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ORIGINAL PAPER

Anxiety-like behaviors and hippocampal nNOS in response to diet-induced obesity combined with exercise

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Abstract

A high-fat diet (HFD) and overweight status can induce hippocampal dysfunction, leading to depression and anxiety. Exercise has beneficial effects on emotional behaviors. We previously reported that exercise training rescues HFD-induced excess hippocampal neuronal nitric oxide synthase (nNOS) expression, which is a key regulator of anxiety. Here, we investigated anxiety-like behaviors and hippocampal nNOS expression in response to HFD combined with exercise. Mice were assigned to standard diet, HFD, or HFD with exercise groups for 12 weeks. We found that exercise during the final 6 weeks of the HFD regime improved 12 weeks of HFD-induced defecation, accompanied by rescue of excess nNOS expression. However, anxiety indicators in the elevated plus maze were unchanged. These effects were not apparent after only 1 week of exercise. In conclusion, 6 weeks of exercise training reduced HFD-related anxiety according to one of our measures (defecation), and reversed changes in the hippocampal nNOS/NO pathway.

Keywords High-fat diet · Exercise · Anxiety · Nitric oxide synthase

Introduction

The global prevalence of obesity, an important contributor to the burden of disease, is quickly increasing. Excessive consumption of fat increases the risk of overweight status and obesity, which have been associated with mental disorders [1, 2]. Epidemiological studies have revealed that both consuming a western diet and overweight status are associated with smaller hippocampal volume [3, 4] and mental disorders such as anxiety [2, 5]. However, as stated by Eyres et al., strong evidence for a beneficial effect of diet-induced weight loss on anxiety in individuals with obesity is lacking [6]. Therefore, it appears that additional interventions are needed, not only to ameliorate obesity, but also the symptoms of anxiety in obese individuals.

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Exercise is commonly recommended to combat obesity because an increase in energy expenditure leads to weight loss. Exercise results in adaptive changes in almost all tissues, which contribute to the beneficial effects of exercise on metabolic health. Moreover, exercise has been found to improve not only general metabolic health, but also brain function. Human and animal studies have shown that exercise training has an anxiolytic effect [7–9]. The effects of exercise training are thought to be associated with increases in hippocampal brain-derived neurotrophic factor (BDNF) [10], which enhances hippocampal neurogenesis [11, 12]. Previous studies have examined the effects of a high-fat diet (HFD) or exercise training on brain function, with a particular focus on the hippocampus. Exercise can also reverse the harmful effects on synaptic and behavioral plasticity of 2 months of HFD consumption [13]. Maniam et al. [14] reported that voluntary exercise in combination with a HFD has beneficial effects on behavior in rats exposed to early life stress. Many of these studies applied concurrent HFD and exercise intervention. Given the potential applications for individuals with obesity, the effects of exercise interventions during onset of obesity, or after obesity has developed, are useful research topics.

There is substantial evidence that neuronal nitric oxide synthase (nNOS)-derived nitric oxide (NO), the major NOS isoform in the central nervous system, is a key regulator of affective behavior [15, 16]. A recent study indicated that phosphorylation might be an important mechanism modulating the role of nNOS in NO production [17]. This process is reported to be regulated by the phosphoinositide 3-kinase (PI3 K)/Akt pathway in the brain [18]. Animal studies have shown that mice that genetically lack nNOS or are subject to pharmacologically induced selective nNOS inhibition exhibit fewer anxiety-like behaviors [15, 19]. Moreover, cAMP response element-binding protein (CREB) phosphorylation is essential for the behavioral effects of pharmacological nNOS inhibition [15]. We previously reported that both ageing and HFD consumption increase hippocampal nNOS expression, indicating that there may be a relationship between ageing- and HFD-induced anxiety and activity in the hippocampal nNOS/NO pathway [20, 21]. Interestingly, physical activity completely reversed ageing- and HFD-induced increases in hippocampal nNOS expression compared with controls (young and standard diet groups, respectively). These findings partially explain the beneficial effects of exercise on obesity-related mood regulation dysfunction via the hippocampal nNOS/NO pathway. However, whether exercise-induced changes in nNOS improve HFD-related anxiety has not been established. Furthermore, the role of nNOS phosphorylation via the PI3 K/Akt pathway in mood regulation is poorly understood. Therefore, we investigated the effects of exercise on HFD-related anxiety, hippocampal nNOS expression, and the nNOS/NO pathway.

Materials and methods

Animals

Eighty male C57BL/6 J mice (4 weeks old; Japan SLC, Shizuoka, Japan) were housed in a temperature (23.5 ± 0.7 °C), humidity (34.0 ± 5.7%), and light (12-h light–dark cycle) controlled facility. The animals received standard chow and water ad libitum. All experiments were approved by the Animal Care and Use Committee of Fukuoka University.

HFD and exercise training

The HFD and exercise training protocol were as previously described [21]. Briefly, after 2 weeks of acclimatization, the mice were divided into three groups: the standard diet (SD) group ($n = 24$) was fed a standard diet (CE-7, CLEA Japan, Tokyo, Japan; 62% carbohydrate, 14% fat, and 25% protein), the HFD group ($n = 28$) was fed a HFD (HFD-32, CLEA Japan, Tokyo, Japan; 23% carbohydrate, 57% fat, and 20% protein), and the HFD + Ex ($n = 28$) group was fed the HFD-32 for the 12-week experimental period, and allowed to voluntarily exercise in a running wheel for the final 6 weeks of the experiment. Both the HFD and the HFD + Ex groups were reared in cages with a running wheel, but only the HFD + Ex group was allowed free access to the running wheel during the last 6 weeks of the experiment. Body weight and wheel rotation counts were measured every second day. Total running distance was calculated on the basis of the wheel rotation count. We conducted behavioral tests 2 ($n = 6$/group), 6 ($n = 6$–7/group), 7 (1 week of exercise training, $n = 6$–7/group), and 12 weeks (6 weeks of exercise training, $n = 6$–7/group) into the experimental session, and then sacrificed the mice by decapitation (Fig. 1).

Behavioral testing

We assessed anxiety-like behaviors using the elevated plus maze (EPM), 3 days before sacrifice (Fig. 1). All tests were performed between 13:00 and 16:00 during the light period (80–100 lx). Before each test, the testing apparatus was thoroughly cleaned with 70% (v/v) ethanol and dried to reduce the presence of olfactory cues.

We conducted the EPM test according to the protocol of Nishijima et al. [22]. Briefly, the maze consisted of four arms (each 30 cm long and 5 cm wide) 40 cm above the floor. Two arms contained side and end walls that were 15 cm high (closed arms), and the other two arms had no walls (open arms). We recorded mouse behavior via a video camera. Mice were placed in the center of the maze such that they faced an open arm, and allowed to explore for 5 min. The following parameters were measured manually: (1) number of entries into the open arm; (2) total number of arm entries (total arm entries = open arm entries + closed arm entries, times); (3) percentage of open arm entries (open arm entries/total arm entries × 100); (4) defecation (times).

Tissue preparation

Three days after the EPM, the mice were sacrificed by decapitation following 6 h of fasting. The hippocampus was rapidly collected and homogenized using tweezers to avoid region specificity. Then, portions of hippocampal tissue were frozen in liquid nitrogen for protein analysis. For gene expression analysis, a portion of hippocampal tissue was rapidly immersed in RNA stabilization solution (Ambion, Austin, TX). All specimens were stored at − 80 °C for subsequent biochemical analysis.

Western blot analysis

We performed western blotting to determine nNOS, phosphorylated nNOS^{Ser1412} (pnNOS^{Ser1412}), Akt, phosphorylated Akt^{Ser473} (pAkt^{Ser473}), CREB, and phosphorylated CREB^{Ser133}

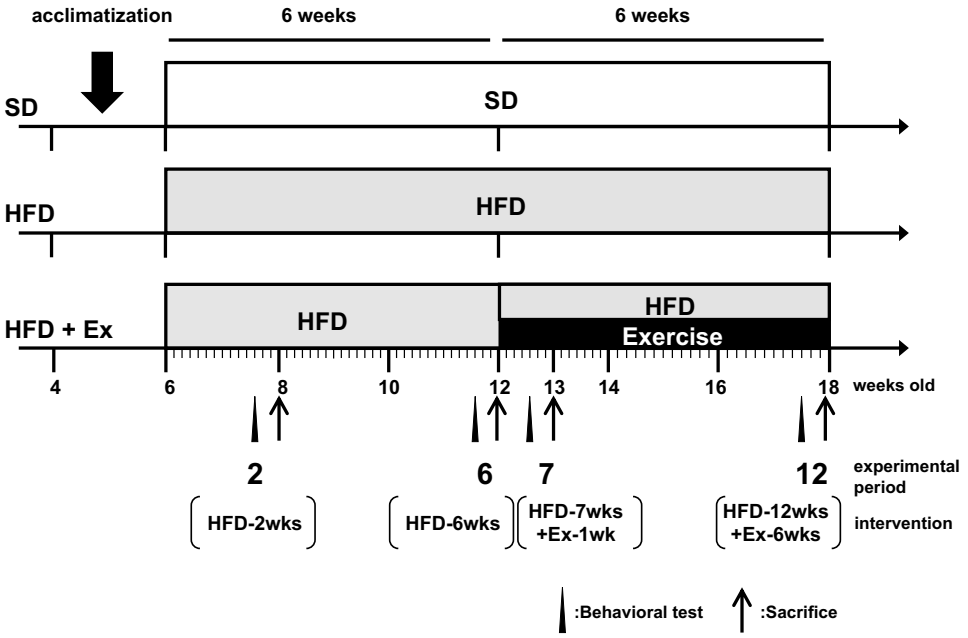

Fig. 1 Schematic view of the experiment. This study design was modified from our previous study [21]. HFD mice were fed a high-fat diet throughout the 12-week experimental period. HFD + Ex mice were fed a high-fat diet throughout the 12-week experimental period and given voluntary access to a running wheel from 12 weeks of age until the end of the experiment. The mice performed behavioral tests at 2, 6, 7, and 12 weeks. Three days after behavioral tests, mice were killed by decapitation

(pCREB^{Ser133}) levels in the hippocampus. Total protein was extracted as previously described [21]. Proteins from each sample (10 µg total protein per lane) were separated via electrophoresis on an 8.0% (w/v) sodium dodecyl sulfate polyacrylamide gel for 40 min at 200 V and transferred to a polyvinylidene fluoride membrane (Millipore, MA, USA) using the semi-dry method. After transfer, the membrane was blocked with 3% (w/v) skim milk at room temperature for 1 h and then incubated overnight with the primary antibodies anti-nNOS (#611852, 1:500; BD Biosciences, San Jose, CA), anti-pnNOS^{ser1417} (#ab5583, 1:500; recognizes mouse nNOS at Ser1412 [17], Abcam, Cambridge, MA), anti-Akt (#4691, 1: 1000; Cell Signaling, Beverly, MA), anti-pAkt^{Ser473} (#4060, 1: 2000; Cell Signaling), anti-CREB (#9197, 1: 1000; Cell Signaling), anti-pCREB^{Ser133} (#9198, 1: 1000; Cell Signaling), and anti-GAPDH (#ACR001P, 1:50,000; Acris Antibodies, Herford, Germany) at 4 °C. Next, the membrane was incubated in horseradish peroxidase-conjugated anti-mouse or -rabbit IgG antibody (#PI-2000 and #PI-1000, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Bound antibodies were detected by ECL Select Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ) and analyzed using an Amersham Imager 600 (GE Healthcare Life Sciences, Tokyo, Japan). The band densities were determined using Image J software (NIH, Bethesda, MD, USA).

Gene expression

nNOS gene expression levels were determined as previously described [21]. Hippocampal tissue from the SD, HFD, and HFD + Ex mice was homogenized using a Polytron homogenizer. Total RNA was extracted from all samples using the Maxwell® 16 LEV System (Promega, Tokyo, Japan). We performed a real-time RT-PCR assay with the Step One Real Time PCR system (Applied Biosystems) using TaqMan Gene Expression Assay probes to analyze nNOS mRNA levels (*Nos1*-Mm00435175_m1; Applied Biosystems, Foster City, CA). nNOS mRNA levels were normalized to GAPDH mRNA levels (*Gapdh*-Mm99999915_g1; Applied Biosystems) and quantified using the ΔΔCt method.

Statistics

Data are shown as the mean ± SE values. All statistical analyses were performed using prism version 7.0 (GraphPad Software, San Diego, CA). We used Student's *t*-tests to assess behavioral parameters, protein levels, and gene expression at 2 and 6 weeks (SD and HFD). Non-parametric data were assessed using the Mann–Whitney *U* test. We used a one-way ANOVA, followed by Bonferroni's post hoc test, to conduct group comparisons for behavioral parameters, protein levels, and gene expression at 7 and 12 weeks (SD, HFD, and HFD + Ex). Non-parametric data were assessed using the Kruskal–Wallis test. Correlations were calculated using Pearson's product–moment correlations. A P value < 0.05 was considered statistically significant.

Results

Effect of HFD and exercise on body weight and fat mass at 2, 6, 7, and 12 weeks

HFD consumption led to a rapid increase in body weight and visceral fat mass by 2 weeks (Fig. 2a, b). This

increase in body weight and fat pad mass in HFD mice continued at 6, 7, and 12 weeks compared with SD mice ($P < 0.01$, Fig. 2). The total running distance in HFD + Ex mice was higher at 12 weeks (158.5 ± 44.2 km) compared with 7 weeks (13.8 ± 5.8 km). At 7 weeks, the single week of exercise in the HFD + Ex mice had not altered body weight or visceral fat mass (Fig. 2a). Finally, at 12 weeks, the 6 weeks of exercise had countered the HFD-induced body weight gain ($P < 0.01$, Fig. 2a). Although not significant, we found that HFD + Ex mice had a lower fat mass compared with HFD mice at 12 weeks ($P = 0.07$, Fig. 2b). These results suggest that HFD consumption rapidly induced body weight gain, and that 6 weeks of exercise had a stronger effect on body weight reduction than other exercise periods.

Effect of HFD on anxiety-like behaviors and the nNOS/NO pathway at 2 weeks

After 2 weeks of HFD consumption, we observed changes in anxiety-like behavior in the EPM (Fig. 3). We found significant differences in the number of open arm entries ($P < 0.05$, Fig. 3a) and the percentage of open arm entries per total arm entries ($P < 0.05$, Fig. 3c). The total number of arm entries was unchanged. The SD and HFD mice did not defecate during the experimental period (Fig. 3d).

Hippocampal nNOS protein, gene expression, and phosphorylation levels were unchanged by HFD consumption at 2 weeks (Fig. 3e–i). pAkt^{Ser473} levels had significantly increased, and CREB and pCREB^{ser133} levels had decreased in HFD mice (Fig. 3j, l, m).

Effect of HFD on anxiety-like behaviors and the nNOS/NO pathway at 6 weeks

At 6 weeks, we found no significant differences in any parameters tested, including open arm entries (Fig. 4a), total arm entries (Fig. 4b), the percentage of open arm entries per total arm entries (Fig. 4c), and defecation (Fig. 4d) between SD and HFD mice. Although 6 weeks of HFD consumption did not affect hippocampal nNOS protein expression or other nNOS/NO related molecules, nNOS gene expression levels were significantly increased (Fig. 4e–m).

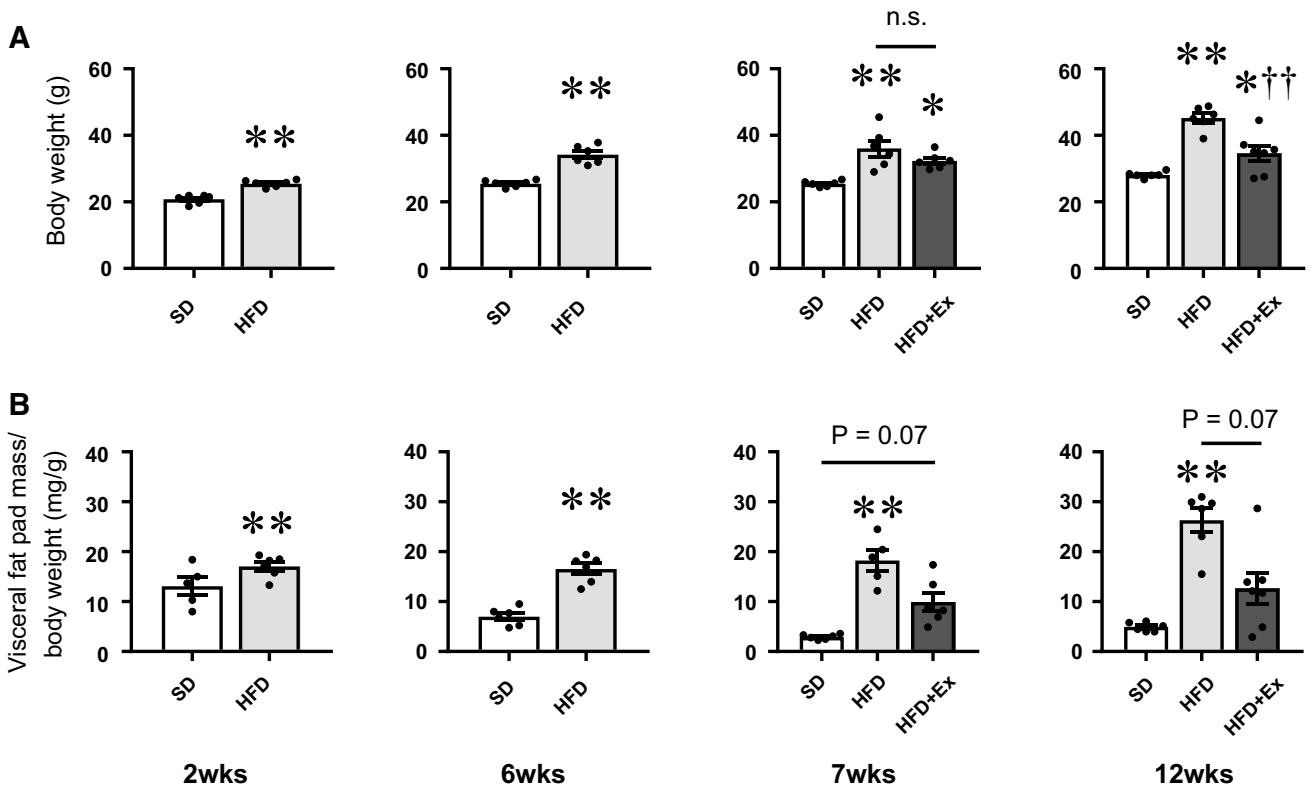

Fig. 2 Body weight (**a**) and relative visceral fat mass (**b**) at 2, 6, 7, and 12 weeks. Open bars, SD group; light gray bars, HFD group; dark gray bars, HFD + Ex group. $^{**}P < 0.01$, $^{*}P < 0.05$ vs SD group, $^{††}P < 0.01$, vs HFD group. *n.s.* non-significant. All data are presented as mean ± SE. Dot plot represents individual data points. $n = 5$–7 per group

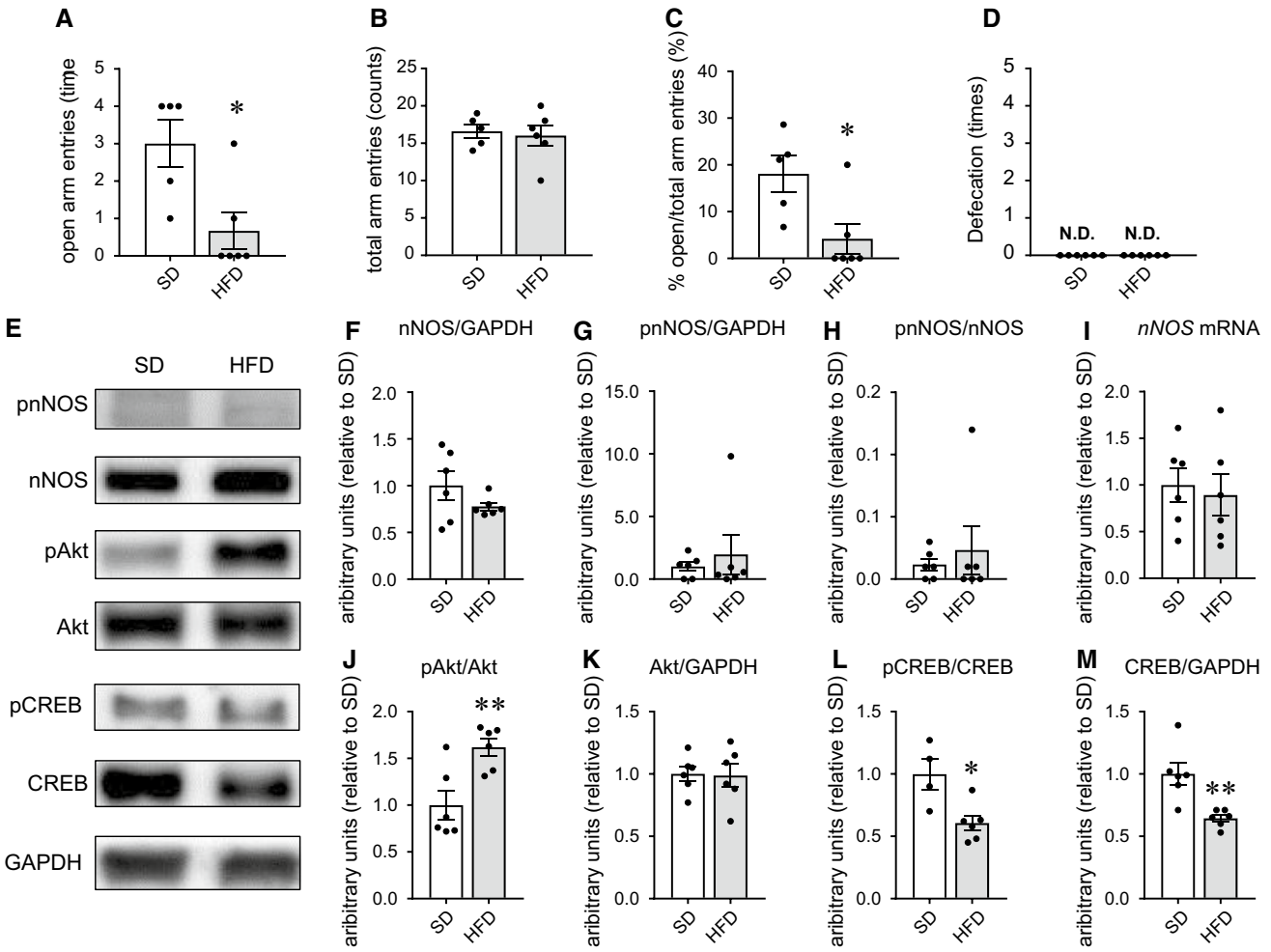

Fig. 3 Behavioral parameters and hippocampal nNOS, Akt, and CREB protein expression and phosphorylation levels after 2 weeks of HFD consumption. Open (**a**) and total arm entries (**b**), percentage of open/total arm entries (**c**), and defecation (**d**). Open arm entries and the percentage of open/total arm entries were significantly decreased in the HFD group compared with the SD group. Representative immunoblots are shown in **e**, and quantified data are presented as nNOS (**f**), pnNOS^{Ser1412}/GAPDH (**g**), pnNOS^{Ser1412}/nNOS (**h**), nNOS gene expression levels (**i**), pAkt^{Ser473} (**j**), Akt (**k**), pCREB^{Ser133} (**l**), and CREB (**m**). Two weeks of HFD consumption led to increased pAkt^{Ser473} and pCREB^{Ser133}, while both nNOS and pnNOS^{Ser1412} levels were unchanged. Open bars, SD group and light gray bars, HFD group. $^{**}P<0.01$, $^{*}P<0.05$ vs SD group. All data are presented as mean ± SE. Dot plot represents individual data points. $n=5$–6 per group

Effect of HFD and 1 week of exercise on anxiety-like behaviors and the nNOS/NO pathway at 7 weeks

Although not statistically significant, compared with the SD group, total arm entries had decreased in the HFD group at 7 weeks ($P=0.07$, Fig. 5b). The percentage of open arm entries per total arm entries had significantly decreased in the HFD + Ex mice (Fig. 5c). We observed defecation in the SD and HFD groups, but not in the HFD + Ex group. However, we found no significant between-group differences (Fig. 5d). These results suggest that the measured behavioral parameters were partially affected by HFD consumption, but not by exercise. At 7 weeks, hippocampal nNOS expression levels had significantly increased in HFD mice ($P<0.05$, Fig. 5f). However, as with the behavioral parameters, nNOS protein expression levels in HFD + Ex mice were also unchanged compared with HFD mice. Moreover, pnNOS^{Ser1412}/GAPDH levels and pAkt^{Ser473} levels had increased with HFD consumption, and decreased with exercise (Fig. 5g, j). pCREB^{Ser133} levels were unchanged in all groups (Fig. 5l).

Effect of HFD and 6 weeks of exercise on anxiety-like behaviors and the nNOS/NO pathway at 12 weeks

At the final time point (Fig. 6, 12 weeks of HFD consumption with 6 weeks of exercise training), the open arm entries and

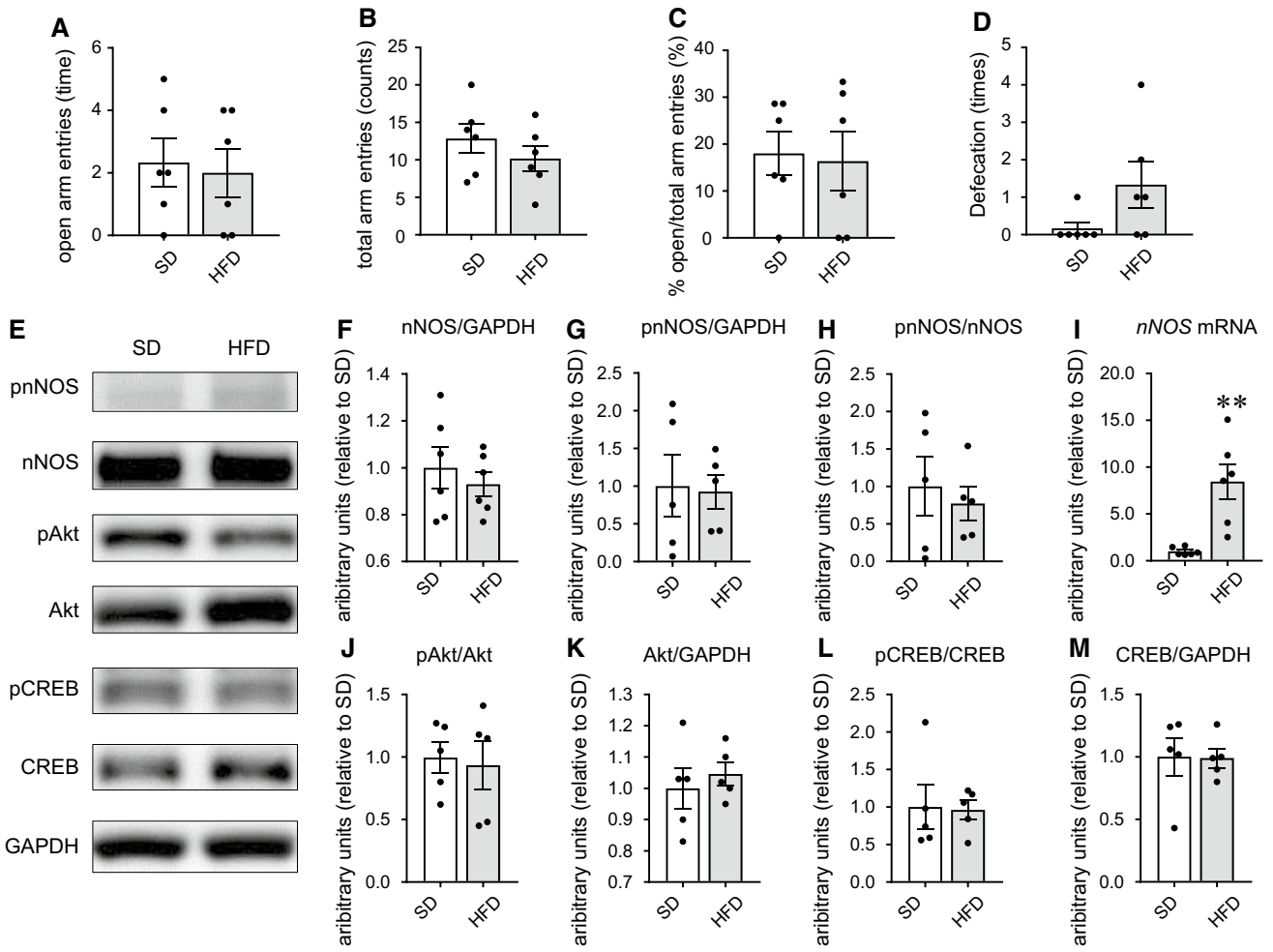

Fig. 4 Behavioral parameters and hippocampal nNOS, Akt, and CREB protein expression and phosphorylation levels after 6 weeks of HFD consumption. Open (**a**) and total arm entries (**b**), percentage of open/total arm entries (**c**), and defecation (**d**). Representative immunoblots are shown in **e**, and quantified data are presented as nNOS (**f**), pnNOS^{Ser1412}/GAPDH (**g**), pnNOS^{Ser1412}/nNOS (**h**), nNOS gene expression levels (**i**), pAkt^{Ser473} (**j**), Akt (**k**), pCREB^{Ser133} (**l**), and CREB (**m**). There were no significant between-group differences after 6 weeks of HFD consumption. Open bars, SD group and light gray bars, HFD group. $^{**}P<0.01$, vs SD group. All data are presented as mean ± SE. Dot plot represents individual data points. $n=5$–6 per group

the percentage of open arm entries per total arm entries were similar in the HFD and SD groups (Fig. 6a, c). However, the percentage of open arm entries per total arm entries ($P<0.05$) had increased in the HFD + Ex group compared with the HFD group (Fig. 6c). Total arm entries were unchanged at this time point. Defecation had also significantly increased in the HFD group ($P<0.01$ vs SD group), and exercise training had reversed the HFD-related increase in defecation ($P<0.01$ vs HFD group). Similar to our previous study [21], 12 weeks of HFD consumption increased hippocampal nNOS expression levels, and these levels were completely repressed in the HFD + Ex group (Fig. 6f). However, nNOS gene expression levels had decreased in the HFD and HFD + Ex mice (Fig. 6i). While nNOS protein expression was altered, phosphorylation of nNOS^{Ser1412} and ^{Akt}Ser473 appeared to have been unchanged by HFD consumption and exercise (Fig. 6g, h, j). Phosphorylation of CREB^{Ser133} in HFD + Ex mice was significantly higher than that in HFD mice (Fig. 6l). These results indicate that 6 weeks of exercise during HFD consumption improves anxiety-like behaviors by modulating the hippocampal nNOS/NO pathway.

Relationship between hippocampal nNOS protein expression levels and body weight or visceral fat mass

Next, we explored possible contributing factors to the observed changes in hippocampal nNOS protein expression levels. At 2 and 6 weeks, nNOS expression levels were not associated with body weight or visceral fat mass (Fig. 7a, b, e, f). However, at 7 weeks, nNOS expression levels were significantly correlated with both body weight ($r=0.59$, $P<0.05$,

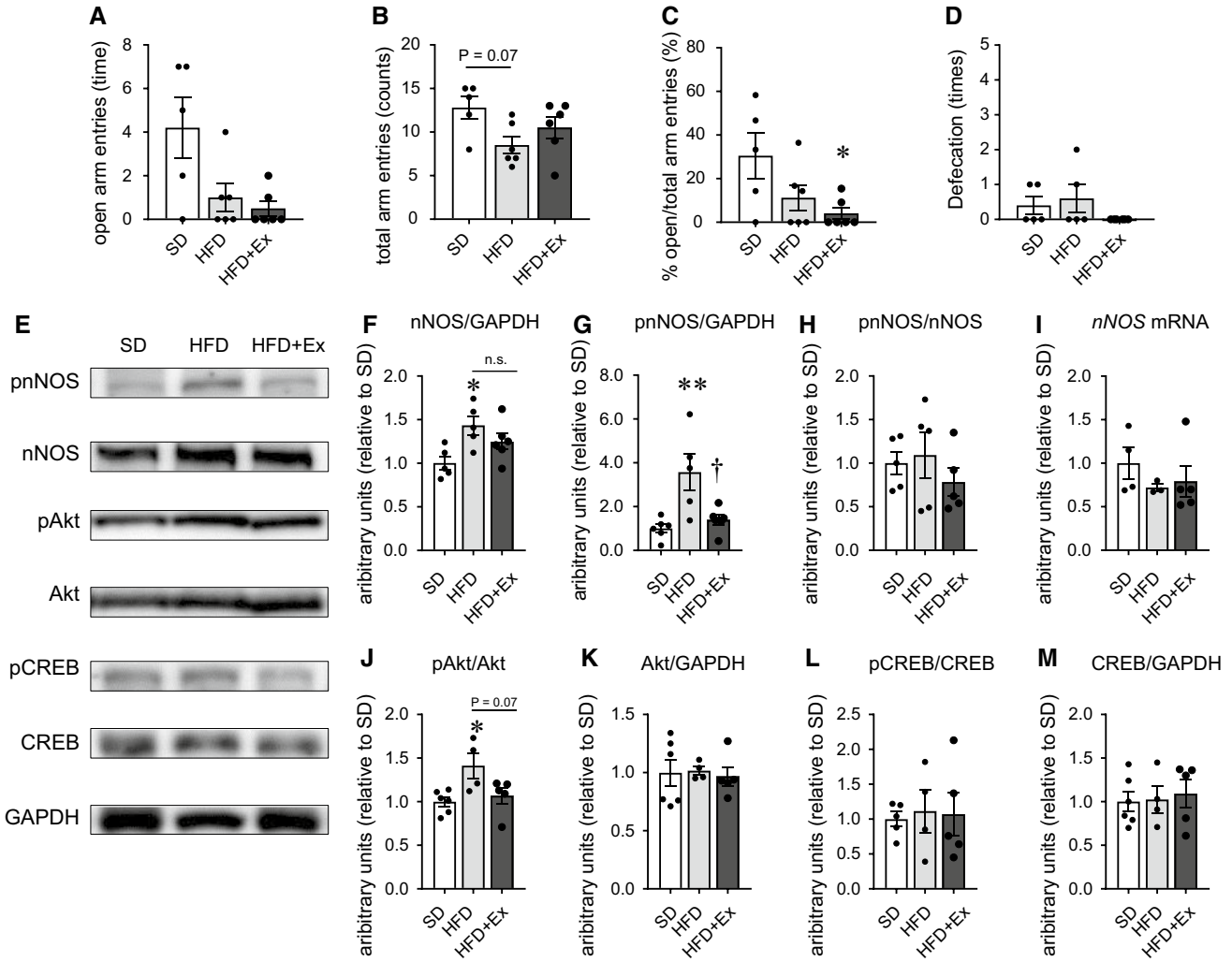

Fig. 5 Behavioral parameters and hippocampal nNOS, Akt, and CREB protein expression and phosphorylation levels after 7 weeks of HFD consumption combined with 1 week of exercise training. Open (**a**) and total arm entries (**b**), percentage of open/total arm entries (**c**), and defecation (**d**). The HFD + Ex group showed a lower percentage of open/total arm entries compared with the SD group. Representative immunoblots are shown in **e**, and quantified data are presented as nNOS (**f**), pnNOS^{Ser1412}/GAPDH (**g**), pnNOS^{Ser1412}/nNOS (**h**), nNOS gene expression levels (**i**), pAkt^{Ser473} (**j**), Akt (**k**), pCREB^{Ser133} (**l**), and CREB (**m**). nNOS expression levels and pnNOS^{Ser1412} levels relative to GAPDH were significantly increased in the HFD group. Exercise normalized pnNOS^{Ser1412} levels, but not nNOS protein expression. Open bars, SD group; light gray bars, HFD group; dark gray bars, HFD + Ex group. $^{**}P < 0.01$, $^{*}P < 0.05$ vs SD group. All data are presented as mean ± SE. Dot plot represents individual data points. $n = 4–7$ per group

Fig. 7c) and relative visceral fat mass ($r = 0.61$, $P < 0.05$, Fig. 7g). Moreover, these associations were also observed at 12 weeks (nNOS vs body weight: $r = 0.50$, $P < 0.05$; nNOS vs visceral fat mass: $r = 0.49$, $P < 0.05$). These data suggest that the accumulation of visceral fat by HFD consumption might contribute to excess nNOS expression in the hippocampus.

Discussion

The major findings of this study were: (1) HFD consumption increased levels of expression of hippocampal nNOS protein; (2) exercise training reversed changes in hippocampal nNOS expression and reduced HFD-related anxiety according to one of our two measures; and (3) hippocampal nNOS expression was correlated with fat weight/body weight at 7 and 12 weeks.

Two weeks of HFD consumption significantly decreased open arm entries and the percentage of open/total arm entries. However, hippocampal nNOS expression levels were unchanged at 2 weeks of HFD consumption. Further, 6 weeks of HFD consumption did not appear to alter the measured behavioral parameters or hippocampal nNOS expression levels. Consistent with our results, Gainey et al. [23] reported that the HFD was associated with increased anxiety-like behaviors at 3 weeks of HFD consumption,

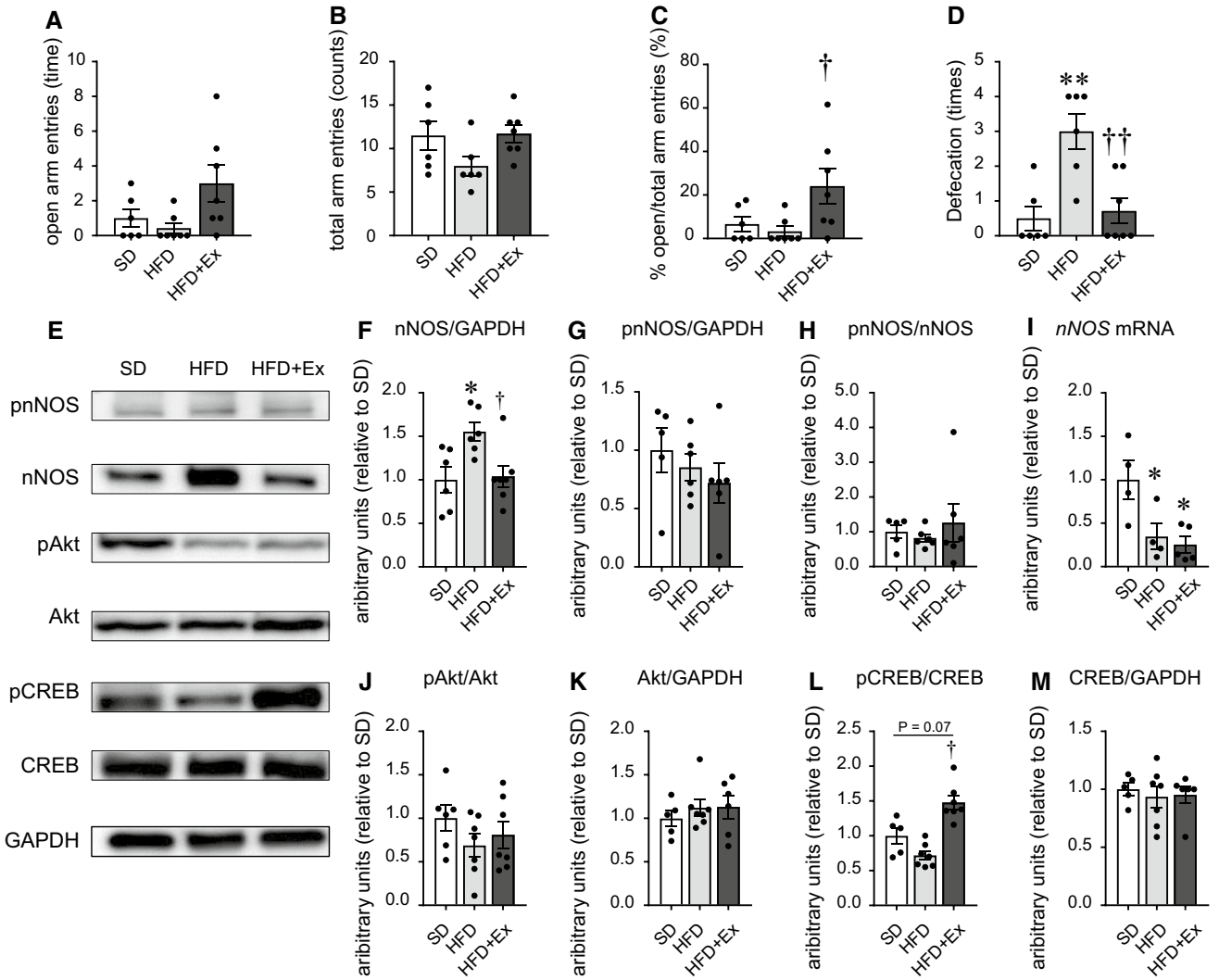

Fig. 6 Behavioral parameters and hippocampal nNOS, Akt, and CREB protein expression and phosphorylation levels after 12 weeks of HFD consumption combined with 6 weeks of exercise training. Open (**a**) and total arm entries (**b**), percentage of open/total arm entries (**c**), and defecation (**d**). Exercise completely restored HFD-induced defecation, and significantly improved open arm entries and the percentage of open/total arm entries. Representative immunoblots are shown in **e**, and quantified data are presented as nNOS (**f**), pnNOS^{Ser1412}/GAPDH (**g**), pnNOS^{Ser1412}/nNOS (**h**), nNOS gene expression levels (**i**), pAkt^{Ser473} (**j**), Akt (**k**), pCREB^{Ser133} (**l**), and CREB (**m**). Exercise rescued HFD-induced excess nNOS expression levels, and improved pCREB^{Ser133} levels compared with the HFD group. Open bars, SD group; light gray bars, HFD group; dark gray bars, HFD + Ex group. $^{**}P < 0.01$, $^{*}P < 0.05$ vs SD group, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs HFD group. *n.s.* non-significant. All data are presented as mean ± SE. Dot plot represents individual data points. $n = 5$–7 per group

but not 1 or 6 weeks. Another study showed that HFD consumption was associated with a decrease in time spent in the open arms of the EPM test at 1 week, but not 3 weeks [24]. Therefore, it appears that transient anxiety was induced during the initial stages of HFD consumption, although the mechanisms underlying this phenomenon are still unclear. Our finding that hippocampal nNOS expression increased with the length of the experimental period indicates that the mechanisms of obesity-induced mood regulation by the nNOS/NO pathway differ between early and late stages of obesity.

We found significantly increased pAkt^{Ser473} levels at 2 weeks of HFD consumption but not at 6 weeks of HFD consumption. This result is difficult to explain. Several recent studies reported similar kinetics in HFD-induced hippocampal pAkt^{Ser473} levels. Abbasnejad et al. showed that a 2-week but not a 3-week HFD increased hippocampal pAkt^{Ser473} levels [25]. Their report that HFD-induced alterations can take place within 1 week might support our finding of substantial differences in hippocampal pAkt^{Ser473} and nNOS expression levels between 6 and 7 weeks of HFD treatment. In addition, their finding that 6 weeks of HFD

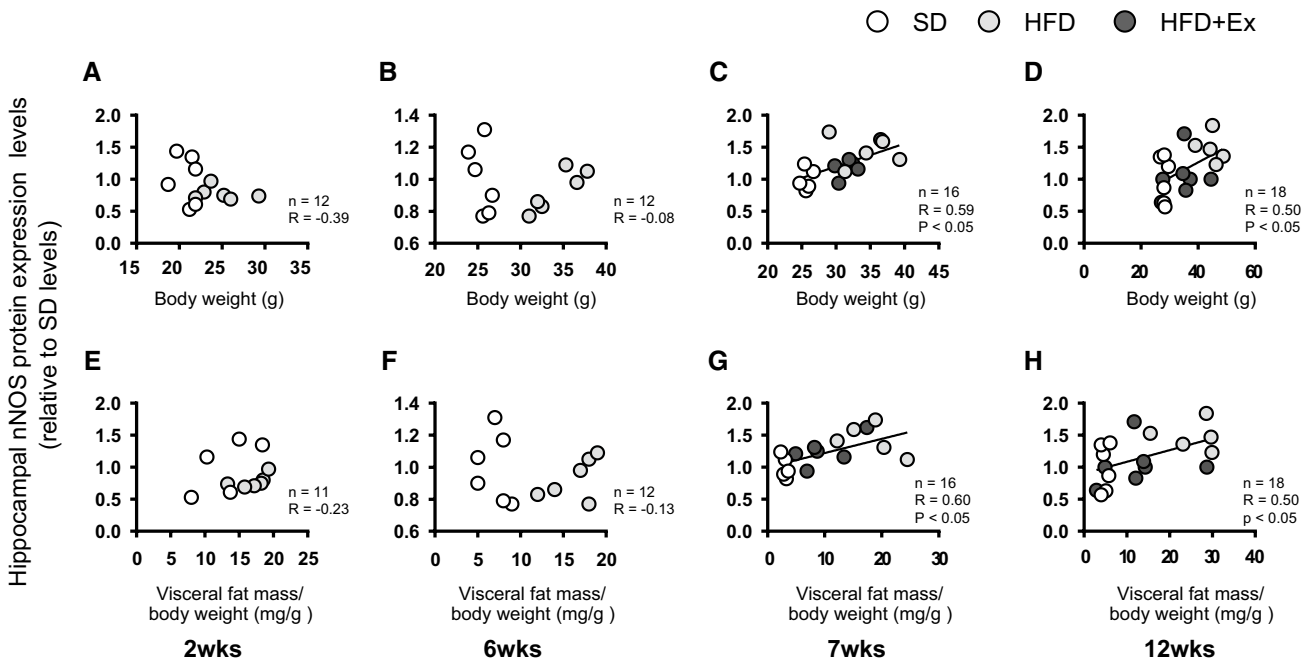

Fig. 7 Correlation between hippocampal nNOS protein expression levels and body weight (**a**, **b**, **c**, and **d**), and relative visceral fat mass (**e**, **f**, **g**, **h**) at 2 weeks (**a**, **e**), 6 weeks (**b**, **f**), 7 weeks (**c**, **g**), and 12 weeks (**d**, **h**). Open circles, SD group; light gray circles, HFD group; dark gray circles, HFD + Ex group. Lines in the scatter plots show significant correlations (by Pearson's product–moment correlations test)

did not alter pAkt^{Ser473} levels was in agreement with our results for the first two time points. Also, previous reports demonstrated that 16 and 20 weeks of a HFD or HFD/high fructose diet inhibited phosphorylation of Akt [26, 27], whereas 26 weeks of a HFD increased pAkt^{Ser473} levels [25]. Although these studies differ from our study in terms of certain methodological factors such as species and dietary composition, the collected findings indicate that HFD-induced phosphorylation of Akt might be bimodal. Another study also showed that pAkt^{Ser473} levels in the hippocampus were increased by a single prolonged stress event [28]. Indeed, acute stress has been found to significantly up-regulate hippocampal pAkt^{Ser473} levels, which then gradually return to pre-stress levels [29]. Therefore, acute-stress-induced increases in hippocampal pAkt^{Ser473} levels might act as a negative regulator. Although not significant, we found a negative correlation between pAkt ^{Ser473} levels and the number of open arm entries, which is a marker of anxiety (data not shown, $P = 0.07$, $r = -0.56$). Thus, it is possible that the PI3 K/Akt pathway regulates mood behavior following the initial stage of HFD consumption.

Consistent with our previous work [21], we demonstrated that 6 weeks of exercise training rescued increases in hippocampal nNOS expression after 12 weeks of HFD consumption (Fig. 6i). At this time point, we found no changes in the number of open arm entries and the percentage of open arm entries after HFD consumption (Fig. 6a, c). However, at 12 weeks of HFD consumption, we found a significant increase in instances of defecation (Fig. 6d), which is frequently used as a measure of anxiety [30–33]. These alterations were completely restored after 6 weeks of exercise training. Therefore, our assessment of behavioral parameters indicated that HFD consumption elicited anxiety-like behaviors in one of our measures, which then decreased after exercise training. Interestingly, 1 week of exercise after 7 weeks of HFD consumption had no effect on behavior or hippocampal nNOS expression (Fig. 5). These results suggest that, compared with 1 week of exercise, greater than 6 weeks of exercise had a stronger effect on anxiety-like behavior via the nNOS/NO pathway. Consistent with this, HFD + Ex mice had higher pCREB^{Ser133} levels and lower nNOS expression levels compared with HFD mice at 12 weeks, while this was not the case at 7 weeks. Zhang et al. [15] clearly showed that hippocampal CREB phosphorylation is essential for the behavioral effects of nNOS. Interestingly, at 12 weeks, pAkt^{Ser473} levels were not significantly different between the groups. Therefore, 6 weeks of exercise during HFD consumption appears to have decreased hippocampal nNOS protein expression levels, which subsequently increased pCREB^{ser133} levels and, despite no alterations in the PI3 K/Akt pathway, led to improvements in anxiety-like behavior.

It is known that nNOS has a "delay action mechanism", although the potential mechanisms are unclear. Zhang et al.

[15] clearly demonstrated the role of nNOS in anxiety-like behavior. They showed that a nNOS selective inhibitor (7-nitroindazole; 7-NI) improved anxiety-like behavior when administered for 28 days, but not when administered for 7 days. Their group also observed that 7-NI administration eliminated chronic mild stress-induced depression-like behaviors 4 weeks, but not 24 h or 2 weeks after the final administration [34]. These findings suggest that significant behavioral changes can occur 4 weeks after modulation of the nNOS/NO pathway. Therefore, it is possible that 7 and 12 weeks of HFD consumption increase hippocampal nNOS expression levels without affecting anxiety-like behaviors measured by the EPM. That we found anxiety-like behaviors in mice after 2 and not 6 weeks of exposure to a HFD supports the idea that the mechanisms of HFD consumption-induced mood behaviors differ in the initial vs late stages of exposure. Further, 6 weeks of exercise training during HFD consumption had significant effects on anxiety-like behaviors and hippocampal nNOS expression (Fig. 6), while this was not the case for 6 weeks of HFD consumption alone (Fig. 4). Thus, changes in anxiety-like behaviors induced by modulation of the nNOS/NO pathway may differ for dietary vs exercise interventions.

Phosphorylation of nNOS is considered to be an important mechanism modulating the role of nNOS in NO production. nNOS phosphorylation is known to occur in skeletal muscles, and nNOS is phosphorylated by muscle contraction and insulin treatment [17, 35]. A recent study showed that nNOS phosphorylation involves glucose uptake in myotubes [36]. As in skeletal muscles, PI3 K/Akt phosphorylates nNOS at Ser1412 in cortical neurons [18] and in the ventromedial hypothalamus [37]. Thus, we anticipated that the HFD or exercise would regulate hippocampal pnNOS ^{Ser1412} via the PI3 K/Akt pathway. We observed that pAkt^{Ser473} levels had increased after 2 and 7 weeks of HFD consumption. However, pnNOS ^{Ser1412} levels had significantly increased only after 7 weeks of HFD consumption, and not after 2 weeks. Moreover, 1 week of exercise restored HFD-induced pnNOS ^{Ser1412} to SD group levels after 7 weeks of HFD consumption. At 12 weeks, we found no significant differences between the groups. In addition, pAkt^{Ser473} levels were correlated with pnNOS ^{Ser1412} levels at 7 weeks (data not shown, $r = 0.58$, $P < 0.05$), but not at 12 weeks. The reason for this discrepancy in the relationship between pAkt^{Ser473} and pnNOS ^{Ser1412} levels at different measurement points is not clear. A previous study suggested that when NO is produced in excessive amounts, it transitions from a physiological neuromodulator to a neurotoxicant [38]. Osuka et al. [39] reported that transient ischemia induces nNOS phosphorylation in the hippocampus. The authors proposed that the presence of pnNOS ^{Ser1412} in the subgranular layer of the dentate gyrus after cerebral ischemia indicates that pnNOS ^{Ser1412} might be a negative regulator of neurogenesis, and that it might be involved in post cerebral ischemic depression. We observed partial phosphorylation of nNOS at Ser1412 at 7 weeks (Fig. 5g). However, the relationship between the behavioral parameters and pnNOS ^{Ser1412} levels does not have a clear pattern. Therefore, it appears that nNOS protein regulation, rather than nNOS phosphorylation, mainly contributes to the regulation of mood behavior by HFD or exercise. Moreover, we demonstrated that pCREB^{Ser133} levels were increased in HFD + Ex mice compared with HFD mice at 12 weeks. This was accompanied by a reduction in nNOS protein but not phosphorylation at Ser1412. Many studies have investigated the effects of acute stimuli on nNOS phosphorylation in the central nervous system (CNS) and muscle tissue, for instance, with respect to transient ischemia [39], a single insulin treatment in cultured myoblast or pituitary cells [36, 40] and in mice [17], and acute oxidative stress exposure in cardiomyocyte cells [41]. Given these reports, phosphorylation of nNOS at Ser1412 might play a role in the regulation of brain function during exposure to acute stimuli, rather than prolonged intervention. Further studies are needed to determine whether acute changes in dietary pattern or single bouts of exercise affect hippocampal nNOS phosphorylation.

We observed that hippocampal nNOS expression levels were significantly correlated with body weight and visceral fat mass at 7 and 12 weeks. Additionally, hippocampal nNOS expression levels were correlated with total energy intake at 7 weeks but not 12 weeks, while total food intake was not associated with hippocampal nNOS expression levels. Given that whole-body nNOS knock-out and wild type mice have a comparable body weight [42, 43], these results suggest that greater nNOS expression in HFD mice was induced by excess energy intake and accumulation of visceral adipose tissue (and subsequently body weight gain), rather than total food intake. This might imply that HFD- or exercise-induced adipokine affects hippocampal nNOS and anxiety regulation. However, it is known that adipokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are associated with impaired neurogenesis [44, 45]. Previously, Arnoldussen et al. [46] suggested that adipokines interact with the CNS, and proposed that in obesity, excessive adipokine production by white adipose tissue mediates characteristic peripheral pathological processes. This consideration supports our findings that hippocampal nNOS expression levels were correlated with fat mass at 7 and 12 weeks, given that longer HFD consumption led to greater accumulation of excessive fat pad mass. Further studies are needed to characterize the relationship between these adipokines and hippocampal nNOS expression levels.

In conclusion, our results demonstrate that 7 and 12 weeks of HFD consumption increased hippocampal nNOS expression levels. Additionally, 6 weeks of exercise training restored hippocampal nNOS expression and

improved 12 weeks of HFD-related anxiety according to a recovery in defecation rate. However, we found no differences in anxiety as measured by the EPM. Furthermore, hippocampal nNOS expression levels were associated with body weight and visceral fat mass in HFD-induced obesity.

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Author contributions YT and YH conceived and designed the experiments. YT, SY, YT, RG, and IK performed the experiments and analyzed data. YT, SR, YU, KK, and YH interpreted the results of the experiments. YT prepared the figures and drafted the manuscript. SR, YU, KK, and YH edited and revised the manuscript. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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