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Circadian rhythms and food anticipatory behavior in Wfs1-deficient mice

Hendrik Luuk [a,b,c,*], Jan Fahrenkrug [a], Jens Hannibal [a]

[a] Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Denmark
[b] Department of Physiology, University of Tartu, Estonia
[c] Centre for Excellence in Translational Medicine, University of Tartu, Estonia

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ABSTRACT

The dorsomedial hypothalamic nucleus (DMH) has been proposed as a candidate for the neural substrate of a food-entrainable oscillator. The existence of a food-entrainable oscillator in the mammalian nervous system was inferred previously from restricted feeding-induced behavioral rhythmicity in rodents with suprachiasmatic nucleus lesions. In the present study, we have characterized the circadian rhythmicity of behavior in Wfs1-deficient mice during *ad libitum* and restricted feeding. Based on the expression of Wfs1 protein in the DMH it was hypothesized that Wfs1-deficient mice will display reduced or otherwise altered food anticipatory activity. Wfs1 immunoreactivity in DMH was found almost exclusively in the compact part. Restricted feeding induced c-Fos immunoreactivity primarily in the ventral and lateral aspects of DMH and it was similar in both genotypes. Wfs1-deficiency resulted in significantly lower body weight and reduced wheel-running activity. Circadian rhythmicity of behavior was normal in Wfs1-deficient mice under *ad libitum* feeding apart from elongated free-running period in constant light. The amount of food anticipatory activity induced by restricted feeding was not significantly different between the genotypes. Present results indicate that the effects of Wfs1-deficiency on behavioral rhythmicity are subtle suggesting that Wfs1 is not a major player in the neural networks responsible for circadian rhythmicity of behavior.

1. Introduction

Mutations in Wolfram syndrome 1 (WFS1) gene are a major cause of Wolfram syndrome (WS), a rare autosomal recessive disorder diagnosed on the basis of early-onset non-autoimmune insulin-dependent diabetes mellitus and progressive optic atrophy [1–4]. WS patients display considerable clinical pleiomorphism, including symptoms like sensoryneural deafness, hypothalamic diabetes insipidus, neurological complications (cerebellar ataxia, myoclonus, epilepsy, nystagmus), renal tract abnormalities, gastrointestinal dysmotility, primary gonadal atrophy, psychiatric disorders, short stature, peptic ulcers and cataract [5–12]. Accumulating evidence suggests that Wfs1 protein is involved in various intracellular processes such as the regulation of Ca^{2+} homeostasis [13,14], regulation of ER-stress response [15–17] and glucose-stimulated insulin release from pancreatic ß-cells [18]. A previous study of the WS mouse model lacking exon 8 of Wfs1 suggests that the lack of Wfs1 protein leads to lower body weight and increased axiety-like behavior [19].

Wfs1 is widely expressed in the central nervous system with enrichment in the central extended amygdala and ventral striatum – brain structures involved in the regulation of anxiety and appetitive behaviors [20]. Among other brain regions, we identified Wfs1 expression also in the compact part of dorsomedial hypothalamic nucleus (DMC), a subregion of dorsomedial hypothalamic nucleus (DMH) – a proposed substrate for food-entrainable circadian rhythms [21–24]. Entrainment of behavioral rhythms to food availability is reflected in the emergence of food anticipatory activity (FAA) upon the introduction of restricted feeding (RF). Rodents having access to food only during a limited period of the subjective day exhibit increased behavioral activity prior to the presentation of the food (i.e. FAA). A number of studies suggest that FAA is dependent on a food-entrainable oscillator most likely located in the hypothalamus (see [25], for a review).

In the present study, we have characterized the circadian rhythmicity of behavior in Wfs1-deficient mice during *ad libitum* feeding and during RF. Assuming that the lack of Wfs1 protein might alter the responsiveness of DMH, it was hypothesized that Wfs1-deficient mice exhibit reduced or altered FAA during the restricted feeding regime. We have also investigated the expres-

Abbreviations: Arc, arcuate nucleus; DD, constant darkness; FAA, food-anticipatory activity; FEO, food-entrainable oscillator; DMH, dorsomedial hypothalamic nucleus; DMC, compact part of DMH; LD, 12 h light/12 h darkness regime; LL, constant light; RF, restricted feeding; SCN, suprachiasmatic nucleus; sem, standard error of mean; VMH, ventromedial hypothalamic nucleus; WS, Wolfram syndrome; wt, wildtype; ZT, *zeitgeber* time.

* Corresponding author at: Department of Physiology, Biomeedikum, Ravila 19, Tartu 50411, Estonia. Fax: +372 737 4332.
E-mail address: hendrik.luuk@gmail.com (H. Luuk).

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sion pattern of Wfs1 protein in the DMH and its colocalization with c-Fos during RF.

2. Material and methods

2.1. Wfs1-deficient mice

Animal care and all experimental procedures were conducted in accordance to the principles of Laboratory Animal Care (Law on Animal Experiments in Denmark, publication 1306, November 23, 2007) and Dyreforsoegstilsynet, Ministry of Justice, Denmark, who issued the licence number 2008/ 561-1445 to JF thereby approving the study. Wfs1-deficient mouse strain was generated as described in [20]. Briefly, Wfs1 targeting construct was created by subcloning a 8.8 kb BamHI fragment from 129SvEv/TacfBr mouse genomic PAC clone 391-J24 (RPCI21 library, MRC UK HGMP Resource Centre, UK) including introns 6–7 and exons 7–8 of Wfs1 gene into pGem11 cloning plasmid (Promega). A 3.7 kb NcoI fragment was replaced by an in-frame NLSLacZNeo cassette, deleting more than 90% of the 8th exon and 60% of the total coding sequence including 8 of the 9 predicted transmembrane domains. Germline chimeras were crossed with wildtype (wt) 129 Sv/EvTac strain to generate heterozygous F1 founder mice. The founders were backcrossed for three generations to the 129 Sv/EvTac background and the progeny was mated to generate a colony of Wfs1-deficient (Wfs1 -/-) mice and their wt littermates for the current experiments. Unless indicated otherwise, the mice were housed in individual cages equipped with running wheels under 12 h/12 h light/dark cycle (lights on at 06:00, light intensity 300 lux) with free access to food and tap water. Prior to the experiments the mice were habituated to the aforementioned housing conditions for at least 2 weeks. The mice were 7–12 weeks old at the beginning of the experiments. For the study of circadian wheel-running under *ad libitum* feeding, 8 Wfs1-deficient (4 male, 4 female) and 8 wt (4 male, 4 female) mice were used. For the restricted feeding experiment, 16 Wfs1-deficient (8 male, 8 female) and 14 wt (7 male, 7 female) mice were used. Four wt mice (2 male, 2 female) were used for Wfs1-immunohistochemistry.

2.2. Recording of circadian activity

Wheel-running activity was monitored by an on-line PC connected via a magnetic switch to the Minimitter Running Wheel activity system (consisting of QA-4 activity input modules, DP-24 dataports and Vital View data acquisition system, MiniMitter Company Inc., Sunriver, OR, USA vers. 4.1) [26]. Wheel revolutions were collected continuously in 10 min bins. Animals were entrained to a 12:12 LD cycle for 7–14 days prior to the initiation of experiments.

2.3. Endogenous period TAU (τ)

Free-running period (τ) was assessed during days 4–18 in constant darkness (DD) or in constant light (LL) after re-entrainment to an LD cycle. TAU was calculated using χ^2 periodogram in ClockLab (ActiMetric Software, Coulbourn Instruments, Wilmette, IL, USA).

2.4. Light induced phase shift using Aschoff type II regime

Light induced phase shift of the circadian rhythm was determined using the Aschoff type II regime as described previously [27]. All animals were light stimulated for 30 min at 300 lux in their home-cages in separate experiments at ZT 16 (ZT 0 denotes lights on, ZT 12 lights off) and ZT 22, respectively, whereafter the lights were turned off for the next 10 d followed by 14 d of re-entrainment in LD before the next light pulse experiment. The light-induced phase shift was determined by the difference in phase from regression lines drawn through the activity onset of the entrained (LD) onset immediately before the day of stimulation and the onset from the free-running phase of activity found typically 2–3 days after light stimulation.

2.5. Restricted feeding experiment

Mice housed individually under 12:12 LD cycle were fed for 14 days on a restricted schedule with food available between ZT 4 and 8 during the subjective day. Running wheel activity was monitored continuously and body weight was recorded every 2 days.

2.6. Tissue fixation

Mice from the RF experiment and 4 wt (2 males and 2 females) under a standard photoperiod were anesthetized using a subcutaneous injection (8 μL/g body weight) of Hypnorm/Midazolam in the middle of the subjective day (ZT 7) and perfused with phosphate buffered saline (PBS) followed by Stefanini fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer, pH 7.6). The brains were removed and post-fixed in the same fixative overnight, cryoprotected in 30% sucrose-PBS for three days, frozen on dry ice and sectioned into 40 μm thick coronal slices in 3 series.

2.7. Immunohistochemistry

Wfs1 immunoreactivity was detected with rabbit antiserum 05149/4 raised against the C-terminal peptide of mouse Wfs1 protein and characterized in [20]. Free-floating tissue slices were incubated for 72 h with the primary antibody (diluted 1:15,000) at 4 °C, followed by room-temperature incubations with 1:800 biotinylated donkey anti-rabbit secondary antibody (711-065-152, Jackson ImmunoResearch Europe, Suffolk, England) for 1 h, 30 min incubation with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA), 30 min incubation with 1:200 biotinylated Tyramide (SAT700, NEN Life Science Products, Belgium) and 30 min incubation with streptavidin-conjugated Cy2 fluorophore (016-220-084, Jackson ImmunoResearch Europe). For double staining with c-Fos, Wfs1 immunoreactivity was visualized with streptavidin-conjugated DyLight 594 fluorophore (016-510-084, Jackson ImmunoResearch Europe) diluted 1:1000. c-Fos immunoreactivity was detected with 1:100 goat anti c-Fos (SC-52-G, Lot# K1109, Santa Cruz Cruz Biotechnologies, CA, USA) followed by 1:1000 donkey anti-goat AlexaFluor 488 (A11055, Molecular Probes, Life Technologies, NY, USA). Stained sections were transferred to glass slides and mounted in 1:1 PBS:glycerol.

2.8. Microscopy

Digital images were acquired using DFC 480 CCD camera (Leica) attached to Leitz DM RB microscope (Leica) equipped with HBO 100 W mercury arc lamp as the light source. Images were captured using Leica Application Suite software (Leica) and adjusted for brightness and contrast in Photoshop CS (Adobe) and assembled into panels in Illustrator CS (Adobe). For consistency, Wfs1 was depicted in green and c-Fos in red (red and green channels were switched in images of Wfs1 c-Fos double staining).

2.9. Statistical analysis

Statistical analysis was carried out in Prism 4 (GraphPad). A p-value <0.05 was considered statistically significant. Since the initial analysis indicated an absence of strong interaction between genotype and sex, the present analysis is based on groups with males and females pooled. Data collection artifacts manifesting

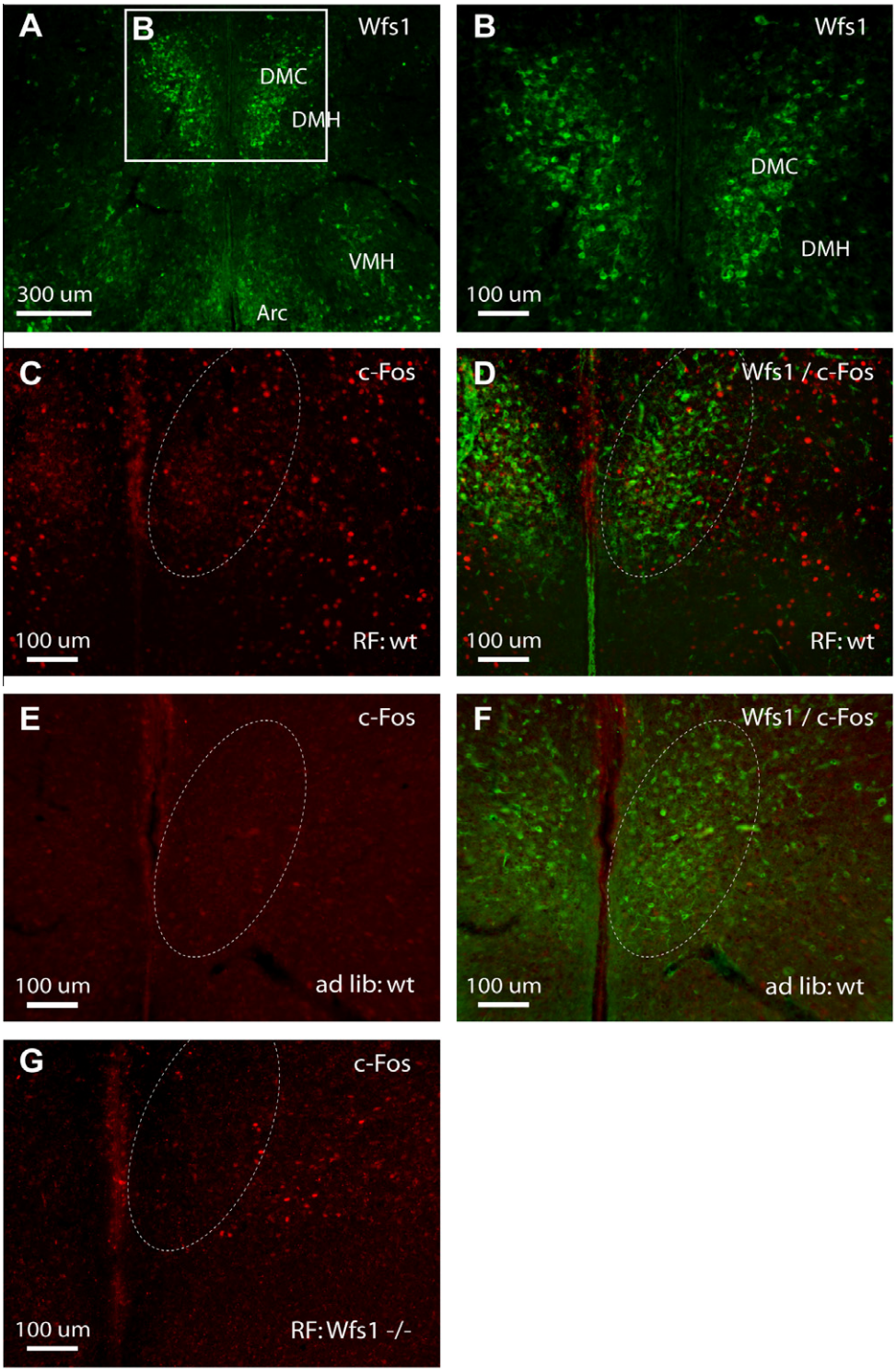

Fig. 1. Wfs1 and c-Fos immunostaining in the dorsomedial hypothalamic nucleus during *ad libitum* feeding and restricted feeding. (A) Wfs1 immunostaining (green) of the hypothalamic section containing DMH under *ad libitum* feeding. (B) Higher magnification of the DMH region in (A). Wfs1-positive neurons are almost exclusively located in DMC. (C) c-Fos immunostaining (red) in the DMH of wt at ZT 7 during restricted feeding (ZT 4–8). c-Fos-positive nuclei are mostly located in the ventral and lateral aspects of DMH. DMC is encircled with a dashed line. (D) Wfs1 (green) and c-Fos (red) double immunostaining of DMH in wt at ZT 7 during restricted feeding. The colocalization of Wfs1 and c-Fos is very limited. DMC is encircled with a dashed line. (E) Lack of c-Fos immunoreactivity in the DMH of a wt mouse at ZT 7 during *ad libitum* feeding. DMC is encircled with a dashed line. (F) Wfs1 (green) and c-Fos (red) double staining in the DMH of a wt mouse at ZT 7 during *ad libitum* feeding. DMC is encircled with a dashed line. (G) c-Fos immunoreactivity in the DMH of a Wfs1-deficient mouse at ZT 7 during restricted feeding (ZT 4–8). DMC is encircled with a dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as >24 h periods of very low or no wheel-running activity due to the failure of the recording device were excluded from the analysis. The number of excluded recordings was as follows: *ad libitum* feeding experiment – 1 wt; RF experiment, *ad-libitum* feeding regime – 2 Wfs1 -/-, 1 wt; RF experiment, restricted feeding regime – 4 Wfs1 -/-, 2 wt.

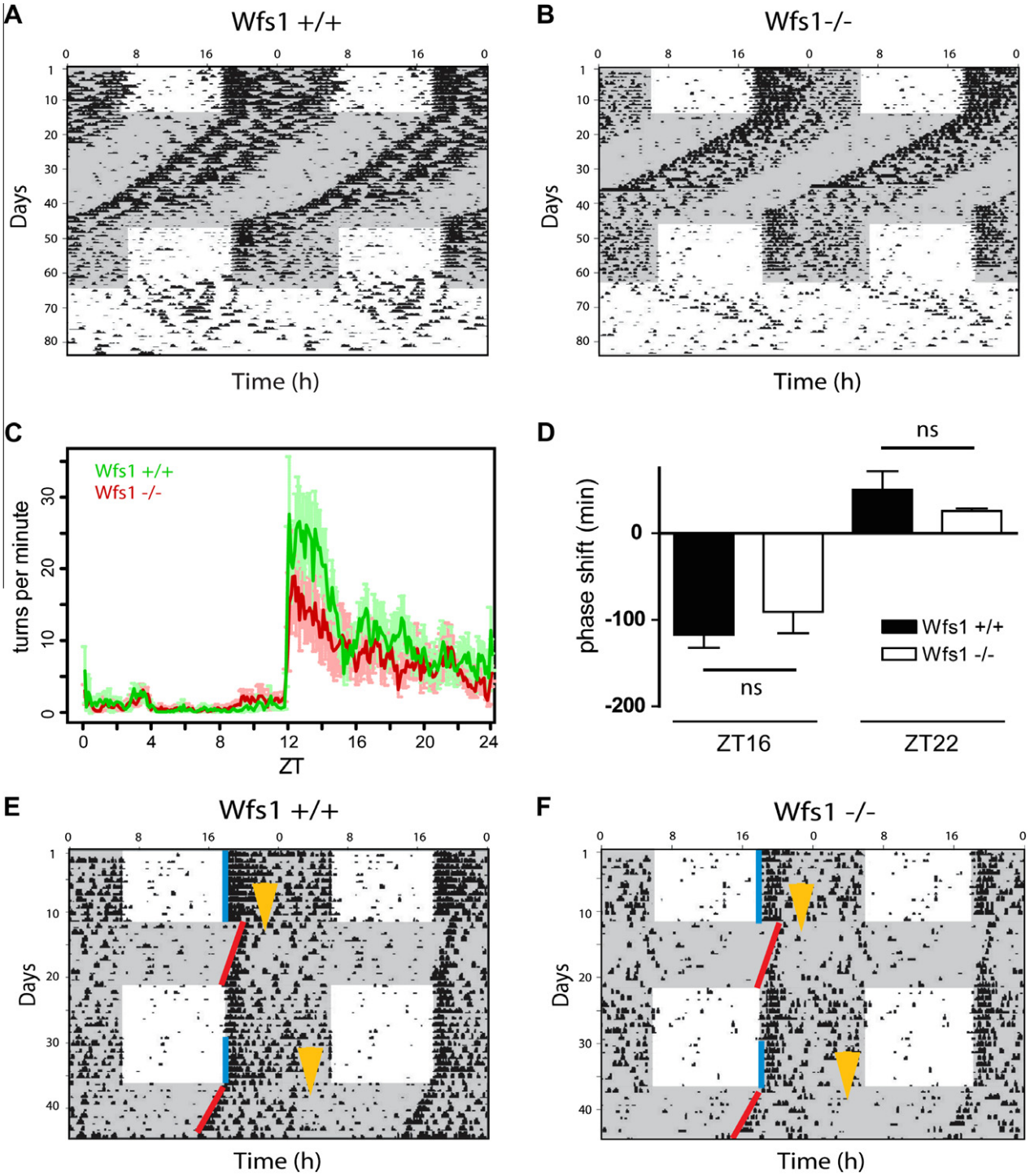

Fig. 2. Circadian behavior of Wfs1-deficient mice during *ad libitum* feeding. (A) Representative actogram of wt mice during LD, DD and LL regimes. Time of day is represented on the horizontal axis. Days are plotted on the vertical axis. Amount of wheel-running activity is represented by the height of the black traces. Shaded regions denote periods of darkness. (B) Representative actogram of Wfs1-deficient mice during LD, DD and LL regimes. Designations of the previous figure apply. (C) Wheel-running activity (mean ± sem) of wt (green, $n = 13$) and Wfs1-deficient mice (red, $n = 14$) in LD during *ad libitum* feeding. The data originate from mice in the restricted feeding experiment 1 week before the onset of restricted feeding. (D) Phase shift in the onset of activity after light-stimulation (mean ± sem). (E) Effect of light-stimulation on the phase of behavioral rhythmicity in wt mice ($n = 7$) at early (ZT 16) and late (ZT 22) subjective night. Arrowheads denote the time of light-stimulation. Blue lines denote the onset of activity during LD prior to light-stimulation. Red lines denote the onset of activity in DD following light-stimulation. (F) Effect of light-stimulation on the phase of behavioral rhythmicity in Wfs1-deficient mice ($n = 8$) at early (ZT 16) and late (ZT 22) subjective night. Designations of the previous figure apply. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

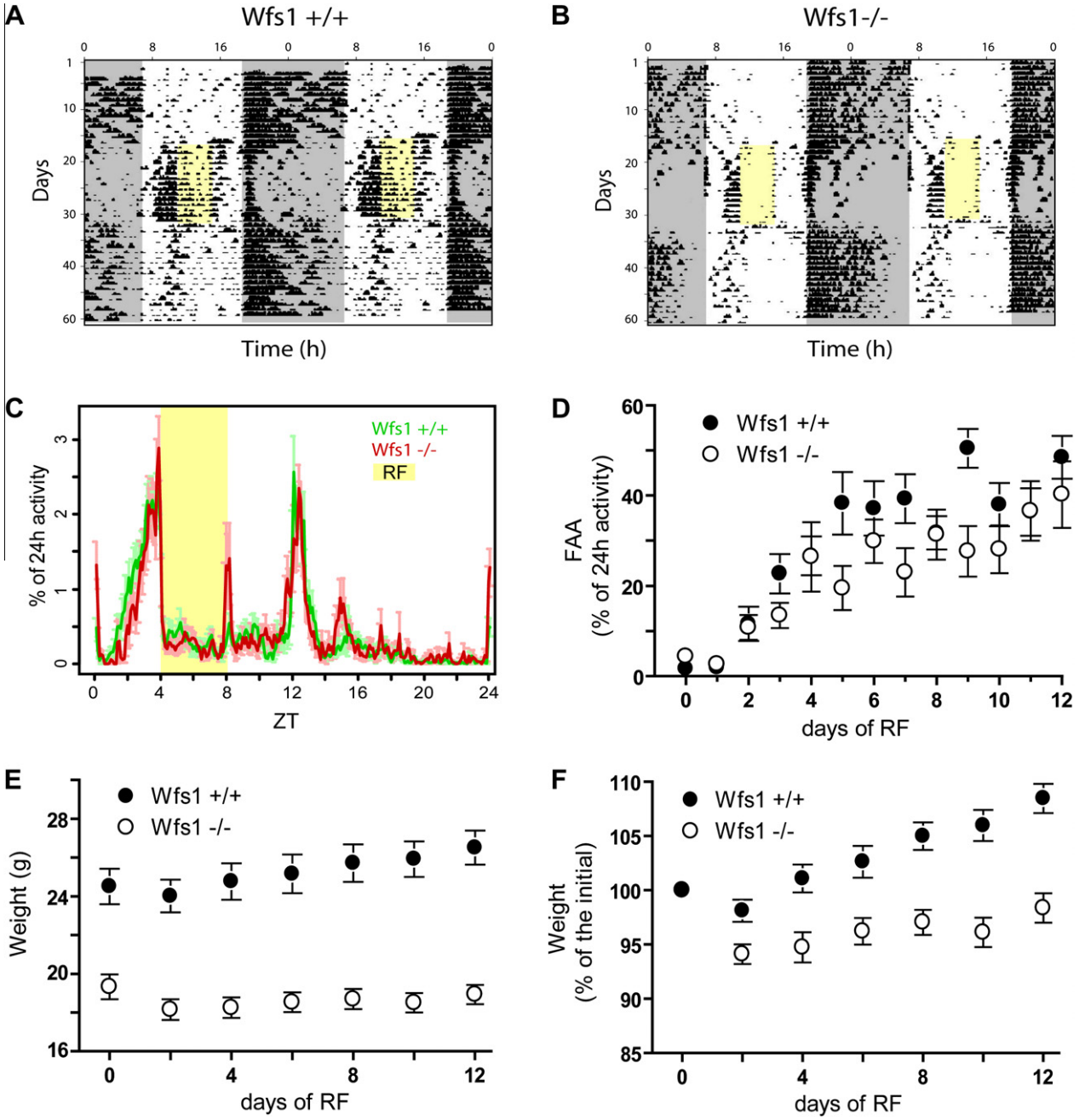

Fig. 3. Circadian behavior of Wfs1-deficient mice during restricted feeding. (A) Representative actogram of wt mice during restricted feeding. Time of day is represented on the horizontal axis. Days are plotted on the vertical axis. Degree of activity is represented by the height of the black traces. Shaded regions denote periods of darkness. Restricted feeding window is marked in yellow. (B) Representative actogram of Wfs1-deficient mice during restricted feeding. Designations of the previous figure apply. (C) Relative wheel-running activity (mean ± sem) of wt (green, $n = 12$) and Wfs1-deficient mice (red, $n = 12$) during restricted feeding. Restricted feeding window is marked in yellow. (D) Daily food anticipatory activity (mean ± sem) in wt ($n = 12$) and Wfs1-deficient mice ($n = 12$). (E) Body weight (mean ± sem) during restricted feeding (wt: $n = 14$, Wfs1 -/-: $n = 16$). (F) Relative body weight (mean ± sem) during restricted feeding (wt: $n = 14$, Wfs1 -/-: $n = 16$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Expression of Wfs1 in the DMH

In agreement with a previous study [20], Wfs1 immunoreactivity in DMH was almost exclusively found in the compact part (Fig. 1A and B) and its intensity varied from weak to moderate. Restricted feeding induced similar expression of c-Fos immunoreactivity in the DMH in wt (Fig. 1C and D) and Wfs1-deficient mice (Fig. 1G) at ZT 7, but not in the *ad libitum* fed mice (Fig. 1E and F). c-Fos immunoreactivity was primarily found in the ventral and lateral aspects of DMH with only a few strongly stained nuclei in the DMC. Since the colocalization of Wfs1 and c-Fos immunoreactivities appeared to be limited to a few neurons, no attempt was made to quantify it.

3.2. Circadian behavior during ad libitum feeding

Wfs1-deficient mice exhibited normal entrainment to the 12:12 h light-dark cycle (Fig. 2A and B) but demonstrated a

significantly lower amount of running wheel activity (mean ± sem: 740.3 ± 140.4, $n = 14$ (Wfs1 -/-) vs 2011 ± 265.5, $n = 13$ (wt); $p < 0.001$, Mann–Whitney test) during the 24 h cycle (Fig. 2C). Endogenous circadian period of Wfs1-deficient mice was similar to that of wild type in constant darkness (mean ± sem: 23.40 ± 0.13, $n = 8$ (Wfs1 -/-) vs 23.44 ± 0.09, $n = 7$ (wt); $p = 0.96$, Mann–Whitney test) whereas in constant light it was significantly longer than in wt mice (mean ± sem: 25.69 ± 0.16, $n = 8$ (Wfs1 -/-) vs 24.68 ± 0.32, $n = 6$ (wt); $p < 0.05$, Mann–Whitney test). The response of Wfs1-deficient mice to light stimulation during early (ZT 16) and late (ZT 22) subjective night was not different from wild type mice (Fig. 2D–F).

3.3. Circadian behavior during restricted feeding

Wfs1-deficient mice were able to entrain to the restricted feeding regime as evidenced by the emergence of food anticipatory activity (FAA) between 1 and 4 h prior to the presentation of food (Fig. 3A and B). The total amount of wheel-running during the RF regime was significantly lower in Wfs1-deficient mice (mean ± sem: 417.3 ± 89.6, $n = 12$ (Wfs1 -/-) vs 1219 ± 131.0, $n = 12$ (wt); $p < 0.001$, Mann–Whitney test), while the circadian profile of relative wheel-running appeared similar to wt (Fig. 3C). The amount of FAA between ZT1-4 was not significantly different between the genotypes (mean ± sem: 29.6 ± 3.9, $n = 12$ (Wfs1 -/-) vs 37.9 ± 3.3, $n = 12$ (wt); $p = 0.24$, Mann–Whitney test). As expected, there was a significant effect of the number of days on RF on FAA (Fig. 3D) when analyzed using 2-way repeated measures ANOVA ($F = 20.16$, df = 12, $p < 0.0001$). The effect of genotype on FAA was insignificant ($F = 3.411$, df = 1, $p = 0.0783$), but there was a trend towards lower FAA in Wfs1-deficient mice. The body weight of Wfs1-deficient mice was significantly lower than in wt (genotype effect: $F = 44.38$, df = 1, $p < 0.0001$, 2-way repeated measures ANOVA) during the experiment (Fig. 3E) and they were not able to regain their initial weight during the RF regime as the wt did (Fig. 3F).

4. Discussion

Since the discovery that an SCN-lesioned animal can anticipate the presentation of food when fed once a day during its rest phase [28,29] there has been considerable effort and controversy regarding the identification of the neural substrate of the hypothesized food entrainable oscillator (FEO). Based on behavioral ablation, rhythm of Per gene expression and induction of c-Fos, the studies of Fuller et al. [22], Gooley et al. [23] and Mieda et al. [24] collectively suggest that the FEO lies in the DMH. On the other hand, a number of studies indicate that DMH is not necessary for the expression of food-entrainable rhythms [30–33]. The controversy appears to be settled by a recent study demonstrating that both the SCN and DMH are relevant but not indispensable for FAA [21], and a notion that the DMH and SCN are just two of possibly many structures that modulate FAA [25,34,35]. With regard to the neurochemical mechanisms of food entrainment, Sutton et al. [36] reported that FAA is significantly attenuated in Mc3r-deficient mice. Similarly, Begriche et al. [37] reported attenuated adaptation of Mc3r-deficient mice to a RF schedule, displaying reduced FAA, reduced food consumption, increased weight loss and reduction of FOS-IR in DMH.

The present study was undertaken to investigate the potential effects of Wfs1-deficiency on FAA during RF. It was hypothesized that due to the lack of Wfs1 expression in DMC Wfs1-deficient mice would show reduced or otherwise altered FAA. As the circadian rhythmicity of behavior was previously uncharacterized in our mouse model it was necessary to study circadian behavior also during *ad libitum* feeding. In line with the very limited number of Wfs1-expressing neurons in the suprachiasmatic nucleus [38], the neural substrate of the light-entrainable oscillator, we found no gross alterations in the circadian rhythmicity of behavior in Wfs1-deficient mice during *ad libitum* feeding despite that Wfs1-deficient mice exhibited an elongated period of wheel-running rhythm under constant light conditions. Given that there was no significant effect of Wfs1-deficiency on FAA, it must be concluded that the FEO has not been eliminated [25].

In line with previous reports [19,39], Wfs1 loss-of-function was associated with significantly lower body weight in the current study. As a novel finding, it was observed that, unlike in wt, the body weight of Wfs1-deficient mice was not restored to the baseline level during the 2 weeks of restricted food intake. A similar phenomenon was observed by Begriche et al. (2012) in Mc3r-deficient mice. In the current study, however, food intake was not measured during RF making it difficult to relate the results with the aforementioned study. We have, however, in an independent experiment observed normal food intake in Wfs1-deficient mice during *ad libitum* feeding (unpublished data). Increased weight loss due to metabolic disturbances cannot be ruled out since decreased stimulus-secretion coupling of insulin [18] and dysregulation of the growth hormone pathway [39] have been observed in Wfs1-deficient mice.

Acknowledgments

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