

Exercise mitigates the effects of a cafeteria diet on antidepressant-like behavior associated with plasma and microbial metabolites in adult male rats

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A cafeteria diet high in saturated fat and sugar has been associated with increased anxiety-like and depressive-like behaviors and memory impairments, whereas exercise has been shown to promote antidepressant-like effects and enhance cognitive function in rodents. The mechanisms underlying the interactions between diet and exercise on mood, anxiety, and memory are not fully understood, but alterations in adult hippocampal neurogenesis (AHN), gut-derived metabolites, or plasma metabolic hormones may play a role. This study investigated whether voluntary exercise could mitigate the effects of concurrent exposure to a cafeteria diet on depression-like, anxiety-like, and cognitive behaviors in young adult male rats. Associated changes in AHN, metabolic hormones, and gut-derived metabolites were examined to identify potential mediators of behavioral changes. We found that exercise mitigated the cafeteria diet-induced increase in immobility in the forced swim test. This antidepressant-like effect of exercise in rats exposed to a cafeteria diet was accompanied by an attenuation of cafeteria diet-induced changes in plasma insulin and leptin, as well as in the abundance of caecal metabolites anserine, indole-3-carboxylate, and deoxyinosine. Exercise modestly improved spatial learning in the Morris water maze, promoted AHN and increased circulating levels of GLP-1, and these effects were blunted in animals exposed to a cafeteria diet suggesting that dietary composition plays a role in modulating the effects of exercise. Correlation analyses revealed that specific caecal metabolites were associated with depression- and cognition-related behaviors, independent of diet and exercise, highlighting the potential role of gut-derived metabolites in antidepressant-like behavior and cognitive function. Together these findings provide insight into potential metabolite and hormone-mediated mechanisms underlying the effects of a cafeteria diet and exercise on brain and behavior.

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Introduction

Increased availability of ultra-processed, energy-dense foods (1) and the prevalence of sedentary lifestyles (2) contributes to rising global ill health. A Western-style diet high in saturated fats and sugar, combined with inactivity, alters metabolic hormone concentrations (3–5), which causes obesity and increases the risk of depression, anxiety (6), and cognitive impairment (7, 8).

Rodent studies show that cafeteria (high-fat and high-sugar) diets, which mimic human Western-style diets, increase depression-like and anxiety-like behavior (9, 10), and impair recognition (11) and spatial memory (12). Conversely, exercise reduces anxiety-like and depression-like behavior and enhances pattern separation and spatial learning and memory (13–17). However, it remains unclear whether exercise can attenuate cafeteria diet-induced effects on depression-like, anxiety-like, and cognitive behaviors.

Adult hippocampal neurogenesis (AHN), the birth of new neurons in the dentate gyrus (DG) of the hippocampus, regulates anxiety-like behavior (18), pattern separation (19, 20), and spatial memory (21, 22), and is required for responses to antidepressants (23, 24). AHN is sensitive to external factors including diet and exercise (25), but these effects may be associated with age or sex. For example, adolescent-initiated cafeteria diet decreases AHN in male rats (26, 27), whereas the effects of adult-initiated cafeteria diet on AHN are unclear. However, the pro-neurogenic effects of exercise are well characterized in adult rodents (17, 28, 29). Importantly, both diet and exercise alter concentrations of metabolic hormones leptin (30), ghrelin (31), insulin (32), glucagon-like peptide 1 (GLP-1) (33), and fibroblast growth factor 21 (FGF-21) (34). These hormones influence AHN, suggesting possible mechanisms of lifestyle-mediated regulation of AHN and associated behaviors.

Diet and exercise are potent modulators of gut microbiota composition and microbial metabolism (35–39). Consumption of a cafeteria diet decreased microbiota diversity (40), and altered caecal metabolite composition in adult male rats (41). Conversely, exercise increased microbiota diversity (42, 43) and short-chain fatty acid (SCFA) production in the caecum (44). Microbial-derived metabolites including SCFAs, essential amino acids, and neurotransmitters (45) are now proposed as key mediators of the microbiota-gut-brain axis, significantly affecting AHN (35, 36). Notably, chronic disruption of the gut microbiota with antibiotics in adult male rats impaired AHN and associated behaviors (37), supporting previous evidence from germ-free mice of a role for gut microbiota in regulating AHN (38). Moreover, depression, anxiety, and Alzheimer's disease are associated with altered composition of gut microbial metabolites, with subsequent influences on AHN (46–49).

It remains unclear however if exercise attenuates the effects of cafeteria diet on AHN and associated behaviors. This study investigated if exposure of young adult male rats to voluntary wheel running exercise altered the effects of a cafeteria diet on AHN, depression-like, anxiety-like, and cognitive behaviors. Results provide insight into potential gut-derived or plasma-mediated metabolic mechanisms through which exercise may mitigate the effects of a cafeteria diet on brain and behavior.

Results

Exercise attenuated cafeteria diet-induced increases in body weight gain and adipose tissue

Both diet and exercise significantly affected body weight (Supplementary Figure S1A). Post-hoc analysis revealed that cafeteria diet increased weight gain in sedentary (CTRL-SED vs. CAF-SED, $p < 0.0001$) and to a lesser extent in exercising (CTRL-EX vs. CAF-EX, $p < 0.01$) animals.

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Interestingly, exercise significantly reduced body weight gain in cafeteria diet-fed animals (CAF-SED vs. CAF-EX, $p < 0.01$) but not in standard chow-fed animals (CTRL-SED vs. CTRL-EX, $p = 0.071$). Repeated measures ANOVA (analysis of variance) showed a significant effect of time and a diet–time interaction on average weekly running distance (Supplementary Figure S1B) but did not reveal a cafeteria diet-induced difference at any given week. Diet, exercise, and their interaction affected epididymal white adipose tissue (eWAT) weight (Supplementary Figure S1C). Cafeteria diet increased eWAT weight (CTRL-SED vs. CAF-SED, $p < 0.0001$; CTRL-EX vs. CAF-EX, $p < 0.01$), while exercise reduced it (CAF-SED vs. CAF-EX, $p < 0.0001$; CTRL-SED vs. CTRL-EX, $p < 0.01$). Finally, both diet and exercise independently affected brown adipose tissue (BAT) weight (Supplementary Figure S1D). Cafeteria diet increased BAT weight only in sedentary animals (CTRL-SED vs. CAF-SED, $p < 0.01$), and exercise reduced BAT weight gain in animals exposed to a cafeteria diet (CAF-SED vs. CAF-EX, $p < 0.05$).

Exercise mitigated a cafeteria diet-induced increase in immobility in the forced swim test and exerted a modest anxiolytic effect irrespective of diet

Because an unhealthy diet has been linked to changes in emotional behaviors, we tested whether exercise could influence the effect of a cafeteria diet on despair, anxiety, anhedonia, and locomotor activity. To measure antidepressant-like behaviors, animals underwent the forced swim test (FST) as previously described (50) (Figure 1B). There was a significant main effect of diet and exercise on the immobility score (Figure 1B, left panel). Cafeteria diet increased immobility in sedentary animals (CTRL-SED vs. CAF-SED, $p < 0.05$) which was mitigated in exercising rats (CAF-SED vs. CAF-EX, $p < 0.05$), suggesting an antidepressant effect of exercise. Swimming behavior was significantly affected by diet and exercise (Figure 1B, middle panel). Post-hoc analysis did not reveal significant differences between groups, although there was a trend to decrease swimming by cafeteria diet in exercising animals (CTRL-EX vs. CAF-EX, $p = 0.060$). Climbing behavior was not significantly affected (Figure 1B, right panel). In the elevated plus maze (EPM), there was a trend for an exercise-induced increase in the percentage of time spent in the open arms (anxiolytic effect, Figure 1C). Similarly in the novelty suppressed feeding (NSF), exercise alone significantly affected latency to eat (Figure 1D). Post-hoc comparisons showed that exercise decreased the latency to eat in standard chow-fed rats (CTRL-SED vs. CTRL-EX, $p = 0.0185$) but not in cafeteria diet-fed animals. To control for food interest which could confound the latency to eat, food intake was measured for each animal during the 30 min posttest (Figure 1E). Cafeteria diet tended to reduce posttest chow consumption in sedentary rats (CTRL-SED vs. CAF-SED, $p = 0.071$) and significantly in exercising rats (CTRL-EX vs. CAF-EX, $p < 0.001$). This suggests that supplementation with cafeteria diet decreased interest in standard chow. The lack of an exercise-induced effect on posttest chow consumption indicates that the reduced latency to eat was not due to increased interest in standard chow, but rather decreased anxiety-like behavior. Finally, animals were tested for anhedonia in the FUST (Figure 1F) and for locomotor activity and anxiety-like behavior in the open field test (OFT) (Figure 1G–H), yielding no significant effects.

There was a modest effect of exercise but not a cafeteria diet on spatial learning and memory

Pattern separation was evaluated in the modified spontaneous location recognition (MSLR) test (Figure 2A). There were no effects of diet or exercise in the large separation (low contextual overlap) test (Figure 2B, left panel), but both interventions tended to affect performance in the small separation [high contextual overlap (pattern separation)] test (Figure 2B, right panel). In the novel object recognition (NOR) test used to assess recognition memory, there were no effects of diet and exercise alone, although a diet–exercise interaction significantly affected the discrimination ratio (Figure 2C). Time and the time–exercise interaction significantly affected the latency to find the platform in the Morris water maze (MWM) over the 4 training days (Figure 2D). During the probe trial of the MWM, there were no effects of diet or exercise on the time spent in the target quadrant (Figure 2E), but there was a significant difference in the latency

to the first visit to target quadrant (Figure 2F). Post-hoc analysis indicated that exercise increased the latency to reach the target quadrant in standard chow-fed (CTRL-SED vs. CTRL-EX, $p < 0.01$) but not in cafeteria diet-fed animals (CTRL-EX vs. CAF-EX, $p < 0.05$). There was a main effect of exercise on average velocity in the probe trial (Figure 2G). Exercise showed a trend to improve spatial learning and search strategies in the MWM in response to cafeteria diet or exercise (see Supplementary Materials and Supplementary Figure S2).

An exercise-induced increase in AHN is reduced by cafeteria diet

Neurogenesis was assessed across the longitudinal axis of the hippocampus using immunohistochemical staining of DCX, a marker of immature neurons in the DG (Figure 3B). There was a significant effect of exercise, and a diet–exercise interaction on the number of DCX⁺ cells/mm² (Figure 3A, left panel). Post-hoc analysis revealed that the diet blunted (although not significantly) the number of DCX⁺ cells/mm² in exercising animals (CTRL-EX vs. CAF-EX, $p = 0.086$). Thus, the neurogenic effect of exercise was observed only in standard chow-fed animals (CTRL-SED vs. CTRL-EX, $p < 0.001$). In the dorsal DG, exercise and the diet–exercise interaction significantly affected the number of DCX⁺ cells/mm² (Figure 3A, middle panel), with post-hoc analysis showing that exercise significantly increased the number of DCX⁺ cells/mm² in chow-fed animals (CTRL-SED vs. CTRL-EX, $p < 0.001$). In the ventral DG, there were significant main effects of diet and exercise on the number of DCX⁺ cells/mm² (Figure 3A, right panel). Post-hoc analysis revealed that cafeteria diet blunted (nonsignificantly) the number of DCX⁺ cells/mm² in exercising animals only (CTRL-EX vs. CAF-EX, $p = 0.068$) and exercise significantly increased the number of DCX⁺ cells/mm² in chow-fed animals (CTRL-SED vs. CTRL-EX, $p < 0.05$).

Exercise attenuated a cafeteria diet-induced increase in the metabolic hormones insulin and leptin in plasma

Changes in metabolic hormones like glucagon-like peptide (GLP) 1, insulin, and leptin are linked with depression, anxiety and cognitive impairment (33, 51, 52). The cafeteria diet significantly increased insulin levels in sedentary animals in the current study (CTRL-SED vs. CAF-SED, $p < 0.001$) (Figure 4A), which was mitigated by exercise (CAF-SED vs. CAF-EX, $p < 0.05$). There was a significant increase in leptin after cafeteria diet in both sedentary (CTRL-SED vs. CAF-SED, $p < 0.0001$) and exercising animals (CTRL-EX vs. CAF-EX, $p < 0.01$) (Figure 4B). Exercise reduced leptin in standard chow (CTRL-SED vs. CTRL-EX, $p < 0.01$) and cafeteria diet-fed groups, (CAF-SED vs. CAF-EX, $p < 0.0001$). Cafeteria diet modestly increased total ghrelin (Figure 4C) and C-peptide (Figure 4D) in sedentary animals, with trends toward significance (ghrelin: CTRL-SED vs. CAF-SED, $p = 0.088$; C-peptide: CTRL-SED vs. CAF-SED, $p = 0.079$). The cafeteria diet increased FGF-21 (Figure 4E) in both sedentary (CTRL-SED vs. CAF-SED, $p < 0.001$) and exercising animals (CTRL-EX vs. CAF-EX, $p < 0.001$). On the other hand, total GLP-1 (Figure 4F) was increased in response to exercise (CTRL-SED vs. CTRL-EX, $p < 0.01$), which was reduced by cafeteria diet (CTRL-EX vs. CAF-EX, $p < 0.05$). Exercise elevated total PYY levels (Figure 4G) but in cafeteria diet-fed animals only (CAF-SED vs. CAF-EX, $p < 0.05$). Finally, glucagon (Figure 4H) was significantly reduced by cafeteria diet in exercising animals (CTRL-EX vs. CAF-EX, $p < 0.01$) and showed a trend toward reduction in sedentary animals (CTRL-SED vs. CAF-SED, $p = 0.085$).

Exercise attenuated a cafeteria diet-induced decrease in caecal metabolites anserine, indole-3-carboxylate, and deoxyinosine

Because a cafeteria diet and exercise can differentially alter gut microbiota compositions (39, 53, 54), we investigated whether the two interventions affected the caecal metabolome. This was also motivated by the observation that diet and exercise significantly affected the caecum weight (Figure 5A).

Principal component analysis from an untargeted metabolomics screen suggested an effect of cafeteria diet on the caecal metabolome (Figure 5B). Differential expression analyses revealed that the diet induced differential expression [false discovery rate (FDR)-adjusted $p < 0.05$] of 100/175 metabolites in sedentary animals (Figure 5C), while in exercising animals, cafeteria diet induced differential expression

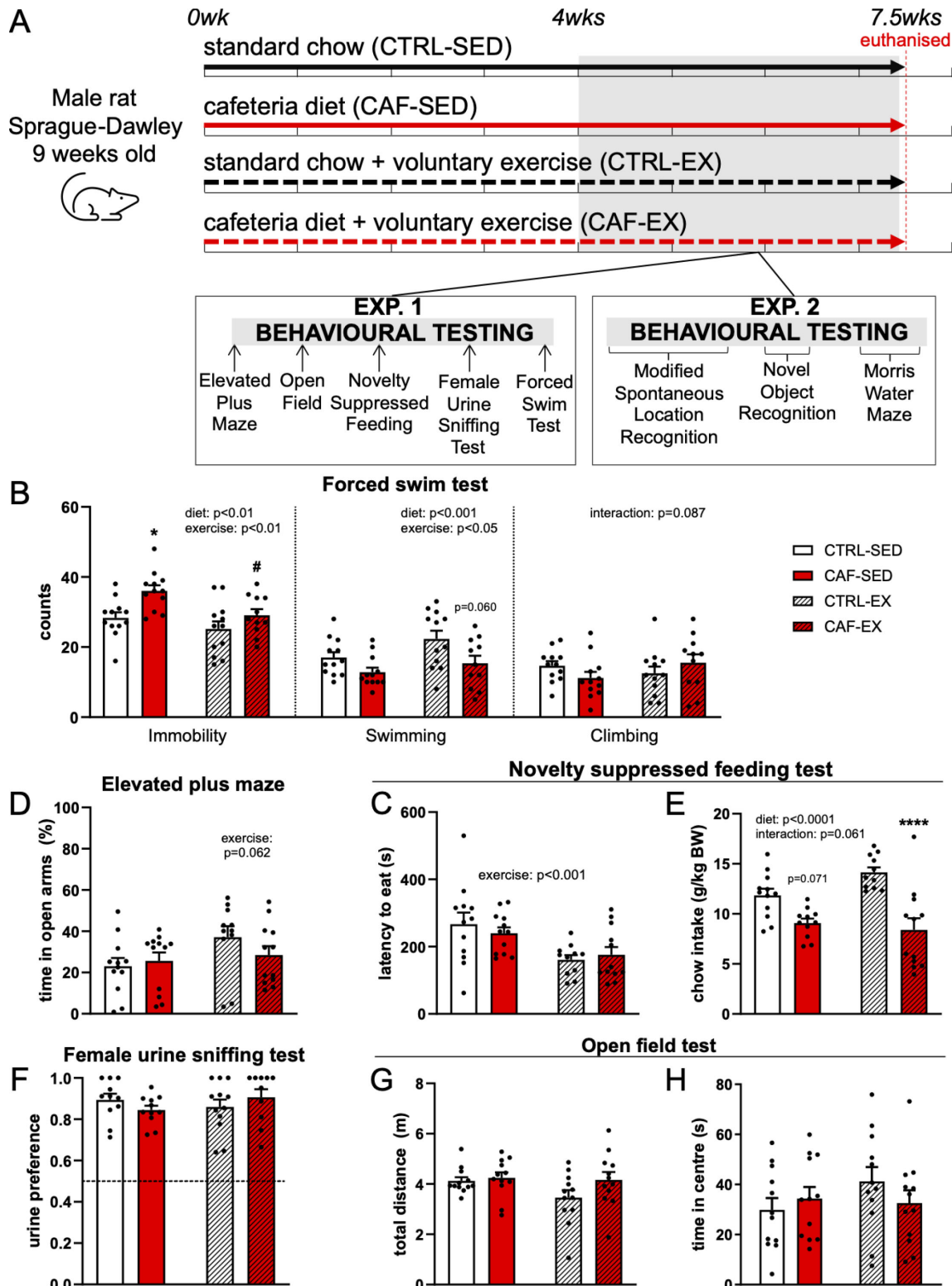


Figure 1. Exercise mitigated a cafeteria diet-induced increase in immobility in the forced swim test and exerted a modest anxiolytic effect irrespective of diet. (A) Experimental design. (B) Immobility: main effect of diet [$F(1,43) = 10.41, p < 0.01$] exercise [$F(1,43) = 7.87, p < 0.01$]; Swimming: main effects of diet [$F(1,43) = 8.80, p < 0.001$] and exercise [$F(1,43) = 4.39, p < 0.05$]; Climbing scores in the FST ($n = 11-12$). (C) Percentage of total time spent in EPM open arms ($n = 11-12$); effect of exercise [$F(1,43) = 3.69, p = 0.062$]; (D) Latency (s) to eat standard chow pellet in the NSF test arena ($n = 11-12$); Main effect of exercise [$F(1,43) = 12.48, p < 0.001$]; (E) Body weight-adjusted standard chow consumption (g) during NSF 30 min posttesting phase ($n = 11-12$); Main effect of diet [$F(1,42) = 30.14, p < 0.0001$], diet-exercise interaction [$F(1,42) = 3.70, p = 0.061$]; (F) Urine preference as time spent sniffing urine/total sniffing time in the FUST ($n = 10-12$). (G) Total distance travelled (m) in the OFT ($n = 12$). (H) Total time (s) spent in the center area of the OFT arena ($n = 12$). Data are expressed as mean $\pm$ SEM, $p = 0.071$, $*p < 0.05$ and $****p < 0.0001$ versus corresponding standard chow-fed group; $p = 0.060, p = 0.058, p = 0.056$ and $*p < 0.05$ versus corresponding sedentary group.

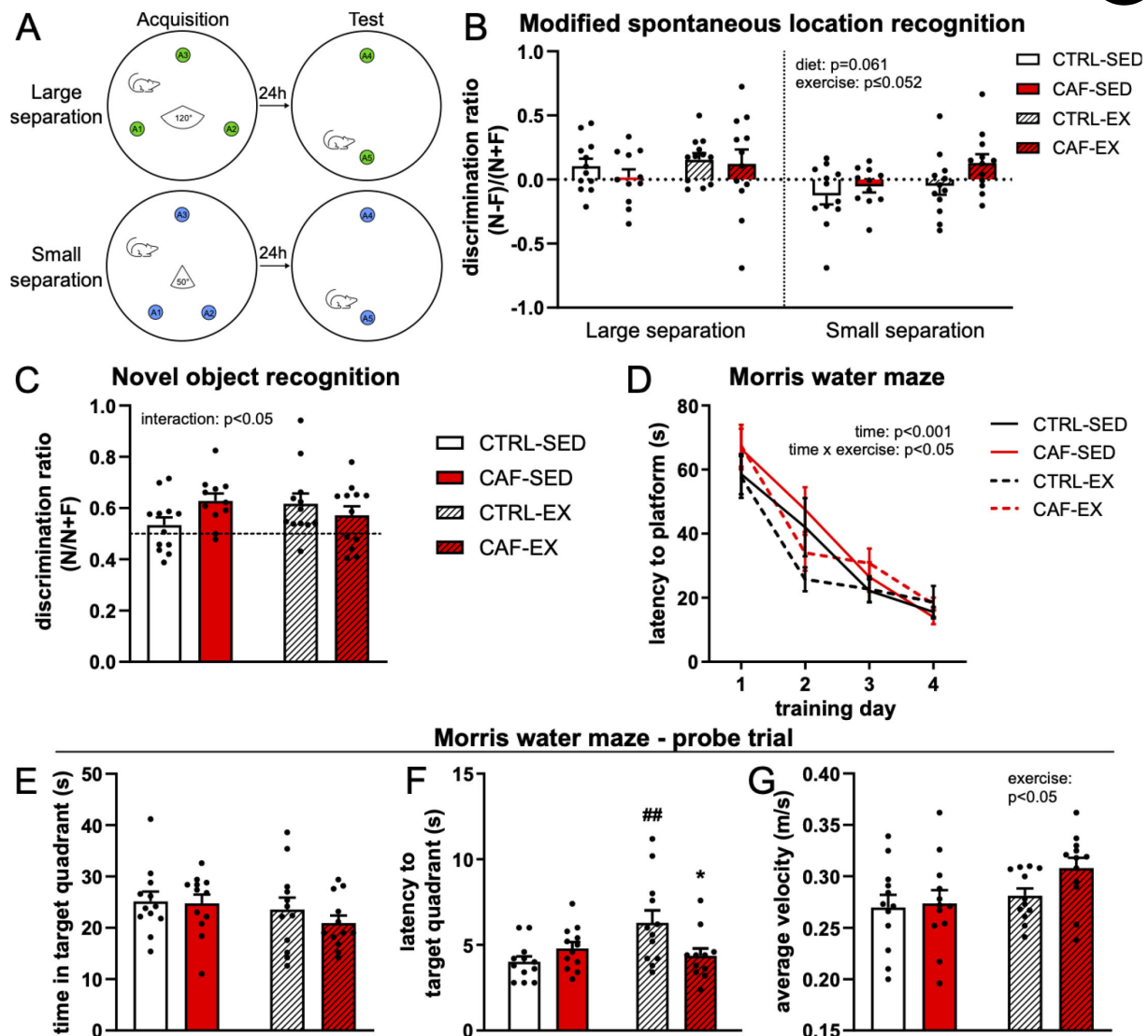


Figure 2. There was a modest effect of exercise but not a cafeteria diet on spatial learning and memory. (A) Schematics of the large and small separation in the MSLR task. (B) Discrimination ratio in the large and small separation of the MSLR ((novel (N) + familiar (F))/(N-F), $n=12$). Small separation effect of diet [$F(1,43)=3.69$, $p=0.061$] and exercise [$F(1,43)=3.98$, $p=0.052$]; (C) Discrimination ratio in the NOR task ($N/(N+F)$, $n=12$), diet-exercise interaction [$F(1,43)=4.17$, $p<0.05$]; (D, E). Average latency (s) to find the platform in the MWM, on (D) all training days; time [$F(3,24)=54.88$, $p<0.001$] and time-exercise interaction [$F(3,24)=3.80$, $p<0.05$]; (E) Cumulative time (s) spent in the target quadrant during the probe trial ($n=12$). (F) Average latency (s) to reach the target quadrant during the probe trial ($n=11-12$); [$H(3)=8.25$, $p<0.05$]; (G) Average swimming velocity during the probe trial ($n=12$), effect of exercise [$F(1,44)=4.51$, $p<0.05$]. Data are expressed as mean $\pm$ SEM, * $p<0.05$ versus corresponding standard chow-fed group; $p=0.074$, ** $p<0.01$ versus corresponding sedentary group.

of 62/175 metabolites (Figure 5D). Conversely, exercise compared to sedentary controls induced differential expression of 5/175 metabolites in standard chow-fed animals (Figure 5E), and 4/175 metabolites in cafeteria diet-fed animals (Figure 5F). Supplemental Table S2 provides a complete list of quantified features.

Three of the top differentially abundant metabolites were notably affected by diet, exercise, and their interaction (Figure 5G-I). Cafeteria diet decreased the abundance of the dipeptide anserine (Figure 5G), the indole derivative indole-3-carboxylate (Figure 5H), and the nucleoside deoxyinosine (Figure 5I) in both sedentary (all $FDR < 0.0001$) and exercising animals ($FDR < 0.0001$ [anserine]; $FDR < 0.01$ [indole-3-carboxylate]; $FDR < 0.05$ [deoxyinosine]). Interestingly, however, for all three metabolites, exercise attenuated the cafeteria diet-related downregulation ($FDR < 0.0001$ [anserine]; $FDR < 0.05$ [indole-3-carboxylate & deoxyinosine]).

The effect of cafeteria diet on the caecal metabolome mainly involved amino acid metabolism and tRNA biosynthesis, as suggested by pathway

enrichment analysis (Supplementary Table S3). Branched-chain amino acid (BCAA), phenylalanine, and tryptophan biosynthesis were all affected by diet in both sedentary and exercising animals. The tryptophan metabolite kynurenine was increased following cafeteria diet but only in sedentary animals ($FDR < 0.05$), while kynurenic acid was increased in both sedentary ($FDR < 0.01$) and exercising ($FDR < 0.05$) animals. Similarly, cafeteria diet increased serotonin, 5-hydroxyindole-3-acetic acid, and n-acetyl-5-hydroxytryptamine in sedentary (all $FDR < 0.0001$) and exercising animals ($FDR < 0.0001$ [n-acetyl-5-hydroxytryptamine & serotonin]; $FDR < 0.001$ [5-hydroxyindole-3-acetic acid]), while kynurenine was increased with exercise but only in standard chow-fed animals ($FDR < 0.05$) (Supplementary Table S2).

Exercise had more limited effects on the metabolome independent of diet. Moreover, we found no pathway enrichment among nominally significant features. Beyond the top three features mentioned earlier (Figure 5G-I), the abundance of the nucleotide CMP (cytidine

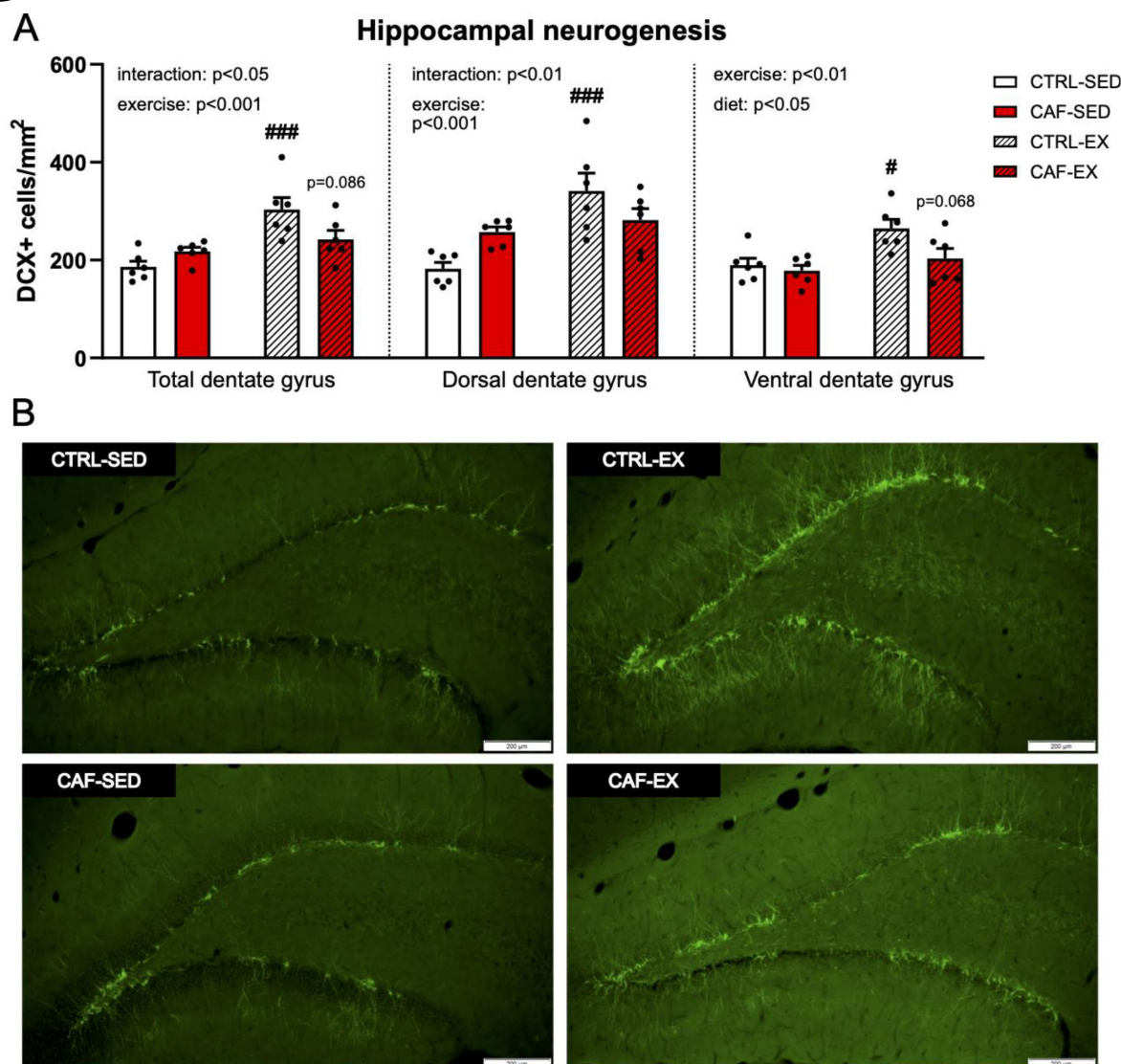


Figure 3. An exercise-induced increase in AHN is reduced by cafeteria diet. (A) Immature neurons in the DG (DCX⁺ cells/mm², $n = 6$). Left panel: Total DG effect of exercise [$F(1,20) = 17.48$, $p < 0.001$] and diet-exercise interaction [$F(1,20) = 7.39$, $p < 0.05$]; middle panel: dorsal DG, effect of exercise [$F(1,20) = 15.33$, $p < 0.001$] and diet-exercise interaction [$F(1,20) = 8.28$, $p < 0.01$]; right panel: ventral DG, effect of diet [$F(1,20) = 4.98$, $p < 0.05$] and exercise [$F(1,20) = 9.29$, $p < 0.01$]. (B) Representative images of DCX⁺ DG neurons taken at 10x magnification. Data are expressed as mean $\pm$ SEM, $p = 0.086$ and $p = 0.068$ versus corresponding standard chow-fed group; # $p < 0.05$ and ### $p < 0.001$ versus corresponding sedentary group.

monophosphate) was decreased by exercise independent of diet (FDR < 0.01 ; Supplementary Table S2). Only three other metabolites were differentially abundant as an effect of exercise in chow-fed animals. The amino acid catabolite 2/3-hydroxybutyric acid was decreased by exercise (FDR < 0.05). Interestingly, it was also strongly decreased by cafeteria diet in both sedentary (FDR < 0.0001) and exercising (FDR < 0.01) animals, possibly related to BCAA metabolism. Finally, the B vitamin pantothenic acid and amino sugar n-acetylneuraminic acid were both increased by exercise (all FDR < 0.05).

Caecal metabolites independently correlate with behavior

To examine whether variations in individual caecal metabolites were associated with behavior, we carried out correlations between all caecal metabolomic features and behavioral task outcome measures.

In general, caecal metabolites were most strongly correlated with immobility time in the FST and the discrimination ratio in the MSLR (Supplementary Table S4). Focusing on the top associations for each read-out (Figure 6A–E), we found a positive relationship between caecal cytosine levels and FST immobility score ($\rho = 0.74$, $p < 0.001$) (Figure 6A).

Conversely, we found significant negative relationships between discrimination ratio in the large separation of the MSLR and caecal levels of 2-aminopimelic acid (more commonly known as aminoadipic acid) ($\rho = -0.66$, $p < 0.01$) (Figure 6B), 1-Phenylethyl acetate ($\rho = -0.68$, $p < 0.01$) (Figure 6C), 4-Vinylguaiacol ($\rho = -0.66$, $p < 0.01$) (Figure 6D), and the serotonin metabolite 5-Hydroxyindole-3-acetic acid ($\rho = -0.64$, $p < 0.01$) (Figure 6E). Some of these caecal metabolites were upregulated by cafeteria diet (Supplementary Table S2).

Discussion

In this study, we found that exercise attenuated cafeteria diet-induced increased immobility in the FST, suggesting that exercise exerted antidepressant-like effects in cafeteria diet-fed animals. Exercise had modest anxiolytic effects and exerted mild improvements in spatial learning in the MWM independent of the dietary intervention, while the cafeteria diet blunted the exercise-induced increase in AHN. In the plasma, exercise attenuated an increase in insulin and leptin resulting from cafeteria diet consumption, and both interventions differentially influenced concentrations of other plasma metabolic hormones. At the level of the

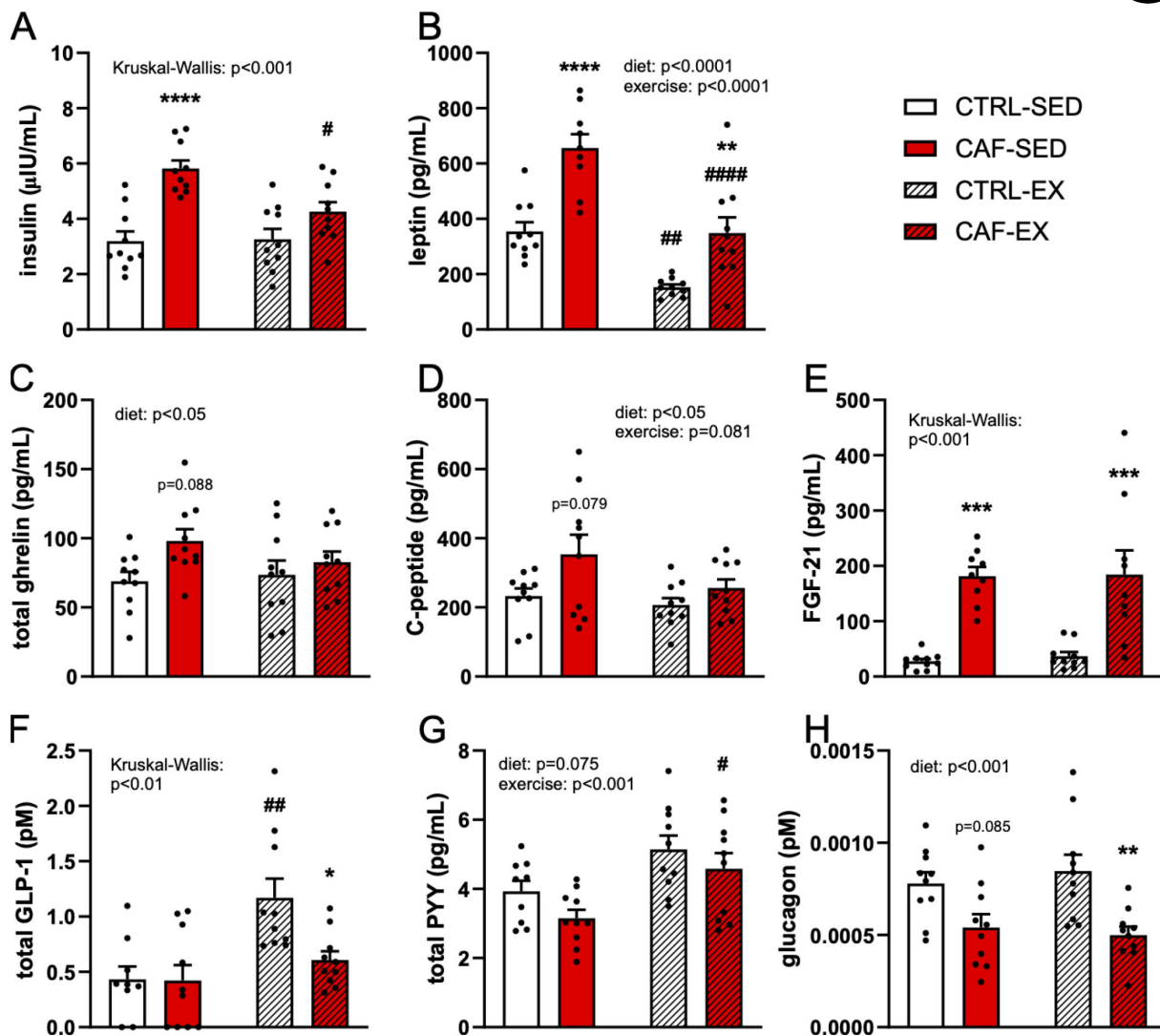


Figure 4. Exercise attenuated a cafeteria diet-induced increase in the metabolic hormones insulin and leptin in plasma. Plasma metabolic hormone concentrations ($n = 9-10$): (A) insulin ($\mu\text{IU/mL}$) [$H(3) = 18.99, p < 0.001$], (B) leptin (pg/mL), diet [$F(1,35) = 36.11, p < 0.0001$], exercise [$F(1,35) = 38.01, p < 0.0001$], (C) total ghrelin (pg/mL), diet [$F(1,36) = 5.11, p < 0.05$], (D) C-peptide (pg/mL) diet [$F(1,36) = 6.15, p < 0.05$] and exercise [$F(1,36) = 3.22, p = 0.081$], (E) FGF-21 (pg/mL) [$H(3) = 25.51, p < 0.001$], (F) total GLP-1 (pM) [$H(3) = 12.61, p < 0.01$], (G) total PYY (pg/mL) diet [$F(1,35) = 3.36, p = 0.075$] and exercise [$F(1,35) = 13.24, p < 0.001$] and (H) glucagon (pM), diet [$F(1,36) = 18.09, p < 0.001$]. Data are expressed as mean $\pm$ SEM, $p = 0.088, p = 0.085, p = 0.079, *p < 0.05, **p < 0.01, ***p < 0.001$ and $****p < 0.0001$ versus corresponding standard chow-fed group; $\#p < 0.05, \#\#p < 0.01$ and $####p < 0.0001$ versus corresponding sedentary group.

caecal metabolome, exercise had few effects but attenuated a cafeteria diet-induced reduction in the abundance of anserine, indole-3-carboxylate, and deoxyinosine. While these effects were not independently associated with behaviors, several other caecal metabolites displayed robust relationships with cognition- and mood-related tasks.

Our findings suggest that the cafeteria diet increased despair-like behaviors as observed by increased immobility in the FST. Previous studies using hypercaloric (normal chow + industrialized animal lard and corn oil) (55) or high-fat diets (56) have yielded varying results in the FST, indicating that the exact diet formulation may play a role. While we did not observe effects of exercise alone on behavior in the FST, in accordance with prior literature (37, 57–59), when rats consumed a cafeteria diet, exercise attenuated immobility in the FST suggesting an antidepressant-like effect. Comparable reductions in FST immobility have been reported following 6–10 weeks treadmill exercise interventions in rodents fed high-fat diets for 20–22 weeks (60, 61). Interestingly, caecal levels of the nucleotide cytosine were positively associated with residualized immobility score in

the FST. Administration of cytidine (the nucleoside form of cytosine) has previously been found to decrease immobility time in the FST (62). This apparent discrepancy may be due to enzymatic metabolism of cytosine to cytidine (63), possibly by gut microbes, accounting for this inverse relationship with FST behavior. While cytidine was detected in our dataset, it did not correlate with FST immobility score, nor did other pyrimidines or their derivatives (data not shown). Thus, the possible role of gut microbiota in mediating a relationship between nucleic acids and mood-related outcomes warrants further study.

Caecal metabolome analysis showed that cafeteria diet increased tryptophan, serotonin, kynurenine, kynurenic acid and 4,8-dihydroxyquinoline-2-carboxylic acid (xanthurenic acid), suggesting enhanced peripheral conversion of tryptophan into metabolites that cannot cross the blood-brain barrier. This likely reduces the availability of tryptophan in the brain for local serotonin production (64). A previous study showed that a 4-week cafeteria diet affects 5HT1A receptor expression in the hippocampus, suggesting an effect of cafeteria diet on serotonin

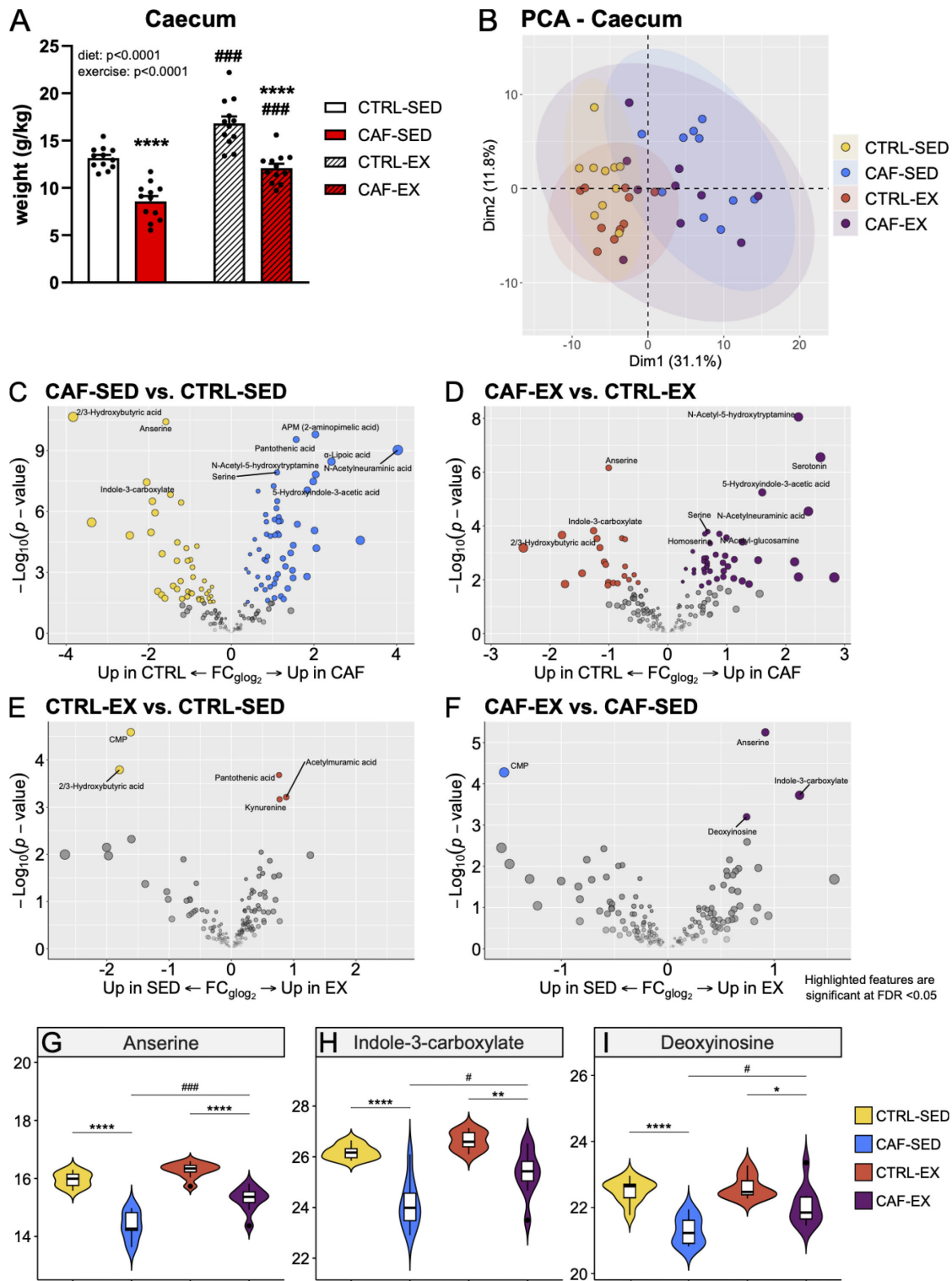


Figure 5. Exercise attenuated a cafeteria diet-induced decrease in caecal metabolites anserine, indole-3-carboxylate, and deoxyinosine. (A) Effects of cafeteria diet and exercise on body weight-adjusted caecum weight (g/kg, $n = 12$), effect of diet [$F(1,44) = 73.80$, $p < 0.0001$] and exercise [$F(1,44) = 43.98$, $p < 0.0001$]. Data are expressed as mean $\pm$ SEM, **** $p < 0.0001$ versus corresponding standard chow-fed group; ### $p < 0.001$ versus corresponding sedentary group. (B) Principal component analysis (PCA) with 95% concentration ellipses showing effects of cafeteria diet and exercise on caecal metabolomes ($n = 10$). (C–F) Volcano plots of quantified caecal metabolites ($n = 175$ features), comparing (C) cafeteria diet (CAF) versus standard chow (CTRL) in sedentary (SED) animals, (D) CAF versus CTRL in exercising (EX) animals, (E) EX versus SED in CTRL-fed animals, and (F) EX versus SED in CAF-fed animals. Highlighted features represent upregulated metabolites (p -value adjusted for false discovery rate (FDR) < 0.05). (G–I) Violin and box- and whisker plot (median represented by horizontal line) of the normalized peak area of caecal metabolites (G) anserine, (H) indole-3-carboxylate, and (I) deoxyinosine (all $n = 10$, FDR-adjusted p -values; * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ versus corresponding standard chow-fed group; $p = 0.094$, # $p < 0.05$, and ### $p < 0.001$ versus corresponding sedentary group). Full list of quantified metabolites available in Supplemental Table S2. Abbreviations: fold change (FC), generalized logarithm base 2 (glog₂).

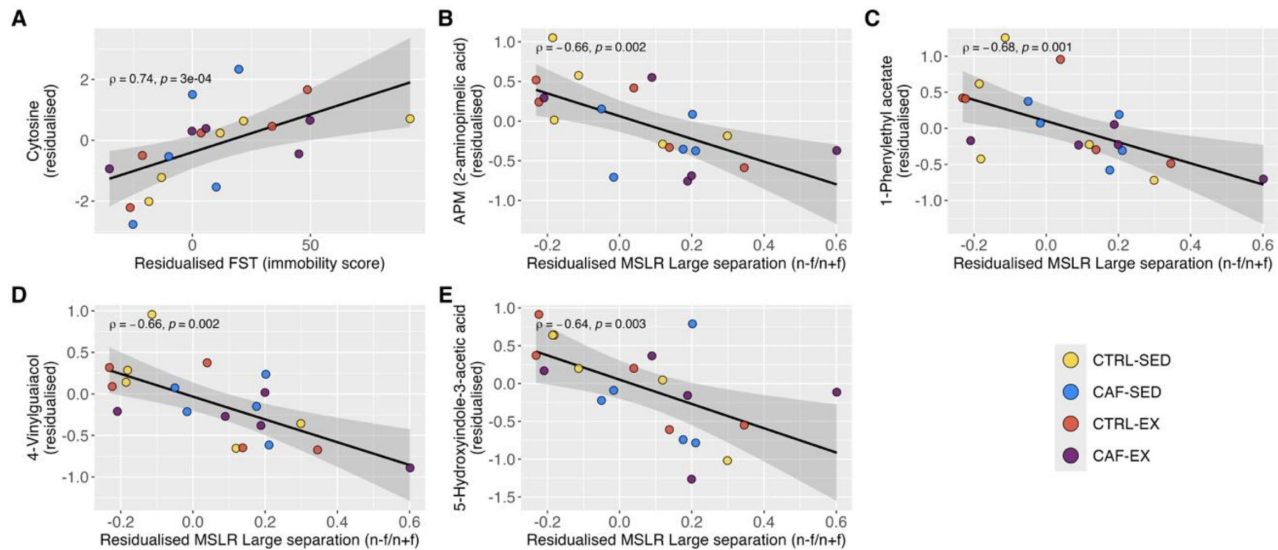


Figure 6. Caecal metabolites independently correlate with behavior. (A–E) Spearman correlations between residualised caecal metabolite abundance (vertical axes) and behavioral readouts (horizontal axes) significant at the FDR < 0.1 level. (A) Caecal cytosine and forced swim test (FST) immobility score. (B) Caecal 2-aminopimelic acid (also known as amino adipic acid) and discrimination ratio in the large separation of the MSLR. (C) Caecal 1-Phenylethyl acetate and discrimination ratio in the large separation of the MSLR. (D) Caecal 4-Vinylguaiacol and discrimination ratio in the large separation of the MSLR. (E) Caecal 5-Hydroxyindole-3-acetic acid and discrimination ratio in the large separation of the MSLR. Data are expressed as model residuals at the original scales (either normalized peak areas for caecal metabolites or stated units for behavioral readouts) after regressing out the effect of experimental condition using linear regression. Spearman's rho and nominal *p*-value are stated for each association.

signaling (65). Our analysis revealed that caecal 5-hydroxyindole-3-acetic acid, the main metabolite of serotonin, negatively associated with discrimination ratio in the MSLR independently of experimental condition, but its abundance also increased with cafeteria diet. Amino adipic acid displayed the same relationship with discrimination ratio and cafeteria diet. Produced peripherally as part of lysine catabolism, amino adipic acid is a substrate of kynurenine aminotransferase, which catalyzes the conversion of kynurenine to kynurenic acid (66). Intrahippocampal amino adipic acid administration decreases endogenous kynurenine levels in rats (66), and amino adipic acid along with BCAA concentrations are increased in plasma of prediabetic, insulin-resistant rats (67). Furthermore, kynurenine metabolites negatively associate with cognitive impairment in middle-aged prediabetic and type-2 diabetic patients (68). In our study, lysine and BCAA metabolism were increased by cafeteria diet, suggesting a greater presence of precursors of amino adipic acid. We speculate that the metabolic state associated with a cafeteria diet alters tryptophan and kynurenine metabolism dynamics, making them less available centrally and contributing to memory deficits. However, to what extent these changes affect serotonin production in the brain remains to be elucidated. While treadmill exercise increases serotonin production in the dorsal raphe nucleus in rats (69), supporting an antidepressant-like effect, further investigation is needed to understand the complex interactions between a cafeteria diet and exercise on tryptophan and serotonin metabolism. Finally, two metabolites of exogenous origin, 4-Vinylguaiacol (70) and 1-Phenylethyl acetate (71), correlated with MSLR discrimination ratio. As these metabolites are used industrially as flavoring agents, the source of exposure is likely from the cafeteria diet. The link to episodic memory is not immediately obvious, and these correlations may instead reflect variations in metabolism that depend on individual physiological factors such as cardiorespiratory fitness, lean body mass, and gut microbiota composition (72), rather than a direct causal relationship with memory performance.

The cafeteria diet did not induce an anxiety-like phenotype in the EPM, NSF test, and OFT. Similarly, a study in male Wistar rats found no effects of a 10-week cafeteria diet on anxiety-like behavior in the EPM (73). Voluntary wheel running decreased the latency to eat in the NSF

test, and showed a tendency to increase the time spent in the open arms of the EPM by standard-chow-fed animals, indicating a potential anxiolytic effect of exercise which affirms previous findings in male rodents (14, 37, 58).

We found that pattern separation (large and small configuration) was not altered by the cafeteria diet, despite a previous study describing that a transgenerational cafeteria diet (eight generations) decreased pattern separation (medium configuration) using touchscreen operant chambers (74). This suggests that long-term dietary exposure may be necessary for cognitive deficits to emerge. Short-term recognition memory has been reported to be impaired by a 6-week cafeteria diet (75), which we did not observe in the NOR in this study. Previous studies similarly report spatial learning and memory impairments in the MWM due to a cafeteria diet (26, 76), but we did not replicate these findings. It is important to note that in these studies, rats also had access to 10% sucrose solutions or soft drinks alongside the diet, whereas in the current study rats had access to water only. Moreover, previous studies comparing the effects of exercise or a high-fat (but not high-sugar) diet on behavior in MWM reported that spatial learning and memory alterations were observed only when interventions began in adolescence and not adulthood (77, 78). This suggests that adult rodents are more resistant to diet and exercise interventions than their younger counterparts (16, 78). However, analysis of search strategies employed by the adult rats in the current study revealed that exercise mitigated a decrease in circling behavior in rats with access to the cafeteria diet. Interestingly, employment of spatially imprecise search strategies has been shown to be related to age and neurogenic capacity (79). While we did not observe effects of a cafeteria diet alone on AHN, exercise increased hippocampal DCX⁺ neuron density in chow-fed animals in line with previous reports (29, 37). The cafeteria diet blunted this pro-neurogenic effect of exercise. As previously reported, a cafeteria diet provided to rats from adolescence decreased AHN (26), and treadmill exercise during adolescence mitigated a high fat diet-induced decrease in AHN (77), suggesting that a cafeteria diet and/or exercise during adolescence rather than in adulthood may have a greater impact on AHN.

In agreement with previous literature (80–83), cafeteria diet-fed sedentary animals displayed elevated plasma insulin and leptin, which



were attenuated by exercise. Previous studies show that markers of insulin resistance were reversed by treadmill or swimming exercise in rats fed a high-fat/high-carbohydrate diet or cafeteria diet (84, 85). While leptin and insulin may promote AHN and cognitive behaviors (30, 51, 52), resistance to these hormones following prolonged elevation has been associated with depression-like behaviors and cognitive impairment (52, 86). Leptin and hippocampal insulin resistance have been shown to increase immobility in the FST (87, 88) and worsen spatial learning in the MWM (84). Therefore, attenuation of cafeteria diet-induced increases in insulin and leptin by exercise may have contributed to the mitigating effects of exercise on cafeteria diet-induced immobility in the FST in the current study. This hormonal normalization likely resulted from exercise-induced decreases in adipose tissue, given that white adipose tissue releases leptin and regulates insulin sensitivity (89, 90). The cafeteria diet did not decrease tGLP-1 in sedentary animals, which contrasts with a previous study (91), potentially due to differences in dietary intervention duration. However, exercise increased plasma tGLP-1 concentrations, mirroring observations in human studies (92, 93), but this was blunted in rats fed a cafeteria diet. Activation of the GLP-1 receptor via exendin-4 has been shown to enhance AHN (33). Thus, it is possible that attenuation of the exercise-induced increase in AHN by a cafeteria diet observed here may be mediated by circulating concentrations of tGLP-1. Exercise also increased plasma PYY concentrations consistent with human studies (94, 95). Increased anxiety-like behaviors in the EPM and immobility in the FST were previously reported in PYY knockout mice (96), and centrally administered PYY decreased anxiety-like behaviors in the EPM (97). Therefore, the exercise-induced increase in PYY in the cafeteria-diet fed animals potentially contributed to its anxiolytic effects observed here.

Studies show that metabolic dysregulation is accompanied by shifts in gut microbiota (98, 99) like increased taxa related to fat deposition (99) and obesity (98, 100). Microbial metabolism is largely determined by gut microbial composition and indeed we found that caecal metabolite abundance was significantly altered by cafeteria diet and attenuated by exercise. Exercise attenuated a cafeteria diet-induced decrease in caecal abundance of the metabolite anserine, a histidine-containing dipeptide which is deficient in aged mice and associated with depression-like behaviors (101). Supplementation with anserine in combination with other dipeptides is associated with a reduction in depression scores (102) and improved cognitive function in humans (103, 104) and improved hippocampal integrity in mice (105). Exercise also attenuated a cafeteria diet-induced decrease in caecal deoxyinosine and indole-3-carboxylate. Interestingly, hippocampal deoxyinosine was reportedly decreased in a mouse model of depression (106), whereas chronic variable stress increased urine concentrations of indole-3-carboxylate (107). Indole-3-carboxylate is biochemically related to tryptophan, a precursor of serotonin, which is negatively correlated with depression scores (108, 109). However, these three metabolites were not associated with behavior independently of diet and exercise in our study. Instead, their regulation may be related to chow/cafeteria diet consumption, since they are food-derived as annotated by the human metabolome database (110).

Taken together, exercise attenuated depression-like behaviours and an increase in plasma concentrations of insulin and leptin in rats fed a cafeteria diet. This was coupled with an exercise-induced attenuation of a reduced abundance of caecal metabolites anserine, deoxyinosine and indole-3-carboxylate due to the cafeteria diet. These circulating hormones and caecal metabolites may be instrumental in mediating the interaction between the effects of a cafeteria diet and exercise on depression-like behaviour. Interestingly, we found that a standard healthy diet is necessary for exercise to increase AHN. These results suggest that dietary quality may determine whether exercise can enhance hippocampal neurogenesis. Finally, the findings provide insight into potential gut-mediated mechanisms and the involvement of circulating metabolic hormones underlying the effects of a cafeteria diet and exercise on hippocampal function. This has important implications for the development of lifestyle interventions targeting mood and cognition.

Methods

Animals

Male Sprague-Dawley rats obtained from Envigo Laboratories (United Kingdom) at approximately 7 weeks old (225–250 g) were housed in groups of four in standard conditions (22 ± 1°C, 50% relative humidity) on a 12-h light-dark cycle (lights on at 7:00 a.m.), with *ad libitum* access to food and water. At the start of the experiment, at approximately 9 weeks old, animals were pair-housed for the duration of the study. All animal procedures were performed under licenses issued by the Health Products Regulatory Authority (AE19130/P123, HPRA, Ireland), in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Animal Experimentation Ethics Committee of University College Cork (2019/025).

Experimental design

At pair-housing, animals were randomly divided into four experimental groups; sedentary animals with access to standard chow (CTRL-SED, *n* = 12), sedentary animals with access to cafeteria diet (CAF-SED, *n* = 12), animals with voluntary access to running wheels and standard chow (CTRL-EX, *n* = 12), and animals with voluntary access to running wheels and cafeteria diet (CAF-EX, *n* = 12, Figure 1A). Standard chow (Envigo, UK) consisted of 6.2% fat (of which 0% saturated fat), 44.2% carbohydrates (of which 0% sugar), 18.6% protein, and 3.5% fibre (Supplementary Table S1). The cafeteria diet consisted of several different food items high in fat and/or sugar, with two high-fat and two high-sugar items given each day in rotation in addition to standard chow, for the duration of the experiment (7.5 weeks), as previously described (111) (Supplementary Table S1). All food was provided in excess, *ad libitum*. Exercising animals had continuous access to a running wheel (Techniplast, UK) for the duration of the experiment. Running distance (km) was recorded in 24 h increments. Weight gain was calculated as % weight gain = $\frac{\text{end weight} - \text{starting weight}}{\text{starting weight}} \times 100$. Weights were not significantly different between groups at the start of the experiment.

Four weeks following intervention onset, anxiety-like behavior was assessed in the EPM, NSF test, and OFT, anhedonia in the female urine sniffing test (FUST), and antidepressant-like behavior in the FST (Figure 1A). In a second cohort of rats randomly allocated to the same experimental groups (CTRL-SED, *n* = 12; CAF-SED, *n* = 12; CTRL-EX, *n* = 12; CAF-EX, *n* = 12), pattern separation, recognition memory, and spatial learning and memory were assessed in the MSLR, NOR and MWM tests, respectively (Figure 1A), 4 weeks following intervention onset.

Behavioral testing

Elevated plus maze Anxiety-like behavior was assessed in the EPM (112). Animals were habituated to a dimly lit (red light, ±5 lux) room 1 h prior to testing. The maze consisted of two opposed open (50 × 10 cm) and two opposed closed (50 × 10 cm, 40 cm walls) arms mounted at 90° angles facing a central platform (10 × 10 cm), elevated 50 cm above the floor. Each animal was placed on the central platform facing an open arm and left to explore freely for 5 min, then returned to its home cage. Behavior was monitored and video tracked. The maze was cleaned using 70% ethanol between each animal to eliminate olfactory cues. Time spent in open arms was scored manually, blinded to experimental groups. Arm entries were recorded when all paws of the animal crossed an arm border. Data are presented as the percentage of total test time in open arms.

Novelty suppressed feeding The NSF test was used to measure anxiety-like behavior (37). The day before the test (6 p.m.), all food was removed from the home cage. Animals were food-deprived for no more than 16 h. The day of the test, animals were habituated to the experimental room for 1 h and then placed in a brightly lit (±1000 lux) circular arena (90 cm diameter) with bedding. A food pellet was placed on a white plastic base in the centre. Latency (s) to begin eating was recorded during 10 min. Once the rat began eating, or the 10-min time limit was reached, the rat was removed from the arena and returned to its home cage with access to preweighed standard chow. After 30 min, chow was removed and weighed to determine the amount consumed adjusted to body weight (g/kg). The arena and food platform were cleaned using 70% ethanol between each animal to eliminate olfactory cues.



Female urine sniffing test Anhedonia was measured using the FUST according to (113). Urine was collected from adult female Sprague-Dawley rats in oestrous. Oestrous cycle was determined by observing vaginal secretion (collected with a plastic transfer pipette, tip diameter < 1 mm) under a light microscope.

Experimental male animals were habituated to the testing room for 15 min, then to a clean, dry cotton bud inside the cage for 45 min. The cotton bud was removed and replaced with a new cotton bud with dH_2O for 3 min, which was again removed. After 45 min, each animal was exposed to another cotton bud with female oestrus urine for 3 min. Each exposure was recorded by video camera. Time spent sniffing the cotton buds was scored manually, blinded to experimental groups. Preference to sniff urine compared to water was calculated as urine preference = $\frac{\text{time spent sniffing urine}}{\text{time spent sniffing urine} + \text{water}}$.

Forced swim test The modified FST was used to measure antidepressant-like behavior (114). In the preswim, rats were individually exposed to a water tank for 15 min (21 cm diameter, filled to 30 cm with 23–25°C water). Twenty-four hours later, rats were placed back in the water tank for 5 min. Behavior during the 5 min test was video recorded. Following testing, rats were removed from the tank, gently towel-dried and returned to their home cage. Active (climbing and swimming) and passive (immobility) behaviors during the test were scored manually, blinded to experimental groups, in 5 s bins as previously described (50).

Open field General locomotor activity was assessed in the OFT. Animals were habituated to the testing room for 1 h before testing. Animals were placed in a brightly lit (± 1000 lux) circular arena (90 cm diameter), allowed to explore for 10 min, and returned to their home cage. Distance moved (m) and time in centre (s) were analyzed using Noldus EthoVision XT 11.5 tracking software. Centre area diameter was set at 45 cm according to (115). The arena was cleaned using 70% ethanol between each animal exposure to eliminate olfactory cues.

Modified spontaneous location recognition test Pattern separation, the ability to discriminate between highly similar memories which is associated with AHN, was assessed in the MSLR test according to (115). Animals were habituated to a dimly lit circular arena (~20 lux, 90 cm diameter) with bedding for 10 min per day for 5 consecutive days prior to testing. External cues were placed in the testing room for spatial navigation. Following habituation, during acquisition, animals were allowed to explore three identical objects (33 cl glass beer bottles, or soda cans) for 10 min, once for the large separation (LS) and once for the small separation (SS) test. For LS, the objects were separated by 120° (Figure 2A). For SS, two of the objects (A1 and A2) were separated by 50° with the third object (A3) at an equal distance between them (Figure 2A). Twenty-four hours after acquisition, animals were allowed to explore two of the objects from the acquisition phase for 5 min. The familiar (A4) was placed in the same location as A3, while the novel (A5) was placed between the locations of A1 and A2 (Figure 2A). Separation order, object type, and object location were randomized across tests. Behavior was recorded and videos were analyzed blinded to experimental groups to determine exploration time with novel and familiar objects. The discrimination ratio (DR) was calculated as $\text{DR} = \frac{(\text{time exploring novel object} - \text{familiar object})}{(\text{time exploring novel object} + \text{familiar object})}$. Objects were cleaned using 70% ethanol between each animal to eliminate olfactory cues.

Novel object recognition Recognition memory was measured in the NOR test. Animals were habituated to a dimly lit circular arena without bedding (~20 lux, 90 cm diameter) for 10 min. The next day, animals were allowed to explore two identical objects (ceramic mug, or 250 ml graduated borosilicate bottle) in the arena for 10 min. Twenty-four hours after acquisition, one object was replaced with a novel object (the object not used during acquisition), and animals were allowed to explore for 5 min. Behavior was recorded and videos were analyzed blinded to experimental groups to determine exploration time with novel and familiar objects in the 5 min test. The DR was calculated as $\text{DR} = \frac{(\text{time exploring novel object} - \text{familiar object})}{(\text{time exploring novel object} + \text{familiar object})}$. The arena and objects were cleaned using 70% ethanol between each animal to eliminate olfactory cues.

Morris water maze Hippocampal-dependent spatial learning and memory were assessed in the MWM as previously described (116). Animals were habituated to the room for 1 h before testing. A circular pool (180 cm diameter) was filled with water ($22 \pm 1^\circ\text{C}$). A transparent platform was placed 1–2 cm below the water surface at a fixed location in the north-west quadrant, 15 cm from the pool wall. External cues were placed in the testing room for spatial navigation. During training (days 1–4), animals were placed in the pool at one of four release points (NE, E, S, and SW), each point used once per day. Animals underwent four daily trials for 4 consecutive days. If the animal found the platform within 120 s, they were left on the platform for 10 s before continuing to the next trial. If the animal failed to reach the platform within 120 s, they were guided to the platform and remained there for 30 s before continuing to the next trial. Trials were recorded and analyzed using Noldus EthoVision XT 11.5 tracking software. On day 5 (probe trial), the platform was removed and animals allowed to explore the pool for 60 s starting at the SE point. Latency to enter and time spent in the platform quadrant (s), and average velocity were analyzed using Noldus EthoVision XT 11.5 tracking software to assess spatial memory. Learning performance and search strategies were assessed as described in Supplementary Methods.

Blood and tissue collection

One day after the last behavioral test, 1–3 h after cessation of the diet and exercise exposures, half of the animals per group ($n = 6$) were weighed and euthanized by rapid decapitation during the light cycle to collect fresh tissues. Trunk blood was collected in 3 mL EDTA-coated tubes (Vacutette, Greiner Bio-One) and centrifuged for 10 min ($3220 \times g$, 4°C) to collect plasma, which was stored at -80°C until used for measurement of metabolic hormones (Supplementary Methods). eWAT and BAT were dissected and weighed. Whole caecum ($n = 12$) was collected and weighed. Caecum content ($n = 10$) was snap frozen using dry ice and stored at -80°C until preparation for metabolomic analysis (Supplementary Methods).

Half of the animals per group ($n = 6$) of the first cohort were weighed and transcardially perfused 1–3 h after cessation of the diet and exercise exposures to assess immature hippocampal neuron counts as a measure of AHN. Sodium pentobarbital (90 mg/kg) was injected intraperitoneally as anaesthetic overdose. Sufficient anesthesia depth was identified by loss of toe pinch reflex. Animals were pinned in a dorsal recumbent position and their thorax was opened to insert a catheter into the heart left ventricle. Ice-cold phosphate-buffered saline (PBS) was perfused by pump (35–40 mL/min) until efflux ran clear, followed by 4% paraformaldehyde (PFA) in PBS. During PBS perfusion, eWAT was dissected and weighed to determine adiposity. Whole brains were postfixed in 4% PFA in PBS for 24 h, transferred to 30% sucrose in PBS until fully sunken, snap frozen in isopentane using liquid nitrogen, and stored at -80°C until sectioning. Brains were sectioned coronally at $40 \mu\text{m}$ using a Leica CM1950 cryostat, collected free-floating in a series of 12 in cryoprotectant (25% 0.1M PBS, 30% ethylene glycol, 25% glycerol, and 20% dH_2O) and stored at -20°C until immunohistochemical staining.

Immunohistochemistry

For immunohistochemical staining of doublecortin (DCX, $n = 6$ per group), a marker of immature neurons, sections were washed in 0.1M PBS (3×5 min) and blocked with 10% donkey serum in 0.5% Triton X-100 in PBS (PBS-T) for 2 h at room temperature (RT). Sections were incubated in primary antibody (rabbit anti-DCX; Abcam, AB18723, 1:5000) for 48 h at 4°C . Antibodies were diluted in 5% donkey serum in 0.5% PBS-T. Sections were washed in 0.5% Tween in PBS (PBS-Tw, 3×20 min), incubated in secondary antibody (Alexa Fluor 488-conjugated donkey anti-rabbit, Invitrogen, AB21206, 1:500) for 2 h at RT and washed in PBS-Tw. Sections were incubated in 4',6-diamidino-2-phenylindole (DAPI) (5 mg/mL, 1:50,000 in PBS) for 3 min, washed in PBS, mounted onto Superfrost Plus slides and cover-slipped using Dako fluorescent mounting media.

Microscopy and image analysis

The DG was imaged using an Olympus BX53 Upright Research Microscope at 20x magnification for DCX. DCX⁺ cells were counted blinded to experimental groups in every 12th section using ImageJ. DG area (mm^2) was



measured using ImageJ on 10x magnification images of DAPI and cell counts were expressed as cells/mm². The dorsal hippocampus (dHi) was defined as anterior-posterior (AP): Bregma – 1.8 to – 5.2, and ventral hippocampus (vHi) as AP: – 5.2 to – 6.7 (112, 117, 118). Sections at AP – 5.2 were only considered vHi if ventral DG was clearly present at the bottom of the section (117). Note: in coronally sectioned rat brain, rostral sections typically only contain dHi, whereas caudal sections containing vHi may also contain portions of dHi and intermediate hippocampus (119, 120). Three dorsal and three ventral sections were analyzed, selecting sections of similar AP coordinates from Bregma for all animals where possible.

Bioinformatic analysis

Differential expression analyses were limited to non-drug-related features (metabolites) annotated at the highest confidence levels 1 and 2a (caecal features, $n = 212$) and performed in R (version 4.1.1). Raw feature peak area values below their associated detection limit (as reported by MS-Omics) were considered missing (i.e., set to “NA”); only features with maximum 25% missingness per condition were retained for quantification (features remaining, $n = 201$). To remove features displaying high technical variance, only metabolites with $< 10\%$ relative standard deviation in the pooled quality control samples were retained (features remaining, $n = 175$). Data were subsequently normalized with variance stabilising normalization (VSN), using the “vsn” package (allowing default 10% outliers). While originally developed for microarray data (121), VSN has successfully been applied in untargeted (122) and simulated (123) metabolomics of comparable dataset size to the one herein, capitalizing on similar mean-variance relationships (124) and error models (125). The generalized logarithm base 2 ($\log_2$) transformation employed in VSN approximates the standard $\log_2$ function for values $\gg 0$. The “limma” package was used for differential expression analysis, with trend = TRUE and robust = TRUE in the eBayes function (126). Resulting feature p values were adjusted for multiple comparisons with the Benjamini-Hochberg method, with a 5% FDR threshold for significance. Pathway over-representation analysis with nominally ($p < 0.05$) differentially abundant caecal metabolites was performed using MetaboAnalystR (v 4.0), with *Rattus norvegicus* Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and hypergeometric tests (127). For correlations between behavioral tasks (20 rats for each behavioral measure) and all caecal metabolomic features, the effect of experimental condition was first regressed out using linear models (“lmFit” in “limma” or “lm” in the “stats” R packages). Spearman correlations were performed with the “Hmisc” package in R. Resulting p values were then adjusted by FDR separately for each readout. Correlations were considered significant at the FDR < 0.1 level. For a complete list of R packages used in this study, see Supplementary Table S5.

Statistical analysis

Data were checked for outliers using the Grubbs outlier test, and for normality using Shapiro-Wilk. Identified outliers were removed from analyses. Except for running distance and MWM training, data were analyzed using two-way ANOVA in GraphPad Prism and SPSS. Running distance data were analyzed using two-way ANOVA with repeated measures, with post-hoc analysis comparing each week using Sidak’s multiple comparisons test. MWM training outcomes were analyzed using two-way ANOVA with repeated measures, with post-hoc analysis on individual training days using two-way ANOVA and Tukey’s multiple comparisons test. Post-hoc analyses of other two-way ANOVAs were performed using Tukey’s multiple comparisons test. Non-normally distributed data were analyzed for differences in group distributions using Kruskal-Wallis with pairwise Mann-Whitney U post-hoc comparisons. Statistical significance was set at $p < 0.05$, with data presented as means $\pm$ standard error of the mean (SEM). For two-way ANOVA results, diet-exercise interactions are reported only when statistically significant.

Data availability

All data are available upon request.

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Author contributions

MHCN conducted and designed experiments, collected and analyzed the data, prepared the figures, acquired funding and wrote the manuscript. SN conducted and designed experiments, collected and analyzed the data, prepared figures and wrote the manuscript. SD-H analyzed the data, prepared figures and wrote the manuscript. EPH conducted experiments. TF conducted experiments. OFO designed experiments, supervised MHCN and EPH, oversaw analysis and interpretation of data, acquired funding and wrote the manuscript. YMN designed experiments, supervised MHCN and SD-H, oversaw analysis and interpretation of data, acquired funding and wrote the manuscript.

The manuscript has been read and approved by all authors. All authors take full responsibility for all data, figures, and text and approve the content and submission of the study. No related work is under consideration elsewhere. All authors state that all unprocessed data are available, and all figures provide accurate presentations of the original data.

Corresponding author: YMN takes full responsibility for the submission process and may be contacted about any aspect of the work.

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Author disclosures

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