral and molecular changes induced by daytime restricted feeding are due to the daily cycle of food deprivation and refeeding, as such, or whether they are due to the fact that food is presented at a time of day when nocturnal rodents are relatively inactive and do not normally eat.

We have shown previously that restricted feeding with a predictable daytime access to the complete meal replacement Ensure Plus (Ensure) or with standard laboratory chow, shifts the rhythms of expression of the clock protein PER2 in limbic forebrain structures involved in motivational and emotional regulation, including the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG) [1,13,26,27]. Moreover, daytime restricted feeding also induces a rhythm of PER2 expression in the dorsomedial hypothalamic nucleus (DMH), an area implicated in the control of food-anticipatory rhythms [8,26]. In contrast to restricted feeding, when a similar daytime access to Ensure was delivered to free-fed rats (restricted treat) it had no effect on PER2 rhythms in any of these brain regions, emphasizing the importance of a negative energy balance in these effects [26,27]. To investigate the importance of time of day, the present study placed food-deprived and free-fed rats on nighttime restricted access to Ensure and assessed the effect on rhythms of running-wheel activity and on rhythms of PER2 expression in the SCN, BNSTov, CEA, BLA, DG,

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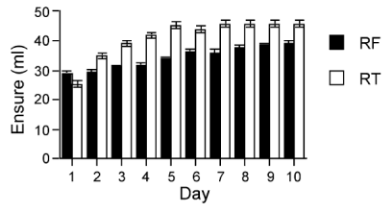

Fig. 1. Mean (±S.E.M.) daily intake (ml) of chocolate Ensure in food deprived (RF, $n = 24$) and free-fed (RT, $n = 24$) rats. Ensure was presented for 2 h each night from ZT16-18 (4–6 h after lights-off) in each group.

and DMH. Preliminary results have been presented in an abstract form [25].

All experimental procedures in this study followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. Every effort was made to reduce the number of animals used and to minimize potential suffering. Male Wistar rats (225–250 g; Charles River Laboratories, St. Constant, QC, Canada) were individually housed in cages equipped with running-wheels, under a 12:12-h light-dark (LD) schedule (∼300 lux at cage level) and had free access to Purina rat chow and water. Running-wheel activity was continuously monitored using VitalView software (Mini Mitter Co. Inc., Sunriver, OR) and analyzed with Circadia software. Following acclimation to the housing environment, one group of rats (group RF; restricted feeding) was food deprived and placed on a nighttime restricted feeding schedule in which Ensure Plus (Ensure; Abbott Laboratories, Abbott Park, IL, USA) was made available for 2 h each night for 10 nights, from zeitgeber time (ZT) 16–18 (ZT12 denotes time of lights off in a 12:12 LD schedule). A second group was given the same nighttime restricted access to Ensure, but had free access to lab chow at all times (group RT, restricted treat). A third group of rats was given *ad libitum* access to normal rat chow only (group AL).

On the day following the last scheduled presentation of Ensure, rats were deeply anesthetized with an overdose of sodium pentobarbital (∼100 mg/kg) at one of six ZTs (ZT1, 5, 9, 13, 17, 21) and perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Serial coronal brain sections (50 μm) were taken using a vibratome. Immunocytochemistry for PER2 was performed as previously described using an affinity purified rabbit polyclonal antibody raised against PER2 (1:800, ADI, San Antonio, TX) [26]. PER2-stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 were counted using the captured images. For analysis, the mean number of PER2-immunoreactive cells per region was calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analyses of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

Fig. 1 shows the amount of Ensure consumed each night during the 2-h access period by rats from the RF and RT groups. It

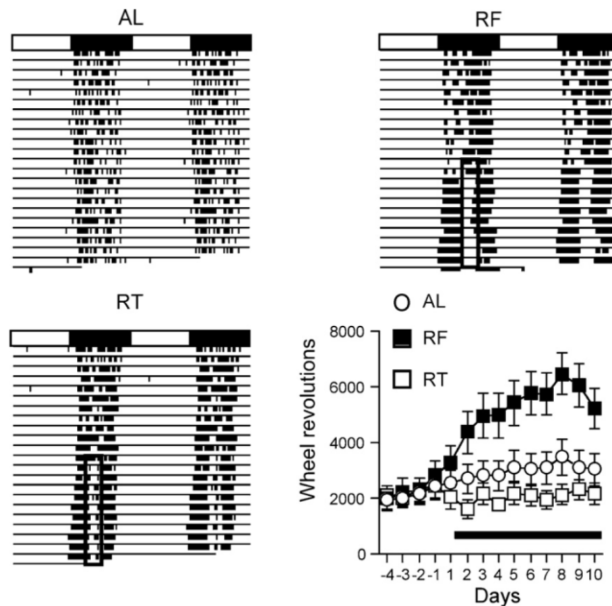

Fig. 2. Double-plotted actograms of wheel-running activity from representative rats from the free-fed, *ad libitum* (AL) control group, the restricted feeding group (RF) and the restricted treat group (RT). The nightly presentation of Ensure occurred from ZT16-18 (4–6 h after lights-off; illustrated by rectangles). All rats were housed under a 12:12 h LD cycle which is illustrated by the bars at the top of each actogram. The vertical marks indicate periods of activity of at least 10 wheel-revolutions/10 min. Successive days are plotted from top to bottom. The graph in the lower right shows mean (±S.E.M.) total daily number of wheel-revolutions per group (ns = 24) starting 4 days before and throughout the 10 days of restricted feeding.

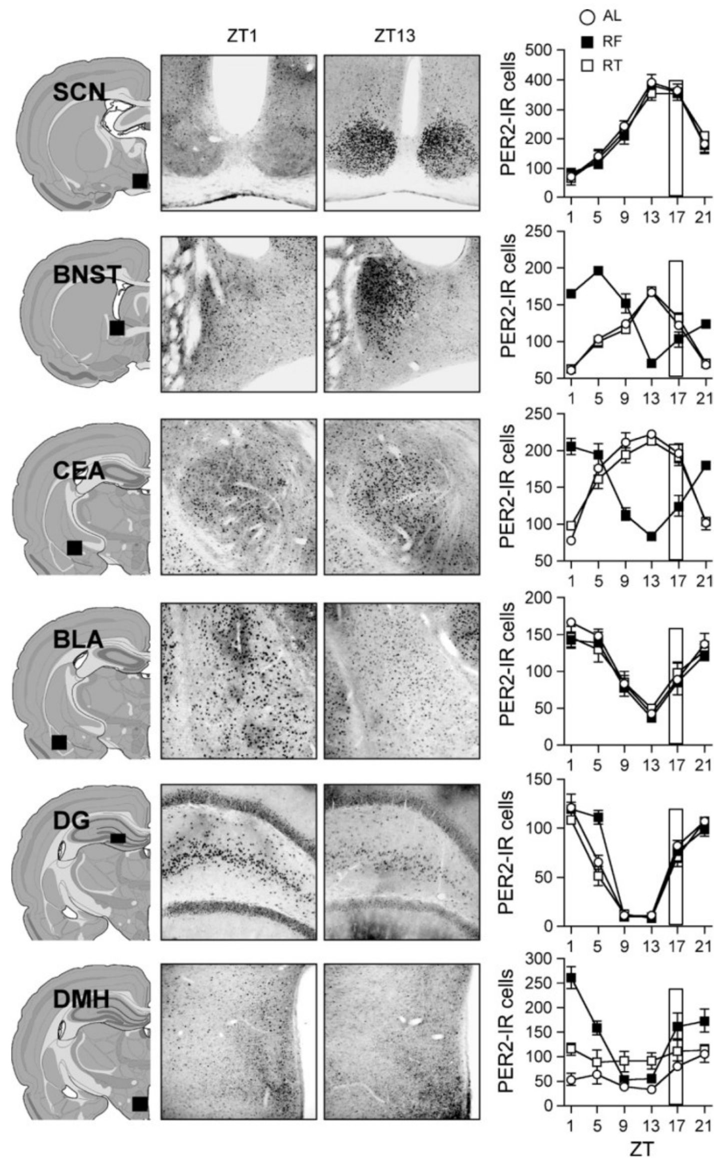

Fig. 3. PER2 expression in control (AL) and in food-deprived (RF) and free-fed (RT) rats under nighttime restricted access to Ensure. Left panel, brain maps showing location of regions under study. The shaded square in each map indicates the area scanned for quantification of PER2 immunoreactivity. Middle panel, examples of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH in AL rats killed at ZT1 or 13. Right panel, graphs showing mean (±S.E.M.) number of PER2-immunoreactive (PER2-IR) nuclei in the SCN, BNSTov, CEA, BLA, DG and DMH as a function of ZT in AL, RF and RT rats ($n = 4$/group). Vertical rectangles inside the graphs indicate the time of Ensure presentation.

can be seen that with the exception of the first night of limited access, rats from the RT group consumed more Ensure than rats from the RF group throughout the experiment ($P < .001$). In addition, rats in the RT group continued to eat chow and continued to gain weight, whereas the RF group lost weight. This points to a fundamental difference in energy balance between these groups. Fig. 2 shows representative double-plotted actograms of wheel-running activity for one rat from each of the three groups (AL, RF and

Table 1
Results from ANOVAs carried out to assess the effect of feeding schedule (AL, NF, NT) and time of day on PER2 expression in each brain area under study

Brain area	Group	Time of Day	Group × time
SCN	$F_{2,54}=0.319$, n.s.	$F_{5,54}=98.9$, $P<0.001$	$F_{10,36}=0.498$, n.s.
BNSTov	$F_{2,54}=34.3$, $P<0.001$	$F_{5,54}=29.6$, $P<0.001$	$F_{10,54}=45.0$, $P<0.001$
CEA	$F_{2,54}=3.35$, $P=0.42$	$F_{5,54}=18.6$, $P<0.001$	$F_{10,54}=37.7$, $P<0.001$
BLA	$F_{2,54}=1.13$, n.s.	$F_{5,54}=34.1$, $P<0.001$	$F_{10,54}=0.314$, n.s.
DG	$F_{2,54}=4.17$, $P=0.021$	$F_{5,54}=136$, $P<0.001$	$F_{10,54}=3.71$, $P=0.001$
DMH	$F_{2,54}=33.8$, $P<0.001$	$F_{5,54}=13.3$, $P<0.001$	$F_{10,54}=6.00$, $P<0.001$

RT). All rats from the RF group showed clear changes in running-wheel patterns, developing a period of reduced running-wheel use during the Ensure presentation, but an overall increase in running-wheel activity over the 24-h day. Rats from the RT group showed reduced running-wheel use during the 2-h Ensure presentation, but no overall increase in running (Fig. 2). ANOVA shows a significant effect of group ($F[2,897]=5.81$, $P<.004$) and a group × day interaction ($F[26,897]=12.95$, $P<.0001$).

The daily patterns of PER2 expression in AL, RF and RT groups are shown in Fig. 3. In both the AL and RT groups PER2 expression was similar and exhibited a circadian rhythm in the SCN, BNSTov and CEA which peaked at ZT13, whereas in the BLA and DG the rhythm peaked at ZT1, as previously described [26]. In the DMH, the expression of PER2 in AL and RT groups differed little as a function of time of day. In contrast, the RF group exhibited rhythms of PER2 expression in the BNSTov and CEA that were shifted and peaked around ZT1-5. The rhythms of PER2 expression in BLA and DG were not shifted in the RF group and resembled those in the AL and RT groups. Finally, nighttime RF induced a robust rhythm of PER2 expression in the DMH. The results from the group x time ANOVAs for each brain area are shown in Table 1.

Restricted feeding is a powerful synchronizer of behavioral and physiological circadian rhythms and of rhythms of expression of clock genes in the brain and periphery in rodents [5,14,16,22]. However, most studies on the circadian effects of such feeding schedules restrict food-availability to a daytime meal. The results of the present study in nighttime fed rats show that, indeed, the time of day meals are presented can play a significant role in the effects of restricted feeding on PER2 rhythms in the limbic forebrain and hypothalamus. We found that contrary to daytime restricted feeding, when rhythms of PER2 expression were shifted in all structures studied, nighttime restricted feeding had no effect on PER2 rhythms in the BLA and DG. This finding indicates that the effect of restricted feeding on PER2 expression in the BLA and DG seen in our previous study on daytime restricted feeding did not result from a negative energy balance, as such, but was dependent on some aspect unique to daytime restricted feeding.

Consistent with our earlier study on daytime restricted feeding, nighttime restricted feeding shifts the rhythm of PER2 expression in the BNSTov and CEA. In both cases PER2 expression peaks ~12 h after the meal. We also found that, as was the case with daytime restricted feeding, nighttime restricted feeding induced a rhythm of PER2 expression in the DMH [15,26]. These results support the conclusion that unlike the BLA and DG, the effect of restricted feeding on PER2 rhythms in the BNSTov, CEA and DMH is strongly linked to a negative energy balance and is independent of the time of day when food is presented. The finding that restricted feeding induces a rhythm of PER2 expression in the DMH is particularly interesting in view of recent evidence implicating both Per2 and the DMH in the expression of certain food-anticipatory rhythms [7,8,15]. Finally, we found that in the absence of food deprivation nighttime restricted access to Ensure had no effect on PER2 expression in any of the brain regions under study. These results add support for the conclusion that the effects of scheduled access to Ensure on PER2 rhythms are linked primarily to its nutritional value and are relatively independent of its incentive properties, per se [26].

The anticipatory behavioral and physiological circadian rhythms associated with restricted feeding are known to be independent of the SCN [23]. However, restricted feeding can affect clock gene expression in the SCN under some circumstances [4,5,12]. Our present findings suggest that some SCN-driven signal could be modulating the daily sensitivity of clock gene expression in the BLA and DG to metabolic cues associated with restricted feeding. Indeed, the finding that restricted feeding shifts PER2 expression in the BLA and DG after daytime but not nighttime restricted feeding is reminiscent of the phase dependency of other synchronizing stimuli, photic as well as non-photic, whose effectiveness is temporally modulated by the SCN clock [17,18,20].

The rhythms of PER2 expression in the BNSTov and CEA are distinct from those in the BLA and DG in several ways, including phase of peak expression and sensitivity to glucocorticoid, thyroid and gonadal hormones [1,2,13,19,21]. The present findings show that the rhythms in these structures are also distinct in their sensitivity to restricted feeding. Specifically, contrary to what was observed in the BLA and DG, PER2 rhythms in BNSTov and CEA were equally affected by daytime and nighttime restricted feeding, suggesting that the sensitivity of the BNSTov and CEA to feeding cues is not gated temporally across the day. Albeit, we are currently unable to explain the nature of the unwavering sensitivity of PER2 rhythms in the BNSTov and CEA to restricted feeding.

In summary, the present findings concerning the effect of nighttime restricted feeding on PER2 rhythms in the limbic forebrain, taken together with our previous work on daytime restricted feeding, point to a complex brain region-dependent interaction between feeding cues and the time of day food is presented. In the BLA and DG the effect of restricted feeding depends on a negative energy balance, but is gated by the time of day. In BNSTov, CEA and DMH the effect of feeding on PER2 expression appears to depend solely on a negative energy balance. The basis and functional consequences of these region-specific differences remain to be determined.

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Appendix D: REPRINT (CHAPTER 3) - Circadian rhythms of PERIOD1 expression in the dorsomedial hypothalamic nucleus in the absence of entrained food-anticipatory activity rhythms in rats

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BEHAVIORAL NEUROSCIENCE

Circadian rhythms of PERIOD1 expression in the dorsomedial hypothalamic nucleus in the absence of entrained food-anticipatory activity rhythms in rats

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Keywords: immunohistochemistry, restricted feeding, suprachiasmatic nucleus, wheel running activity rhythm

Abstract

When food availability is restricted to a single time of day, circadian rhythms of behavior and physiology in rodents shift to anticipate the predictable time of food arrival. It has been hypothesized that certain food-anticipatory rhythms are linked to the induction and entrainment of rhythms in clock gene expression in the dorsomedial hypothalamic nucleus (DMH), a putative food-entrained circadian oscillator. To study this concept further, we made food availability unpredictable by presenting the meal at a random time each day (variable restricted feeding, VRF), either during the day, night or throughout the 24-h cycle. Wheel running activity and the expression of the clock protein, Period1 (PER1), in the DMH and the suprachiasmatic nucleus (SCN) were assessed. Rats exhibited increased levels of activity during the portion of the day when food was randomly presented but, as expected, failed to entrain anticipatory wheel running activity to a single time of day. PER1 expression in the SCN was unchanged by VRF schedules. In the DMH, PER1 expression became rhythmic, peaking at opposite times of day in rats fed only during the day or during the night. In rats fed randomly throughout the entire 24-h cycle, PER1 expression in the DMH remained arrhythmic, but was elevated. These results demonstrate that VRF schedules confined to the day or night can induce circadian rhythms of clock gene expression in the DMH. Such feeding schedules cannot entrain behavioral rhythms, thereby showing that food-entrainment of behavior and circadian rhythms of clock gene expression in the DMH are dissociable.

Introduction

Feeding schedules that restrict food availability to a predictable time each day induce characteristic food-anticipatory circadian rhythms in behavior and physiology in rodents (Richter, 1922; Stephan, 2002; Mistlberger, 2006). Food-anticipatory rhythms appear to rely on endogenous food-entrained circadian oscillators that are independent from the master light-entrained circadian clock in the suprachiasmatic nucleus (SCN; Stephan, 1983; Mistlberger *et al.*, 1996; Marchant & Mistlberger, 1997). The dorsomedial hypothalamic nucleus (DMH), a brain area implicated in the mediation of SCN-driven circadian rhythms (Chou *et al.*, 2003), has recently been proposed to harbor a food-entrainable circadian oscillator that is both necessary and sufficient for the expression of some food-anticipatory rhythms (Gooley *et al.*, 2006; Fuller *et al.*, 2008). This proposal is based on the finding that DMH lesions can block some food-anticipatory rhythms and is supported by the evidence that restricted feeding schedules induce rhythmic expression of clock genes in the DMH (Gooley *et al.*, 2006; Mieda *et al.*, 2006; Verwey *et al.*, 2007, 2008; Fuller *et al.*, 2008). Although there is evidence suggesting that some clock genes play an essential role in food anticipation, evidence linking clock gene rhythms in the DMH, *per se*, and the expression of food-entrained behavioral rhythms is equivocal (Feillet *et al.*, 2006; Fuller *et al.*, 2008; Mistlberger *et al.*, 2008; Storch & Weitz, 2008).

Predictable restricted feeding schedules reliably promote distinct food-anticipatory circadian rhythms in behavior and physiology, and strongly induce the rhythmic expression of clock proteins, such as period1 (PER1) and period2 (PER2), in the DMH (Mieda *et al.*, 2006; Saper & Fuller, 2007; Verwey *et al.*, 2007, 2008; Fuller *et al.*, 2008). Here we describe the rhythmic expression of PER1 in the DMH and wheel-running activity rhythms in rats exposed to unpredictable feeding schedules where food was restricted to a different time each day. Such variable restricted feeding (VRF) schedules reproduce the daily fluctuations in energy balance normally associated with restricted feeding but, due to their unpredictable nature, preclude the emergence and entrainment of precise food-anticipatory circadian rhythms (Escobar *et al.*, 2007). In the present experiment, food was presented at variable and unpredictable times during the 12-h day (daytime VRF), the 12-h night (nighttime VRF) or anytime across the entire 24-h day (anytime VRF) for a period of 10 days.

Materials and methods

Animals and housing

All experimental procedures followed the guidelines set out by the Canadian Council on Animal Care (http://www.ccac.ca/) and were

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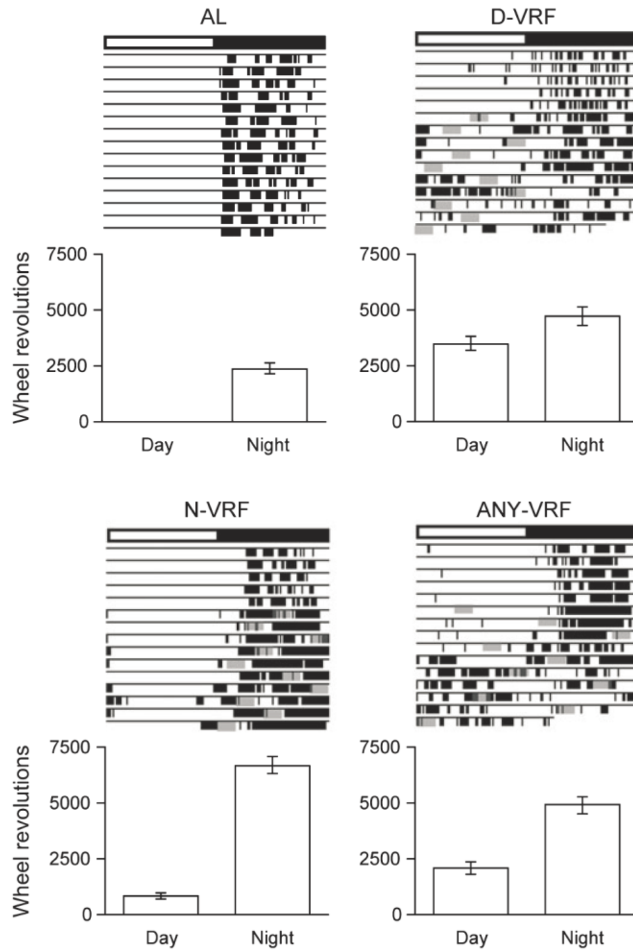

FIG. 1. Wheel running activity records for a single representative rat from the *ad libitum* (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups. Below each actogram, bar graphs show the average wheel running activity in the day and night for Days 5–9 (inclusive) for all rats in each group (n = 15–16/group). Actograms illustrate the 12 : 12 h LD cycle at the top of each record and sequential days are plotted from top to bottom. Vertical marks indicate periods of activity of at least 5 wheel-revolutions/10 min. Semi-transparent rectangles indicate time of Ensure availability.

approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada). All efforts were made to minimize the number of rats used; a total of 63 male Wistar rats (Charles River Laboratories, St Constant, QC, Canada) weighing 225–250 g at the beginning of the study were used. All rats were housed individually in cages equipped with running wheels under a 12 : 12 h light–dark cycle (LD cycle; ~300 lux at cage level) and had free access to standard rodent diet (#5075; Charles River Laboratories) and water for at least 2 weeks before each experiment. Wheel running activity was recorded continuously by computer (VITALVIEW; Minimitter, Bend, OR, USA).

VRF

Rats were randomly assigned to one of four groups: daytime VRF; nighttime VRF; anytime VRF; or *ad libitum* (AL) chow. In accordance with the Animal Care and Use Committee at Concordia University, all VRF groups were fasted and received the highly palatable complete meal replacement, chocolate Ensure Plus (Ensure, 1.5 Cal/mL; see complete nutritional facts at http://ensure.com/) for 2 h each day; experimental feeding schedules lasted 10 days. The daytime VRF group received access to Ensure starting at zeitgeber time (ZT; ZT0 = when environmental lights turn on) 6, 3, 10, 4, 1, 7, 10, 2, 5 and 0, for Days 1–10, respectively. The nighttime VRF group received access to Ensure starting at ZT (ZT12 = environmental lights turn off) 18, 15, 22, 16, 13, 19, 22, 14, 17 and 12, for Days 1–10, respectively. Rats in the anytime VRF group received access to Ensure starting at ZT4, 13, 21, 7, 14, 10, 19, 6, 15 and 0, for Days 1–10, respectively.

Tissue preparation and immunohistochemistry

On the last day of the experiment, rats were deeply anesthetized with sodium pentobarbital (Somnotol, 100 mg/kg) at one of four ZTs (ZT1, 7, 13 or 19). Rats were perfused transcardially with 300 mL of cold saline (4°C; 0.9% NaCl in distilled water) followed by 300 mL of cold

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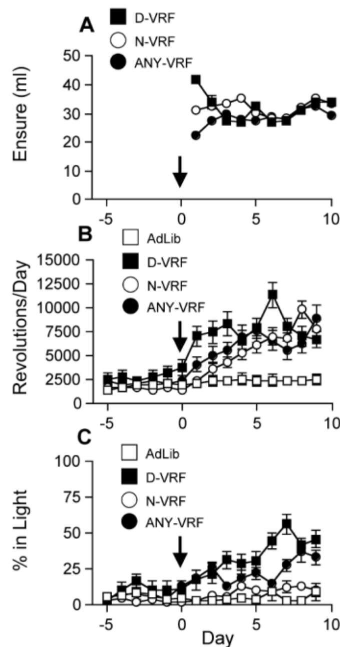

FIG. 2. (A) Ensure consumption for each group across the 10-day variable restricted feeding (VRF) schedules, (B) total number of wheel running revolutions per 24 h for each day of the experiment, and (C) percentage of total wheel running activity that took place during the 12 h of light. Symbols and vertical lines indicate mean ± SEM (n = 15–16/group). Arrows indicate the start of restricted feeding schedules.

paraformaldehyde solution (4°C; 4% paraformaldehyde in 0.1 M phosphate buffer), and brains were post-fixed for 24 h in cold 4% paraformaldehyde solution. Serial coronal sections (50 μm) containing the regions of interest were collected using a vibratome and stored in Watson's cryoprotectant at −20°C until processing (Watson *et al.*, 1986).

Immunohistochemistry was performed using established protocols (Amir *et al.*, 2004). Briefly, brain sections containing the SCN and DMH were incubated (40 h, 4°C) in a primary solution with polyclonal rabbit antibodies for PER1 (1 : 24 000; generous gift from Dr S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) and 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada) in 5% milk buffer in a Triton Trizma-buffered saline solution (0.3% Triton, 50 mM Trizma buffer, 0.9% saline). Free-floating sections were then incubated in a secondary antibody solution with biotinylated anti-rabbit IgG made in goat (1 : 200; Vector Laboratories), followed by an incubation in an Avidin–Biotin-Peroxidase solution (Vectastain Elite ABC Kit; Vector Laboratories). Sections were then rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution, and immunoreactive (IR) cells were finally stained with a solution containing 0.5% DAB with 0.01% H_2O_2 and 8% $NiCl_2$. Blocking experiments performed by adding the PER1 peptide (1 mg/mL in phosphate-buffered saline) to the primary incubation solution prevented PER1 staining. Brain sections were mounted on gelatin-coated slides, dehydrated with alcohols, cleared with Citrisolv and glass coverslips were fixed in place with permount.

Microscopy and data analysis

PER1-stained sections were examined under a light microscope, and images of the SCN and DMH were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA) and image SXM software (v1.6, S D Barrett; http://www.ImageSXM.org.uk). IR cells were counted for each image using a 400× 400 μm template, and means were calculated for each brain area based on the six unilateral images with the highest number of IR cells. Differences between groups were determined with analysis of variance (ANOVA), where the alpha level was set at 0.05.

Results

Food intake and wheel running activity

Wheel running activity records for representative rats from the AL, daytime VRF, nighttime VRF and anytime VRF groups as well as bar graphs showing the average day/night activity for each group for the last 5 days of the VRF schedule are shown in Fig. 1. Graphs showing daily Ensure consumption as well as the daily totals and daily distributions of wheel running activity are shown in Fig. 2. With the exception of the first few days of the experiment, Ensure consumption was virtually the same in all VRF groups (Fig. 2A). However, throughout the course of the entire 10-day protocol, differences in weight loss were observed between groups**.** Specifically, whereas the daytime VRF group lost 85 ± 5 g (mean ± SEM) or 24% of their starting weight, the nighttime and anytime VRF groups lost 58 ± 3 g (~17%) and 61 ± 4 g (~17%) of their starting weight, respectively. VRF schedules increased the total amount of daily wheel running activity by two–three-fold relative to daily running activity in AL controls (Fig. 2B). Moreover, VRF schedules increased daytime running (Fig. 2C) as compared with the AL control group. Daytime VRF was associated with the largest increase in daytime running. Nighttime VRF also resulted in a small increase in the percentage of daytime activity, while anytime VRF exhibited an intermediate increase in daytime running. Significantly, none of the VRF schedules led to the emergence of characteristic food-entrained circadian rhythms in anticipatory wheel running activity.

PER1 expression

Photomicrographs showing examples of PER1 expression in the SCN and DMH of rats from the AL, daytime VRF, nighttime VRF and anytime VRF groups are shown in Fig. 3, and graphs showing mean PER1 expression as a function of ZT for all VRF groups relative to AL values are shown in Fig. 4. In all groups, PER1 expression in the SCN was rhythmic (ANOVA$_{TIME}$; AL: $F_{3,12}$ = 17.6, P < 0.001; daytime VRF: $F_{3,11}$ = 18.9, P < 0.001; nighttime VRF: $F_{3,12}$ = 28.8, P < 0.001; anytime VRF: $F_{3,12}$ = 30.6, P < 0.001), peaking at about ZT13 (see Fig. 4). In contrast, in the DMH, PER1 expression varied as a function of feeding schedule (see Fig. 4). The expression of PER1 in the DMH in AL fed rats was arrhythmic (ANOVA$_{TIME}$, $F_{3,12}$ = 1.76, P = 0.21). In contrast, daytime VRF induced a circadian rhythm of PER1 expression in the DMH that peaked at about ZT13 (ANOVA$_{TIME}$, $F_{3,11}$ = 37.9, P < 0.0001). Under nighttime VRF, PER1 expression in the DMH was also rhythmic but, contrary to daytime VRF, the peak expression occurred at about ZT1 (ANOVA$_{TIME}$, $F_{3,12}$ = 8.68, P < 0.002). When VRF occurred anytime across the day and night, PER1 expression in the DMH was arrhythmic (ANOVA$_{TIME}$, $F_{3,12}$ = 1.48, P = 0.26), although overall levels were higher than those seen in the AL group (ANOVA$_{GROUP}$, $F_{1,24}$ = 5.6; P < 0.05).

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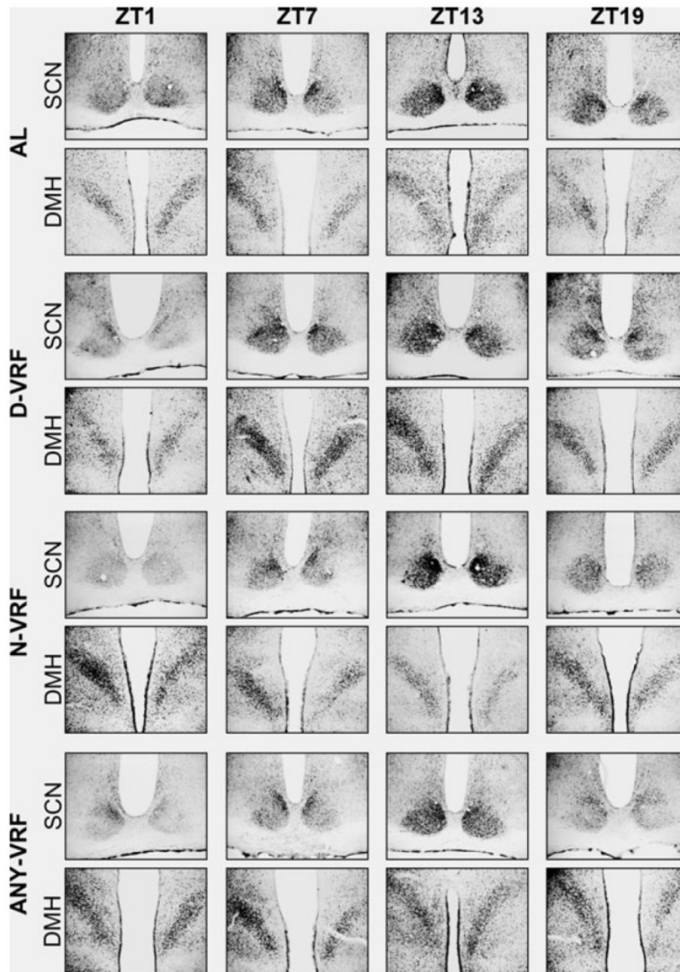

FIG. 3. Photomicrographs showing examples of PER1-immunostaining in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) across the day in rats from the *ad libitum* (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups.

Discussion

Predictable restricted feeding schedules, in which food is presented at the same time each day, lead to the emergence of entrained food-anticipatory rhythms in behavior. Furthermore, such predictable schedules induce and entrain circadian rhythms of PER1 and PER2 expression in the DMH (Mieda *et al.*, 2006; Verwey *et al.*, 2007; Fuller *et al.*, 2008). The DMH appears to contribute to the expression and control of some food-anticipatory rhythms, such as the rhythm in body temperature (Gooley *et al.*, 2006; Fuller *et al.*, 2008). Consequently, it has been proposed that the induction of a circadian rhythm in PER expression in this region is intimately linked to the emergence and entrainment of food-anticipatory rhythms under restricted feeding. In the present study we used unpredictable feeding schedules in which food was presented at a different time each day. Such schedules lead to gradual loss of body weight of between 17 and 24%, roughly the same magnitude of weight loss seen in rats subjected to 10 days of predictable feeding schedule with Ensure (2 h/day). The levels and distribution of daily wheel running activity were differentially affected by these VRF schedules but, because of the unpredictability of the time of feeding, distinct food-entrained anticipatory rhythms in wheel running activity did not emerge. However, despite the unpredictability of the feeding times, the daytime VRF group showed the largest increase in wheel running activity during the day, the nighttime VRF group showed the majority of wheel running activity during the night, and the anytime VRF group demonstrated an intermediate distribution (Fig. 2C). Circadian rhythms of PER1 expression in the SCN were not affected

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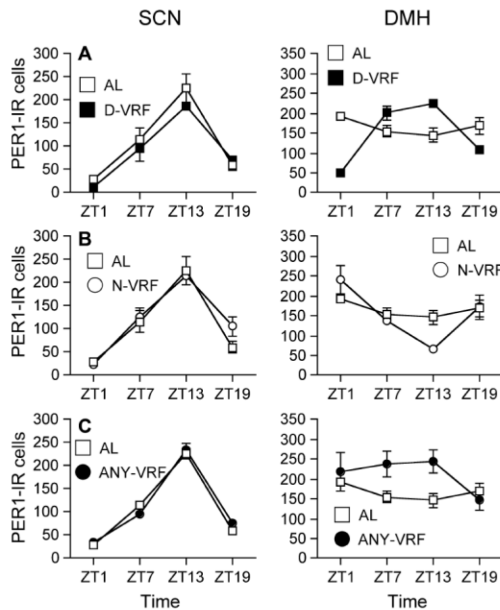

FIG. 4. (A–C) Graphs showing mean (± SEM) number of PER1-immunoreactive (PER1-IR) nuclei in the suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DMH) as a function of ZT for the *ad libitum* (AL), daytime variable restricted feeding (D-VRF), nighttime variable restricted feeding (N-VRF) and anytime variable restricted feeding (ANY-VRF) groups (n = 3–4/group).

by any of the VRF feeding schedules, consistent with results showing lack of effect of predictable restricted feeding schedules with Ensure on PER2 expression in this region (Verwey *et al.*, 2007, 2008). In contrast, PER1 expression in the DMH, which was arrhythmic in freely fed rats, became rhythmic under daytime and nighttime VRF schedules. When the daily access to food was limited to the daytime, peak PER1 expression in the DMH was observed between ZT7 and ZT13, and an opposite rhythm was observed when food was restricted to the nighttime. However, PER1 expression in the DMH remained arrhythmic when food was presented at random times throughout the 24-h day. These results demonstrate that in addition to predictable restricted feeding schedules (Mieda *et al.*, 2006; Verwey *et al.*, 2007, 2008; Fuller *et al.*, 2008), VRF can also induce circadian rhythms of PER1 expression in the DMH. Furthermore, they show that the pattern and phase of the rhythm of PER1 expression induced in the DMH depends on whether food is given during the daytime or the nighttime, as previously shown for PER2 under predictable restricted feeding schedules (Verwey *et al.*, 2007, 2008). Together, these results show that the induction of a circadian rhythm in PER1 expression in the DMH can be dissociated from the emergence of food-entrained behavioral rhythms in rats.

An important question that emerges from these findings concerns the critical factor involved in the induction of the different patterns and phase of the circadian rhythms of PER1 expression in the DMH. One possibility is that the observed rhythm in PER1 expression is dependent on the time of the last meal before the rats were killed. This is unlikely, however, because in the present study the daytime and anytime VRF groups were both presented with their last meal at ZT0, but resulted in decidedly different daily profiles of PER1 expression in the DMH. Whereas the daytime VRF group exhibited rhythmic PER1 expression in the DMH that peaked during the day, in the group that received food randomly throughout the 24-h day (anytime VRF) PER1 expression in the DMH was arrhythmic, albeit elevated. These data, together with our previous observation that exposure to a single episode of food deprivation and refeeding has no effect on PER2 expression in the DMH (Verwey *et al.*, 2007, 2008), suggest that it is the entire 10-day VRF schedule and the portion of the day when food becomes available (daytime, nighttime or anytime) that determines subsequent circadian rhythms in PER1 expression and not simply the time the last meal was presented.

A second issue to be considered is the relation between the induction of PER rhythms in the DMH and the emergence of food-anticipatory rhythms. We found that daily restricted feeding can induce a rhythm in PER1 expression in the DMH, even when the feeding time is unpredictable and animals cannot accurately anticipate the precise time of the daily meal. These results show that the induction of PER1 rhythms in the DMH and the food-entrainment of activity rhythms are dissociable. Another example of such a dissociation comes from previous studies using restricted 'treats'. Restricted treat schedules provide a daily highly palatable treat to a freely fed rat and induce treat-anticipatory activity (Mistlberger & Rusak, 1987; Mendoza *et al.*, 2005a,b; Angeles-Castellanos *et al.*, 2008), but fail to induce a circadian rhythm of PER2 expression in the DMH (Verwey *et al.*, 2007, 2008). Similarly it has been shown that food-entrained behavioral rhythms and the rhythm of expression of Per1 in digestive organs in rats are also dissociable (Davidson *et al.*, 2003).

The present results add in an important way to the current debate about the role of the DMH as a food-entrained circadian oscillator driving food-anticipatory rhythms. The mammalian circadian clock is based on daily oscillations in the expression of several clock genes (Reppert & Weaver, 2002). Among them, the *Bmal*1 gene controls the expression of *Per* genes and plays an essential role in the generation of circadian rhythms. Bmal1 and Per2 mutant mice each exhibit pronounced deficits in the circadian modulation of behavior, and either mutation has the capacity to disrupt circadian clocks at the molecular level (Bunger *et al.*, 2000; Shearman *et al.*, 2000; Bae *et al.*, 2001). Both Bmal1 and Per2 mutants have been reported to exhibit deficient or disrupted food-anticipatory rhythms under daily scheduled restricted feeding (Feillet *et al.*, 2006; Fuller *et al.*, 2008). Furthermore, restoring Bmal1 in the DMH was found to rescue food-anticipatory rhythms of body temperature in Bmal1 mutant mice (Fuller *et al.*, 2008). Although subsequent PER expression in the DMH of mutant mice was not assessed, it was suggested that a full complement of clock genes in the DMH is important for the expression of food-anticipatory changes in the rhythm of core body temperature. Contrary to these findings, other studies have shown that both Bmal1 and Per2 mutant mice exhibit clear food-anticipatory activity (Mistlberger *et al.*, 2008; Storch & Weitz, 2008), suggesting that the food-entrainment of behavior may be independent of certain clock genes. Future studies will need to focus on the self-sustainability of the restricted feeding-induced rhythms in clock gene expression in the DMH, as well as the extent to which the full complement of canonical clock genes are being expressed within this structure. The present results show that although the expression of *Per* genes in the DMH is sensitive to restricted feeding schedules, the induced rhythm does not depend on food being presented at a particular, predictable time each day. Rather, a rhythm of PER1 expression in the DMH can be induced when food is restricted to a certain portion of the day (i.e.

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daytime or nighttime). Together, these results indicate that the induction of a rhythm of PER expression in the DMH can be dissociated from and can exist in the absence of stable, food-entrained rhythms in behavior.

In summary, the present study shows that the induction of circadian PER1 expression in the DMH and circadian food-anticipatory rhythms in wheel running activity are dissociable. Furthermore, they confirm previous evidence indicating that the daily pattern of PER expression in the DMH induced by restricted feeding varies as a function of circadian time of food presentation. Functional consequences of the circadian clock gene expression in the DMH remain unclear, but we would suggest that perhaps rhythms in the DMH are particularly relevant to the control of core body temperature (Dimicco & Zaretsky, 2007). The DMH is clearly affected by restricted feeding and appears to mediate at least some food-anticipatory circadian rhythms (Gooley *et al.*, 2006; Fuller *et al.*, 2008). However, VRF schedules were sufficient to induce a circadian rhythm of PER1 expression in the DMH, suggesting that food-entrainment, *per se*, is not a requirement for circadian rhythms of clock gene expression in the DMH (Landry *et al.*, 2006, 2007; Verwey *et al.*, 2007, 2008).

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Abbreviations

AL, *ad libitum*; DAB, 3,3-diaminobenzidine; DMH, dorsomedial hypothalamic nucleus; Ensure, chocolate ensure plus; IR, immunoreactive; LD, light–dark; PER1, period1; PER2, period2; SCN, suprachiasmatic nucleus; VRF, variable restricted feeding; ZT, zeitgeber time.

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Appendix E: REPRINT (CHAPTER 4) - Variable restricted feeding disrupts the daily oscillations of PERIOD2 expression in the limbic forebrain and dorsal striatum in rats

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Variable Restricted Feeding Disrupts the Daily Oscillations of Period2 Expression in the Limbic Forebrain and Dorsal Striatum in Rats

Michael Verwey · Shimon Amir

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Abstract Predictable restricted feeding schedules limit food availability to a single meal at the same time each day, lead to the induction and entrainment of circadian rhythms in food-anticipatory activity, and shift daily rhythms of clock gene expression in areas of the brain that are important in the regulation of motivational and emotional state. In contrast, when food is delivered under a variable restricted feeding (VRF) schedule, at a new and unpredictable mealtime each day, circadian rhythms in food-anticipatory activity fail to develop. Here, we study the effects of VRF on the daily rhythm of plasma corticosterone and of clock gene expression in the limbic forebrain and dorsal striatum, of rats provided a 2-h access to a complete meal replacement (Ensure Plus) at an unpredictable time each day. VRF schedules varied the mealtimes within the 12 h of light (daytime VRF), the 12 h of dark (nighttime VRF), or across the 24 h light–dark cycle (anytime VRF). Our results show that contrary to the synchronizing effects of predictable restricted feeding, VRF blunts the daily corticosterone rhythm and disrupts daily rhythms of PER2 expression in a region-specific and mealtime-dependent manner.

Keywords Circadian rhythms · Clock genes · Corticosterone · Irregular mealtimes · Bed nucleus of the stria terminalis · Amygdala · Dentate gyrus · Suprachiasmatic nucleus

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Introduction

Circadian rhythms can be entrained by restricted feeding cycles that provide a single meal at the same time each day (Stephan et al. 1979; Mistlberger 1994).In rodents, these predictable cycles of feeding and fasting will induce and entrain characteristic food-anticipatory rhythms in behavior and physiology (Richter 1922), and synchronize the rhythms of clock gene expression in the periphery and brain (Damiola et al. 2000; Hara et al. 2001; Le Minh et al. 2001; Wakamatsu et al. 2001; Davidson et al. 2002, 2003; Angeles-Castellanos et al. 2007; Verwey and Amir 2009).In contrast, little is known about the circadian effects of variable restricted feeding (VRF) cycles, which provide a single meal at a new and unpredictable time each day and thus cannot entrain rhythms of food-anticipatory behavior. There is some evidence that VRF cycles disrupt daily behavioral rhythms as well as daily rhythms in metabolite and hormone levels (Valle 1981; Escobar et al. 2007; Verwey et al. 2009), but the effect of VRF on daily rhythms of clock gene expression has not been systematically examined.

In the present study, we assessed the effects of VRF on circulating levels of corticosterone and daily rhythms of the clock protein, Period2 (PER2). Specifically, PER2 expression was measured in the suprachiasmatic nucleus (SCN), the master circadian clock, and in several forebrain nuclei important in stress, motivation, and emotional regulation. The dorsal striatum, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA), and the dentate gyrus (DG) have all been shown to exhibit daily rhythms in PER2 expression in rats (Amir et al. 2004; Lamont et al. 2005; Hood et al. 2010).Moreover, predictable restricted

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feeding cycles have been shown to entrain the daily rhythms of PER2 expression in the limbic forebrain, without shifting the daily rhythm of PER2 expression in the SCN (Verwey et al. 2007, 2008).In the present study, we show that contrary to the synchronizing effects of predictable feeding schedules, VRF schedules disrupt the daily rhythms of PER2 expression in a region-specific and mealtime-dependent manner.

Materials and Methods

Animals and Housing

All experimental procedures were approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada) and adhered to the rules and recommendations set out by the Canadian Council on Animal Care (http://www.ccac.ca/). Male Wistar rats weighing between 225 and 250 g at the start of the experiment (n=63; Charles River Laboratories, St. Constant, QC, Canada) were individually housed in cages equipped with running wheels, in a 12:12-h environmental light–dark cycle. Rats had free access to standard rat chow (Rodent diet #5075; Charles River Laboratories, St. Constant, QC, Canada) and water during the initial 2-week entrainment period. The ad libitum (AL) control group continued with free access to rat chow, but food availability was restricted to a 2-h meal/day for rats assigned to the VRF groups (see next section). Running wheel records and complete descriptions of the behavioral patterns under each VRF condition were reported previously (Verwey et al. 2009).

Variable Restricted Feeding

Variable restricted feeding schedules varied the time of food arrival within the 12 h light phase (daytime VRF), the 12 h dark phase (nighttime VRF), or across the entire 24-h light–dark cycle (anytime VRF). Under all VRF conditions, the meals consisted of a 2-h access to the complete meal replacement chocolate Ensure Plus (Ensure, http://ensure.com/), each day for 10 days. The daytime VRF group received a meal starting at zeitgeber time (ZT; ZT0 is when environmental lights turn on) 6, 3, 10, 4, 1, 7, 10, 2, 5, and 0, for days 1–10, respectively. The nighttime VRF group received a meal starting at ZT (ZT12=Environmental lights turn off) 18, 15, 22, 16, 13, 19, 22, 14, 17, and 12, for days 1–10, respectively. Rats in the anytime VRF group received a meal starting at ZT 4, 13, 21, 7, 14, 10, 19, 6, 15, and 0, for days 1–10, respectively.

Perfusions and Immunocytochemistry

Perfusions were carried out at ZT1, ZT7, ZT13, or ZT19. At each time, rats from each group were injected with sodium pentobarbital (Somnotol, 100 mg/kg) and perfused transcardially with 300 mL of cold saline (4°C; 0.9% NaCl in distilled water) followed by 300 mL of cold paraformaldehyde solution (4°C; 4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed from the skull and post-fixed for 24 h (4% paraformaldehyde solution, 4°C). Alternate coronal brain sections (50 µm) containing the brain areas of interest were sliced on a vibratome and stored in cold Watson's cryoprotectant (−20°C) until staining (Watson et al. 1986). One set of brain sections were used to analyze PER1 expression in the SCN and DMH, and the data were published separately (Verwey et al. 2009).The present experiment used the second set of brain sections to examine PER2 expression in the SCN, limbic forebrain, and dorsal striatum.

Immunohistochemistry for PER2 was carried out as previously described (Amir et al. 2004).Briefly, sections were incubated (40 h, 4°C) in a primary antibody solution containing polyclonal rabbit antibodies for PER2 (1:1,000; Alpha Diagnostics International, San Antonio, TX, USA) and 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada) in triton (0.3%) trizma-buffered saline (50 mM trizma buffer with 0.9% NaCl). After rinsing the free-floating tissue in trizma-buffered saline, sections were incubated in a secondary antibody solution with biotinylated anti-rabbit IgG made in goat (1 h, 4°C; 1:200; Vector Laboratories), rinsed again, then incubated in an avidin–biotin solution (2 h, 4°C; Vectastain Elite ABC Kit; Vector Laboratories). The tissue was then bathed in a 0.5% 3,3-diaminobenzidine solution and immunoreactive cells were stained with a solution containing 0.5% 3,3-diaminobenzidine, 0.01% H_2O_2, and 8% $NiCl_2$. Brain sections were mounted on gelatin-coated slides, dehydrated in a series of alcohols, cleared with Citrisolv and glass coverslips were fixed in place with permount glue (Fisher Scientific, Ottawa, ON, Canada).

Plasma Sampling and Corticosterone Assay

Blood samples were collected prior to each perfusion, within 90 s of first contact with the home cage. Plasma was isolated by centrifugation (10,000 rpm, 10 min, 4°C) and stored at −80°C until the corticosterone assay was performed. Enzyme-linked immunosorbent assays were carried out according to the instructions provided (Corticosterone EIA Kit 900-097; Assay Designs Inc., Ann Arbor, MI, USA). Each sample was assayed in duplicate, and values were averaged between wells containing the same sample (intra-assay variability <5%).

Microscopy and Data Analysis

Using a light microscope, brain sections were studied and images of the SCN, BNSTov, CEA, BLA, DG, and dorsal striatum were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA), and image SXM software (v1.6, S D Barrett, http://www.ImageSXM.org.uk). PER2-immunoreactive (PER2-IR) cells were counted for each brain area using either a 400×400-μm (SCN, BNSTov, CEA, BLA, dorsal striatum) or 400×200-μm (DG) template, and means were calculated for each brain area based on the six unilateral images with the highest immunoreactivity. Differences between groups were determined with analysis of variance (ANOVA) where the alpha level was set at 0.05.

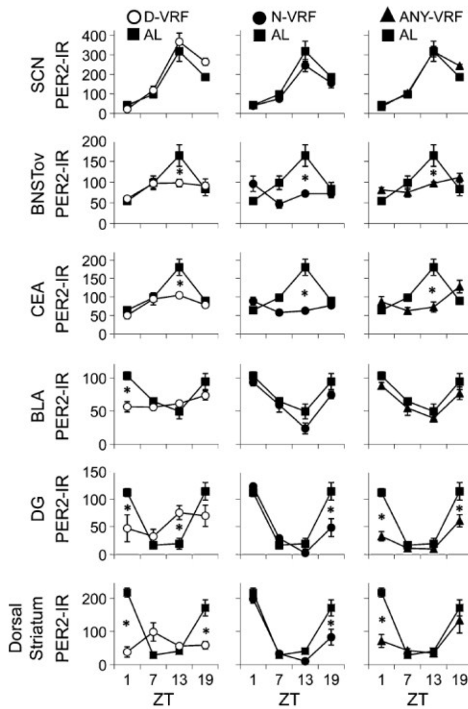

Fig. 1 Daily patterns of PER2 expression under daytime VRF (*D-VRF*; *left column*), nighttime VRF (*N-VRF*; *middle column*), and anytime VRF (*ANY-VRF*; *right column*), compared to AL controls. Each brain area is graphed separately and is listed on the far left of each row. Graphs illustrate the mean (±SEM) PER2-immunoreactivity (*PER2-IR*) in each brain area, according to the zeitgeber time (*ZT*) when the rats were killed. *Asterisks* represent a statistically significant difference ($p<0.05$) between feeding conditions at a single time point

Results

On average, over the course of the 10-day experiment, each VRF group consumed a similar amount of Ensure (daytime VRF=47.8±0.8 kcal/day; nighttime VRF 46.9±0.7 kcal/day; anytime VRF=43.2±0.6 kcal/day). In response to these hypocaloric diets, the daily pattern of PER2 expression was modulated in several brain areas, and the daily profiles of PER2-IR are shown in Fig. 1, where each VRF group is graphed with respect to AL controls. A robust daily rhythm in PER2 expression was observed in the SCN of all groups (Table 1), where peak PER2 expression was seen around ZT13 and trough PER2 expression around

Table 1 Daily patterns of PER2 expression in each brain area, under each feeding condition

Condition/structure	$ANOVA_{ZT}$
AL controls	
SCN	$F_{3,12}=63.6$, $p<0.001$
BNSTov	$F_{3,12}=16.8$, $p<0.001$
CEA	$F_{3,12}=60.0$, $p<0.001$
BLA	$F_{3,12}=22.3$, $p<0.001$
DG	$F_{3,12}=82.2$, $p<0.001$
Dorsal striatum	$F_{3,12}=41.1$, $p<0.001$
D-VRF	
SCN	$F_{3,11}=28.2$, $p<0.001$
BNSTov	$F_{3,11}=1.9$, ns
CEA	$F_{3,11}=4.5$, $p<0.05$
BLA	$F_{3,11}=2.6$, ns
DG	$F_{3,11}=1.4$, ns
Dorsal striatum	$F_{3,11}=2.2$, ns
N-VRF	
SCN	$F_{3,12}=19.1$, $p<0.001$
BNSTov	$F_{3,12}=2.8$, ns
CEA	$F_{3,12}=4.3$, $p<0.05$
BLA	$F_{3,12}=13.9$, $p<0.001$
DG	$F_{3,12}=56.1$, $p<0.001$
Dorsal striatum	$F_{3,12}=35.2$, $p<0.001$
ANY-VRF	
SCN	$F_{3,12}=69.1$, $p<0.001$
BNSTov	$F_{3,12}=4.1$, $p<0.05$
CEA	$F_{3,12}=4.4$, $p<0.05$
BLA	$F_{3,12}=8.0$, $p<0.01$
DG	$F_{3,11}=11.4$, $p<0.01$
Dorsal striatum	$F_{3,12}=4.2$, $p<0.05$

ns not significant

Table 2 Feeding conditions and the time of day (ZT) both modulate PER2 expression, compared to AL controls

Condition/structure	$ANOVA_{Feeding}$	$ANOVA_{ZT}$	$ANOVA_{Feeding \times ZT}$
D-VRF vs. AL			
SCN	$F_{1,23}$=4.1, ns	$F_{3,23}$=74.0, p<0.001	$F_{3,23}$=1.7, ns
BNSTov	$F_{1,23}$=2.6, ns	$F_{3,23}$=13.0, p<0.001	$F_{3,23}$=4.6, p<0.05
CEA	$F_{1,23}$=18.1, p<0.001	$F_{3,23}$=33.1, p<0.001	$F_{3,23}$=7.7, p<0.001
BLA	$F_{1,23}$=18.8, p<0.001	$F_{3,23}$=14.6, p<0.001	$F_{3,23}$=10.4, p<0.001
DG	$F_{1,23}$=1.1, ns	$F_{3,23}$=12.3, p<0.001	$F_{3,23}$=9.7, p<0.001
Dorsal striatum	$F_{1,23}$=20.7, p<0.001	$F_{3,23}$=11.6, p<0.001	$F_{3,23}$=25.4, p<0.001
N-VRF vs. AL			
SCN	$F_{1,24}$=6.2, p<0.05	$F_{3,24}$=67.4, p<0.001	$F_{3,24}$=1.3, ns
BNSTov	$F_{1,24}$=11.9, p<0.01	$F_{3,24}$=6.9, p<0.01	$F_{3,24}$=12.1, p<0.001
CEA	$F_{1,24}$=60.0, p<0.001	$F_{3,24}$=20.5, p<0.001	$F_{3,24}$=41.1, p<0.001
BLA	$F_{1,24}$=10.3, p<0.01	$F_{3,24}$=32.2, p<0.001	$F_{3,24}$=0.9, ns
DG	$F_{1,24}$=10.1, p<0.01	$F_{3,24}$=118, p<0.001	$F_{3,24}$=14.3, p<0.001
Dorsal striatum	$F_{1,24}$=10.8, p<0.01	$F_{3,24}$=72.8, p<0.001	$F_{3,24}$=3.7, p<0.05
ANY-VRF vs. AL			
SCN	$F_{1,24}$=1.6, ns	$F_{3,24}$=130, p<0.001	$F_{3,24}$=1.9, ns
BNSTov	$F_{1,24}$=1.6, ns	$F_{3,24}$=14.3, p<0.001	$F_{3,24}$=10.6, p<0.001
CEA	$F_{1,24}$=7.3, ns	$F_{3,24}$=10.0, p<0.001	$F_{3,24}$=19.0, p<0.001
BLA	$F_{1,24}$=8.0, p<0.01	$F_{3,24}$=25.1, p<0.001	$F_{3,24}$=0.2, ns
DG	$F_{1,24}$=66.5, p<0.001	$F_{3,24}$=71.0, p<0.001	$F_{3,24}$=16.0, p<0.001
Dorsal striatum	$F_{1,24}$=11.0, p<0.01	$F_{3,24}$=23.9, p<0.001	$F_{3,24}$=7.3, p<0.001

ns not significant

ZT1, and this SCN rhythm was unaffected by VRF(Fig. 1; Table 2). Consistent with previous findings (Amir et al. 2004; Lamont et al. 2005; Hood et al. 2010), rats that were freely fed with standard rat chow also exhibited robust daily oscillations of PER2 expression in the BNSTov, CEA, BLA, DG, and dorsal striatum (Table 1). Specifically, in the BNSTov and CEA, PER2 expression peaked around ZT13 and troughed around ZT1 (Fig. 1). In contrast, the BLA, DG, and dorsal striatum, each exhibited a daily oscillation of PER2 expression that peaked around ZT1 and troughed around ZT13 (Fig. 1). It was these daily rhythms of PER2 expression, outside of the SCN, which were strongly affected by VRF.

The effect of VRF on PER2 expression in the limbic forebrain and dorsal striatum is shown in Fig. 1, and statistical analyses are shown in Table 2. In the daytime VRF group, the daily rhythm of PER2 expression was dampened in all of the extra-SCN areas, and the CEA was the only structure that continued to exhibit a daily oscillation in PER2 expression (Table 1). In the nighttime VRF group, the circadian rhythm of PER2 expression in the BNSTov and CEA was dampened as compared to AL controls (Fig. 1; Table 2), although PER2 expression in the CEA continued to show a significant daily oscillation (Table 1). The BLA, DG, and dorsal striatum continued to exhibit robust circadian rhythms in the nighttime VRF group (Table 1), comparable to AL controls. In the anytime VRF group, all extra-SCN structures continued to show a daily oscillation in PER2 expression (Table 1). However, under anytime VRF, the daily patterns of PER2 expression in the BNSTov, CEA, DG, and dorsal striatum were each blunted compared to AL controls (Fig. 1; Table 2).

Daily patterns of plasma corticosterone are shown in Fig. 2. The AL controls were the only group that showed a statistically significant daily oscillation (Table 3). The daily pattern of corticosterone in the daytime VRF group was significantly different from the AL controls (Table 3). The nighttime VRF group exhibited corticosterone levels that were generally higher than the AL controls (Table 3). Finally, while the anytime VRF group was not statistically different from AL controls overall, it failed to show a significant daily oscillation (Table 3).

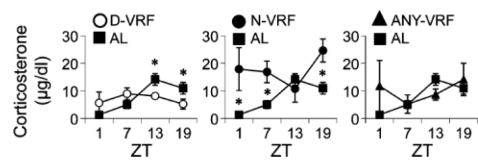

Fig. 2 Daily patterns of corticosterone under daytime VRF (*D-VRF*; *left*), nighttime VRF (*N-VRF*; *middle*), and anytime VRF (*ANY-VRF*; *right*), compared to AL controls. Graphs illustrate the mean (±SEM) corticosterone concentration in blood plasma (micrograms per deciliter) according to the zeitgeber time (*ZT*) when the blood plasma samples were collected. *Asterisks* represent a statistically significant difference (p<0.05) between feeding conditions at a single time point

Table 3 Daily patterns in the amount of corticosterone under each condition, as well as how the corticosterone levels under each VRF schedule, compared to AL controls

Feeding condition	$ANOVA_{ZT}$	$ANOVA_{Feeding}$	$ANOVA_{Feeding \times ZT}$
AL	$F_{3,11}=12.7$, $p<0.001$		
D-VRF	$F_{3,11}=0.7$, ns		
N-VRF	$F_{3,12}=1.1$, ns		
ANY-VRF	$F_{3,09}=0.5$, ns		
D-VRF vs. AL	$F_{3,22}=5.0$, $p<0.01$	$F_{1,22}=0.4$, ns	$F_{3,22}=4.5$, $p<0.05$
N-VRF vs. AL	$F_{3,23}=1.6$, ns	$F_{1,23}=11.0$, $p<0.01$	$F_{3,23}=2.2$, ns
ANY-VRF vs. AL	$F_{3,20}=1.9$, ns	$F_{1,20}=0.6$, ns	$F_{3,20}=1.6$, ns

ns not significant

Discussion

The present results show that VRF schedules, which provide an unpredictable meal each day, can disrupt daily rhythms of corticosterone release (Fig. 2) and of PER2 expression in the dorsal striatum and certain regions of the limbic forebrain. While the daily rhythm of PER2 expression in the SCN failed to respond to the VRF schedules and remained in synchrony with the light–dark cycle, the effects of VRF on PER2 expression outside of the SCN varied according to the brain area and the phase of the light–dark cycle when meals were given. The daily rhythms of PER2 expression in the dorsal striatum and limbic forebrain were blunted under daytime VRF (Fig. 1). In contrast, nighttime VRF blunted the daily patterns of PER2 expression in the BNSTov and CEA, but daily rhythms were relatively undisturbed in the BLA, DG, and dorsal striatum (Fig. 1). Daily patterns of PER2 expression under anytime VRF were similarly blunted in the BNSTov and CEA, but also showed some disruption in the DG and dorsal striatum (Fig. 1), perhaps due to the daytime meals provided under this schedule (on days 1, 4, 6, 9, and 10 of the 10-day anytime VRF schedule).

Phase-dependent effects of feeding, on daily rhythms of clock gene expression, have also been observed under other feeding schedules and in other brain areas. Predictable restricted feeding schedules that provide a daytime meal will shift the daily rhythm of PER2 expression in the BLA and DG, but such a shift is not observed if a predictable nighttime meal is provided (Verwey et al. 2007, 2008). Thus, clock gene expression in the BLA and DG appears to respond differentially to daytime and nighttime meals, regardless of whether they are given according to a predictable or unpredictable schedule. Moreover, VRF induces yet another pattern of responding in the dorsomedial hypothalamic nucleus (DMH), a nucleus where clock gene expression is potentially involved in food entrainment (Fuller et al. 2008; Mistlberger et al. 2008). Instead of blunting the daily profile of clock gene expression, as was observed in the BNSTov and CEA, daytime and nighttime VRF were shown to induce a daily rhythm of Period1 (PER1) expression in the DMH (Verwey et al. 2009). These differential effects are probably intrinsic to the brain areas, rather than specific to PER1 or PER2 expression, because both clock genes were similarly unaffected in the SCN. Nucleus-specific effects of feeding, on daily rhythms of PER1/PER2 expression, reaffirm that circadian clock genes are regulated in a decidedly nucleus-specific manner.

When rats are freely fed, the daily rhythm of corticosterone is important in sustaining daily rhythms of PER2 expression in the BNSTov and CEA (Segall et al. 2006, 2009). In contrast, under predictable RF, the daily rhythm of corticosterone becomes food-entrained and will exhibit a food-anticipatory release before the daily mealtime (Ahlers et al. 1980; Honma et al. 1983). But even after adrenalectomy, restricted feeding can still entrain daily rhythms of PER2 expression in the BNSTov and CEA (Segall et al. 2008). Such results demonstrate that glucocorticoids are one (Le Minh et al. 2001; Yoshida et al. 2006), but not the only, link between feeding and circadian rhythms. Instead, feeding-related and fasting-related hormones or other metabolic factors might also be involved in the modulation of PER2 expression, corticosterone release, and behavior. For instance, the orexigenic hormone ghrelin, which typically increases with hunger and decreases in response to feeding, can influence corticosterone release as well as behavioral anxiety (Asakawa et al. 2001).

Feeding-responsive hormones, such as ghrelin, exert an important influence on the daily rhythms of corticosterone and behavior. It is interesting to note, however, that while ghrelin can influence light-induced FOS expression in the SCN (Yi et al. 2008), this was the only brain area where PER2 rhythms failed to respond to VRF. Alternatively, factors that are directly involved in metabolism, such as peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) and sirtuin1 (SIRT1), have been shown to influence clock gene expression (Canaple et al. 2006; Liu et al. 2007; Asher et al. 2008; Nakahata et al. 2008). Thus, the nucleus-specific effects we observe on PER2 expression may rely on the metabolic factors that could be specific to each of these brain areas. In addition, the effects that we observe on the daily rhythm of

corticosterone release probably rely on hormonal changes that influence the brain circuitry underlying stress.

If the link between restricted feeding and daily rhythms in clock gene expression were simply due to metabolic factors, however, the effects of a meal should not depend on the time of day when it is provided. Our previous studies with predictable restricted feeding and our current study with unpredictable VRF, both provide evidence that this pattern of response is not always true. Restricted feeding entrains PER2 rhythms in the BNSTov and CEA, so they peak approximately 12 h after the predictable meal, regardless of whether meals are provided mid-day or midnight (Verwey et al. 2007, 2008; Verwey and Amir 2009). Moreover, the daily pattern of PER2 expression in the BNSTov and CEA was similarly blunted by both daytime VRF and nighttime VRF in the present study. Because the effects of restricted feeding seem to be independent from the time of day when food is given, we would propose that metabolic factors, like PGC-1alpha and SIRT1 (Canaple et al. 2006; Liu et al. 2007; Asher et al. 2008; Nakahata et al. 2008), could play an important role in mediating the effects of restricted feeding on clock gene expression in the BSNTov and CEA. In contrast, changes in the daily rhythm of PER2 expression in the BLA, DG, and dorsal striatum depend on the time of day when meals are provided. Daytime feeding, whether it is predictable or unpredictable, seems to have strong effects on the daily rhythm of PER2 expression in the BLA, DG, and dorsal striatum, while nighttime meals leave these rhythms largely intact. Thus, we would also propose that metabolic factors, like PGC-1alpha and SIRT1, might be less important in the BLA, DG, and dorsal striatum. Collectively, these findings would seem to emphasize the importance of studying metabolic factors and circadian clock gene expression concurrently.

The present study supports the view that irregular meals not only affect behavioral and physiological rhythms, as previously shown (Escobar et al. 2007; Verwey et al. 2009), but also bring about additional disruptions of daily rhythms in clock gene expression in the brain as well as daily rhythms in corticosterone release. Circadian disruptions and unstable entrainment have diverse consequences that, in various experiments, disrupt neural architecture and cognitive flexibility, as well as lead to cardiac and renal diseases (Martino et al. 2008; Karatsoreos et al. 2011). A growing body of literature suggests a fundamental difference between daytime and nighttime food consumption (Arble et al. 2009; Fonken et al. 2010). Light exposure during the night disrupts the circadian system of mice and not only leads to an increase in daytime food consumption but also an increase in body mass and a decrease in glucose tolerance (Fonken et al. 2010). If food is provided only during the 12 h of light, nocturnal mice will gain more weight than if food is provided instead during the 12 h of dark (Arble et al. 2009). Causal links between clock gene expression, hormone release, and behavior remain to be clearly demonstrated, but these findings continue to support an important link between metabolism and the circadian system at behavioral, hormonal, and molecular levels (Turek et al. 2005; Canaple et al. 2006; Liu et al. 2007; Asher et al. 2008; Nakahata et al. 2008).

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Appendix F: REPRINT (CHAPTER 5) - Nucleus-specific effects of meal duration on daily profiles of Period1 and Period2 protein expression in rats housed under restricted feeding.

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NUCLEUS-SPECIFIC EFFECTS OF MEAL DURATION ON DAILY PROFILES OF PERIOD1 AND PERIOD2 PROTEIN EXPRESSION IN RATS HOUSED UNDER RESTRICTED FEEDING

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Abstract—Restricted feeding (RF) schedules provide a cycle of fasting and feeding each day and induce circadian rhythms in food-anticipatory activity. In addition, daily rhythms in the expression of circadian clock genes, such as rhythms in Period1 (PER1) or Period2 (PER2), are also shifted in many brain areas that are important for the regulation of motivation and emotion. In order to differentiate brain areas that respond to the time of food presentation from areas that are sensitive to the degree of restriction, the present study compared RF schedules that provided rats with either a 2 h-meal (2hRF) or a 6 h-meal (6hRF) each day. As expected, 2hRF was associated with less food-consumption, more weight-loss, and more food-anticipatory running-wheel activity than 6hRF. In association with these metabolic and behavioral differences, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), which has been proposed to be integral to the generation and/or maintenance of food-anticipatory activities, peaked earlier in the 2hRF group and later in the 6hRF group. Because both RF groups exhibited approximately synchronous food-anticipatory activity, but phase shifted rhythms of PER1 and PER2 expression in the DMH, it suggests that the phase of food-anticipatory activity is not directly regulated by this brain area. Next, daily rhythms of PER2 expression in the limbic forebrain responded to each RF schedule in a nucleus-specific manner. In some brain areas, the amplitude of the PER2 rhythm was differentially adjusted in response to 2hRF and 6hRF, while other areas, responded similarly to both RF schedules. These findings demonstrate that daily rhythms of clock gene expression can be modulated by the motivational state of the animal, as influenced by meal duration, weight loss and food-consumption. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: circadian clock gene, dorsomedial hypothalamic nucleus, oval nucleus of the bed nucleus of the stria terminalis, central nucleus of the amygdala, basolateral amygdala, dentate gyrus.

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Abbreviations: AL, *ad libitum* food access; BLA, basolateral amygdala; BNSTov, oval nucleus of the bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DG, dentate gyrus of the hippocampus; DMH, dorsomedial hypothalamic nucleus; IR, immunoreactivity; PER1, Period1 protein; PER2, Period2 protein; Ppargc1a, peroxisome proliferative activated receptor gamma coactivator-1alpha; RF, restricted feeding; SCN, suprachiasmatic nucleus; Sirt1, Sirtuin1; ZT, zeitgeber time; 2hRF, restricted feeding: 2 h daily meal; 6hRF, restricted feeding: 6 h daily meal.

Restricted feeding (RF) schedules that provide a single meal at the same time each day induce robust circadian rhythms in behavior and physiology (Richter, 1922; Mistlberger, in press). In rodents, these rhythms in food-anticipatory activity are associated with alterations in the daily expression rhythms of circadian clock genes and proteins, such as Period1 (PER1) and Period2 (PER2), in many peripheral tissues and brain nuclei (Hara et al., 2001; Wakamatsu et al., 2001; Minami et al., 2002; Kudo et al., 2004; Mieda et al., 2006; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). In recent years there has been considerable interest in the role that these tissue- and nucleus-specific rhythms might play in the generation of food-anticipatory activities as well as how they might interact with local metabolic processes (Feillet et al., 2006; Shirai et al., 2007; Sonoda et al., 2007; Asher et al., 2008; Belden and Dunlap, 2008; Fuller et al., 2008; Nakahata et al., 2008; Challet et al., 2009; Escobar et al., 2009; Pendergast et al., 2009; Storch and Weitz, 2009; Karatsoreos et al., 2011). However, the factors that influence the effect of RF on clock gene expression in the periphery and brain have not been fully explored. For example, we have shown that the effect of RF on the daily rhythm of PER2 expression in the rat forebrain varies as a function of whether food is presented during the light phase or dark phase of the light-dark cycle (Verwey et al., 2007, 2008) and whether it is given at the same or different time each day (Verwey et al., 2009; Verwey and Amir, 2011). The duration of the daily meal influences food-consumption, weight-loss, hunger, arousal, and food-anticipatory activity (Honma et al., 1983; Stephan and Becker, 1989), but whether or not it is important in the modulation of the daily pattern of clock gene expression has not been determined.

Daily PER2 rhythms, which are responsive to RF, have been reported in regions of the limbic forebrain that are important in the regulation of motivational and emotional state (Amir et al., 2004; Lamont et al., 2005a,b; Waddington Lamont et al., 2007; Amir and Stewart, 2009). These regions include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus (DG). Furthermore, the daily pattern of PER1 and PER2 expression in the dorsomedial hypothalamic nucleus (DMH), an area that has been linked to the regulation of feeding and arousal, and to food-anticipatory activity in some studies, is also affected by RF (Mieda et al., 2006; Verwey et al., 2007, 2008; Fuller et al., 2008). Therefore, to study the impact of meal duration on clock gene expression in these brain regions, we provided rats

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with either a 2 h-meal (2hRF) or a 6 h-meal (6hRF) at the same time each day. The results show that meal duration plays a key role in the regulation of food-anticipatory activity and circadian rhythms of clock gene expression in the forebrain, and underscore the importance of motivational factors in the entrainment of behavioral and molecular circadian rhythms by RF in rats. Preliminary results have been presented in abstract form (Verwey and Amir, 2010).

EXPERIMENTAL PROCEDURES

Animals, housing and restricted feeding

All experimental procedures were approved by the Animal Care Committee at Concordia University (Montréal, QC, Canada) and followed the guidelines set out by the Canadian Council on Animal Care. Male Wistar rats (72 rats; 250–275 g at the start of each experiment) were individually housed in cages equipped with running wheels, and kept in light-proof and sound-attenuated chambers. Running wheel activity was continuously recorded by computer (Vitalview, Minimitter, OR, USA) and inspected with circadia software. All rats were kept in a regular 12 h-light (~300 lx at cage level):12 h-dark cycle, and had free access to food (Rodent diet #5075, Charles River Laboratories, St. Constant, QC, Canada) and water. After a two-week acclimation period, rats in the restricted feeding groups received either a single 2 h meal/day (2hRF) or a 6 h meal/day (6hRF) for 10 days. In both groups the meals began at zeitgeber time 4 (ZT4), 4 h after the environmental lights turned on (ZT0 denotes time of light on). Accordingly, the 2hRF group had access to food from ZT4–6, while the 6hRF group had access to the food from ZT4–10. The *ad libitum* (AL) fed group had free access to food throughout the experiment.

Immunohistochemistry

At the end of the restricted feeding schedules, rats were injected with an overdose of euthanyl (~150 mg/kg, CDMV, St. Hyacinthe, QC, Canada) and perfused transcardially around the clock (ZT 1, 5, 9, 13, 17, 21; n=4/timepoint) with 300 ml of cold saline (4 °C; 0.9% NaCl in distilled water) followed by 300 ml of cold paraformaldehyde solution (4 °C; 4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and post-fixed for ~24 h in paraformaldehyde solution. Coronal brain sections (50 μm) were sliced on a vibratome, and brain sections containing the regions of interest were collected and stored at −20 °C, in Watson's cryoprotectant, until staining (Watson et al., 1986). Sections containing the suprachiasmatic nucleus (SCN), BNSTov, CEA, BLA, DG and the DMH were immunostained for PER2 protein. Whereas, a second set of brain sections that contained only the DMH was stained for the PER1 protein.

Immunohistochemistry was performed as previously described (Amir et al., 2004; Verwey et al., 2009). Briefly, polyclonal antibodies for either PER1 (1:24000; made in rabbit; generous gift from Dr. S. M. Reppert, University of Massachusetts Medical School, Worcester, MA, USA) or PER2 (1:800; made in goat; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Both primary antibody solutions were made with trizma-buffered saline (TBS; 50 mM trizma buffer with 0.9% NaCl) and contained triton (0.3%). The PER1 antibody solution was also milk-buffered and contained 2% normal goat serum (Vector Laboratories, Burlington, ON, Canada), whereas the PER2-containing solution was not milk-buffered and contained 2% normal horse serum (Vector Laboratories, Burlington, ON, Canada). After rinsing the free-floating tissue in fresh TBS, brain sections were placed into a secondary incubation solution containing either biotinylated anti-rabbit IgG made in goat (in the case of PER1-staining; 1 h, 4 °C; 1:200; Vector Laboratories) or biotinylated anti-goat IgG made in horse (in the case of PER2-staining; 1 h, 4 °C; 1:400; Vector Laboratories). Brain sections were rinsed in fresh TBS, and then incubated in an avidin-biotin solution for 2 h (4 °C; Vectastain Elite ABC Kit; Vector Laboratories). Next, to visualize the immunoreactive cells, sections were rinsed in a 0.5% 3,3-diaminobenzidine solution (10 min), followed by a solution containing 0.5% 3,3-diaminobenzidine, 0.01% H_2O_2 and 8% $NiCl_2$ (10 min). All sections were then mounted on gelatin-coated slides, underwent serial alcohol dehydration and cleared with citrisolv. Glass coverslips were then glued in place with permount (Fisher Scientific, Ottawa, ON, Canada).

Microscopy and data analysis

Slides were examined under a light microscope (Leitz Laborlux S) using a 20× objective. 400×400 μm^2 (SCN, BNSTov, CEA, BLA, DMH) or 400×200 μm^2 (DG) images were captured and analyzed using a Sony XC-77 camera (Sony, Tokyo, Japan), a Scion LG-3 Frame Grabber (Scion Corporation, Frederick, MD, USA) and Image SXM software (v.1.6, SD Barrett, http://www.imagesxm.org.uk). The mean number of stained nuclei in each brain region was determined by computing the average count from the six sections containing the greatest number of stained nuclei out of all the images taken of a given structure. Differences in clock gene expression between groups were determined with an analysis of variance (ANOVA) where the alpha level was set at 0.05. Running-wheel activity, food consumption and body weight were analyzed with two-way repeated measures ANOVAs, and followed with post-hoc Tukey's tests.

RESULTS

Food consumption, body weight and running wheel activity

When all rats were freely fed (day 1–5), there was no significant difference in food consumption between the groups. As expected, during RF (days 6–14), the 2hRF and 6hRF groups ate less food than the AL group (P<0.01; Fig. 1, top graph). Moreover, during this period of RF, the 2hRF group ate less food than the 6hRF group (P<0.01). For example, on the 9th day of the RF schedule (day 14), the 2hRF group ate only 49% of their baseline food consumption, while the 6hRF group ate 71% on the same day. As a consequence of these differences in food-consumption, at the end of the 10-day schedules, the 2hRF group had lost 21% of their initial body weight while the 6hRF group had only lost 10% (Fig. 1, Bottom graph). In contrast, the AL group did not change their daily food consumption and, from day 6 to day 15, gained an additional 10% in body weight (Fig. 1).

Representative actograms, which illustrate the daily patterns of running-wheel activity before and after the initiation of the RF schedules for an individual in each group, can be seen in Fig. 2. Based on all of the running-wheel activity records, both RF groups developed food-anticipatory activity, at a time-of-day when AL controls were normally inactive (Fig. 3). The total daily activity was increased in both RF groups, as compared to the AL group (P<0.01; Fig. 4, top graph), and the 2hRF group ran more than the 6hRF group (P<0.01). The 2hRF group also exhibited more food-anticipatory running-wheel activity, from ZT1–4, than the 6hRF group (P<0.01; Fig. 4, middle graph). Accounting for the differences in total activity (Fig. 4, bottom graph), the 2hRF group still exhibited a higher

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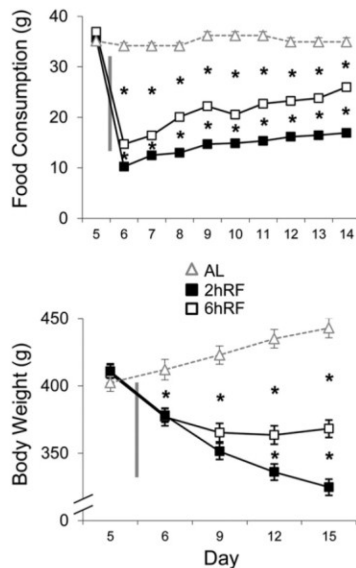

Fig. 1. Food consumption (top graph) and body weight (bottom graph) for each group over the course of the experiment (daily mean±SEM; n=24/group). RF schedules lasted from day 6–15 and the vertical gray lines mark the day food was restricted in the 2hRF and 6hRF groups. Day 15 has been omitted from the top graph because some rats were perfused before the end of the 10th meal.

proportion of activity during the food-anticipatory period than the 6hRF group (e.g. day 14, 2hRF:12.9±0.1%, 6hRF:8.4±0.1%).

PER2 expression in the SCN, limbic forebrain and DMH

Robust daily oscillations of PER2 expression were observed in the SCN and limbic forebrain under AL conditions (Fig. 5). In the AL group, PER2 expression in the SCN, BNSTov and CEA was highest around ZT13 and lowest around ZT1. Whereas, in the BLA and DG, anti-phase oscillations were observed that peaked around ZT1 and troughed around ZT13, which is consistent with previous reports (Amir et al., 2004; Lamont et al., 2005b). The DMH also exhibited a low-amplitude rhythm under AL conditions, with a peak at ZT21 (P<0.05). RF had no effect on the rhythm of PER2 expression in the SCN, but daily rhythms were adjusted by RF in all other regions (Table 1, Fig. 5).

In general, all areas continued to exhibit a daily rhythm of PER2-IR under most feeding conditions (P<0.05). The only exceptions were the BNSTov under 6hRF and the BLA under 2hRF, which failed to show a daily oscillation in PER2-IR (P>0.05). Specifically, we found that the rhythm of PER2 expression in the BNSTov (Fig. 5) was modulated differentially by the two RF schedules (Table 1). In particular, PER2 expression in the BNSTov was elevated at ZT17 in both RF groups compared to the AL group (P<0.05), but was lower in the 6hRF group compared to the 2hRF group (P<0.05). Moreover, while a robust daily rhythm in PER2 expression was observed in the BNSTov of 2hRF rats, the daily pattern was arrhythmic in the 6hRF group. In the CEA, peak PER2 expression was also shifted to ZT17 under 2hRF and 6hRF (Fig. 5), but both daily profiles remained rhythmic. At ZT17, however, the 2hRF exhibited higher PER2 expression in the CEA than the 6hRF group (P<0.05), which is consistent with a higher-amplitude rhythm under 2hRF (Table 1). Finally, the RF-induced rhythm of PER2 expression in the DMH also

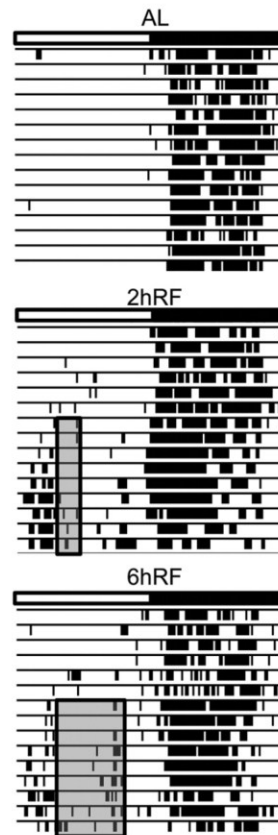

Fig. 2. Single-plotted actograms illustrating the daily pattern of running-wheel activity for a representative rat from each group. Each horizontal line graphs 24 h of running-wheel activity (data are binned every 10 min), while the white and black rectangles along the top of each record illustrate the 12 h:12 h light-dark cycle. The rat in the AL group had free access to food throughout the entire record. In contrast, the rats in the 2hRF and 6hRF groups also had free access to food for the first 6 d of each record, and then the RF mealtimes are illustrated by the shaded rectangles within each plot for the last 9 d of each record.

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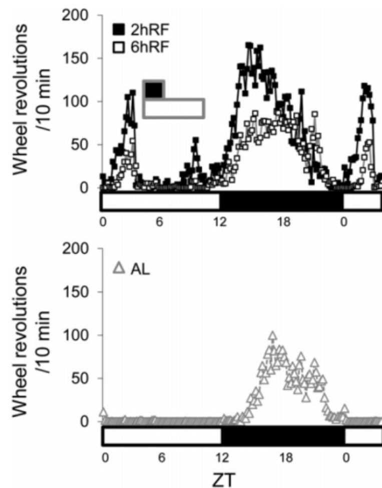

Fig. 3. Mean running-wheel activity for each group (n=24/group; 10-min bins), from ZT0 on day 14 until ZT4 on day 15. The light-dark cycle is illustrated along the bottom of each graph, along with ZT. The 9th daily meal is illustrated by horizontal rectangles within the top graph (filled=2hRF, open=6hRF) and the graph ends immediately before the 10th and last meal. In contrast, the AL group in the bottom graph had free access to food at all times.

depended on the length of the daily meal. Specifically, less PER2-IR was observed at ZT9 in the 6hRF than the 2hRF group, which could suggest a phase delay of the DMH rhythm.

In contrast to the differential effects of 2hRF and 6hRF that we observed in the BNSTov and CEA, both RF groups lead to a similar shift in the rhythm of PER2 expression in the BLA. The daily rhythm of PER2 expression in the BLA peaked around ZT17–21 in the 6hRF group instead of around ZT1 in AL controls. In the DG, the main effect of 2hRF and 6hRF on the daily rhythm of PER2 expression was a reduction in the number of cells expressing PER2 at ZT1 as compared to the AL group at the same time point ($P<0.01$). Thus, 2hRF and 6hRF had differential effects on the amplitude of daily oscillations of PER2 expression in the BNSTov, CEA, and DMH, but similar effects on the daily rhythms of PER2 expression in the BLA and DG (Table 1).

PER1 expression in the DMH

Even though the PER1 and PER2 genes are homologous, there are also many differences in their regulation and consequences. For these reasons, we also evaluated PER1 expression in the DMH in the same rats. Consistent with earlier studies, PER1 expression in the DMH exhibited a low-amplitude rhythm under AL conditions ($P<0.05$; Fig. 6). In contrast, robust daily rhythms in PER1 expression were observed under both RF schedules ($P<0.01$; Fig. 6). Importantly, RF increased the amplitude and shifted the daily rhythm of PER1 expression in the DMH, so that there was a statistically significant interaction between time and feeding group (Table 2). PER1-IR was significantly higher at ZT13 in both RF groups compared to controls ($P<0.01$). Moreover, at ZT9, the 2hRF group exhibited higher PER1-IR than the 6hRF group ($P<0.01$), which is consistent with a phase delay of this rhythm in the 6hRF group with respect to the 2hRF group.

DISCUSSION

RF schedules modulate daily rhythms of clock gene expression in the brain and periphery (Hara et al., 2001; Wakamatsu et al., 2001; Minami et al., 2002; Kudo et al., 2004; Mieda et al., 2006; Angeles-Castellanos et al., 2007; Waddington Lamont et al., 2007). By contrasting 2hRF and

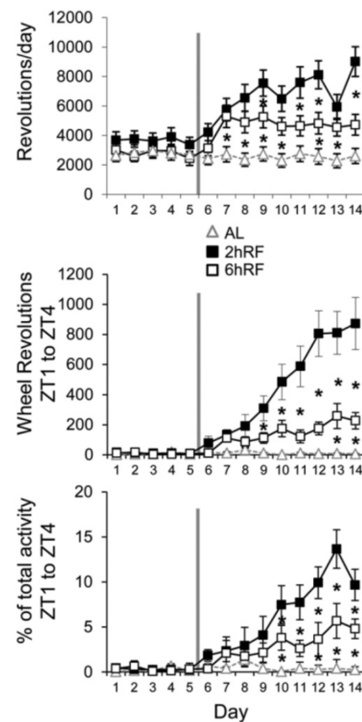

Fig. 4. Mean (±s.e.m.) running-wheel revolutions for each group (n=24/group) for each day of the experiment (top graph) and during the food-anticipatory period (ZT1–4, middle graph). The proportion of the total activity that occurred during the food-anticipatory period is also shown (bottom graph). All groups had unrestricted food-access from day 1–5, but food was restricted in the 2hRF and 6hRF group from day 6–14. The onset of restricted food-access is marked by the vertical grey lines in each graph. Day 15 has been omitted from all graphs, because it was the last day of the experiment and incomplete data were available.

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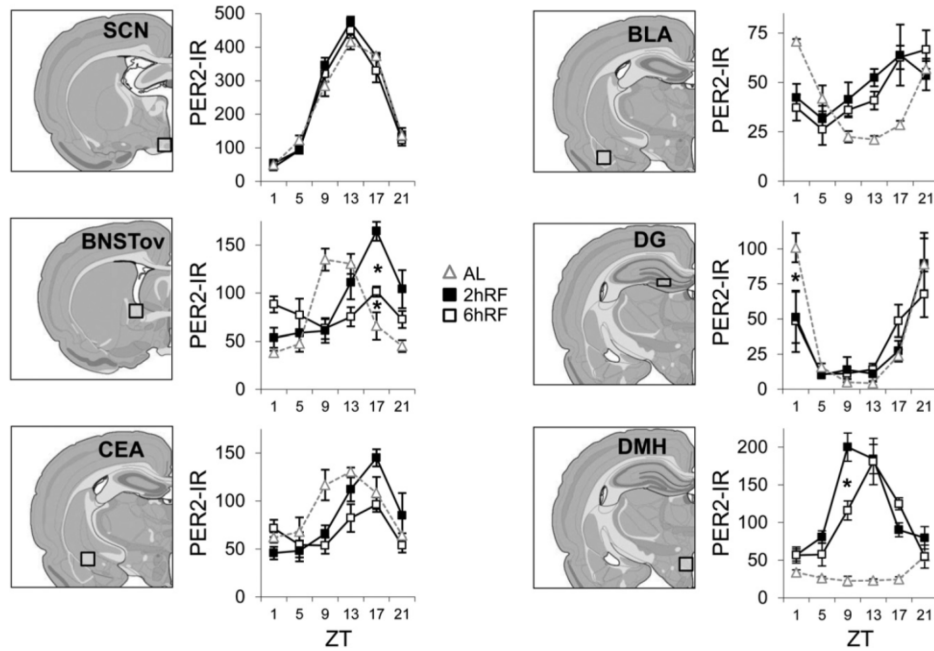

Fig. 5. Schematics of the location of the SCN, BNSTov, CEA, BLA and DG (left column) and the mean PER2-immunoreactivity (PER2-IR±s.e.m.; n=4/group/ZT) in each structure across the 24 h light-dark cycle.

6hRF the present experiment has demonstrated that, depending on the brain area, these effects can be dependent or independent of the meal duration. Meal duration influences the amount of food rats are able to consume each day, and therefore also influences body weight (Honma et al., 1983; Stephan and Becker, 1989). These differences in body weight, arguably, influence the motivation for food, which could be reflected in the finding that the 2hRF group exhibited significantly more food-anticipatory running-wheel activity than the 6hRF group. However, these RF schedules also had general effects that were not specifically linked to the mealtime. For instance, the overall daily activity was highest in the 2hRF group, intermediate in the 6hRF group, and lowest in the AL group. In parallel with

Table 1. ANOVA of PER2 expression in the SCN, limbic forebrain and DMH

Brain region	Two-way ANOVA	Statistical significance
SCN	$ANOVA_{TIME}$	$F_{5,54}$=232, P<0.01
	$ANOVA_{GROUP}$	$F_{2,54}$=1.9, ns
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=1.3, ns
BNSTov	$ANOVA_{TIME}$	$F_{5,54}$=9.7, P<0.01
	$ANOVA_{GROUP}$	$F_{2,54}$=2.6, ns
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=8.7, P<0.01
CEA	$ANOVA_{TIME}$	$F_{5,54}$=14.1, P<0.01
	$ANOVA_{GROUP}$	$F_{2,54}$=5.9, P<0.01
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=3.0, P<0.01
BLA	$ANOVA_{TIME}$	$F_{5,54}$=22.7, P<0.01
	$ANOVA_{GROUP}$	$F_{2,54}$=0.6, ns
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=1.8, ns
DG	$ANOVA_{TIME}$	$F_{5,54}$=7.6, P<0.01
	$ANOVA_{GROUP}$	$F_{2,54}$=1.7, ns
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=4.8, P<0.01
DMH	$ANOVA_{TIME}$	$F_{5,54}$=19.3, P<0.001
	$ANOVA_{GROUP}$	$F_{2,54}$=71.7, P<0.001
	$ANOVA_{TIME\times GROUP}$	$F_{10,54}$=9.7, P<0.001

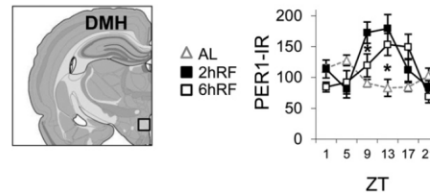

Fig. 6. Schematic of the location of the DMH (left) and mean PER1-immunoreactivity (PER1-IR±s.e.m.; n=4/group/ZT) in this structure across the 24 h light-dark cycle (right). Asterisks (*) denote a statistically significant difference between two groups (P<0.05).

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Table 2. Two-way ANOVA of PER1 expression in the DMH

Two-way ANOVA	Statistical significance
$ANOVA_{TIME}$	$F_{5,54}=5.5$, $P<0.01$
$ANOVA_{GROUP}$	$F_{2,54}=6.0$, $P<0.01$
$ANOVA_{TIME \times GROUP}$	$F_{10,54}=5.3$, $P<0.01$

this finding, nocturnal onset of activity also appeared to be earliest in the 2hRF group, intermediate in the 6hRF group and relatively late in the AL group. Because the daily rhythm of PER2 expression was unchanged in the SCN, we suggest that this effect on the phase angle of entrainment is not related to the entrainment of the master clock, per se, and could instead be related to the non-specific changes in the amount of running-wheel activity. Finally and most importantly, where the daily rhythms of PER1 and PER2 expression in the DMH as well as PER2 expression in the BNSTov and CEA were each differentially responsive to 2hRF and 6hRF, PER2 rhythms in the BLA and DG responded similarly to both RF schedules. These findings reflect an ever-growing complexity of tissue-specific effects of feeding on daily rhythms of PER1 and PER2 protein expression in the rat forebrain and hypothalamus.

One factor that could mediate some of this tissue-specificity could be the adrenal stress hormone corticosterone. When rats are freely-fed, the daily rhythm of corticosterone release is fundamentally important to sustain daily rhythms of PER2 expression in the BNSTov and CEA but not in the BLA and DG (Amir et al., 2004; Lamont et al., 2005b; Segall et al., 2006, 2009). In contrast, the importance of this stress hormone in modulating clock gene expression in the DMH is not well understood. In addition, RF schedules that provide a short daily meal produce a robust food-anticipatory release of corticosterone, whereas RF schedules that provide a long daily meal do not produce this food-anticipatory release (Honma et al., 1983). Thus, these differential patterns of corticosterone release, coupled with region-specific effects on the daily patterns of PER2 expression, could be important for the changes in PER2 rhythms that we observe in response to 2hRF and 6hRF. However, RF has also been shown to modulate the daily rhythms of clock gene expression in the brains of adrenalectomized rats (Segall et al., 2008), suggesting that this hormone is not the only factor influencing these rhythms.

Core metabolic factors could also be modulating daily rhythms of clock gene expression. Metabolic factors like peroxisome proliferative activated receptor gamma coactivator-1alpha (Ppargc1a) and sirtuin1 (Sirt1), have both been shown to influence clock gene expression (Rodgers et al., 2005, 2008; Liu et al., 2007; Asher et al., 2008; Belden and Dunlap, 2008). In particular, Sirt1 expression has been reported in the BNST and DMH (Ramadori et al., 2008), and provides a metabolic influence on PER2 expression (Asher et al., 2008). However, RF-induced changes of Sirt1 expression have also been observed in the SCN (Satoh et al., 2010), a brain area where in the present study PER2 expression was unaffected by RF. We should note that Asher et al. (2008) demonstrated an influence of SIRT1 on PER2 expression in the liver and in cell culture. Thus, we propose that the importance of SIRT1 may vary in a nucleus-specific manner and that, in the SCN of freely moving rats, the environmental LD cycle could supersede the influence of SIRT1 on the daily rhythm of PER2 expression. Consistent with these nucleus-specific responses, while Sirt1 expression is increased in the hypothalamus by fasting, this modulation has not been observed in the forebrain (Ramadori et al., 2008). In contrast, Ppargc1a is also expressed in several key brain areas that are involved in energy homeostasis (Sarruf et al., 2009), many of which interact and influence the brain nuclei in the present study. However, the nucleus-specific circadian rhythms in the expression of these factors, and the way this daily pattern might change under conditions of RF, remain topics for future study.

The DMH has been an area of great interest in the regulation of circadian rhythms in food-anticipatory activity (Gooley et al., 2006; Landry et al., 2006; Mieda et al., 2006; Fuller et al., 2008; Mistlberger et al., 2008, 2009). Instead of being necessary or sufficient for rhythms in food-anticipatory activity, it was recently suggested that, the DMH might be involved in modulating the amount of food-anticipatory activity (Mistlberger, in press). In the present study, the rats in the 2hRF and 6hRF groups both exhibited different amounts of synchronous food-anticipatory activity. In contrast, the daily rhythms of PER1 and PR2 expression, under each RF schedule, were out of synchrony between the two groups. Because the DMH rhythm under 6hRF appeared to be phase delayed compared the rhythm under 2hRF, this is another demonstration that daily rhythms of PER1 and PER2 expression in this brain area are dissociable from the timing of food-anticipatory activity. Consistent with this dissociation, variable RF schedules that provide an unpredictable meal each day, fail to induce circadian rhythms in food-anticipatory activity, but robust daily rhythms of PER1 expression in the DMH are still observed (Verwey et al., 2009). Nor does this observed dissociation, between rhythms in clock gene expression and rhythms in food-anticipatory activity, appear to be limited only to PER1. We have previously reported that daily rhythms in anticipatory running-wheel activity, induced by highly palatable treat-access, do not induce daily rhythms of PER2 expression in the DMH (Verwey et al., 2007, 2008). Thus, even though some reports have suggested that daily rhythms in food-anticipatory activity rely on an intact canonical circadian feedback loop (Feillet et al., 2006; Fuller et al., 2008), other studies suggest that we must start to look beyond this prototypical molecular clock (Storch and Weitz, 2009; Pendergast et al., 2009).

The present study has demonstrated that the amount of food that is consumed is able to modulate, in a nucleus-specific manner, the daily rhythms of clock gene expression in the limbic forebrain and hypothalamus. Because a daily treat will induce some food-anticipatory activities but fail to adjust daily rhythms in PER2 expression (Verwey et al., 2007, 2008), we have known for some time that a

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negative energy balance is important. However, this study demonstrates that the degree of food-restriction is also important in determining the daily rhythms of clock gene expression in the brain. This intimate link, between circadian rhythms and metabolism, has been emerging for several years (Damiola et al., 2000; Wakamatsu et al., 2001; Liu et al., 2007; Asher et al., 2008; Lamia et al., 2008; Escobar et al., 2009; Karatsoreos et al., 2011), but it is only more recently that we have started to appreciate the remarkable tissue-specificity of these effects.

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Appendix G: REPRINT (APPENDIX A) –

Food-entrainable circadian oscillators in the brain

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REVIEW ARTICLE
Food-entrainable circadian oscillators in the brain

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Keywords: clock gene expression, dorsomedial hypothalamic nucleus, limbic forebrain, restricted feeding

Abstract

Circadian rhythms in mammalian behaviour and physiology rely on daily oscillations in the expression of canonical clock genes. Circadian rhythms in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus but are also observed in many other brain regions that have diverse roles, including influences on motivational and emotional state, learning, hormone release and feeding. Increasingly, important links between circadian rhythms and metabolism are being uncovered. In particular, restricted feeding (RF) schedules which limit food availability to a single meal each day lead to the induction and entrainment of circadian rhythms in food-anticipatory activities in rodents. Food-anticipatory activities include increases in core body temperature, activity and hormone release in the hours leading up to the predictable mealtime. Crucially, RF schedules and the accompanying food-anticipatory activities are also associated with shifts in the daily oscillation of clock gene expression in diverse brain areas involved in feeding, energy balance, learning and memory, and motivation. Moreover, lesions of specific brain nuclei can affect the way rats will respond to RF, but have generally failed to eliminate all food-anticipatory activities. As a consequence, it is likely that a distributed neural system underlies the generation and regulation of food-anticipatory activities under RF. Thus, in the future, we would suggest that a more comprehensive approach should be taken, one that investigates the interactions between multiple circadian oscillators in the brain and body, and starts to report on potential neural systems rather than individual and discrete brain areas.

Introduction

Circadian rhythms are observed in many aspects of animal behaviour and physiology, and include daily rhythms in motivated behaviours, hormone release, body temperature, cognition and mood (Hastings *et al.*, 2007; Lamont *et al.*, 2007; Mendoza, 2007; Schibler, 2007; Wirz-Justice, 2008). At the molecular level, circadian clocks rely on daily oscillations in the transcription and translation of canonical clock genes (Reppert & Weaver, 2002). In mammals, circadian oscillations in clock gene expression are observed in the master circadian clock, the suprachiasmatic nucleus (SCN), and are fundamentally linked to the generation and regulation of circadian rhythms within this structure (Fuller *et al.*, 2008; Gavrila *et al.*, 2008). Circadian rhythms in clock gene expression are also observed in many brain areas outside the SCN, areas with diverse roles in behaviour and physiology (Guilding & Piggins, 2007). Tissue-specific clock gene expression is thought to modulate many essential cellular, homeostatic and metabolic processes (Rutter *et al.*, 2002; Challet *et al.*, 2003; Pardini & Kaeffer, 2006; Hastings *et al.*, 2007). In turn, it is becoming increasingly clear that metabolic signals influence clock gene expression (Liu *et al.*, 2007; Belden & Dunlap, 2008; Nakahata *et al.*, 2008; Rodgers *et al.*, 2008; Grimaldi *et al.*, 2009). Thus, patterns of food intake not only affect satiety and hunger but also have the potential to influence clock gene expression throughout the brain and body.

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To probe the interaction between metabolism and clock gene expression, in rodents, restricted feeding (RF) schedules have been used. RF schedules limit food availability to a single meal each day and, in response, animals gradually develop a number of food-anticipatory activities (see Fig. 1). Specifically, increases in locomotor activity, body temperature and hormone release are observed before the predictable daily meal (Stephan, 2002). Most importantly, RF schedules also shift or entrain the daily rhythm of clock gene expression in many brain areas (Wakamatsu *et al.*, 2001; Mieda *et al.*, 2006; Abe *et al.*, 2007; Angeles-Castellanos *et al.*, 2007; Girotti *et al.*, 2009). Thus, RF provides a powerful tool with which to study the neural and molecular bases of food-anticipatory activities as well as the nature and function of daily oscillations of clock gene expression in the brain.

Circadian clock gene expression in the brain

The basis for self-sustained circadian rhythms is a highly conserved molecular autoregulatory feedback loop that oscillates with an ~24-h period (Reppert & Weaver, 2002; Yoo *et al.*, 2004; Kornmann *et al.*, 2007; Liu *et al.*, 2007; Asher *et al.*, 2008; Belden & Dunlap, 2008). Briefly, at the core of this loop the genes *Clock* and *Bmal1* encode proteins that dimerize and act as a transcription factor. CLOCK:-BMAL1 heterodimers then enhance the transcription of *period* (*Per1*, *Per2* and *Per3*) and *cryptochrome* (*Cry1* and *Cry2*) genes (Gekakis *et al.*, 1998; Hogenesch *et al.*, 1998). PER and CRY expression oscillate across the day and the proteins feed back into the nucleus,

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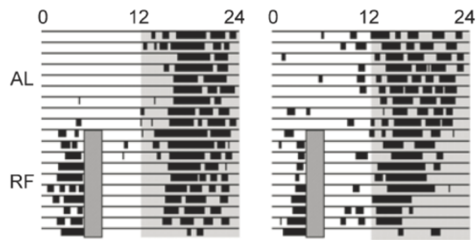

FIG. 1. Actograms illustrating the daily pattern of running-wheel activity for two representative rats that each received *ad libitum* food access (AL; first 9 days of the record) followed by restricted feeding (RF; last 10 days of the record). Each horizontal line plots 24 h and sequential days are arranged from top to bottom. Rats were housed in a 12 : 12-h light : dark schedule, illustrated by the empty (light phase) and shaded (dark phase) areas in each actogram. Numbers above the actograms indicate zeitgeber time (ZT). Under RF, rats received 2 h of access to food each day (ZT4-6), illustrated by the shaded rectangle. As expected, RF produced characteristic food-anticipatory running-wheel activity in the few hours leading up to the predictable mealtime.

and interfere with the transcriptional activity of CLOCK:BMAL1 (Kume *et al.*, 1999; Vitaterna *et al.*, 1999; Shearman *et al.*, 2000). In essence, these genes and their protein products are the gears and motors that allow circadian clocks to 'tick'. Thus, daily oscillations in clock gene expression (e.g. PER1 and PER2) allow for the identification of brain areas that express crucial circadian clockwork.

Circadian rhythms in clock gene expression have been found in many brain regions outside the SCN including the hippocampus, piriform cortex, cingulate cortex, prefrontal cortex, striatum, paraventricular hypothalamic nucleus, arcuate nucleus, olfactory bulb, nucleus accumbens and pituitary (Yamamoto *et al.*, 2001; Abe *et al.*, 2002; Gillespie *et al.*, 2003; Kriegsfeld *et al.*, 2003; Shieh, 2003; Granados-Fuentes *et al.*, 2004; Shieh *et al.*, 2005; Angeles-Castellanos *et al.*, 2007). Furthermore, we recently identified robust rhythms of PER2 expression in select regions of the limbic forebrain, areas that are important in the regulation of stress, motivation and emotion (Amir *et al.*, 2004; Lamont *et al.*, 2005b). These limbic areas include the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus of the hippocampus (DG). Although circadian oscillations in clock gene expression in most brain areas are either directly or indirectly under the control of the SCN, rhythms in brain regions outside the SCN are also sensitive to diverse hormonal and behavioural manipulations, including restricted feeding (Wakamatsu *et al.*, 2001; Lamont *et al.*, 2005a; Amir & Robinson, 2006; Perrin *et al.*, 2006; Segall *et al.*, 2006; Angeles-Castellanos *et al.*, 2007; Verwey *et al.*, 2007, 2008, 2009; Waddington Lamont *et al.*, 2007).

Brain areas sensitive to restricted feeding

There are two reasons to study circadian rhythms of gene expression under RF. The first reason is to determine which brain areas are involved in the regulation and entrainment of food-anticipatory activities *per se*. The second reason is to study of the properties and functions of putative circadian oscillators outside the SCN. Our recent work has been concerned primarily with the latter issue and we have recently started to define some of the hormonal and behavioural influences that affect clock gene expression in the limbic forebrain (Amir *et al.*, 2004; Lamont *et al.*, 2005b; Amir & Stewart, 2009). To carry out these studies, we and other have studied immediate–early gene expression (e.g. cFOS) and the expression of clock genes (e.g. PER1, PER2) across the circadian cycle (Angeles-Castellanos *et al.*, 2004, 2007; Mendoza *et al.*, 2005b; Verwey *et al.*, 2007).

Immediate–early genes, such as cFOS, are readily expressed throughout the brain in response to diverse stimuli. Although cFOS expression might be elevated in a particular brain area, clock gene expression may be unaffected in the same region. Our own research has illustrated this point by contrasting rats on an RF schedule with rats on a restricted treat (RT) schedule (Verwey *et al.*, 2007). Specifically, a restricted daily meal of highly palatable complete meal replacement, chocolate Ensure Plus (Ensure), was given to fasted (RF) and freely-fed (RT) rats. Although both groups consumed similar amounts of Ensure, only 37% of the RT group exhibited anticipatory running wheel activity in the hours leading up to the Ensure access while 100% of the RF group anticipated the daily mealtime. Of note, in response to Ensure, both groups exhibited increases in cFOS expression in the limbic forebrain and hypothalamus. In contrast, the daily pattern of PER2 expression in the same brain structures was only affected by RF, while the RT group and the *ad libitum*-fed control groups were indistinguishable. Thus, this dissociation between cFOS and PER2 expression in response to RF and RT emphasizes the importance of distinguishing conclusions based on immediate–early gene expression from conclusions based on clock gene expression. Moreover, the differential effects of RF and RT on PER2 expression suggest that clock gene expression in the limbic forebrain and hypothalamus is relatively insensitive to the incentive value of food and, instead, requires the metabolic challenges associated with fasting under RF.

The limbic forebrain

The limbic forebrain contributes to the regulation of motivation and emotion, and includes brain areas such as the bed nucleus of the stria terminalis (BNST), amygdala and hippocampus. Collectively, these areas modulate neuroendocrine, autonomic and behavioural responses to different types of stress and to drugs of abuse (Loewy, 1991; Gray, 1993; Erb *et al.*, 2001; Nijsen *et al.*, 2001). Moreover, these brain areas also modulate fear and anxiety, learning and memory, reproductive and maternal behaviours, and ingestive behaviours (Casada & Dafny, 1991; Van de Kar & Blair, 1999; Stefanova & Ovtscharoff, 2000; Walker *et al.*, 2001, 2003; Figueiredo *et al.*, 2003). Under *ad libitum* feeding conditions, PER2 is expressed with a circadian rhythm in the BNSTov, CEA, BLA and DG (Amir *et al.*, 2004; Lamont *et al.*, 2005b). On a 12 : 12-h light : dark schedule, daily oscillations of PER2 expression in the BNSTov and CEA peak around the time of transition from day to night (dusk), a daily expression profile which is in synchrony with PER2 expression in the SCN (Amir *et al.*, 2004; Lamont *et al.*, 2005b). In contrast, peak PER2 expression in the BLA and DG is observed around the time of transition from night to day (dawn; Lamont *et al.*, 2005b), opposite to the oscillation observed in the BNSTov, CEA and SCN (see Fig. 2). Consistent with other extra-SCN circadian oscillators (Sakamoto *et al.*, 1998), no circadian rhythm is observed in PER2 expression in the limbic forebrain after the SCN is lesioned (Amir *et al.*, 2004; Lamont *et al.*, 2005b). Additionally, daily rhythms of PER2 expression in the BNSTov and CEA are also sensitive to hormonal signals. In the rat, adrenal stress hormones (corticosterone), gonadal hormones (Oestrogen, testosterone) and thyroid hormones all modulate PER2 expression in the BNSTov and CEA though, importantly, these hormones do not modulate PER2 expression in the SCN, BLA or DG (Amir *et al.*, 2004; Lamont *et al.*, 2005b; Amir & Robinson, 2006; Perrin *et al.*,

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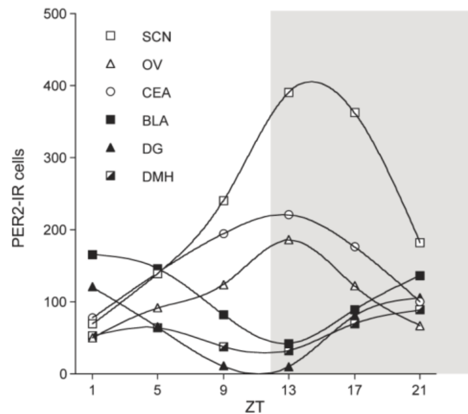

FIG. 2. Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of *ad libitum*-fed rats. Each line illustrates the estimated mean of PER2 immunoreactivity in a particular structure, plotted according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The 12 : 12-h light : dark cycle is also illustrated by the shaded and unshaded areas of the graph. With the exception of the DMH, all structures exhibited robust daily rhythms in PER2 expression. Peak PER2 expression was observed in the SCN, BNSTov and CEA at ZT13 whereas peak PER2 expression was observed in the BLA and DG at ZT1 (redrawn from data published in Verwey *et al.*, 2007).

2006). In particular, the daily rhythm of corticosterone, the main adrenal glucocorticoid in rats, is critical for daily rhythms in PER2 expression in the BNSTov and CEA (Segall *et al.*, 2006, 2009). Interestingly, in contrast to many other manipulations that fail to modulate PER2 expression in the BLA and DG, the daily rhythm of PER2 expression in these areas is affected by RF schedules.

In our studies on the effect of RF on PER2 expression in the limbic forebrain we found that, when food is restricted to the middle of the day when rats normally do not eat (daytime RF), the daily rhythms of PER2 expression in the BNSTov, CEA, BLA and DG shift such that peak PER2 expression in all of these structures is observed ~12 h after the daytime meal (Verwey *et al.*, 2007). In contrast, RF schedules that provide a nighttime meal (nighttime RF), a more appropriate mealtime for nocturnal rats, shift the daily rhythms of PER2 expression in the BNSTov and CEA but not in the BLA and DG (see Fig. 3). As was the case for daytime RF, peak PER2 expression was observed in the BNSTov and CEA ~12 h after the predictable mealtime; however, PER2 expression in the BLA and DG was unaffected by nighttime RF and similar to that in *ad libitum*-fed controls (Verwey *et al.*, 2008). Thus clock gene expression in the BLA and DG is affected only by daytime RF whereas, PER2 expression in the BNSTov and CEA peaks ~12 h after both daytime RF and nighttime RF. Thus clock gene expression in the BNSTov and CEA would appear to be food-entrained.

The changes in clock gene expression seen in response to RF vary as a function of the gene studied. Differences between PER1 and PER2 expression have been observed in the limbic forebrain. In *ad libitum*-fed rats, PER1 expression is reportedly rhythmic in the BNST and hippocampus but not rhythmic in the CEA or BLA (Angeles-Castellanos *et al.*, 2007) whereas, as previously noted, PER2 expression is rhythmic in all of these brain areas under *ad libitum* feeding (Amir *et al.*, 2004; Lamont *et al.*, 2005b). Under a daytime RF schedule, PER1 expression was reported to be unchanged in the CEA and BLA but altered in the BNST and hippocampus (Angeles-Castellanos *et al.*, 2007). In contrast, under similar conditions we found robust shifts in PER2 expression in all these regions (Amir *et al.*, 2002; Verwey *et al.*, 2007, 2008; Waddington Lamont *et al.*, 2007). These data suggest key differences in the regulation of PER1 and PER2 expression in the limbic forebrain by RF, but these two genes have not been studied concurrently in the same experiment so a note of caution is necessary. Indeed, whereas we quantified PER2 expression specifically in the BNSTov, Angeles-Castellanos *et al.* (2007) quantified PER1 immunoreactivity in the BNST as a whole. Similarly, we quantified PER2 expression specifically in the DG whereas Angeles-Castellanos *et al.* (2007) examined PER1 expression throughout the hippocampus. Both the BNST and hippocampus are heterogeneous structures that contain many distinct subregions with different functions and these subregions should be analysed individually. Nevertheless, there is also evidence that PER1 and PER2 are differentially expressed in the limbic forebrain in the mouse (Feillet *et al.*, 2008).

The SCN

The SCN also exhibits changes in the expression of clock genes in response to RF schedules but, compared to the large shifts observed in the limbic forebrain, these changes are usually subtle. For example, the daily rhythm of expression of Per1 mRNA in the SCN shifts in response to RF, but these changes are relatively small. The rhythms of Per1 in the SCN did not become entrained by the RF schedule (Mendoza *et al.*, 2005a). In our studies we have not observed RF-induced changes the expression of PER2 in the SCN (Verwey *et al.*, 2007, 2008). One exception to this generalization occurs when rats are housed in constant light. Under these conditions, circadian locomotor activity rhythms become disrupted and PER2 expression in the SCN does not exhibit a circadian rhythm. Remarkably, when placed on an RF schedule in these constant light conditions, both locomotor activity and PER2 rhythms are reinstated and are entrained to the time of feeding (Lamont *et al.*, 2005a).

Some of the most robust effects on clock gene expression in the SCN have been observed in response to caloric restriction (Challet *et al.*, 1996, 1998; Andrade *et al.*, 2004; Mendoza *et al.*, 2005d). In mice, hypocaloric feeding schedules can entrain SCN-driven circadian rhythms and affect clock gene expression in this brain area. Importantly, this effect has been attributed to the hypocaloric feeding *per se* rather than the reorganization of circadian activities and the development of food-anticipatory activities. Specifically, delivering a single hypocaloric meal each day leads to the development of food-anticipatory activities. However, the delivery of six meals per day do not lead to the same development of coordinated food-anticipation and yet still affect clock gene expression in the SCN (Mendoza *et al.*, 2008). This work clearly demonstrates that certain changes in clock gene expression can be brought on simply by reduced caloric intake rather than food-entrainment *per se*.

The dorsomedial hypothalamic nucleus (DMH)

The DMH is an important relay in the transmission of signals from the SCN to the rest of the brain and body. This structure also appears to integrate metabolic signals with inputs it receives from the SCN (Elmquist *et al.*, 1998) and provides important outputs to brain areas that regulate sleep, such as the ventrolateral preoptic nucleus, and arousal, such as the lateral hypothalamus (Chou *et al.*, 2003). Under *ad libitum* feeding conditions the DMH does not exhibit robust daily

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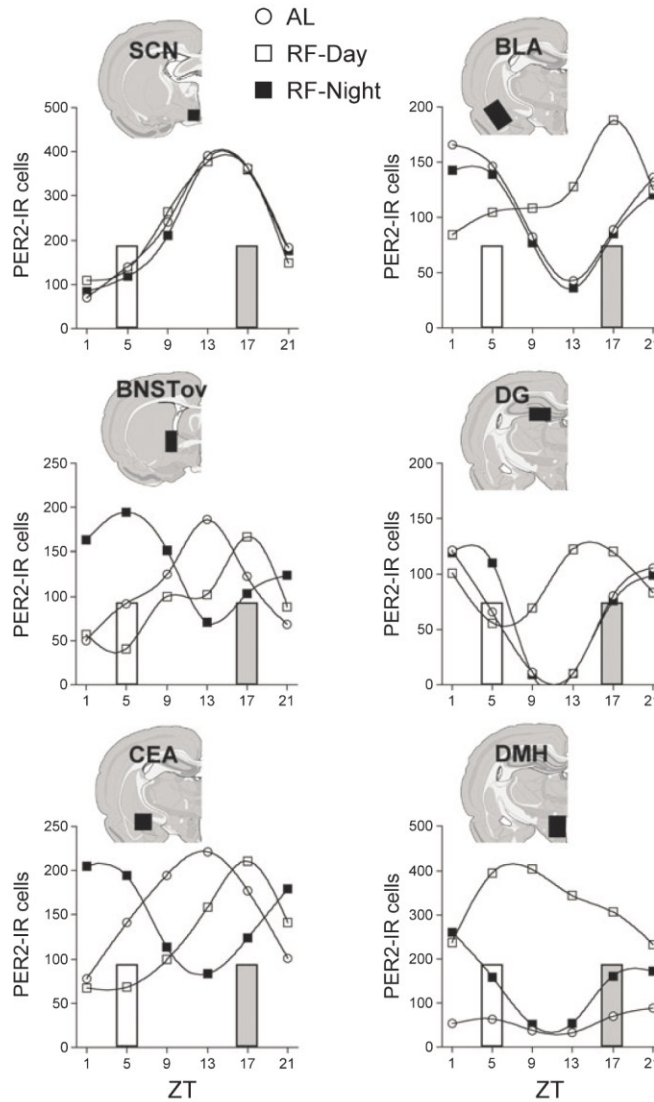

FIG. 3. Daily patterns of PER2 expression in the SCN, BNSTov, CEA, BLA, DG and DMH of rats under RF and *ad libitum* (AL) feeding conditions. Mean PER2 immunoreactivity is plotted for each structure, according to zeitgeber time (ZT; ZT0, lights on; ZT12, lights off). The daily meal of Ensure was provided either in the middle of the day (RF-day; ZT4–6; open rectangle) or middle of the night (RF-night; ZT16-18; shaded rectangle). The daily pattern of PER2 expression in the BNSTov, CEA and DMH was affected by both RF schedules. In contrast, the daily pattern of PER2 expression in the BLA and DG was only shifted under RF-day conditions. Finally, PER2 expression in the SCN was unaffected by either RF schedule (redrawn from data published in Verwey *et al.*, 2007 and 2008).

oscillations in clock gene expression but, under RF, large-amplitude circadian rhythms in Bmal1, PER1 and PER2 expression emerge (Mieda *et al.*, 2006; Verwey *et al.*, 2007, 2008; Fuller *et al.*, 2008; Moriya *et al.*, 2009). These daily oscillations in clock gene expression in the DMH depend on the time of day when meals are provided. Specifically, under a daytime RF schedule, peak PER2 expression is observed in the DMH soon after the mealtime. However, under a nighttime RF schedule, peak PER2 expression is observed in the

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DMH several hours after the mealtime (Verwey *et al.*, 2007, 2008). This interaction between the time when food is given and the time of peak expression suggests that, although clock gene expression in the DMH is affected by RF schedules, it may not be exclusively food-entrained. To date, studies of clock gene expression in the DMH have been carried out in SCN-intact rats and mice and the DMH has strong connections to the SCN (Chou *et al.*, 2003). An intriguing question, therefore, is whether clock gene expression in the DMH could be food-entrained if the SCN were lesioned.

The circadian basis of food-anticipatory activities

The characteristics of food-anticipatory activities are consistent with an underlying food-entrained circadian oscillator. For example, when rats are fasted for 2–4 days after being on an RF schedule, food-anticipatory activities continue to appear each day around the predicted mealtime (Boulos *et al.*, 1980). An hourglass model, whereby rats anticipate subsequent food arrival a fixed number of hours after the last meal, would not account for daily food anticipation under a 72-h fast. Also consistent with a circadian mechanism, food-anticipatory activities show limits of entrainment. Although rodents accurately predict 24-h food intervals, 18-h intervals do not lead to characteristic food-anticipatory activities (Stephan *et al.*, 1979a,b). If food-anticipatory behaviours were based on an hourglass mechanism, 18- and 24-h schedules should represent similar challenges. Based on these findings, putative circadian oscillators throughout the brain and body are thought to contribute to the regulation and entrainment of food-anticipatory activities. Although some aspects of food anticipation persist when canonical circadian clock genes are mutated or knocked out, deficits are also observed (Feillet *et al.*, 2006; Fuller *et al.*, 2008; Storch & Weitz, 2009).

Light-entrained and food-entrained circadian oscillators are viewed as separate and relatively independent systems (Mistlberger, 1994; Stephan, 2002). Consistent with this hypothesis, there are generally subtle effects of RF on clock gene expression in the SCN, and SCN lesions do not interfere with the development of food-anticipatory activities (Stephan *et al.*, 1979a,b). However, because no single brain lesion eliminates all food-anticipatory activities, the specific areas and tissues of the brain and body that are involved in circadian food-entrained anticipatory activities remain unclear. Food-entrained and light-entrained oscillators affect the expression of overlapping canonical clock genes in many brain areas and so the focus of experiments should start to shift towards uncovering networks of RF-sensitive brain areas.

The DMH and food-anticipatory activity

It has been suggested that clock gene expression in the DMH is necessary for the expression of food-anticipatory activities (Gooley *et al.*, 2006; Fuller *et al.*, 2008). Specifically, mutant mice with a targeted disruption of the Bmal1 gene are reported to exhibit deficient circadian rhythms and disruptions in their ability to show food-anticipatory increases in core body temperature (Fuller *et al.*, 2008). Moreover, food anticipation has been reported to be restored when exogenous Bmal1 is transfected to the DMH (Fuller *et al.*, 2008). Lesions of the DMH have also been reported to interfere with the expression of food-anticipatory activities (Gooley *et al.*, 2006). Clearly, these studies suggest that the DMH could be important in the expression of certain food-anticipatory rhythms. However, there are also a number of other studies that contradict this conclusion (Landry *et al.*, 2006, 2007; Moriya *et al.*, 2009; Storch & Weitz, 2009). For example, it has recently been reported that some food-anticipatory behaviours persist in Bmal1-mutant mice (Pendergast *et al.*, 2009; Storch & Weitz, 2009). Furthermore, there are a number of reports of robust food-anticipatory activities in DMH-lesioned rats (Landry *et al.*, 2006, 2007). These discrepancies have led to active debate in the literature (Gooley *et al.*, 2006; Landry *et al.*, 2006; Fuller *et al.*, 2008; Mistlberger *et al.*, 2008) and it is clear that a more comprehensive understanding of the control of food-anticipatory rhythms will depend on further experimentation and replication.

Accurate food-anticipatory activities are not required for robust circadian rhythms in clock gene expression in the DMH. Variable restricted feeding schedules provide an unpredictable meal each day, and inherently prohibit accurate food-anticipation (Escobar *et al.*, 2007; Verwey *et al.*, 2009). When the mealtime is varied within either the 12 h of light or the 12 h of darkness, large-amplitude rhythms in PER1 expression are clearly observed in the DMH despite a lack of food-entrained running wheel activity (Verwey *et al.*, 2009). In contrast, when food availability is varied throughout the entire 24-h cycle, PER1 expression in the DMH is elevated across the day as compared to controls, though no daily rhythm is observed (Verwey *et al.*, 2009). Thus, robust circadian rhythms in PER1 expression can be induced by RF schedules even without the entrainment of food-anticipatory activity. Conversely, food-anticipatory activities have also been observed in the absence of circadian rhythms in clock gene expression in the DMH. Food-anticipatory activities have been reported in rats receiving a daily chocolate treat (Mistlberger & Rusak, 1987; Mendoza *et al.*, 2005c) but, in those rats anticipating the daily treat, PER2 expression in the DMH is similar to *ad libitum*-fed controls (Verwey *et al.*, 2007, 2008). Collectively, these findings demonstrate that the food entrainment of running wheel activity and PER expression in the DMH are dissociable. However, the induction and entrainment by RF of daily rhythms in clock gene expression in the DMH certainly represents an interesting and important area of study, whether or not it is crucial to all food-anticipatory behaviours.

Potential mechanisms

In the anticipation of food, canonical clock proteins do not operate in isolation. Homeostatic signals, learning and as yet undiscovered circadian mechanisms all may contribute to the expression of food-anticipatory rhythms. Furthermore, in spite of the observation that certain food-anticipatory activities persist in mice with circadian gene mutations, extra-SCN clock gene expression remains an important area of study in the context of restricted feeding. Indeed, several lines of evidence, mostly from work in the periphery, have made it abundantly clear that clock gene expression has an important influence on tissue-specific metabolism and physiology (Fu *et al.*, 2002; Oster *et al.*, 2006; Chen-Goodspeed & Lee, 2007; Winter *et al.*, 2007). Fundamentally, understanding the regulation and consequence of clock gene expression in different regions of the brain is not only important for the elucidation of mechanisms underlying RF but also essential to understanding the interaction between the circadian system and physiology.

Several pathways influence circadian clock gene expression throughout the brain and body. Daily rhythms of clock gene expression in the limbic forebrain and hypothalamus are selectively sensitive to RF and not RT. Thus, rather than pathways associated with reward and feeding *per se*, metabolic pathways associated with fasting are probably involved in the regulation of clock gene expression in these areas. One metabolic pathway whereby RF schedules could influence circadian oscillators in the brain involves two interacting proteins that relate directly to cellular metabolism and clock gene expression, peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1a) and sirtuin1 (SIRT1; Rodgers *et al.*, 2005; Liu

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et al., 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008). Specifically, the expression of PGC-1a in peripheral tissues such as liver and muscle follows a circadian rhythm (Liu *et al.*, 2007). In turn, PGC-1a has been found to induce and regulate the rhythmic expression of clock genes in peripheral tissues and, significantly, to affect behavioural and physiological circadian rhythms (Canaple *et al.*, 2006; Liu *et al.*, 2007). In contrast, SIRT1 is directly sensitive to food availability and it deacetylates PER2, thus also affecting the circadian feedback loop (Asher *et al.*, 2008; Nakahata *et al.*, 2008). These mechanisms have been studied in the periphery (Liu *et al.*, 2007; Asher *et al.*, 2008; Nakahata *et al.*, 2008) but additional research is needed to demonstrate their importance in the brain. PGC-1a mRNA is expressed in many brain areas, including the olfactory bulb, cerebral cortex, septal nucleus, striatum, hippocampus and substantia nigra (Tritos *et al.*, 2003; Cowell *et al.*, 2007). Furthermore, the expression of PGC-1a in cortical cells has been shown to be regulated by neuronal activity (Meng *et al.*, 2007), raising the intriguing possibility that PGC-1a may mediate the effects of signals arising from daily restricted feeding and from other perturbations of physiology and behaviour on the expression of clock genes in the brain.

A second pathway whereby RF schedules could influence clock gene expression in the limbic forebrain is through the action of glucocorticoids. Glucocorticoids modulate clock gene expression in the periphery, and daily rhythms in glucocorticoid release are needed to sustain at least certain brain oscillators (Segall *et al.*, 2006). The daily rhythm of glucocorticoid release is also changed by RF, and a food-anticipatory release of this stress hormone is well documented (Ahlers *et al.*, 1980). Although glucocorticoids do not appear to influence PER2 expression in the limbic forebrain under RF (Segall *et al.*, 2008), the RF modulation of glucocorticoid release could have a role in the modulation in the brain of clock gene expression, especially of PER1, which contains a glucocorticoid-responsive element in its promoter region (Yamamoto *et al.*, 2005).

A third pathway whereby RF schedules could influence clock gene expression is through body temperature. The daily rhythm of body temperature is entrained by RF and has been studied extensively in the context of the DMH. Circadian rhythms in cerebral temperature also occur (Boudreau *et al.*, 2008), but their importance to clock gene expression remains unknown. In the periphery, circadian rhythms in body temperature and the associated induction of heat shock proteins appear to have some influence on clock gene expression (Kommann *et al.*, 2007). Through a combination of inputs, some of which have been discussed here, RF schedules are able to influence clock gene expression in a region-dependent manner. Elucidating the mechanisms whereby RF is able to alter clock gene expression in some brain areas and not others, and uncovering the consequences of these effects, should be a major focus of future research.

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Abbreviations

BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; BNSTov, oval nucleus of the BNST; CEA, central nucleus of the amygdala; DG, dentate gyrus of the hippocampus; DMH, dorsomedial hypothalamic nucleus; Ensure, chocolate Ensure Plus; PER1, period1 protein; PER2, period2 protein; PGC-1a, peroxisome proliferator-activated receptor gamma coactivator 1a; RF, restricted feeding; RT, restricted treat; SCN, suprachiasmatic nucleus.

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