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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Running Title: PER1 expression after variable restricted feeding
Pages: 23
Figures: 4
Tables: 0

Word Count
(i) Entire Manuscript: 4419
(ii) Abstract: 250
(iii) Introduction: 379

Keywords: Restricted Feeding, Suprachiasmatic Nucleus, Immunohistochemistry, Running wheel activity rhythm

PER1 expression after variable restricted feeding

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 24hr cycle, for 10 days. Wheel running activity and the expression of the clock protein, Period1 (PER1), in the compact part of the DMH and the suprachiasmatic nucleus (SCN) were assessed. Although rats exhibited increased levels of activity during the portion of the day when food was randomly presented, as expected, distinct food-entrained activity rhythms were absent. 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 24hr 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; Fuller *et al.*, 2008; Verwey *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 PER1 and PER2, in the DMH

(Mieda *et al.*, 2006; Saper & Fuller, 2007; Verwey *et al.*, 2007; Fuller *et al.*, 2008; Verwey *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 12hr day (daytime VRF), the 12hr night (nighttime VRF) or anytime across the entire 24hr day (anytime VRF) for a period of ten 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 approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada). All efforts were made to minimize the number of rats used and their potential suffering. 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 12hr:12hr light-dark cycle (LD cycle; ~300 lux at cage level) and had free access to standard rodent diet (#5075; Charles River Laboratories, St. Constant, QC,

Canada) and water for at least 2 weeks before each experiment. Running wheel activity was recorded continuously by computer (Vitalview, Minimitter, Bend, OR, USA).

Variable restricted feeding

Rats were randomly assigned to one of 4 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 2hr 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 anaesthetized with sodium pentobarbital (Somnotol, 100 mg/kg) at one of four ZTs (ZT1, 7, 13, or 19). Rats were perfused transcardially with 300ml of cold saline (4°C; 0.9% NaCl in distilled water) followed by 300 ml of cold paraformaldehyde solution (4°C ; 4% paraformaldehyde in 0.1M phosphate buffer) and brains were post-fixed for 24hrs

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 (40hrs, 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, 50mM 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, Burlington, ON, Canada), followed by an incubation in an Avidin-Biotin-Peroxidase solution (Vectastain Elite ABC Kit; Vector Laboratories, Burlington, ON, Canada). Sections were then rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution and immunoreactive 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 PBS) 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

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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). Immunoreactive (IR) cells were counted for each image using a 400x400μm template and means were calculated for each brain area based on the 6 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 are shown in Fig. 1, and graphs showing daily Ensure consumption as well as the total amount and distribution of daily running-wheel 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 running-wheel activity by 2-3 fold relative to daily running activity

in AL controls (Fig. 2b). Moreover, VRF schedules increased daytime running (Fig. 2c) as compared to 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 around ZT13 (see Figs. 4, 5). In contrast, in the DMH, PER1 expression varied as a function of feeding schedule (see Figs. 4, 5). 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 which peaked around 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 around 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).

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 17-24%, roughly the same magnitude of weight loss seen in rats subjected to 10 days of predictable feeding schedule with Ensure (2hr/day). The levels and distribution of daily running wheel activity were differentially affected by these VRF schedules, but because of the unpredictability of the time of feeding, distinct food-entrained anticipatory rhythms in running wheel activity did not emerge. However, despite the unpredictability of the feeding times, the daytime VRF group showed the largest increase in running wheel activity during the day, the nighttime VRF group

showed the majority of running wheel 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 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 24hr day. These results demonstrate that in addition to predictable restricted feeding schedules (Mieda *et al.*, 2006; Verwey *et al.*, 2007; Fuller *et al.*, 2008; Verwey *et al.*, 2008), variable restricted feeding 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 result 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 24hr 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 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 (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. 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 running wheel 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; Landry *et al.*, 2007; Verwey *et al.*, 2007; 2008).

Acknowledgements

Supported by Fonds de la recherche en santé Québec (FRSQ), Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council of Canada (NSERC), the Concordia University Research Chairs Program (CRUC). We would like to thank Dr. S.M. Reppert for his generous gift of PER1 antibody and Dr. J. Stewart for helpful comments on the manuscript.

Abbreviations:

AL, ad libitum; ANOVA, analysis of variance; DMH, dorsomedial hypothalamic nucleus; Ensure, chocolate ensure plus; IR, immunoreactive; LD, light-dark; PER1, period1; PER2, period2; VRF, variable restricted feeding; SCN, suprachiasmatic nucleus; ZT, zeitgeber time

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PER1 expression after variable restricted feeding

Figure 1:

Running wheel 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. Actograms illustrate the 12hr:12hr 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 2:

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

Figure 3:

Photomicrographs showing examples of PER1-immunostaining in the SCN and 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.

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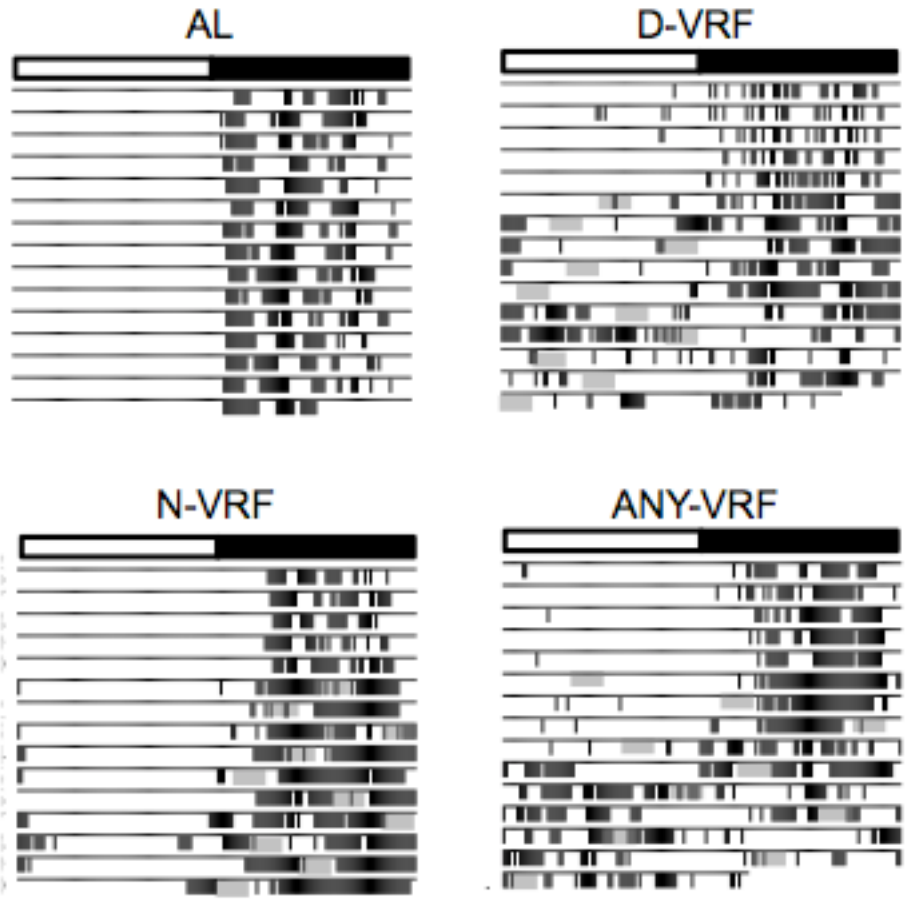
Figure 4:

Graphs showing mean (±SEM) number of PER1-immunoreactive (PER1-IR) nuclei in the SCN and DMH as a function of ZT for the AL, daytime VRF, nighttime VRF and anytime VRF groups (n=3-4/group).



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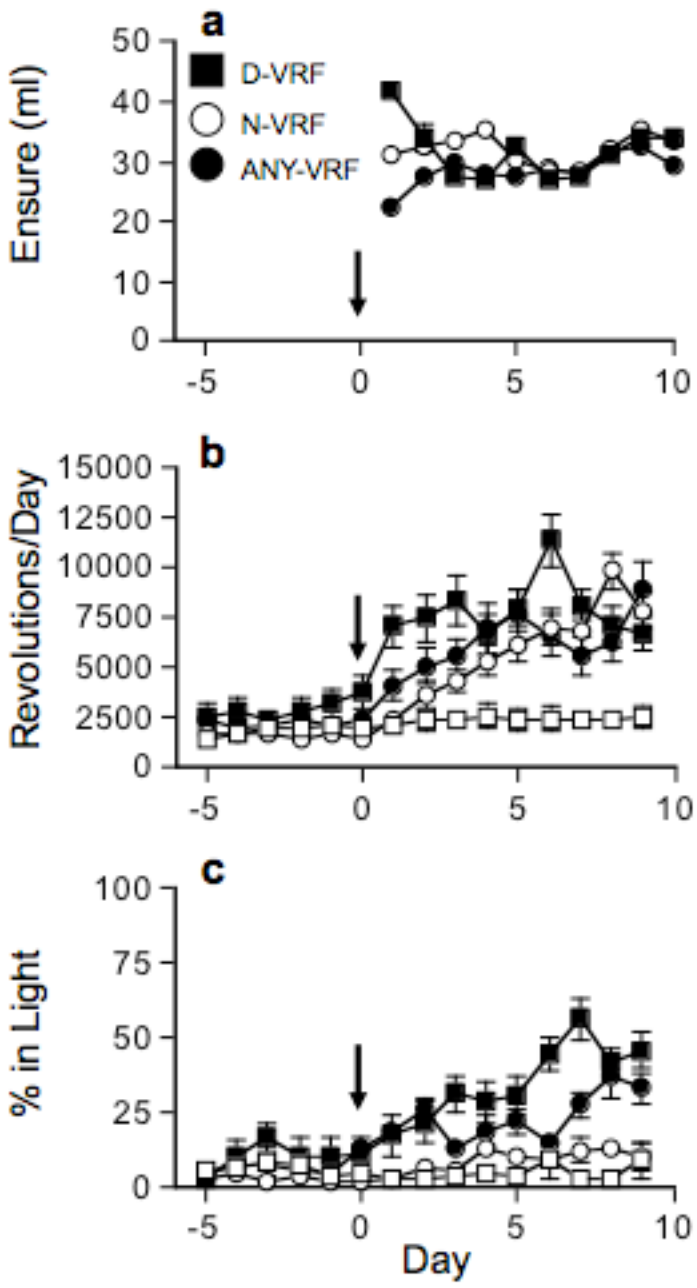
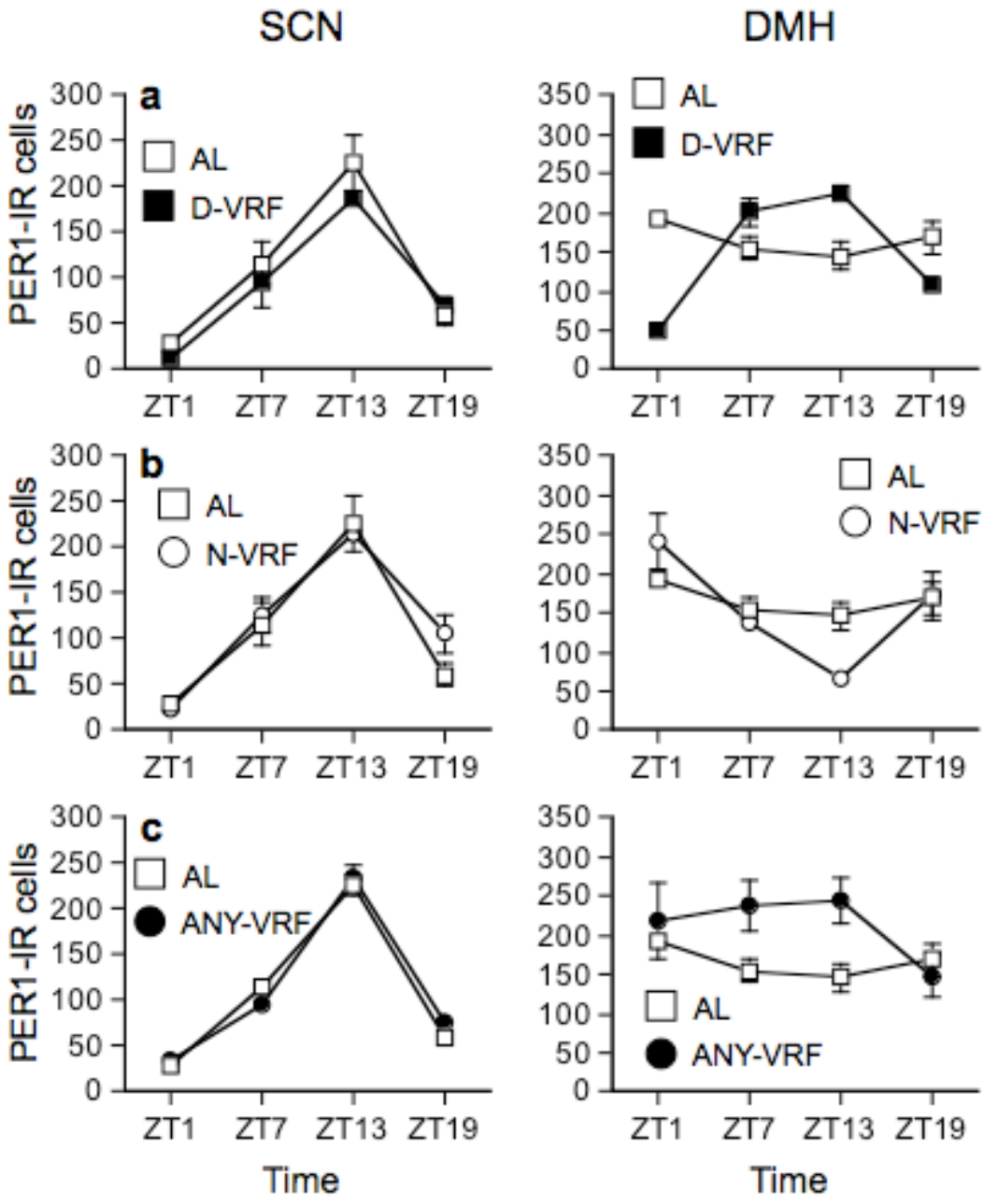


PER1 expression after variable restricted feeding



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