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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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List of Abbreviations

Abbreviation	Definition
2hRF	Restricted feeding: 2h daily meal
6hRF	Restricted feeding: 6h daily meal
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

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.

Keywords: 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

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, 2011). 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, Verwey et al., 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, Lamont et al., 2005b, 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, Fuller et al., 2008, Verwey et al., 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 and Amir, 2010).

Experimental Procedures

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-light (~300 lux at cage level):12h-dark cycle, 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 (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, 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 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 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 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 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 3). The total daily activity was increased in both RF groups, as compared to the AL group ($p<0.01$; Figure 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 ($p<0.01$; Figure 4, middle graph). Accounting for the differences in total activity (Figure 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). 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; Figure 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) 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 (Figure 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 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 ($p<0.05$; Figure 6). In contrast, robust daily rhythms in PER1 expression were observed under both RF schedules ($p<0.01$; Figure 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 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 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, Segall et al., 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, Liu et al., 2007, Asher et al., 2008, Belden and Dunlap, 2008, Rodgers et al., 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, Mistlberger et al., 2009). Instead of being necessary or sufficient for rhythms in food-anticipatory activity, it was recently suggested that, instead, 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 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, Verwey et al., 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, Verwey et al., 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 (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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Figure Captions

Figure 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 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 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.

Figure 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.

Figure 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.

Figure 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 ($p<0.05$).

Acknowledgements

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Figure 1
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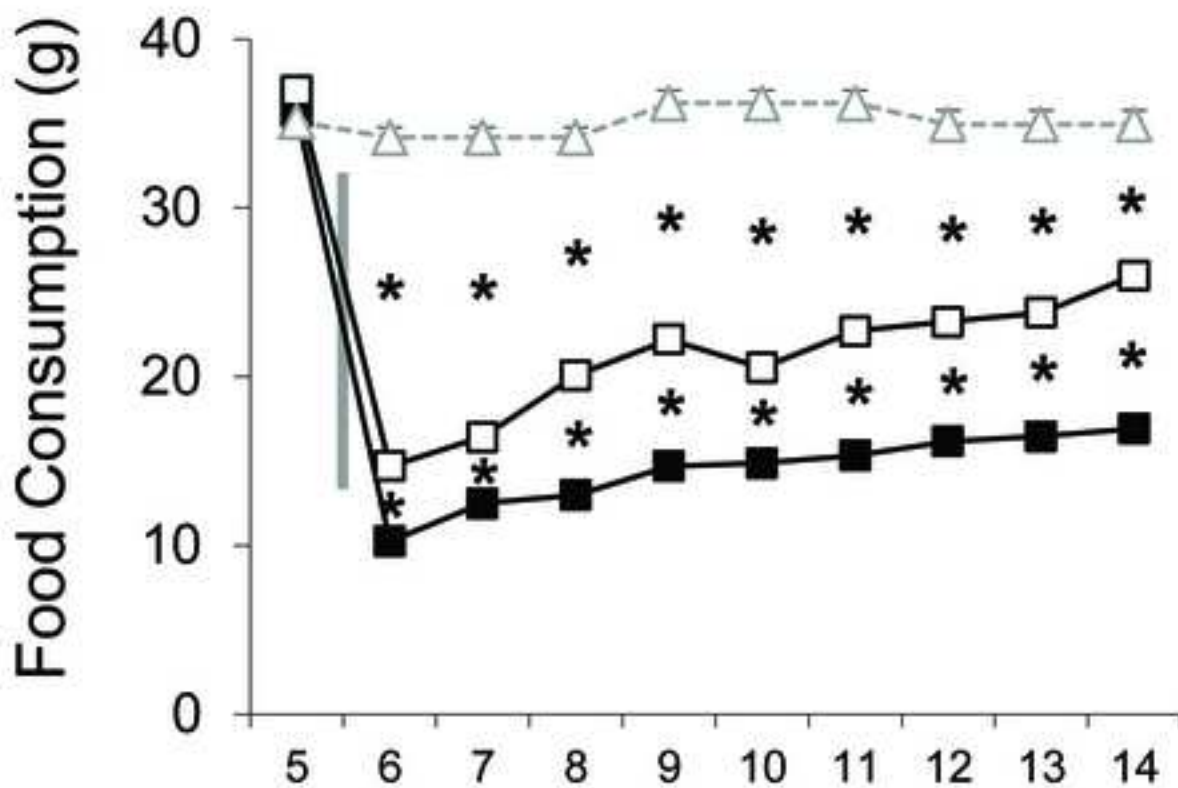

Figure 2

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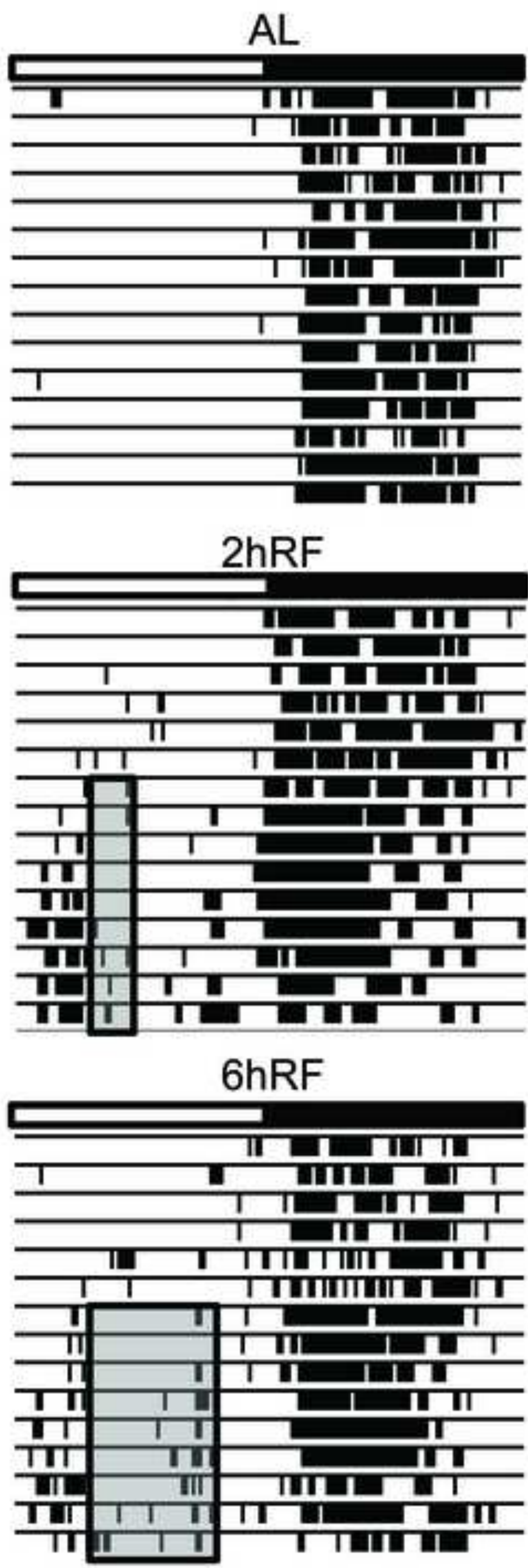

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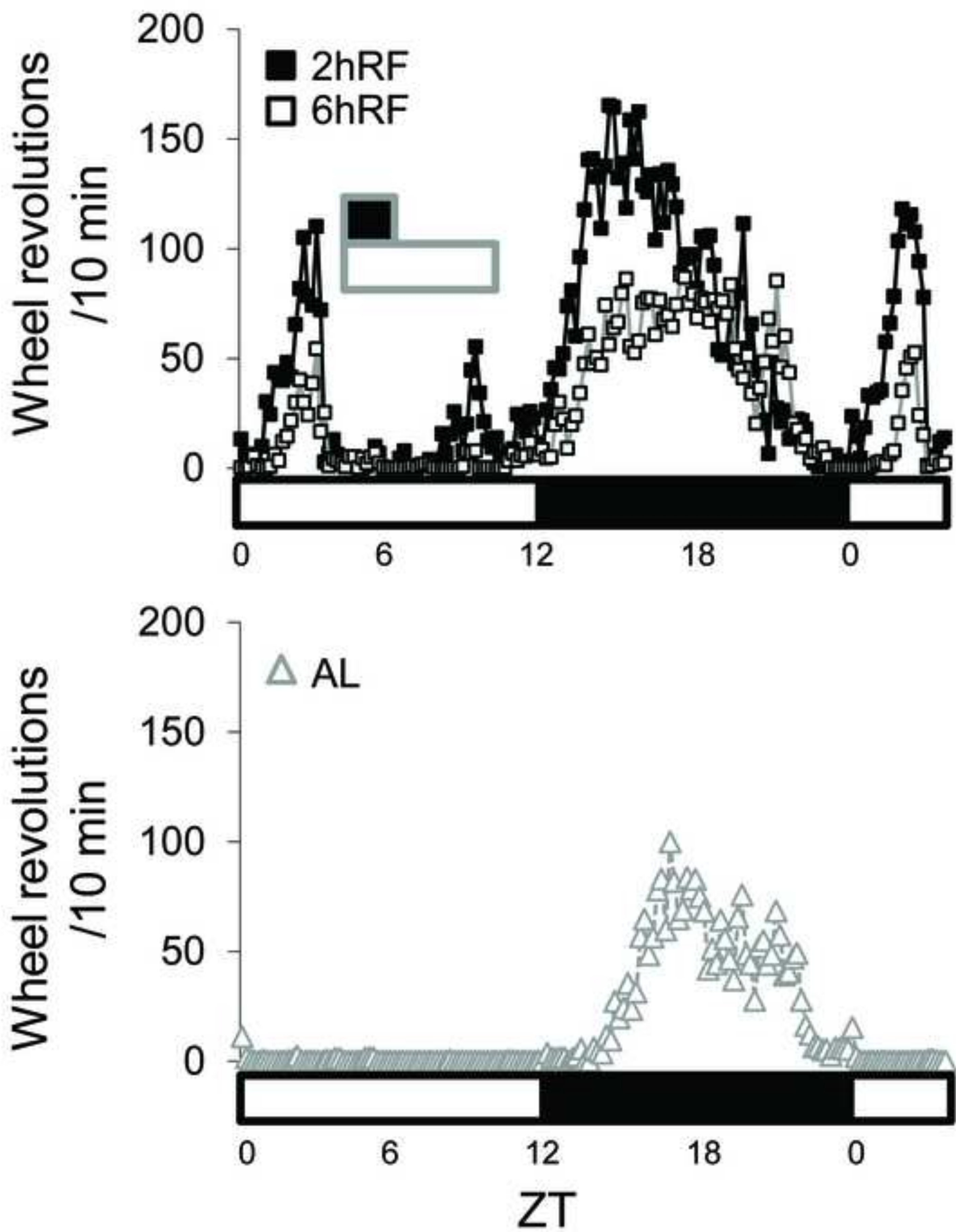

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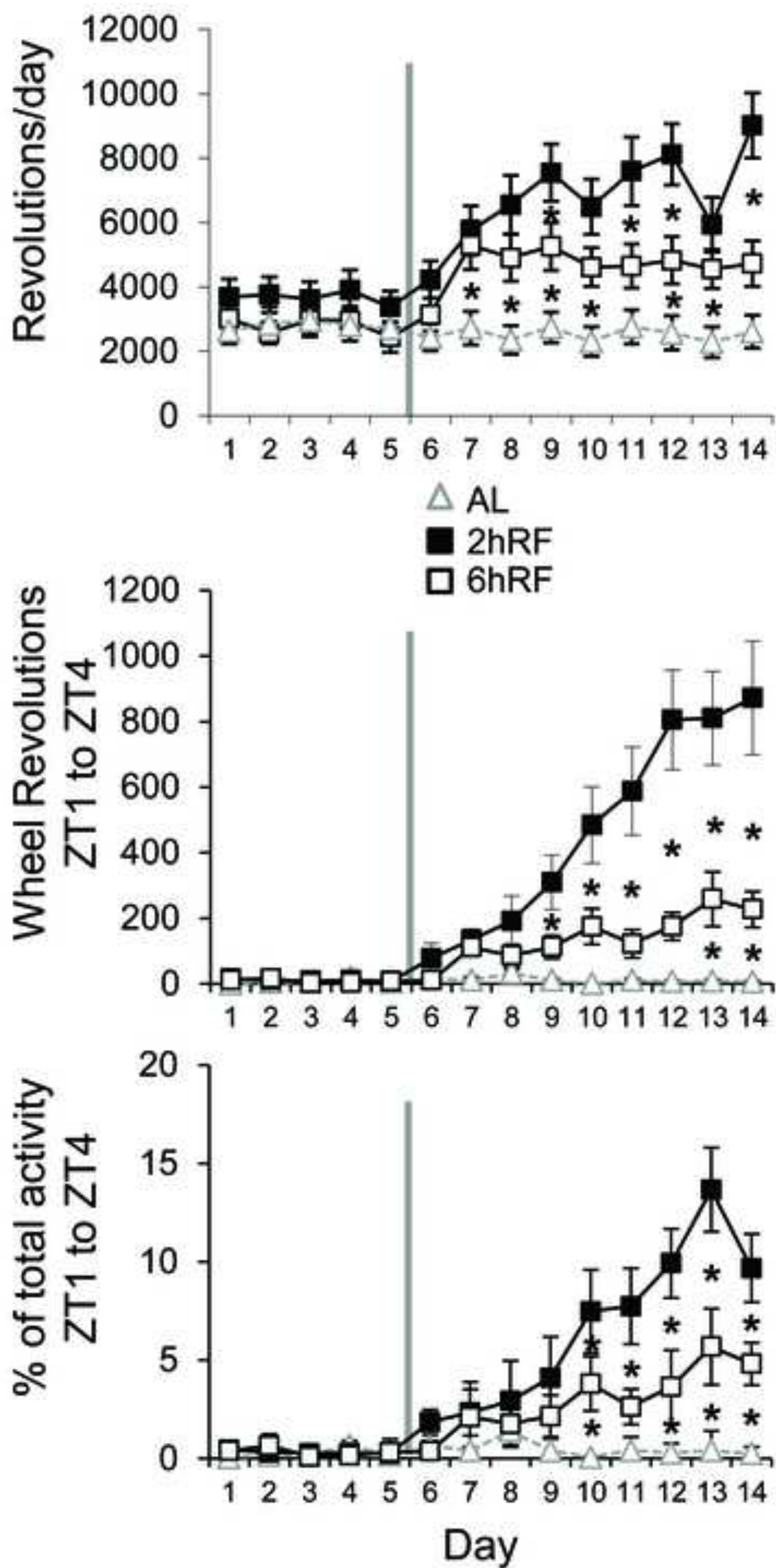

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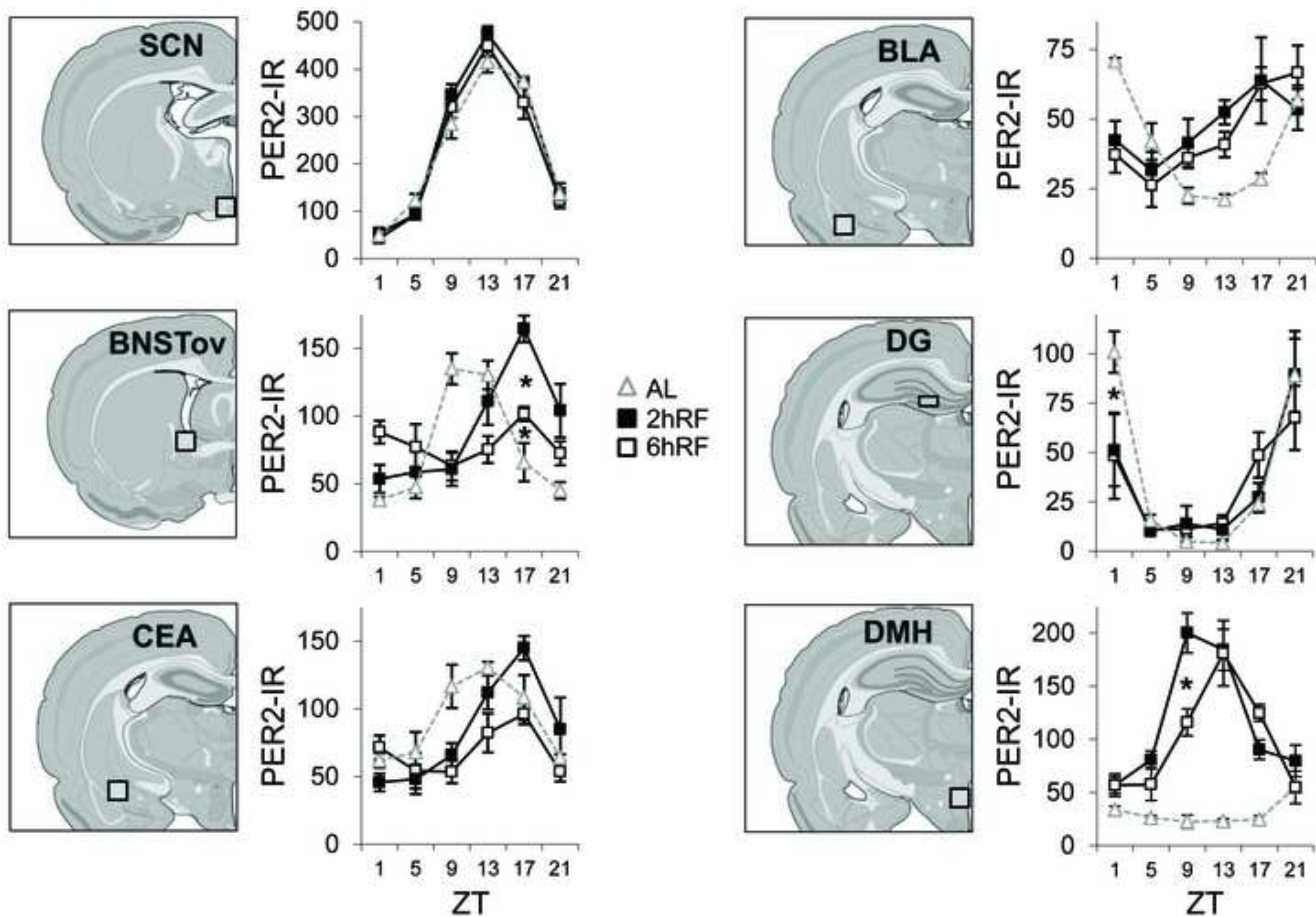

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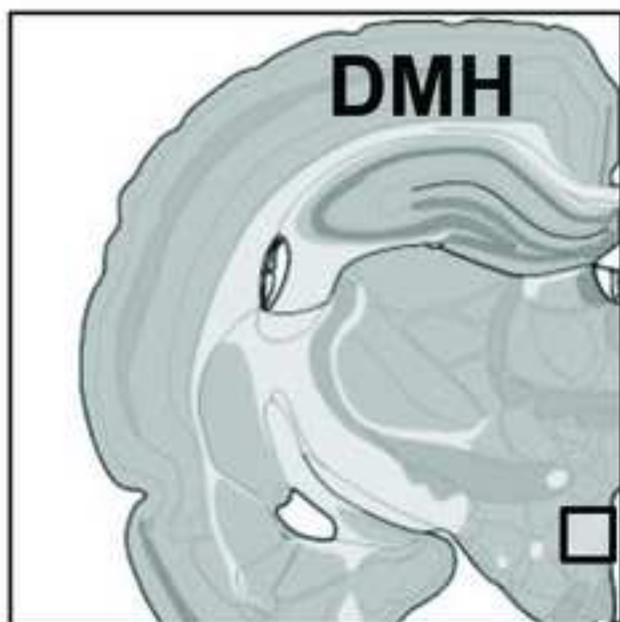

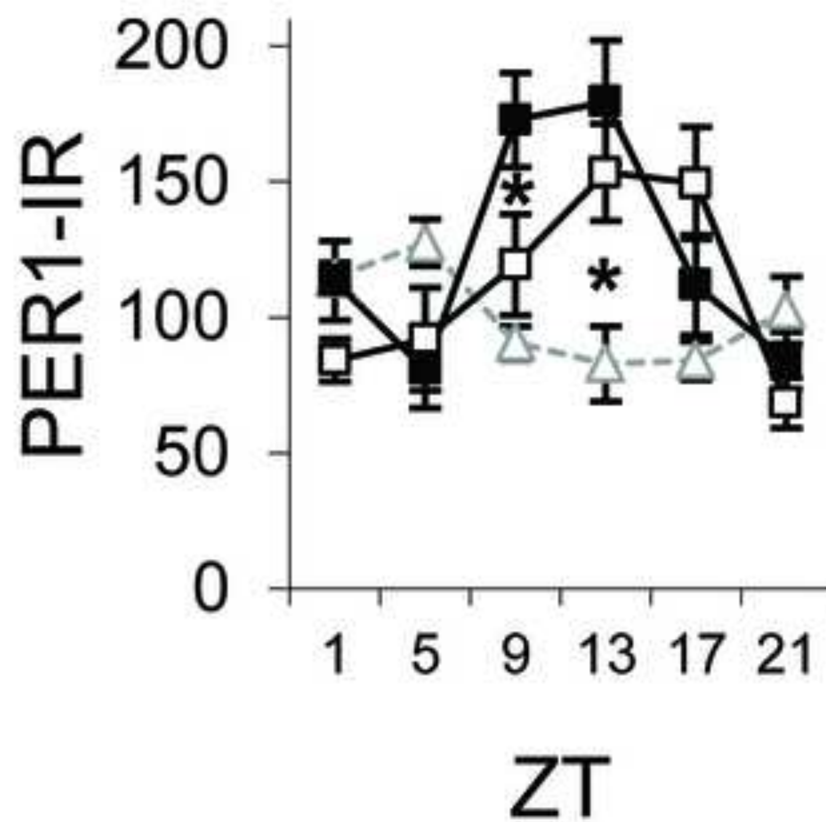

Table 1

TABLE 1: ANOVA of PER2 expression in the SCN, limbic forebrain and DMH

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

Table 2

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, $p<0.01$
$ANOVA_{TIMExGROUP}$	$F_{10,54}$=5.3, $p<0.01$