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RESEARCH ARTICLE

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Evaluating the daily modulation of FADD and related molecular markers in different brain regions in male rats

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Abstract

Fas-Associated protein with Death Domain (FADD), a key molecule controlling cell fate by balancing apoptotic versus non-apoptotic functions, is dysregulated in post-mortem brains of subjects with psychopathologies, in animal models capturing certain aspects of these disorders, and by several pharmacological agents. Since persistent disruptions in normal functioning of daily rhythms are linked with these conditions, oscillations over time of key biomarkers, such as FADD, could play a crucial role in balancing the clinical outcome. Therefore, we characterized the 24-h regulation of FADD (and linked molecular partners: p-ERK/t-ERK ratio, Cdk-5, p35/p25, cell proliferation) in key brain regions for FADD regulation (prefrontal cortex, striatum, hippocampus). Samples were collected during Zeitgeber time (ZT) 2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20, and ZT23 (ZT0, lights-on or inactive period; ZT12, lights-off or active period). FADD showed similar daily fluctuations in all regions analyzed, with higher values during lights off, and opposite to p-ERK/t-ERK ratios regulation. Both Cdk-5 and p35 remained stable and did not change across ZT. However, p25 increased during lights off, but exclusively in striatum. Finally, no 24-h modulation was observed for hippocampal cell proliferation, although higher values were present during lights off. These results demonstrated a clear daily modulation of FADD in several key brain regions, with a more prominent regulation during the active time of rats, and suggested a key role for FADD, and molecular partners, in the normal physiological functioning of the brain's daily rhythmicity, which if disrupted might participate in the development of certain pathologies.

KEYWORDS
brain regions, cell proliferation, circadian clock, neural plasticity, rat

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1 | INTRODUCTION

In the so-called extrinsic apoptotic pathway, cell death is initiated trough Fas receptor activation, followed by the signaling of Fas-Associated protein with Death Domain (FADD) with other intracellular proteins (see García-Fuster et al., 2016; Ramos-Miguel et al., 2012, and references within). Previous evidence has depicted this signaling pathway, and particularly FADD, as a key molecule that controls cell fate by balancing apoptotic versus non-apoptotic functions (e.g., Park et al., 2005; Tourneur & Chiocchia, 2010), therefore suggesting a key role for this protein in balancing neurotoxic versus neuroplastic events taking place in the brain under pathophysiological conditions. In this context and over the past 15 years our research group has extensively worked on characterizing the role of FADD in post-mortem brain samples of subjects with psychopathologies (e.g., major depression: García-Fuster et al., 2014; drug addiction: García-Fuster et al., 2008) and/or clinical dementia (e.g., Ramos-Miguel et al., 2017), as well as in the brains of animal models capturing certain aspects of these disorders, in physiological processes such as aging (Hernández-Hernández et al., 2018a), and through the administration of several pharmacological agents (e.g., García-Cabrerizo & García-Fuster, 2019; García-Fuster & García-Sevilla, 2015, 2016; García-Fuster et al., 2007, 2009, 2011; Hernández-Hernández et al., 2018a, 2018b; Ledesma-Corvi & García-Fuster, 2022). The main results presented FADD as a great pharmacological target since treatments with prototypical drugs for these disorders reverted, in some cases, FADD brain changes in key brain regions (mainly studied in prefrontal cortex, striatum and hippocampus) (some data reviewed by García-Fuster et al., 2016; Ramos-Miguel et al., 2012). However, all of these experiments were performed during the light phase of the day (lights-on: inactive period for rodents) and did not consider the impact of time-of-day and/or the role of daily fluctuations in FADD regulation.

In this context, it is well known that the daily rhythm has a great influence on the regulation of cellular homeostasis and physiology (Godinho-Silva et al., 2019; Huang et al., 2011; Kinouchi & Sassone-Corsi, 2020; Kon et al., 2017; Serin & Acar Tek, 2019), and persistent disruptions in its normal functioning could be behind different pathologies (e.g., McEwen & Karatsoreos, 2015), for instance those related to psychopathologies such as major depressive disorder (e.g., Bedrosian & Nelson, 2017; Homolak et al., 2018; Leng et al., 2019; Sato et al., 2022) and/or neurodegeneration (Duncan, 2020; Maiese, 2021; Sharma et al., 2021; Standlee & Malkani, 2022; Wang & Li, 2021). Therefore, the oscillations over time of certain biomarkers such as FADD might be playing a crucial role in maintaining health and/or in the development of these pathologies. Moreover, given the importance of understanding how daily rhythms might influence drug efficacy and/or toxicity during pharmacological studies to ensure an optimal treatment response (reviewed by Gaspar et al., 2019), our goal was to characterize, for the first time, the 24-h regulation of FADD in key brain regions in which FADD has been shown to be altered in some of the aforementioned clinical outcomes (e.g., prefrontal cortex, striatum, hippocampus; see Ramos-Miguel et al., 2012 for FADD regulation across brain regions in the male rat).

Significance

The normal physiological functioning of the brain's daily rhythmicity is of great importance, since its disruption could lead to certain psychopathologies (i.e., depression). Evaluating how key biomarkers oscillate over time is key to balance the clinical outcome. In particular, Fas-Associated protein with Death Domain (FADD), which controls cell fate by balancing apoptotic versus non-apoptotic functions, is dysregulated in post-mortem brains of depressed-patients, in animal models capturing certain aspects of the disorder, and by several antidepressants. This study proved a clear 24-h modulation of FADD in several key brain regions of importance to depression, which if disrupted might participate in the development of depression.

Moreover, to deepen our understanding on the daily modulation of FADD at the cellular level, some key molecular markers, with certain links to FADD, were selected for this study. In particular, given the direct involvement of members of the MAPK signaling (i.e., ERK1/2) in the regulation of FADD by certain pharmacological agents (e.g., García-Fuster et al., 2007), and the regulation of circadian genes by components of this pathway (e.g., Akashi et al., 2008; Eckel-Mahan et al., 2008; reviewed by Wang et al., 2020), we evaluated the daily regulation of p-ERK/t-ERK. Additionally, cyclin-dependent kinase-5 (Cdk-5) has been ascribed several functions in the nervous system, ranging from neuronal migration, neuronal outgrowth, axonal guidance, and synaptic plasticity (reviewed by Pao & Tsai, 2021), and has been shown to be modulated in parallel to FADD following desipramine treatment (see Ledesma-Corvi & García-Fuster, 2022), and to crosstalk with MEK–ERK signaling (e.g., Ramos-Miguel & García-Sevilla, 2012). Also, Cdk-5, which has been implicated in the regulation of circadian clocks under physiological conditions (e.g., Brenna et al., 2019; Kwak et al., 2013; Ripperger et al., 2022), is known to depend on co-factors p35 and p25 for its activity (Patrick et al., 1999; Tsai et al., 1994). In fact, the dysregulation of Cdk-5 activity by p25 accumulation can lead to neurotoxicity and/or various neurodegenerative disorders through constitutive activation and misplacement of Cdk-5 (e.g., Cheung & Ip, 2012). Finally, since Cdk-5 has a role on hippocampal neurogenesis (e.g., Lagace et al., 2008), and brain FADD (protein and mRNA) was increased in the hippocampus of rats with impaired cell proliferation rates (Ki-67+ mitotic progenitor cells; García-Fuster et al., 2011), the present study also investigated whether cell proliferation in the hippocampus was subjected to a 24-h modulation.

2 | EXPERIMENTAL PROCEDURES

2.1 | Animals and brain samples collection

This study used 42 Sprague–Dawley male adult rats (about 400–425 g) that were bred in the animal facility of the University of the

Balearic Islands. Rats were housed in groups of 2–3 rats under controlled environmental conditions (22°C, 70% humidity, 12-h light and dark cycles, lights on at 8:00 a.m., Zeitgeber time 0, ZT0, and lights off at 8:00 p.m., ZT12) with *ab libitum* access to a standard diet and tap water. Rats were acclimatized to the experimenters by handling them for at least 2 days prior to beginning of the procedure, which complied with the ARRIVE Guidelines (Percie du Sert et al., 2020), the EU Directive 2010/63/EU and the Spanish Royal Decree 53/2013 for animal experiments, and was approved both by the Local Bioethical Committee and the Regional Government. Unfortunately, no female rats were available at the time when this experiment was performed, and therefore the effect of sex as a biological variable could not be included in the experimental design.

Groups of allocated rats ($n = 5–6$ per group; see Figure 1a) were sacrificed every 3 h by decapitation during a period of 24 h (i.e., ZT2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20, and ZT 23). Brains were removed and dissected freshly. Initially, the whole prefrontal cortex was freshly dissected, and after separating both hemispheres, the striatum, and hippocampus were also collected from the right hemisphere. Samples were immediately frozen in liquid nitrogen, and stored at −80°C for posterior western blot analysis of key cell markers (see Figure 1b). The left hemisphere was quickly frozen in isopentane at −30°C and stored at −80°C until the hippocampal region was entirely cryostat-cut (from approximately −1.72 to −6.80 mm from Bregma) in 30 μm sections that were slide mounted (see more details in García-Cabrerizo et al., 2020; García-Cabrerizo & García-Fuster, 2016; García-Fuster et al., 2010), and kept at −80°C until the proliferation of novel cells (Ki-67 + cells) was evaluated by immunohistochemical analysis in the dentate gyrus region.

2.2 | Western blot analysis

Total homogenates were prepared as previously described (e.g., Boronat et al., 2001; García-Cabrerizo & García-Fuster, 2016; García-Fuster et al., 2007) and brain proteins (40.5 μg of total protein which was quantified through BCA assay, Thermo Fisher Scientific, cat #23225) of each brain region of study were loaded into 10%–12% acrylamide gels (depending on the molecular weight of the protein under evaluation), separated by electrophoresis, transferred to nitrocellulose membranes, and incubated overnight at 4°C with the following primary antibodies: (1) anti-FADD (H-181) (sc-5559, batch D0109; 1:5000; Santa Cruz Biotechnology, CA, USA); (2) anti-p-ERK1/2 (p44/p42) (9101; 1:1000; Cell Signaling, MA, USA); (3) anti-t-ERK1/2 (CEMI0112011, Clone 631122; 1:1000; Cell Signaling); (4) anti-Cdk-5 (DC17) (sc-249, 1:5000; Santa Cruz Biotechnology); and (5) p35-p25 (C64B10) (2680, 1:1000; Cell Signaling). The next day, membranes were incubated with the appropriate secondary antibodies (anti-rabbit or anti-mouse IgG linked to horseradish peroxidase) for 1 h at room temperature (1:5000 dilution; Cell Signaling). The immunoreactivity of each target protein was detected with ECL reagents (Amersham, Buckinghamshire, UK), visualized by exposure to an autoradiographic film (Amersham ECL Hyperfilm) for 1–60 min, and quantified with a GS-800 Imaging Calibrated Densitometer (Bio-Rad, CA, USA). In each gel, individual values were compared to control male rats (animals sacrificed at ZT2) to estimate the % magnitude of change. Note that ZT2 was selected as our "control group" to which the other samples refer to, since it corresponds to the time of day which we usually perform all experiments (10:00 h; see e.g., García-Cabrerizo & García-Fuster, 2015). Each sample was evaluated at least 2–3 times in different gels, and the mean value for each rat was used to calculate the mean value per treatment group. β-actin was used as a loading control since it was not regulated in a daily fashion in any brain region (see representative immunoblots in Figure 2).

2.3 | Immunohistochemical analysis

Labeling cell expressing the endogenous marker Ki-67 was used to assess the rate of hippocampal cell proliferation. To do so, three slides per rat containing eight sections each (24 sections per rat), from the anterior, middle, and posterior part of the hippocampus

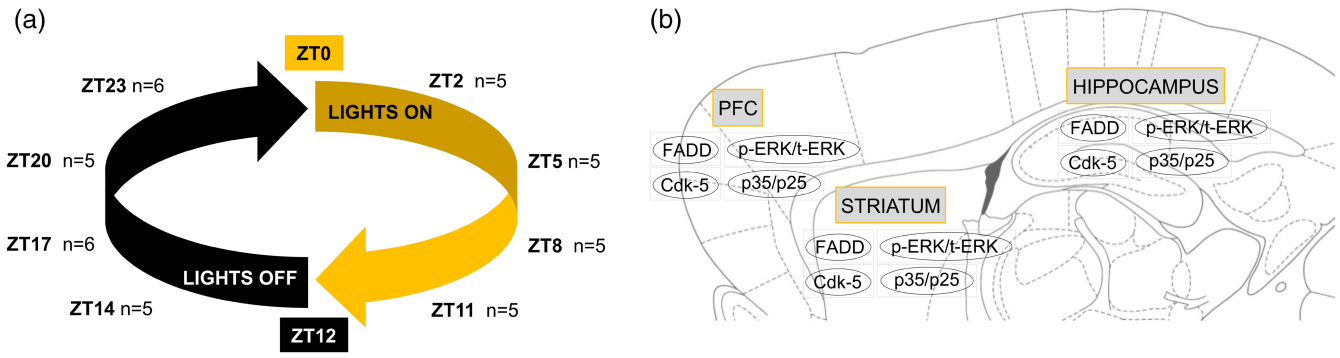

FIGURE 1 (a) Experimental design. Brain samples from male Sprague–Dawley rats were collected during the light/dark cycle period of 24 h (lights on vs. lights off). Groups of treatment: Zeitgeber time (ZT) 2 ($n = 5$), ZT5 ($n = 5$), ZT8 ($n = 5$), ZT11 ($n = 5$), ZT14 ($n = 5$), ZT17 ($n = 6$), ZT20 ($n = 5$) and ZT23 ($n = 6$) (ZT0, lights-on or inactive period; ZT12, lights-off or active period). (b) Schematic representation of the molecular markers evaluated in the prefrontal cortex (PFC), striatum and hippocampus. Figure modified and adapted from García-Cabrerizo and García-Fuster (2015).

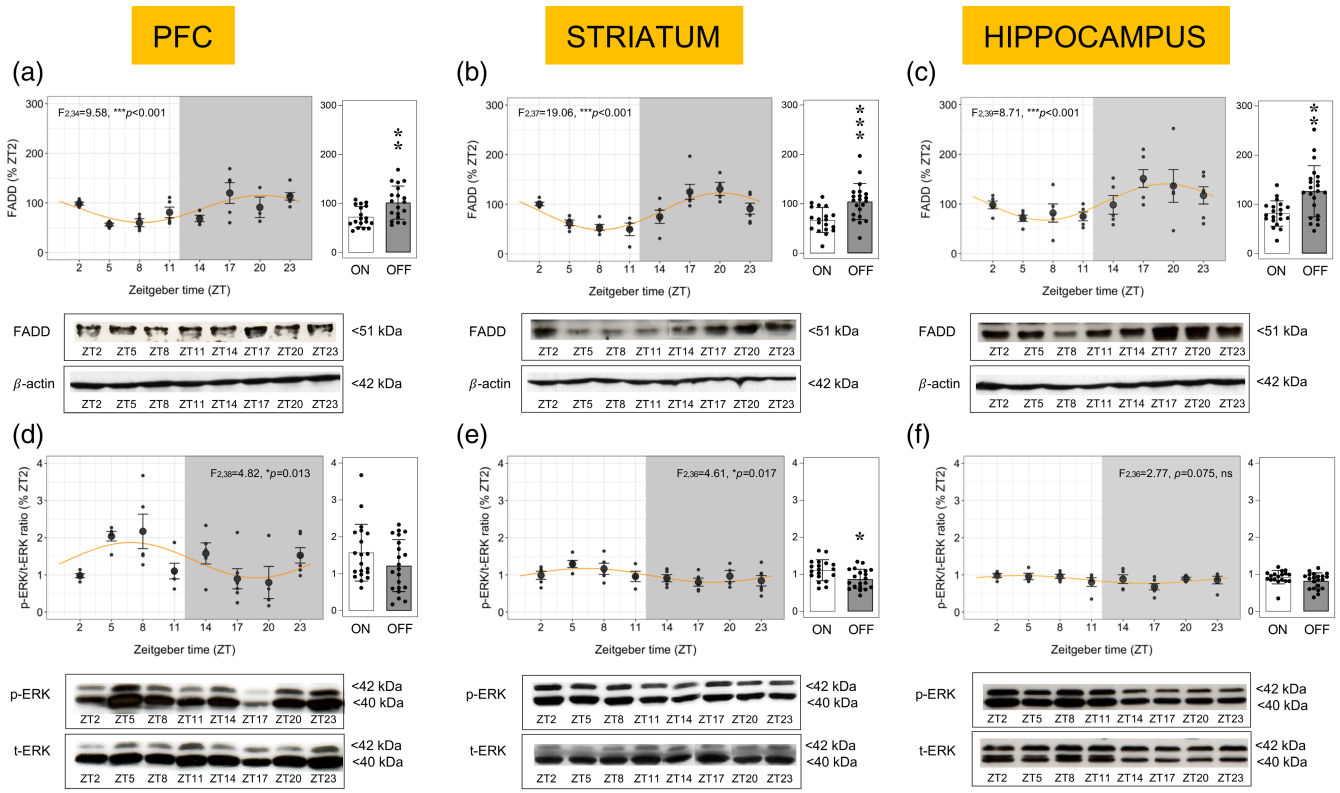

FIGURE 2 Daily modulation of FADD (a–c) and p-ERK/t-ERK ratio (d–f) in rat brain pre-frontal cortex (PFC) (a, d), striatum (b, e) and hippocampus (c, f). Groups of treatment: Zeitgeber time (ZT) 2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20 and ZT23 (ZT0, lights-on or inactive period; ZT12, lights-off or active period). See Table S1 for the particular number of data points (*n*) per marker and time point analyzed. Columns represent mean ± SD of n experiments per group. Individual symbols are shown for each rat. Cosinor analyses were performed for each protein of study at each brain region to assess 24-h rhythmicity. Comparisons between lights on versus lights off were assessed with two-tailed Student *t*-tests. Bottom panels: Representative immunoblots depicting labeling of FADD, β-Actin, p-ERK and t-ERK are shown for each set of experiments. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; ns: no statistical significance ($p > .05$).

were fixed in 4% paraformaldehyde. Antigen exposure was carried out using 10% sodium citrate (pH 6.0, 90°C, 1 h). Subsequently, the samples were incubated for 30 min with a .3% H_2O_2 solution (60 mL 3% stock + 540 mL phosphate buffer saline, PBS) to inhibit endogenous peroxidase. After consecutive washes with PBS, the rectangular area surrounding the brain slices was sealed with a waterproof pen to prevent the liquid from escaping. Then, samples were blocked for 1 h with 500 μL of bovine serum albumin (BSA), incubated overnight with the Ki-67 polyclonal antibody (1:20,000, provided by Professors Huda Akil and Stanley J. Watson, Michigan Neuroscience Institute, University of Michigan, USA), and then for 1 h with the secondary anti-rabbit antibody (1:1000, Cell Signaling). Positive cells were detected through signal amplification by incubating slides with an Avidin/Biotin complex and a DAB chromogen, generating a brown signal. To be able to differentiate the proliferating cells from the rest, a final nuclei staining was done with cresyl blue-violet. Subsequently, tissue dehydration was carried out in a battery of alcohols of ascending degrees, and slides were coverslipped following an immersion in xylene with a drop of adhesive. Positive cells were quantified manually in the whole dentate gryus of all sections by an experimenter blind to the treatment groups with a Leica DMR light microscope (63× objective lens) and focusing through the thickness of the section (30 μm). The total number of positive cells was multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker (e.g., García-Cabrerizo et al., 2020).

2.4 | Data and statistical analysis

Results are expressed as the mean value ± standard deviation (SD), and for each rat, individual symbols are shown, as described in recent guidelines for reporting data in experimental biology (Michel et al., 2020). The number of data points (*n*) per marker, time point and brain structure included in the analyses for graphs in Figures 2–4 is included in Table S1. Cosinor analyses were performed to assess 24-h rhythmicity for each protein of study at each brain region. To do so, data was analyzed with R Studio (Version 3.3.0) using custom R scripts available (Bastiaanssen et al., 2023). To assess the overall differences between day and night (lights on vs. off), data from ZT2-ZT11 or ZT14-ZT23 were combined and analyzed with GraphPad Prism, Version 9.4.1 (GraphPad Software, CA, USA) through two-tailed Student *t*-tests. The level of significance was set at $p \leq .05$. Data will be available upon request to the corresponding author.

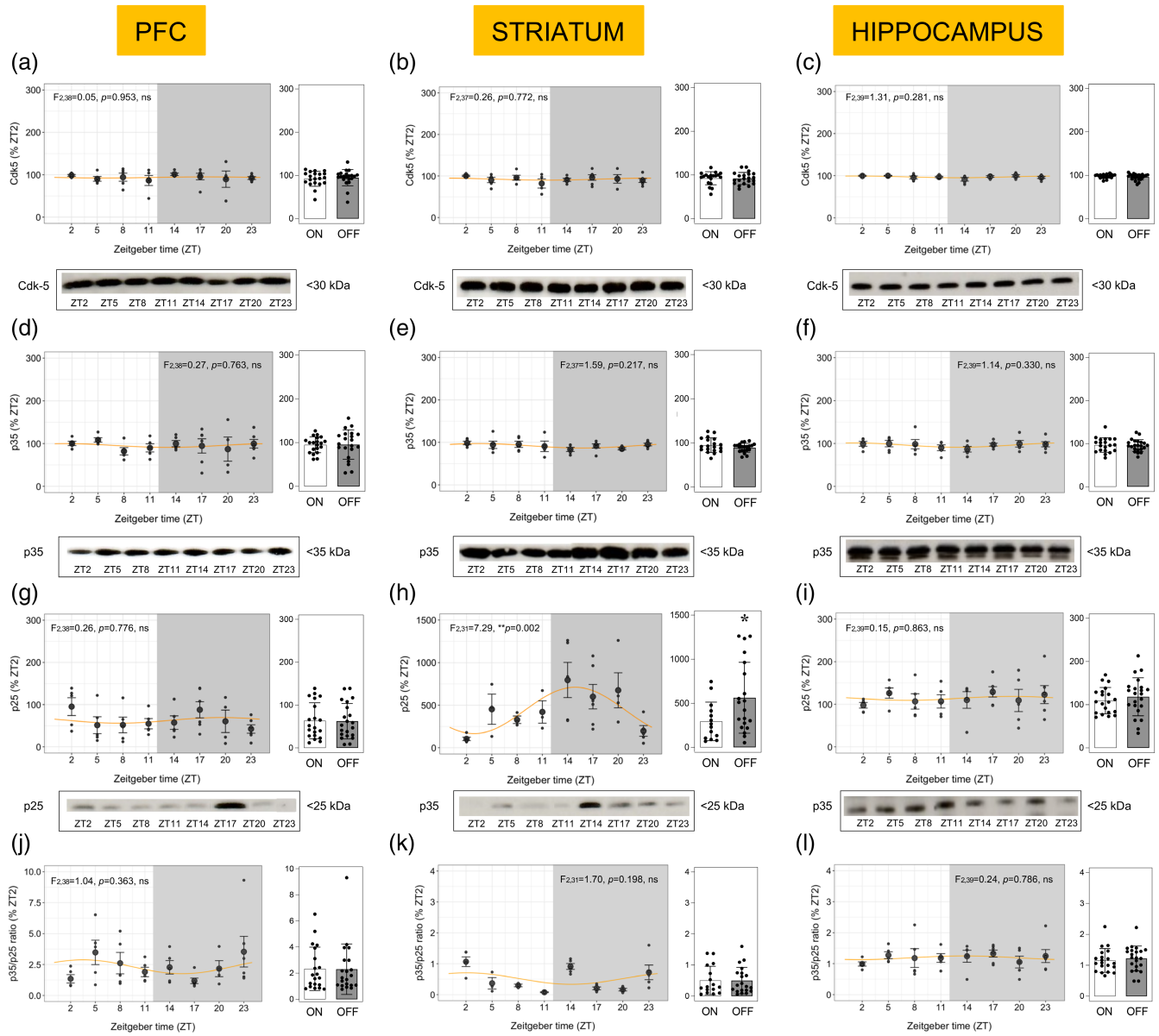

FIGURE 3 Daily modulation of Cdk-5 (a–c), p35 (d–f), p25 (g–i) and p35/p25 ratio (j–l) in rat brain pre-frontal cortex (PFC) (a, d, g, j), striatum (b, e, h, k) and hippocampus (c, f, i, l). Groups of treatment: Zeitgeber time (ZT) 2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20 and ZT23 (ZT0, lights-on or inactive period; ZT12, lights-off or active period). See Table S1 for the particular number of data points (*n*) per marker and time point analyzed. Columns represent mean ± SD of *n* experiments per group. Individual symbols are shown for each rat. Cosinor analyses were performed for each protein of study at each brain region to assess 24-h rhythmicity. Comparisons between lights on versus lights off were assessed with two-tailed Student *t*-tests. Bottom panels: representative immunoblots depicting labeling of Cdk-5, p35 and p25 are shown for each set of experiments. $^{*}p < .05$; $^{**}p < .01$; ns: no statistical significance ($p > .05$).

3 | RESULTS

3.1 | Daily modulation of FADD and of p-ERK/t-ERK across brain regions

The regulation of FADD protein content showed similar daily fluctuations in all brain regions analyzed, as reported by cosinor analyses (prefrontal cortex: $F_{2,34} = 9.58$, $p < .001$; striatum: $F_{2,37} = 19.06$, $p < .001$; hippocampus: $F_{2,39} = 8.71$, $p < .001$; Figure 2a–c; see further details in Table S2). When combining rats from the day or night periods (lights on vs. lights off), there was an overall upregulation of FADD for all brain regions during their active time (lights-off period, prefrontal cortex: $t = 3.12$, $df = 35$, $^{**}p = .004$; striatum: $t = 3.70$, $df = 38$, $^{***}p < .001$; hippocampus: $t = 3.52$, $df = 40$, $^{**}p = .001$: Figure 2a–c).

As for the regulation of the p-ERK/t-ERK ratio, the results showed that it varied across brain regions, showing certain rhythmicity both in prefrontal cortex ($F_{2,38} = 4.82$, $p = .013$: Figure 2d) and striatum ($F_{2,36} = 4.61$, $p = .017$; Figure 2e), but not in hippocampus ($F_{2,36} = 2.77$, $p = .075$; Figure 2f; see further details in Table S2). Moreover, when comparing groups from day or night periods, p-ERK/t-ERK was downregulated during their active time (i.e., dark phase), although only significantly in striatum ($t = 2.66$, $df = 37$, $^{*}p = .011$; Figure 2e),

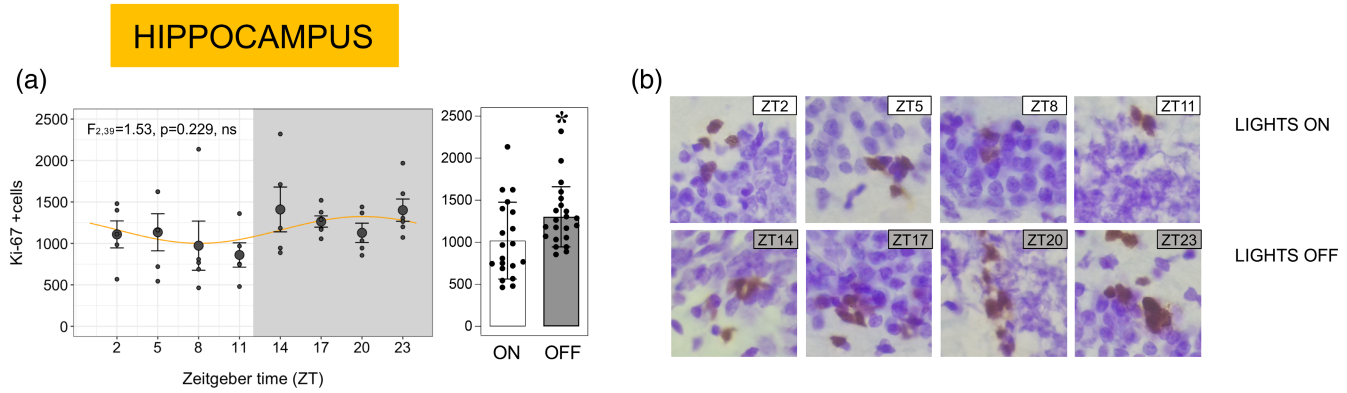

FIGURE 4 Daily modulation of adult hippocampal cell proliferation (Ki-67+cells). (a) Groups of treatment: Zeitgeber time (ZT) 2 ($n=5$), ZT5 ($n=5$), ZT8 ($n=5$), ZT11 ($n=5$), ZT14 ($n=5$), ZT17 ($n=6$), ZT20 ($n=5$) and ZT23 ($n=6$) (ZT0, lights-on or inactive period; ZT12, lights-off or active period). Columns represent mean ± SD of the number of+cells quantified in every 8th section throughout the entire extent of the hippocampal dentate gyrus and multiplied by the sampling factor 8 providing an estimate of the total number of positive cells per marker. Individual symbols are shown for each rat. A cosinor analysis was performed to assess 24-h rhythmicity. The comparison between lights on versus lights off was assessed with a two-tailed Student *t*-test. (b) Representative images showing individual Ki-67+cells (brown labeling in the blue granular layer) taken with a light microscope (63× objective lens). Lower magnification images (5×) are shown in Figure S1, which included the square areas represented in this magnified version. $^*p<.05$; ns: no statistical significance ($p>.05$).

since no statistical differences were observed in prefrontal cortex ($t=1.54$, $df=39$, $p=.132$; Figure 2d) or hippocampus ($t=1.52$, $df=37$, $p=.138$; Figure 2f).

3.2 | Daily modulation of Cdk-5 and p35/p25 across brain regions

Both Cdk-5 and p35 values remained quite stable during the different hours evaluated, as well as showed no changes in their regulation when comparing day versus night periods for all brain regions of study (Figure 3a–f; statistical results reported in Table S2). Moreover, no daily changes were observed for p25 and p35/p25 ratio in prefrontal cortex ($F_{2,38}=.26$, $p=.776$ and $F_{2,38}=1.04$, $p=.363$; Figure 3g,j) or hippocampus ($F_{2,39}=.15$, $p=.863$ and $F_{2,39}=.24$, $p=.786$; Figure 3i,l); also, when comparing day versus night periods (prefrontal cortex: $t=.05$, $df=39$, $p=.957$; Figure 3g,j; hippocampus: $t=.54$, $df=40$, $p=.593$; Figure 3i,l). However, in striatum, p25 showed a daily significant fluctuation ($F_{2,31}=7.29$, $p=.002$; Figure 3h). Moreover, p25 showed an overall significant higher expression during the lights-off than the lights-on period in this region ($t=2.27$, $df=32$, $^*p=.030$; Figure 3h). Still, and in line with the results in prefrontal cortex and hippocampus, the calculation of the p35/p25 ratio showed no significant variations throughout the study in striatum ($F_{2,31}=1.70$, $p=.198$; Figure 3k). In this sense, p35/25 ratio showed no differences in expression between the lights-off than the lights-on period in this region ($t=.02$, $df=34$, $^*p=.986$; Figure 3k).

3.3 | Daily modulation of adult hippocampal cell proliferation

Although no significant rhythmicity was observed in hippocampal cell proliferation in rats across time ($F_{2,39}=1.53$, $p=.229$; Figure 4a), the results showed a higher rate of cell proliferation during their active time (i.e., lights-off, $t=2.26$, $df=40$, $^*p=.029$) with a mean increase of 284 Ki-67+cells and as compared to the lights-off phase (Figure 4a).

4 | DISCUSSION

The regulation of FADD protein content and p-ERK/t-ERK showed similar daily fluctuations in all brain regions analyzed, with higher overall values during the active time of rats (i.e., lights off). Both Cdk-5 and p35 values remained quite stable during the different hours evaluated, as well as showed no changes in their regulation when comparing day (lights-on) versus night (lights-off) periods for all brain regions of study. However, p25 showed an overall significant higher expression during the active time of rats (i.e., lights off), but exclusively in striatum. Finally, the regulation of hippocampal cell proliferation did not follow a significant 24-h modulation, although higher overall values were observed during the active time of rats (i.e., lights off). In conjunction, these results demonstrated clear daily modulations of some of these markers in several regions of the brain (i.e., FADD oppositely regulated than p-ERK/t-ERK in prefrontal cortex and striatum), suggesting a more generalized role for them in the brain, while others were regulated in a region-specific manner (e.g., p25 in striatum, and Ki-67 in hippocampus), suggesting their involvement in more specific roles that deserve future studies.

The present results proved a clear 24-h modulation of FADD in several key brain regions, with a more prominent regulation during the active time of rats. All prior experiments evaluating FADD regulation in disease and/or by pharmacological drugs ascertained changes during the inactive time of rats (lights on). Interestingly, in all brain regions analyzed FADD followed a cosinor-based rhythmometry, which should be taken into consideration when future

experiments are designed so all samples are taken at similar hours during the day. Moreover, the increased FADD levels observed during the active time of rats suggested a key role for FADD in the normal physiological functioning of the daily rhythmicity of the animal. As for the meaning of showing increased levels associated with activity, rats that were bred for higher locomotion activity in a novel environment (bred high-responder, bHR; Stead et al., 2006) also showed significant basal differences in brain (cortical and hippocampal) FADD content, displaying bHR rats' higher contents of FADD as compared to bLR rats (García-Fuster et al., 2009), which goes along with the present data. Moreover, although traditionally an apoptotic and/or neurotoxic role was established for higher values of brain FADD, mainly evaluated in the context of an acute modulation of psychostimulant drugs (i.e., García-Fuster et al., 2009, 2011), particular conditions in which neurotoxic effects were expected showed normal FADD levels (i.e., following repeated drug treatments: García-Cabrerizo & García-Fuster, 2015; García-Fuster et al., 2016, 2009, 2011) and/or even decreased contents (e.g., García-Fuster et al., 2007). Additionally, in a recent study, even though we initially speculated an increase in FADD with the presence and severity of multiple age-related neuropathologies, the results showed that decreased cortical FADD protein was associated with clinical dementia and cognitive decline (Ramos-Miguel et al., 2017). Moreover, during the aging process, which is one of the physiological conditions most affected by daily rhythm alterations (reviewed by Duncan, 2020), FADD progressively decreased with age in the hippocampus of rats (Hernández-Hernández et al., 2018a). Overall, the fact that dysregulations of FADD have been implicated in several behavioral and/or pathological changes, together with the present findings, showing the regulation of FADD in several brain centers in a daily manner, ascribes an important physiological role for this protein that deserves future studies, especially since the potential dysregulation of its rhythmicity could lead to the development of future brain disorders.

In the context of evaluating some key molecular markers that might be regulated in parallel to FADD, we first explored ERK1/2 (ratio between p-ERK and t-ERK), since it has a direct link with FADD activation (e.g., García-Fuster et al., 2007). The results showed that the regulation of the p-ERK/t-ERK ratio varied across brain regions, following a cosinor-based rhythmometry both in prefrontal cortex and striatum, as compared to hippocampus where no overall changes were reported. In particular, when performing the comparisons combining all groups from the lights-on versus lights-off periods, p-ERK/t-ERK ratio was decreased during the lights-off period (higher expression during the inactive time of the rats, lights on), although it was only statistically significant in striatum. Interestingly, this course regulation was opposite to the one observed for FADD, suggesting that during their active time (lights off), rats showed higher values of FADD and lower values of p-ERK/t-ERK ratios. Prior studies that evaluated the regulation of circadian genes by components of the MAPK pathway (e.g., Akashi et al., 2008; Eckel-Mahan et al., 2008; reviewed by Wang et al., 2020) also showed that their activity peaked in certain brain regions during the day (inactive period for the mice evaluated; Eckel-Mahan et al., 2008). Moreover, since this disruption of p-ERK oscillations was linked to an impaired memory persistence (Eckel-Mahan et al., 2008), the 24-h modulation of p-ERK/t-ERK observed in association with FADD fluctuations might be key for normal physiological activities (e.g., Gaspar et al., 2019; Goode et al., 2022; McCauley et al., 2020; Patke et al., 2020).

The next marker we explored was Cdk-5, which is known to participate in the regulation of circadian clocks under physiological conditions (e.g., Brenna et al., 2019; Kwak et al., 2013; Ripperger et al., 2022), and co-factors p35 and p25, needed for its activity (Patrick et al., 1999; Tsai et al., 1994). The results showed no daily regulation for Cdk-5 and p35, since their levels remained unchanged across time. Conversely, p25 showed a 24-h regulation exclusively in striatum, with increased levels during the active time of rats (i.e., lights off), in line with prior rhythms in transcripts described across the human striatum (Ketchesin et al., 2021). Prior results have shown that the dysregulation of Cdk-5 activity by p25 accumulation can lead to neurotoxicity and/or various neurodegenerative disorders through constitutive activation and misplacement of Cdk-5 (e.g., Cheung & Ip, 2012). However, although no overall changes were observed in Cdk-5 contents across time, Cdk-5 signaling in the dorsal striatum have been shown to alter microcircuits implicating the association of pathologies with circadian behavior in mice (Zhou et al., 2022). These effects might be mediated by the phosphorylation of certain proteins involved in regulating the 24-h clock, since Cdk-5 has been shown to regulate the function of CLOCK (Kwak et al., 2013) and/or that of Period 2 (PER2; Brenna et al., 2019) proteins by direct phosphorylation. These data support prior reports suggesting that Cdk-5 is critically involved in the regulation of the circadian clock and may represent a link to various diseases affected by a derailed daily regulation, probably through the activation of its co-factor p25, and in parallel to FADD regulation (e.g., Ledesma-Corvi & García-Fuster, 2022).

Finally, the present study investigated whether cell proliferation in the hippocampus was subjected to a daily modulation. The results showed that the regulation of hippocampal cell proliferation did not follow a significant 24-h modulation, although higher overall values were observed during the active time of rats (i.e., lights off). In line with our results, a prior study in mice evaluated whether proliferation of hippocampal progenitors was subjected to a daily modulation, at 3 h intervals during 24 h, and found that the number of dividing cells remained constant through the light–dark cycle (van der Borght et al., 2006). Moreover, prior studies also found that mice expressed higher proliferation rates in the middle of the dark period (Bouchard-Cannon et al., 2013; Fredrich et al., 2017; Holmes et al., 2004; Tamai et al., 2008), which suggested that activity was associated with increased neurogenesis, similarly to our data, and probably in relation to the daily regulation of certain hippocampal-dependent functions, such as memory (e.g., Snider et al., 2018) or neurological impairments (e.g., Li et al., 2016), among others.

Overall, these results demonstrated a clear 24-h modulation of FADD in several key brain regions, with a more prominent regulation during the active time of rats, and suggested a key role for FADD in the normal physiological functioning of the brain's daily rhythmicity,

together with the general opposite fluctuations observed in p-ERK/t-ERK ratios. The brain regions studied are widely known for their implications in different and highly diverse processes that regulate homeostasis, and therefore any disruptions in these biomarkers might participate in the development of brain pathologies in which they have a role (e.g., major depression, clinical dementia). Future studies should increase the number of rats included at each time-point, since some of our current groups were comprised by few animals, which was caused by availability at the time of the procedure. Moreover, although our study evaluated a relatively small number of markers, our hypothesis-driven candidates have been previously implicated in processes that balance cell death versus neuroplasticity for particular psychopathologies. Therefore, knowing their daily rhythmicity is key to understanding their potential role and/or target regulation for later developing future treatments.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, F.Y.-G., R.G.-C., E.H.-H., G.S, C.B.-H., and M.J.G.-F; *Investigation*, F.Y.-G., L.G.-M., and S.L.-C; *Formal Analysis*: F.Y.-G., L.G.-M., S.L.-C., R.G.-C., and M.J.G.-F; *Writing – Original Draft*, M.J.G.-F.; *Writing – Review & Editing*: F.Y.-G., R.G.-C., E.H.-H., G.S., C.B.-H., L.G.-M., and S.L.-C.; *Funding Acquisition*: M.J.G.-F.; *Supervision*: M.J.G.-F.

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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DECLARATION OF TRANSPARENCY

The authors, reviewers, and editors affirm that in accordance with the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

TABLE S1. Number of data points (*n*) per marker, time point and brain structure for graphs in Figures 2–4.

TABLE S2. Statistical cosinor analysis for graphs in Figures 2–4.

FIGURE S1. Representative images of entire blots for the analyzed proteins (FADD, beta-actin, p-ERK, t-ERK, Cdk-5, p35 and p25) for each brain region studied. Please note that these full gels do not necessarily represent the images cropped and shown in Figures 2 and 3.

FIGURE S2. Representative images showing individual Ki-67 + cells (brown labeling in the blue granular layer) in the whole dentate gryus that were taken with a light microscope (5× objective lens). The square areas represent a magnified version of the positive cells taken with the 63× objective lens.

Data S1.

Data S2.

Data S3.

Data S4.

Data S5.

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