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Social stress alters sleep in FGF21-deficient mice

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ABSTRACT

Although several previous studies have suggested a relationship between sleep and the stress response, the mechanism underlying this relationship remains largely unknown. Here, we show that fibroblast growth factor 21 (FGF21), a lipid metabolism-related hormone, may play a role in this relationship. In this study, we examined differences in the stress response between FGF21 knockout (KO) mice and wild-type (WT) mice after social defeat stress (SDS). When the amount of non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep and wakefulness were averaged over the dark period after SDS, only KO mice showed significant differences in NREM sleep and wakefulness. In the social interaction test, KO mice seemed to be more prone to social avoidance. Our real-time (RT) -PCR results revealed that the mRNA expression of the stress- and sleep-related gene gamma-aminobutyric acid A receptor subunit alpha 2 was significantly lower in WT mice than in KO mice. Moreover, KO mice showed lower plasma levels of ketone bodies, which also affect sleep/wake regulation, than WT mice. These results suggested that FGF21 might influence sleep/wake regulation by inducing production of an anti-stress agent and/or ketone bodies, which may result in resilience to social stress.

1. Introduction

Fibroblast growth factor 21 (FGF21) is a hormone known to regulate energy homeostasis in animals and humans (Kharitonenkov et al., 2005; Lewis et al., 2019). This hormone is widely expressed in the liver, pancreas, adipose tissue and testis (Tacer et al., 2010). In recent years, the role of FGF21 in the central nervous system (CNS) has been explored. In rodents, FGF21 can cross the blood–brain barrier (BBB) (Hsuchou et al., 2007), while in humans, FGF21 is found in cerebrospinal fluid (CSF) at levels correlated with those in plasma (Tan et al., 2011). Furthermore, its receptors, which consist of FGFR1 and β-Klotho, are specifically expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus, the site that controls circadian rhythms (Bookout et al., 2013). The known roles of FGF21 in the CNS include energy homeostasis and the stress response. In terms of the stress response, previous studies have mainly focused on food deprivation stressors (Badman et al., 2007; Inagaki et al., 2007). While the FGF21 level is known to increase during food deprivation, it also drives ketogenesis (Badman et al., 2007; Potthoff et al., 2009) and gluconeogenesis (Fisher et al., 2011; Liang et al., 2014; Potthoff et al., 2009) partly via peroxisome proliferator-activated receptor α (PPARα) (Badman et al., 2007; Inagaki et al., 2007). A more recent study in mice showed that psychological stress also increases the level of FGF21 (Usui et al., 2021). These researchers exposed male mice to social defeat stress (SDS), a model that induces depression-like behaviors such as social avoidance (Golden et al., 2011). In humans, there is a negative correlation between CSF levels of FGF21 and depression scores (Liu et al., 2017).

Along with the change in metabolic biomarkers, including FGF21, stress is known to induce immediate and gradual alternations at the behavioral level. The influence of stress on sleep is particularly well studied. Since social stress is the most common type of stress experienced by social animals, including humans, many studies have investigated the relationship between social stress, sleep, and psychiatric disorders. In humans, sleep disruptions are one of the core symptoms of social stress-related psychiatric disorders such as depression and anxiety (Difrancesco et al., 2019). In rodents, previous studies have reported

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that chronic social stress impacts sleep, which eventually leads to depression-like behaviors (Henderson et al., 2017; Olini et al., 2017; Wells et al., 2017). Furthermore, several studies have suggested that even acute social stress can alter sleep/wake patterns, and these studies have shown that sleep deprivation after social stress alters social behaviors (Feng et al., 2020; Fujii et al., 2019; Meerlo et al., 2001; Nagai et al., 2020). Thus, it is clear that sleep plays a critical role in the stress response. However, the processes that link stress and sleep are not completely understood.

Here, we hypothesized that FGF21 might be involved in the stress response by regulating sleep. Our previous study showed that knockout of PPARα, an upstream factor of FGF21, caused sleep alterations as well as changes in the plasma level of ketone bodies in mice (Kondo et al., 2020); moreover, ketone bodies themselves impact the quality of sleep (Chikahisa et al., 2014). Moreover, FGF21 is known to induces a drop in body temperature (Inagaki et al., 2007), which resembles the phenomenon observed in social stress-induced sleep-like inactivity (Nagai et al., 2020).

Therefore, in this study, we aimed to investigate the role of FGF21 in the stress response, especially sleep alterations, by comparing FGF21-deficient mice with wild-type mice.

2. Materials and methods

2.1. Animals

Adult male FGF21 knockout (KO) mice (C57BL/6N background, provided by Kyoto University) and male WT mice that were littermates of FGF21 KO mice were used for sleep recording experiments, behavioral and biochemical analysis at 18–32 weeks old (Body weight: 35.5 ± 1.1 g (KO), 33.3 ± 0.5 g (WT); there was no difference in body weight between KO mice and WT mice throughout the experiments). Age-matched adult male C57BL/6 N mice (Japan SLC, Shizuoka, Japan) were used for plasma FGF21 measurement. For the SDS experiment, male ICR retired mice (Japan SLC, Shizuoka, Japan) were used as aggressors. Mice were housed individually, fed ad libitum and maintained on a 12-hour light-dark (L/D) cycle with constant temperature (23 ± 1 °C) and humidity. The total numbers of mice used in the sleep recording/voluntary movement, core body temperature and behavioral experiments were WT: n = 7 and KO: n = 7 and in the biochemical analyses WT: n = 19 and KO: n = 7. All procedures were performed in accordance with the Guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan and were approved by the Animal Study Committee of Tokushima University (License No. T30–51). All experimental procedures were done under the Guidelines for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. This study was carried out in compliance with the updated Animal Research: Reporting of In Vivo Experiments (ARRIVE 2.0) guidelines.

2.2. Sleep recording

2.2.1. Surgery in preparation for sleep recording

A cocktail of ketamine (100 mg/kg) and xylazine (25 mg/kg) was used to anesthetize the mice. A total of four stainless steel miniature screw electrodes, two anterior to the bregma (1.5 mm rostral and 1.5 lateral) and two posterior to the bregma (2.5 mm caudal and 2.5 lateral), were implanted in the skull for electroencephalogram (EEG) recording. Teflon-coated stainless-steel wires were implanted on both sides of the trapezius muscles for electromyogram (EMG) recording. An implantable telemetric device (Nano tag, Kissei Comtec, Matsumoto, Japan) was embedded in the peritoneal cavity for locomotor activity and body temperature recording.

2.2.2. Sleep recording

After a two-week recovery period from surgery, the mice were transferred to plastic cages (20 × 12.5 × 30 cm) in a soundproof recording chamber and habituated for at least two days. The EEG/EMG electrodes were connected to computer-assisted data recording software (Vital Recorder, Kissei Comtec, Matsumoto, Japan). Polysomnographic recordings of EEG/EMG were acquired during baseline, post-cage transfer and post-SDS, along with locomotor activity and body temperature.

2.2.3. Sleep data analysis

All sleep data were analyzed using data analysis software (SleepSign, Kissei Comtec, Matsumoto, Japan). The vigilance states were classified manually into 10-second epochs of wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep based on EEG patterns and EMG signals. Epochs containing artificial noise were excluded from further analysis. NREM sleep was characterized by continuous, slow, high-voltage EEG and low-voltage EMG activity. On the other hand, REM sleep was characterized by continuous, low-voltage theta EEG and low-voltage EMG activity. Fast Fourier transform was performed to calculate the EEG power spectrum in the epoch. The EEG delta frequency band was set at 0.5–4.0 Hz. Slow-wave activity (SWA) was calculated by the percentage of delta power (0.5–4.0 Hz) to the total power (0.5–50 Hz) and used for an indicator of sleep depth and sleepiness. Locomotor activity, body temperature and time spent sleeping (REM or NREM) and awake were averaged over hourly intervals.

2.3. Social defeat stress (SDS)

The SDS model has been used as an animal model for depression-like behaviors. This model is based on the resident-intruder paradigm, and a male ICR mouse was used as a resident and an aggressor in this experiment. Male ICR mice were screened for latency and frequency of attack toward a novel male C57BL/6 N mouse. Male FGF21 KO mice or WT mice were transferred to the home cage of an ICR mouse for 3 min during ZT 10–12 (“SDS”). As a control condition, mice were transferred to an empty novel cage for 3 min during the same ZT (“cage transfer” or “CON”). In the sleep recording experiment, sessions were performed for three consecutive days during the same ZT. As major differences in sleep, locomotor activity and body temperature between WT mice and KO mice were observed after the first session and attenuated by the third session, a single session was performed in subsequent experiments to focus on the early stress response.

2.4. Measurement of plasma FGF21 and corticosterone levels

Blood was collected from the facial vein using a disposable animal lancet (Goldenrod Animal Lancet, MEDIpoint, NY, USA) an hour after cage transfer (“CON”) or stress exposure (“SDS”), which was at ZT 12. For basal level measurements, blood was collected from undisturbed mice at ZT 12. Blood samples were collected in tubes containing EDTA-2Na (1 mg/ml) and were centrifuged at 4000 rpm for 15 min at 4 °C to separate the plasma. Both plasma FGF21 levels and corticosterone levels were measured using an enzyme immunoassay (ELISA) kit. A Mouse/Rat FGF-21 Quantikine ELISA Kit (R&D Systems, MN, USA) was used for FGF21 level measurement, and a Corticosterone EIA Kit (Yanaihara Institute Inc., Fujinomiya, Japan) was used for corticosterone measurement.

2.5. Measurement of plasma glucose, triglyceride, free fatty acid total cholesterol and ketone body levels

Trunk blood was collected immediately after decapitation. Blood samples were collected in tubes containing EDTA-2Na (1 mg/ml) and were centrifuged at 4000 rpm for 15 min at 4 °C to separate the plasma. Plasma glucose, triglycerides (TGs) free fatty acids (FFAs) and total cholesterol (TC) were measured by HK-UV (glucose), GPOHDAOS (TGs), ACS-ACOD (FFAs) and COD-POD (TC) enzyme assays using an

automatic biochemical analyzer system (HITACHI 7180, Hitachi, Tokyo, Japan and JCA-DM2250, JEOL, Tokyo, Japan). Ketone bodies were measured using an automatic JCA-BM12 analyzer system (JEOL, Tokyo, Japan) with enzymatic assay kits (Kainos Laboratories, Tokyo, Japan).

2.6. Social interaction test

A social interaction test was performed to determine anxiety and social behavior. Note that the social interaction test was performed with mice that were used for sleep recording experiments that also had undergone three SDS sessions. The test was performed 24 h after the third sessions. Male FGF21 KO mice or WT mice were placed in a behavioral open-field chamber (20 × 20 cm) with a metal-mesh enclosure (10 × 10 × 11 cm) at the center. Two sessions of 150 s each were conducted with an interval of 60 s during ZT 10–12. The enclosure at the center was empty during the first session for habituation, whereas a male ICR mouse was placed in the enclosure for the second session. The test was performed twice: the following day after transfer to the novel empty cage ("CON") and after exposure to stress ("SDS"). The ICR mice used for this test were the same as those used for SDS. The time spent in the center region was defined as the time that the KO mice or WT mice spent in 30 % of the chamber's central area, whereas the time spent in the corner region was defined as the time those mice spent in the four corners of the chamber.

2.7. Gene expression analysis

2.7.1. RNA extraction

One hour after transfer to the novel empty cage or after exposure to stress, the mice were euthanized by rapid cervical dislocation. Brain tissues were dissected immediately after decapitation, frozen in liquid nitrogen, and kept at − 80 °C until use. Total RNA was extracted from the hypothalamus, hippocampus, cortex and amygdala using an RNA extraction kit (QIAGEN, Hilden, Germany).

2.7.2. Microarray

For labeling, cyanine-3 (Cy3)-labeled cRNA was prepared from 150 ng of RNA using the One-Color Low Input Quick Amp Labeling kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions, followed by RNAeasy column purification (QIAGEN, Valencia, CA, USA). Dye incorporation and cRNA yield were checked with a NanoDrop ND-1000 spectrophotometer. For hybridization, 600 ng of Cy3-labeled cRNA (specific activity > 6 pmol Cy3 /μg cRNA) was fragmented at 60 °C for 30 min in a reaction volume of 25 μl containing 25 x Agilent fragmentation buffer and 10 x Agilent blocking agent following the manufacturer's instructions. Upon completion of the fragmentation reaction, 25 μl of 2 x Agilent hybridization buffer was added to the fragmentation mixture and hybridized to Agilent SurePrint GE Unrestricted Microarrays (G2519F) for 17 h at 65 °C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed for 1 min at room temperature with GE Wash Buffer 1 (Agilent Technologies, Santa Clara, CA, USA) and 1 min at 37 °C with GE Wash buffer 2 (Agilent Technologies, Santa Clara, CA, USA) and then were dried immediately. For scanning, slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505C) using one color scan setting for 8x60K array slides (scan area 61 × 21.6 mm, scan resolution 3 μm, dye channel set to green and green PMT set to 100 %). The scanned images were analyzed with Feature Extraction Software 10.7.1.1 (Agilent Technologies, Santa Clara, CA, USA) using default parameters (protocol GE1_107_Sep09 and grid: 028282_D_F_20110531) to obtain background-subtracted and spatially detruded processed signal intensities. Features flagged in Feature Extraction as Feature Nonuniform outliers were excluded.

2.7.3. Real-time (RT)-PCR

A high-capacity cDNA transcription kit (Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesis. RT–PCR was performed using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Gene-specific and predesigned TaqMan primer sets, probes (Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (Roche Applied Science, Mannheim, Germany) were used in this process. All results were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All procedures were performed according to the guidelines provided by the manufacturer.

2.8. Statistical analysis

All results are expressed as the mean ± SEM. For sleep recording, locomotor activity, body temperature, and behavioral experiments, the results were analyzed using two-way repeated-measures ANOVAs or mixed-effects analysis followed by Bonferroni's multiple comparisons test. Analysis of FGF21 and corticosterone levels involved Friedman tests followed by Dunn's multiple comparisons test. For plasma levels of biomarkers aside from FGF21 and corticosterone, Student's t-test was used for analysis. For gene expression analysis, unpaired Welch's t-test with the Holm-Sidak correction for multiple comparisons was used. $P < 0.05$ was assumed to be statistically significant. All statistical tests were carried out using the software package GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Sleep/wake patterns, locomotor activity and body temperature in FGF21 KO mice after social defeat stress (SDS)

FGF21 KO mice showed no major differences in the durations of sleep or wakefulness at baseline (Supplementary Fig. S1a,b,c). Slow-wave activity (SWA) during non-rapid eye movement (NREM) sleep was slightly higher in KO mice, but the difference was not significant (Supplementary Fig. S1d). FGF21 KO mice showed no major differences in locomotor activity or body temperature at baseline (Supplementary Fig. S1e,f).

However, averaging the duration of NREM sleep, REM sleep and wakefulness over the dark period revealed that only KO mice showed a significant increase in NREM sleep and a decrease in wakefulness after stress compared to the control condition (Fig. 1a, Supplementary Fig. S2, wakefulness: $F(2, 23) = 3.774$, $P = 0.0420$ for CON vs. SDS [KO]; NREM sleep: $F(2, 35) = 5.315$, $P = 0.0311$ for baseline vs. SDS [KO], $P = 0.0297$ for CON vs. SDS [KO]). SWA increased in both WT and KO mice after stress, while in KO mice, SWA also showed an increase after the control condition (i.e., cage transfer) (Fig. 1a, $F(2, 23) = 15.78$, $P = 0.0324$ for CON vs. SDS [WT], $P < 0.0001$ for baseline vs. SDS [KO], $P = 0.0240$ for CON vs. SDS [KO], $P = 0.0418$ for baseline vs. CON [KO]). There was no significant difference in the duration of REM sleep among mice or conditions (Fig. 1a, $F(2, 23) = 2.753$, $P > 0.05$ in every comparison). The lack of significant difference between WT mice and KO mice might indicate that pathways other than FGF21 are involved; these pathways should be explored in follow-up studies. While body temperature decreased in WT mice after SDS (Fig. 1b, Supplementary Fig. S4, $F(2, 26) = 5.585$, $P = 0.0490$ for CON vs. SDS [WT]), locomotor activity decreased in WT and KO mice after social stress (Fig. 1b, Supplementary Fig. S4, $F(2, 26) = 9.305$, $P = 0.0014$ for baseline vs. SDS [WT], $P = 0.0136$ for CON vs. SDS [WT], $P = 0.0487$ for CON vs. SDS [KO]). A decline in body temperature in WT mice after SDS might be due to the slight decrease in locomotor activity during SDS, although this decrease was not significant (Supplementary Fig. S3).

3.2. Plasma FGF21 levels after SDS

To determine the involvement of FGF21 in the stress response, the plasma level of FGF21 was measured. Indeed, SDS increased plasma

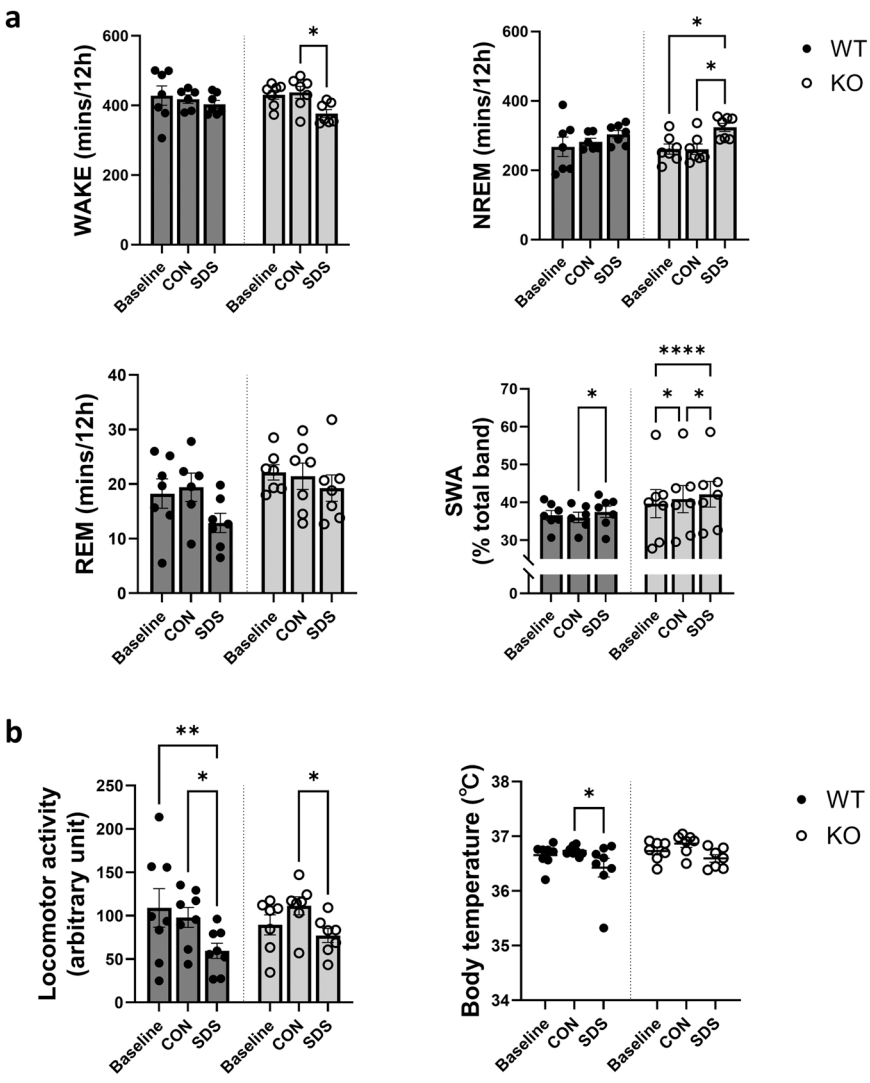

Fig. 1. Changes in sleep/wake regulation, locomotor activity and body temperature in wild-type (WT) and fibroblast growth factor 21 (FGF21) knockout (KO) mice after social defeat stress (SDS). The durations of wakefulness, NREM sleep, and REM sleep; slow-wave activity (SWA) (a); and locomotor activity and body temperature (b) at baseline, after cage transfer ("CON") and after social defeat stress ("SDS"). Closed circles indicate WT mice, and open circles indicate KO mice. All data are expressed as the mean ± SEM (n = 6–7/group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (baseline vs. CON vs. SDS) according to two-way repeated-measures ANOVA or mixed-effects analysis followed by Bonferroni's multiple comparisons test.

FGF21 levels dramatically (Fig. 2a, $P = 0.0324$ for baseline vs. SDS, $P = 0.0026$ for CON vs. SDS). When the FGF21 level was measured in the same group of mice one week after SDS, it had decreased to the level observed at baseline (Supplementary Fig. S5, $P = 0.0099$ for CON vs. SDS, $P = 0.0212$ for SDS vs. recovery).

3.3. Plasma corticosterone levels after SDS

SDS also increased plasma corticosterone levels in both WT and KO mice (Fig. 2b, $P = 0.0035$ for baseline vs. SDS [WT], $P = 0.0005$ for baseline vs. SDS). This result suggested that social defeat caused initial corticosterone responses via the hypothalamic–pituitary–adrenal (HPA) axis, regardless of FGF21 levels.

3.4. Plasma levels of ketone bodies after SDS

To determine the role of ketone bodies in sleep alterations in KO mice, plasma levels of ketone bodies (acetoacetate [AcAc], β-hydroxybutyrate [BHB], and total ketones) as well as glucose, TGs, FFAs and TC were measured after SDS. As expected due to previous studies (Badman et al., 2007; Potthoff et al., 2009), the plasma level of ketone bodies was higher in WT mice than in KO mice (Fig. 2c, $P = 0.0392$ for WT vs. KO [AcAc]). On the other hand, there was no significant difference in the plasma levels of glucose, TGs, FFAs and TC between WT and KO mice. Interestingly, the plasma level of ketone bodies was positively correlated with the plasma level of corticosterone (Supplementary Fig. S6, $R^2 = 0.8856$ and $P = 0.0170$ for WT, $R^2 = 0.7916$ and $P = 0.007$ for KO). This result indicates changes in the relationship between stress and lipid metabolism.

3.5. Social interaction test

Next, we examined the effect of SDS on social behavior with a social interaction test. All mice, both WT and KO, showed social avoidance of a target ICR mouse and a decrease in exploratory activity after stress (Fig. 3a, b, total distance traveled: $F(1, 13) = 113.1$, $P < 0.0001$ for CON vs. SDS [WT], $P < 0.0001$ for CON vs. SDS [KO], duration in center region: $F(1, 13) = 18.64$, $P = 0.0491$ for CON vs. SDS [WT], $P = 0.0073$ for CON vs. SDS [KO]). However, while WT mice had a mild tendency to increase their duration in corner regions after stress (Fig. 3c, $F(1, 13) = 23.88$, $P = 0.1049$ for CON vs. SDS [WT]), KO mice had an obvious difference in their duration in corner regions before and after stress (Fig. 3c, $F(1, 13) = 23.88$, $P = 0.0008$ for CON vs. SDS [KO]). Notably, the mice used in the social interaction test underwent three consecutive sessions. Nonetheless, this result suggested that KO mice were more vulnerable to stress.

3.6. Gene expression

In addition to plasma biomarkers, gene expression was assessed to

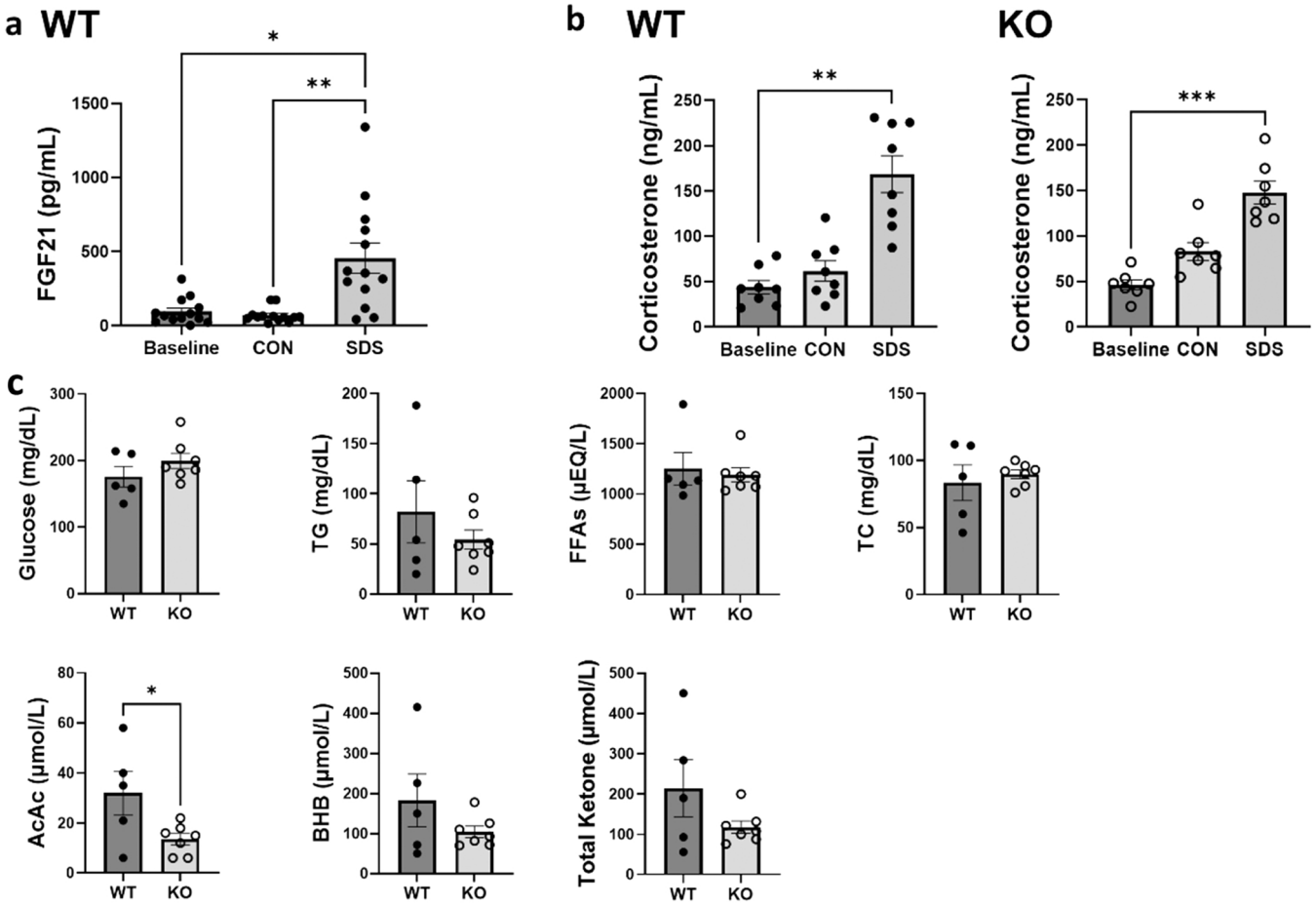

Fig. 2. Plasma levels of biomarkers in wild-type (WT) and fibroblast growth factor 21 (FGF21) knockout (KO) mice. Plasma FGF21 levels in WT mice at baseline, after cage transfer ("CON") and after social defeat stress ("SDS") (a). Plasma corticosterone levels in WT mice and KO mice at baseline, after cage transfer ("CON") and after social defeat stress ("SDS") (b). Plasma levels of glucose, triglycerides (TGs), free fatty acids (FFAs), total cholesterol (TC), acetoacetate (AcAc), β-hydroxybutyrate (BHB) and total ketones after SDS (c). Closed circles indicate WT mice, and open circles indicate KO mice. All data are expressed as the mean ± SEM (a: n = 13; b: n = 8 (WT), 7 (KO); c: n = 5 (WT), 7 (KO)). *p < 0.05, * *p < 0.01, * **p < 0.001 (a-b: baseline vs. CON vs. SDS, c: WT vs. KO) according to a Friedman test followed by Dunn's multiple comparisons test (a-b) and unpaired Student's t-test (c).

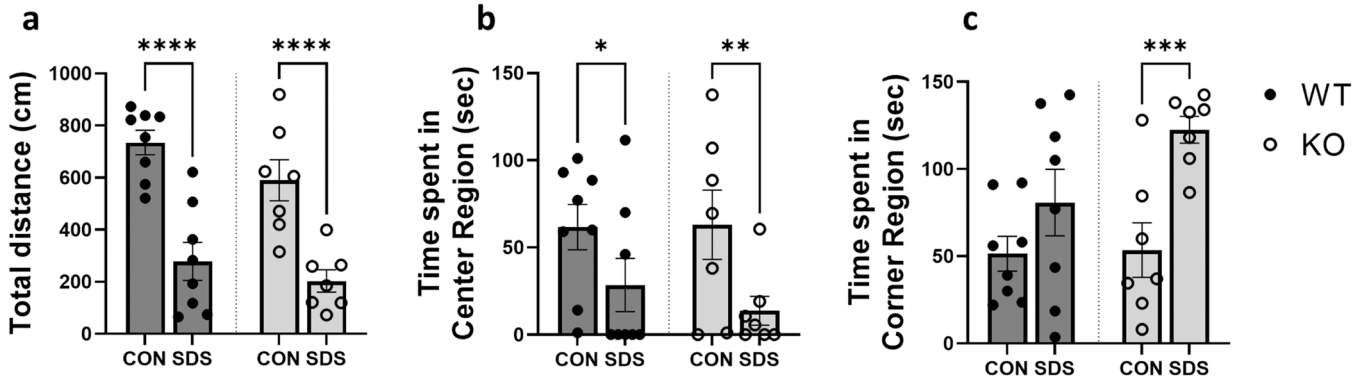

Fig. 3. Social interaction test. Total distance traveled during the session with ICR mice (a). Duration in the center region (b) and corner region (c). Closed circles indicate WT mice, and open circles indicate KO mice. All data are expressed as the mean ± SEM (n = 7–8/group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (CON vs. SDS) according to two-way repeated-measures ANOVAs followed by Bonferroni's multiple comparisons test.

investigate the mechanisms underlying changes in behavior. First, gene expression microarrays were performed to assess comprehensive differences in gene profiles between WT and KO mice after social stress. We selected the hypothalamus, hippocampus, cortex, and amygdala as they are involved in the stress response, sleep/wake regulation, thermoregulation, fear conditioning and learning. The analysis identified over 20 genes each that were differentially expressed in WT mice compared to KO mice (Supplementary Table S1). Two genes were selected for further analysis based on their known contributions to the stress response and sleep/wake regulation. These genes were *gamma-aminobutyric acid* (*GABA*) *A receptor subunit alpha 2* (*gabra2*) (Engin et al., 2012) and *granulin* (*grn*) (Cui et al., 2019). Then, real-time (RT)-PCR was

performed. Comparison of gene expression revealed that the expression level of *gabra2* was suppressed in WT mice after stress regardless of the brain region (Fig. 4, $P = 0.005738$ for WT vs. KO [SDS; hypothalamus], $P = 0.024123$ for WT vs. KO [SDS; hippocampus], $P = 0.001528$ for WT vs. KO [SDS; cortex], $P = 0.009263$ for WT vs. KO [SDS; amygdala]), in contrast to findings in KO mice. There was no significant difference in the expression of *grn* between WT and KO mice, regardless of condition.

4. Discussion

In this study, we investigated the role of FGF21 in the relationship between the stress response and sleep/wake regulation. To achieve this goal, differences in the stress response of WT mice and FGF21 KO mice, i.e., changes in sleep, locomotor activity, body temperature, plasma biomarkers and social interactions, were explored in after SDS.

Although an increase in the duration of NREM sleep, an increase in SWA and a decrease in the duration of wakefulness after exposure to social stress have been reported in several previous studies, we found, for the first time, that these changes were greater in mice lacking FGF21 (Fig. 1a). The duration of REM sleep decreased slightly, but this difference was not significant. In the control condition, i.e., cage transfer (CON), no change in the durations of NREM sleep, wakefulness or REM sleep was observed, even though both the CON and SDS procedures were performed at the end of the light period, which is the resting period for mice. SDS also caused a drop in body temperature (Supplementary Fig. S4) during the first few hours of the dark period. In fact, in WT mice, we confirmed that FGF21 levels increased significantly after stress (Fig. 2a). Social stress increased the plasma levels of other biomarkers, such as corticosterone (Fig. 2b). The levels of this stress hormone increased after stress in both WT and KO mice, which indicated that the initial stress response via activation of the HPA axis occurred regardless of the presence of FGF21. However, in the social interaction test, KO mice were more prone to social avoidance than their WT peers and remained in the sheltered area of an open-field chamber for a longer duration (Fig. 3c). Taken together, these findings demonstrate that FGF21 plays a role in stress vulnerability by influencing sleep and wakefulness. We assume that some stress coping mechanisms were triggered by FGF21 in WT mice but obviously not in KO mice.

Interestingly, after three consecutive days of exposure to SDS, the duration of wakefulness decreased even in WT mice (Supplementary Fig. S8, S9), while plasma levels of FGF21 slightly decreased (Supplementary Fig. S10). This result might indicate that although the effect of acute stress was attenuated by FGF21, repeated exposure to stress disrupted sleep homeostasis and increased the duration of NREM sleep in WT mice.

Next, we aimed to investigate the molecular mechanism underlying the differences in the stress response between WT and KO mice. Prior to RT–PCR, a microarray assay was performed to comprehensively assess the gene expression profile after SDS. The RT–PCR results revealed significantly suppressed expression of *gabra2* in WT mice compared to KO mice after stress (Fig. 4). The α2 subunit of the $GABA_A$ receptor is encoded by *gabra2*; this receptor constitutes 15–20 % of all GABA receptors. $GABA_A$ receptors are involved in various CNS disorders, from anxiety to schizophrenia (Engin et al., 2012). Alcohol dependence (AD) is one such disorder, and it has been shown that variations in *gabra2* are strongly associated with the risk of AD (Edenberg et al., 2004). Indeed, *gabra2* variants interact with stress to increase alcohol use (Kiive et al., 2017). Notably, FGF21 is also associated with increased alcohol consumption (Søberg et al., 2017), and a recent study in rodents showed that FGF21 expression itself suppressed alcohol consumption (Flippo et al., 2022). However, we cannot conclude that the $GABA_A$ receptor α2 subunit and FGF21 are directly and/or indirectly linked, as it is possible that they are both part of the anti-stress system, which determines vulnerability to the social stress-induced problematic alcohol use that eventually causes AD. In addition, α2 $GABA_A$ receptors are known to mediate sleep alterations by benzodiazepines, which are widely used to treat anxiety disorders (Kopp et al., 2004). While the presence of relationship between *gabra2* suppression and GABAergic activation remains inconclusive, the differences stress-induced sleep alteration between WT mice and KO mice might be partly due to changes in the expression of *gabra2*. This finding may eventually lead to the identification of a novel role of FGF21 in the GABAergic system after social stress exposure.

Another possibility is that ketone bodies are involved. Ketone bodies, mainly AcAc and BHB, are intermediates generated during lipogenesis or lipolysis. During food deprivation, FGF21 expression is induced; FGF21 expression is thought to be one of the pathways that stimulates ketogenesis (Badman et al., 2007; Potthoff et al., 2009). In the present study, we showed that the plasma levels of ketone bodies were higher in WT mice than in KO mice (Fig. 2c). Since ketone bodies are also involved in the maintenance of wakefulness and locomotor activity (Chikahisa

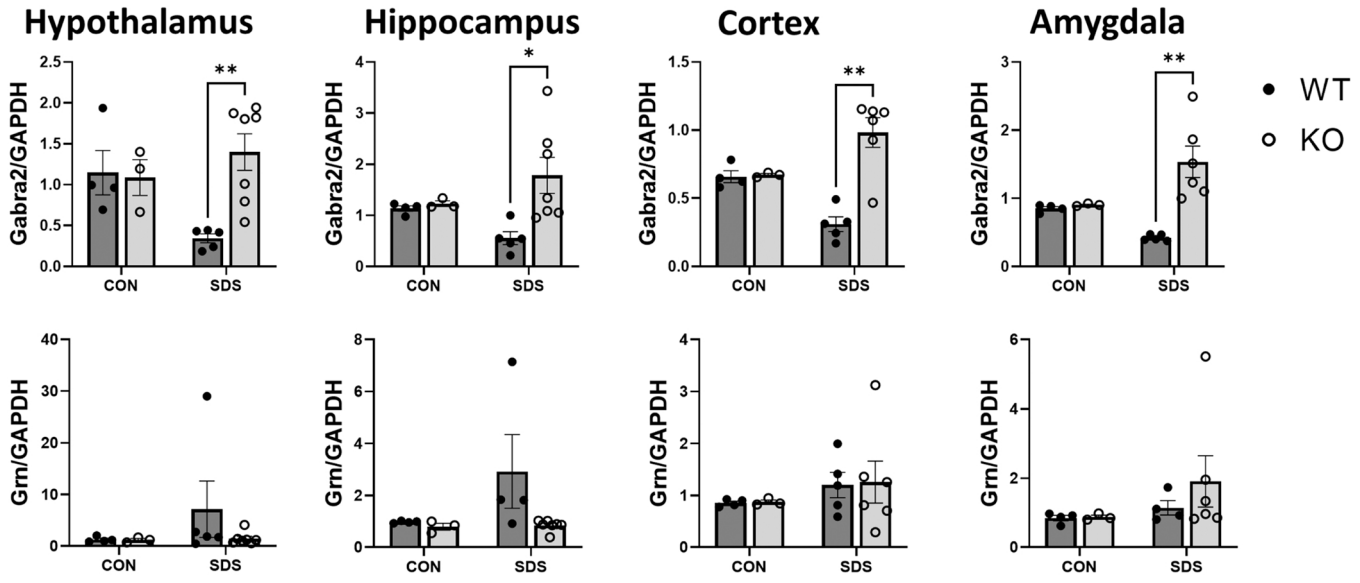

Fig. 4. Expression levels of sleep/stress-related genes in the hypothalamus, hippocampus, cortex, and amygdala of wild-type (WT) and fibroblast growth factor 21 (FGF21) knockout (KO) mice after cage transfer (CON) and after social defeat stress (SDS). All data are expressed as the mean ± SEM (n = 3–7/group). *p < 0.05, **p < 0.01 (CON vs. SDS) according to an unpaired Welch's t-test with the Holm-Sidak correction for multiple comparisons.

et al., 2014; Kondo et al., 2020), it would not be surprising if the upregulation of ketogenesis by FGF21 is responsible for the sleep alterations observed after social stress exposure. Interestingly, we found that the levels of BHB and total ketones were strongly correlated with plasma corticosterone levels (Supplementary Fig. S6), and the AcAc level was strongly correlated with the expression level of *gabra2* in the cortex (Supplementary Fig. S7). Although further studies are needed, these findings might indicate that ketone bodies are involved in the regulation of stress- and/or sleep-related gene expression. Overall, these results also support the possibility that ketone bodies play a role in the stress response.

5. Conclusions

In conclusion, social stress increased the duration of NREM sleep and decreased the duration of wakefulness in mice lacking FGF21, resulting in social avoidance, while FGF21 might have triggered a stress coping mechanism in WT mice. These results suggest that FGF21 may facilitate resilience to social stress, possibly by regulating the expression of anti-stress agent(s).

While these results are encouraging, the nature of the social stress paradigm used in this study prevented assessment of female mice. It is widely known that depression and sleep disorders are more frequent females than in males. Therefore, future work is needed to explore the role of FGF21 in the stress coping of female mice using a novel social stress model that is applicable to female mice.

Nonetheless, our study provides novel insight into the interaction between the stress response, sleep/wake regulation and metabolism. These findings could eventually lead to new strategies for treating social stress-related diseases, including anxiety disorders, adjustment disorders and depression.

Competing interests

The authors declare no competing interests.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

All authors are responsible for the study concept and design. S.H. and S.C. planned and performed all of the experiments and the data analysis. Y.N., M.K. and N.I. generated, maintained and provided FGF21 knockout mice. All authors discussed the results. S.H. wrote the first draft of the manuscript. All authors revised the manuscript and gave final approval for the submitted manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2022.10.005.

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