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.

Keywords: Circadian rhythms, restricted feeding, PER2, Suprachiasmatic nucleus, Oval nucleus of the bed nucleus of the stria terminalis, Central nucleus of the amygdala, Dentate gyrus, Dorsomedial hypothalamic nucleus.

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, 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 (225-250g; 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 6 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 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, 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 x 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 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 remains to be determined.

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Figure captions

Fig. 1

Mean (±sem) 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.

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 12h: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/10 min. Successive days are plotted from top to bottom. The graph in the lower right shows mean (±SEM) total daily number of wheel-revolutions per group (ns=24) starting four 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 (±SEM) 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.

Table 1

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 x 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

Figure 1

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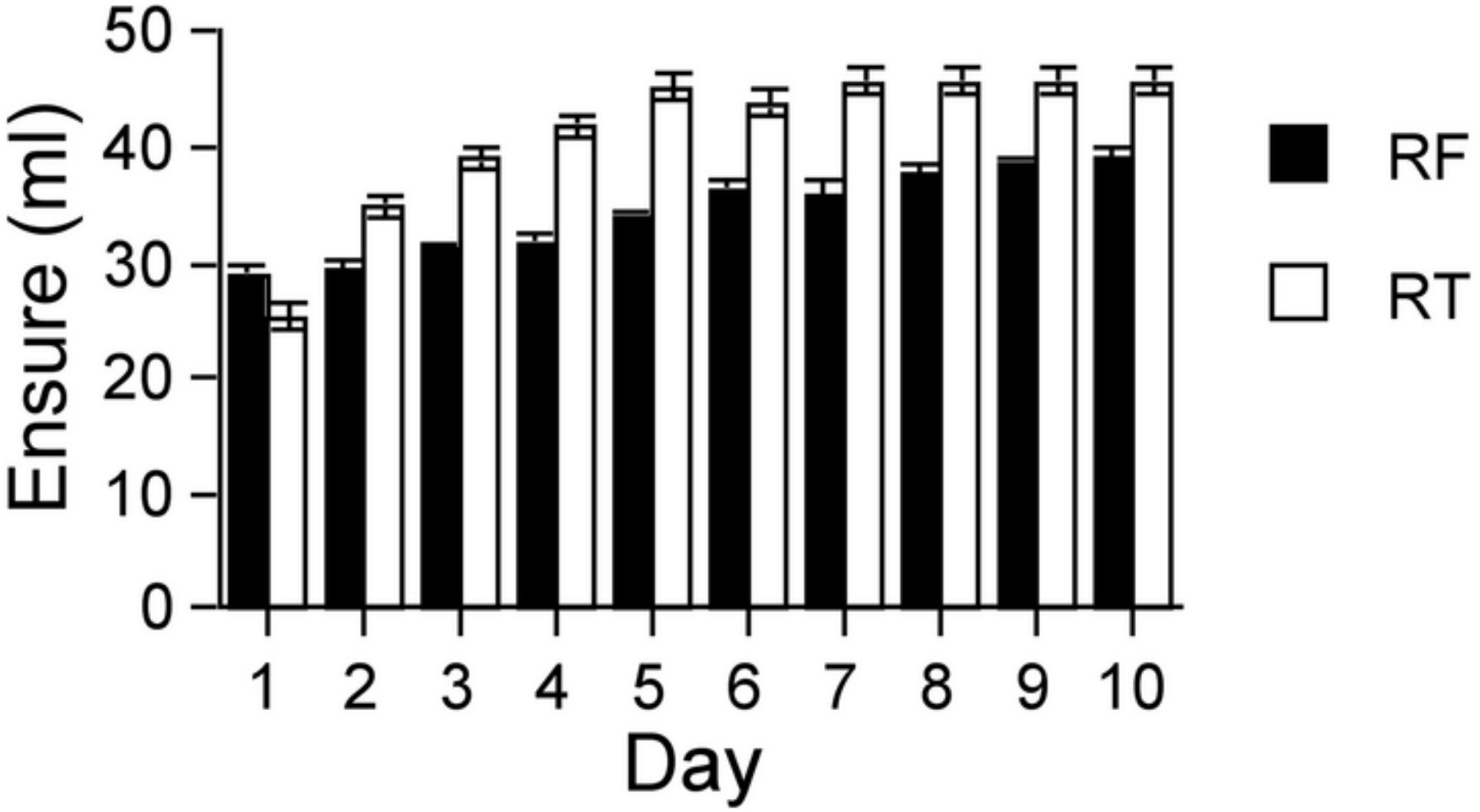

Figure 2

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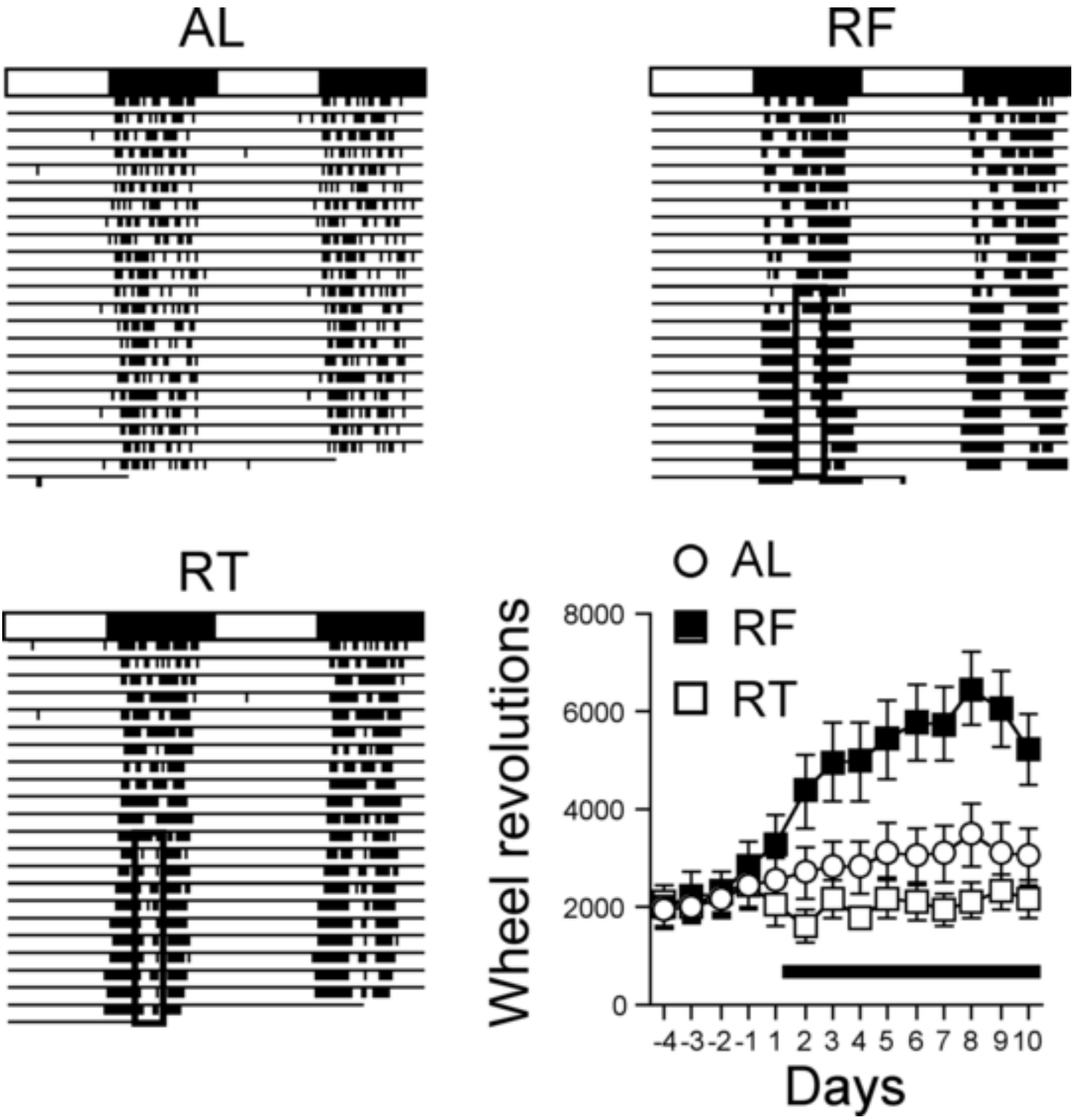

Figure 3

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