Original research

Gut microbial modulation of 3-hydroxyanthranilic acid and dopaminergic signalling influences attention in obesity

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ABSTRACT

Background Obesity-related alterations in the gut microbiota have been linked to cognitive decline, yet their relationship with attention remains poorly understood.

Objective To evaluate the possible relationships among gut metagenomics, plasma metabolomics and attention.

Design We conducted faecal shotgun metagenomics and targeted plasma tryptophan metabolomics across three independent cohorts (n=156, n=124, n=804) with functional validations in preclinical models, including three faecal microbiota transplantation (FMT) experiments in mice and *Drosophila melanogaster*.

Results Obesity was consistently associated with reduced attention. Metagenomics analyses identified *Proteobacteria* species and microbial functions related to tryptophan biosynthesis from anthranilic acid (AA) as negatively associated with attention in obesity. Plasma tryptophan metabolic profiling and machine learning revealed that 3-hydroxyanthranilic acid (3-HAA) was positively associated with attention, particularly in obesity, while AA showed a negative association. Bariatric surgery improved attention and enriched microbial species linked to attention. In mice, diet-induced obesity (DIO) and microbiota depletion reduced 3-HAA and 5-hydroxy-indole acetic acid (5-HIAA) concentrations in the prefrontal cortex (PFC), which were restored by FMT. Global metabolic profiling (>600 metabolites) of PFC from the FMT group identified 3-HAA and the tryptophan and tyrosine pathways among the most significant in mice receiving microbiota from high-attention donors. A second FMT experiment also revealed a consistent enrichment of the tryptophan and tyrosine metabolism at the transcriptional level in the PFC, with *Haao* (3-hydroxyantrhanilic acid dioxygenase) and *Aox4* (aldehyde oxidase 4), key in 3-HAA and 5-HIAA degradation, among the significantly regulated genes. In a third FMT study, attentional traits were transmitted from humans to mice alongside modulation of serotonergic and dopaminergic pathways. In *Drosophila*, mono-colonisation with *Enterobacter cloacae* and DIO induced attention deficit-like behaviours, which were mitigated by 3-HAA supplementation.

Conclusions We have identified the microbiota and 3-HAA as potential therapeutic targets to improve attention, especially in obesity.

WHAT IS ALREADY KNOWN ON THIS TOPIC;

⇒ Obesity is a known determinant of the gut microbiota composition and individuals with obesity perform poorly on tasks assessing memory, executive function and attention.

WHAT THIS STUDY ADDS

⇒ Patients with obesity had worse attention performance that was accompanied by higher levels of species from the Proteobacteria and Parabacteroides phyla and lower levels of Actinobacteria, Synergistetes and Verrucomicrobia species. 3-hydroxyanthranilic acid (3-HAA) was positively associated with attention in patients with obesity in three different cohorts. Alterations in the gut microbiota using antibiotics or faecal microbiota transplantation significantly modulated the brain concentrations of 3-HAA or the brain expression of 3-hydroxyanthranilic acid dioxygenase (*Haao*), responsible for the degradation of 3-HAA, in mice. In *Drosophila*, diet-induced obesity and mono-colonisation with *Enterobacter cloacae* induced attention-deficit–like behaviours, which were also triggered by AA supplementation, whereas 3-HAA supplementation alleviated deficits.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Targeting the gut microbiota, 3-HAA or *Haao* offers a promising avenue to improve attention performance in humans.

INTRODUCTION

Obesity is well known to be associated with declined cognitive ability in the context of low-degree systemic chronic inflammation. The latter is also linked to alterations in the tissues most enriched in lipids, the adipose tissue and the brain.[1–3] Attentional function is an integral component of cognition, interconnected with memory and brain activation. Attentional processes are essential to be able to function in daily life. Individuals with obesity show important deficits in attention.[4] This is important because attention and related executive functions (planning and organisational skills) are crucial for successful adherence to diet and physical activity programmes.[5 6] This cognitive process is mainly mediated by the prefrontal cortex (PFC) and modulated by neurotransmitter systems.[7 8] Interestingly, attention-deficit hyperactivity disorder (ADHD) and obesity seem to co-aggregate.[5 6 9]

Obesity also affects the gut microbiota, the diverse community of micro-organisms (including bacteria, archaea, viruses and fungi) that inhabit the gastrointestinal tract, leading to a dysregulated microbial state recently found to be associated with impaired short-term memory.[10] However, little is known about the potential involvement of the gut microbiota in other cognitive processes. The relationship between attention and the gut microbiome in humans has been explored in the context of ADHD, with inconsistent results.[11] For instance, the gut microbiota from patients with ADHD differed from healthy individuals with an increased abundance of *Bifidobacterium* and decreased *Dialister* and *Faecalibacterium* genera found in the former. *Bifidobacterium* was also enriched in individuals with ADHD in other studies.[12 13] However, the differences between individuals with and without ADHD could be attributed to contrasting dietary patterns or drug intake.

In this study, we aimed to explore the potential relationship between attentional function and the gut microbiota composition and functionality in subjects with and without obesity. We hypothesised that attentional function would be associated with both obesity status and a specific gut microbiome profile. For this purpose, we studied the metagenomics profiles of the IRONMET (n=157) and IRONMET-CGM (n=124) cohorts in whom we also performed a comprehensive targeted quantification of tryptophan-related metabolites. In addition, we also explored the effect of sleeve gastrectomy surgery after a 1-year follow-up to better substantiate the involvement of the gut microbiota in attention. Additionally, we further explored the role of tryptophan metabolism in attention performance through a targeted metabolomics in a validation cohort (Health Imageomics, n=804). We assessed the role of the gut microbiota on tryptophan metabolism in mice and whether human attention performance could impact the mouse brain transcriptome in several faecal microbiota transplantation (FMT). Finally, we assessed the causal effect of *Enterobacter cloacae* and tryptophan-related metabolites in attention deficit/hyperactivity-related traits in *Drosophila melanogaster*.

MATERIALS AND METHODS

Human cohorts

IRONMET cohort (baseline, n=157; 1-year follow-up: n=62)

Human subjects were recruited as part of the IRONMET cohort, a case–control study to investigate the associations between glucose metabolism, brain iron content, cognitive function, physical activity and the composition of the gut microbiota. The cohort included n=157 subjects with obesity (n=83, body mass index (BMI)≥30 kg/m^2), overweight (n=35, 30>BMI>25 kg/m^2) and normal weight (n=39, BMI≤25 kg/m^2) age 22–66 years (47 males and 110 females) (online supplemental table S1). When the cohort is divided solely into two subgroups based on obesity status, individuals without obesity are defined as non-obese (n=74, BMI<30 kg/m^2) and with obesity (n=83, BMI≥30 kg/m^2). A total of n=62 subjects without obesity (n=32, BMI<30 kg/m^2), with obesity (n=22, BMI≥30 kg/m^2) and n=8 who underwent bariatric surgery were followed for 1 year (27–66 years, 18 males and 45 females) (online supplemental table S32). The exclusion criteria were serious systemic disease unrelated to obesity, systemic diseases with intrinsic inflammatory activity, clinical symptoms and signs of infection in the previous month, use of antibiotics, antifungal or antivirals in the previous 3 months, pregnancy or lactation, severe disorders of eating behaviour major psychiatric antecedents, excessive alcohol intake (≥40 g/day in women or 80 g/day in men) and history of trauma or injured brain. All subjects gave written informed consent, validated and approved by the Ethics committee of the Hospital Dr Josep Trueta (Project Code PI15/01934).

IRONMET-CGM (n=124)

Patients were recruited as part of the IRONMET CGM, a case–control study to explore the associations between glucose metabolism, cognitive function, brain iron content and gut microbiota composition. The cohort included 124 subjects (23–67 years, 38 males and 86 females, BMI 33.04±10.05 kg/m^2, online supplemental table S2) with obesity (n=63, BMI≥30 kg/m^2), overweight (n=19, 30>BMI>25 kg/m^2) and normal weight (n=42, BMI≤25 kg/m^2). The exclusion criteria were serious systemic disease unrelated to obesity, systemic diseases with intrinsic inflammatory activity, clinical symptoms and signs of infection in the previous month, under anti-inflammatory chronic treatment (with steroidal and/or non-steroidal anti-inflammatory drugs), use of antibiotics, antifungal or antivirals in the previous 3 months, pregnancy or lactation, severe disorders of eating behaviour major psychiatric antecedents, excessive alcohol intake (≥40 g/day in women or 80 g/day in men), history of disturbances in iron balance and major psychiatric antecedents. Written informed consent was obtained from all subjects, validated and approved by the Ethics committee of the Hospital Dr Josep Trueta (https://clinicaltrials.gov/ct2/show/NCT03889132).

Health Imageomics (n=804)

The Health Imageomics study is a multicentric observational study including participants residing in the province of Girona (Northeast Catalonia, Spain). It includes a representative population of the province of Girona (n=804) aged ≥16 and ≤ 52 years old recruited in public primary care centres. It includes n=700 participants without obesity (BMI<30 kg/m^2) and 104 with obesity (BMI≥30 kg/m^2) (online supplemental table S25). Selection criteria included no history of infection during the last 15 days and consent to be informed of potential incidental findings. The exclusion criteria were inflammatory bowel disease, irritable bowel syndrome, colorectal cancer and any other intestinal diseases. Subjects gave written informed consent, validated and approved by the Ethics Committee (Project Code GO03-001848).

Samples and data from participants included in this study were provided by the IDIBGI Horizontal Aging Program and the IDIBGI Biobank from the Healthy Imageomics study. They were processed following standard operating procedures with

the appropriate approval of the Ethics and Scientific Committees. The Healthy Imageomics Study is an observational study including participants of the province of Girona (Northeast Catalonia, Spain).

All subjects gave written informed consent and were performed in accordance with the Declaration of Helsinki.

Neurophysiological assessment

Attention was evaluated using the Digit Forward test, a subtest of the Digit Span task, which is a subset of the Wechsler Adult Intelligence Scale-III (WAIS-III).[14] The Digit Span test comprises seven pairs of randomly ordered sequences of numbers in increased length. During the Forward Span, the examiner reads aloud each sequence, and the participant is asked to repeat the sequence in the same order. In contrast, the Backward Span requires the participant to repeat the digits in reverse order.

Two trials at each span length are presented. The maximum span is defined as the longest number of digits the patient can correctly repeat on at least one of the two trials. Forward and backward performance can be scored in two ways: either as subscores (the number of correct items of each type) or as span scores (the maximum number of digits correctly repeated). In our study, we used the subscore method for evaluation.

The Digit Forward Span is primarily considered a measure of attentional capacity. Higher scores indicate better attentional ability. It has a relatively narrow range, with 89% of individuals scoring within the 5–8 digit span range.[15] The average forward digit span in healthy individuals typically falls within 7 ± 2.[16]

In contrast, the Digit Backward Span is not a pure measure of attention. It requires additional cognitive processes and relies more on working memory and is considered an executive task. As such, forward and backward span represent a qualitatively different type of tasks assessing distinct cognitive abilities and should be considered separately.[17] In fact, patients with Alzheimer's disease often exhibit normal digit span forward despite significant memory impairments and the Digit Span Forward has also been shown to be insensitive to the cognitive impairments found in a mixed clinical population.[18] All assessments were administered under standardised conditions by trained neuropsychologists with extensive experience in cognitive testing.

Statistical analysis

Statistical analyses were performed using R (V.4.1.1). First, normality and homogeneity of variances were tested visually and using Shapiro-Wilk test of normality. Overall differences between study groups were assessed using the Kruskal-Wallis test. The Wilcoxon test was used to compare differences between groups. Spearman's analysis was used to determine the correlation between quantitative variables.

Metagenomics analyses

We filtered microbial taxa and molecular functions so that only those with >10 reads in at least 10% samples were selected. Differential abundance analyses for taxa and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues associated with the digit forward test scores were calculated using the ANCOM-BC methodology.[19] We adjusted the models for age, gender, BMI and years of education in the baseline analyses and for age, gender, years of education and the digit forward score at baseline for the follow-up analyses. The p values were adjusted for multiple comparisons using a Sequential Goodness of Fit[20] as implemented in the 'SGoF' R package. Unlike false discovery rate (FDR methods, which decrease their statistical power as the number of tests increases, SGoF methods increase their power with increasing number of tests. SGoF has proven to behave particularly better than FDR methods with high number of tests and low sample size, which is the case of omics large datasets. KEGG functional enrichment or over-representation analysis of differentially expressed genes was performed using the clusterProfiler R package,[21] based on hypergeometric distribution. For multiple testing correction control, clusterProfiler q-values were estimated (Storey correction) and significance was set at q-value <0.1.[22]

Metabolomics analyses

To identify metabolites associated with attention, we employed a machine learning variable selection approach on the ranked residuals of the digit forwards scores and metabolites after controlling for age, gender, BMI and education years. In the case of the mice studies, metabolomic data was normalised using a probabilistic quotient normalisation approach using the R package KODAMA. Then, we performed a multiple random forest-based algorithm as implemented in the Boruta R package.[23] This consists of four steps: randomisation, model building, statistical testing and iteration until the status of all features is decided. For statistical testing, we used a Bonferroni corrected two-tailed binomial test to select relevant features and assess whether these features are important (significantly higher, confirmed) or unimportant (significantly lower, rejected). The algorithm was run with a confidence level cut-off of 0.005 for the Bonferroni adjusted p values and 5000 trees.

Machine learning models are referred to as 'black-boxes' models due to their nature, complexity and difficult interpretation. To facilitate model explainability, we used sophisticated tools based on game-theory Shapley values to compute the exact Shapley Additive exPlanations (SHAP) scores and determine the contribution of each selected metabolite for each individual in the model.[24] The exact computation of SHAP values guarantees that explanations are always consistent and locally accurate. By contrasting the model prediction with and without the metabolite for each individual, SHAP values establish the significance of a given value in a given metabolite. A given feature may have different SHAP values for different individuals depending on the interactions with other features for each specific individual. The R packages 'treeshap' and 'SHAPforXGBoost' were used to calculate and plot the SHAP scores.

RNA sequencing analysis

Analysis of differentially expressed genes associated with the donors' digit forward scores in the recipient mice medial PFC (mPFC) was performed on gene counts using the 'limma' R package.[25] Low expressed genes were filtered so that only genes with >10 counts in at least two samples were selected. RNA sequencing data were then normalised for RNA composition using the trimmed mean of M-value as implemented in the edgeR package.[26] Normalised counts were then converted to log2 count per million with associated precision weights to account for variations in precision between different observations using the 'voom' function with donor's age, gender, BMI and education years. A robust linear regression model adjusted for the previous covariates was then fitted to the data using the 'lmFit' function with the option method = 'robust', to limit the influence of outlying samples. Finally, an empirical Bayes method was applied to borrow information between genes with the 'eBayes' function. P values were adjusted for multiple comparisons using the SGoF. A cut-off of <0.05 was used as

a threshold for statistical significance. The functional roles of differentially expressed genes were characterised using over-representation analyses based on the KEGG databases. Pathway significance was assessed using a hypergeometric test and Storey correction was applied for multiple testing correction. Statistical significance was set at <0.1.

RESULTS AND DISCUSSION

Attention is negatively associated with obesity, sulfate-reducing bacteria, but positively with species from the phyla Actinobacteria, Verrucomicrobia and Synergistetes

We assessed attention in two independent cohorts of patients with and without obesity (IRONMET, n=157; IRONMET-CGM, n=124; online supplemental tables S1 and S2) using the digit forward test included in the WAIS. In both cohorts, we found a consistent decrease in attention according to the obesity status, with patients with obesity having the lowest scores (figure 1A,B). These findings remained significant after controlling for age, gender and the years of education as confounder variables (online supplemental figure S1). To explore the potential involvement of the gut microbiota in these associations, we next performed faecal shotgun metagenomics analyses. Then, gut microbial species associated with attention were identified using the ANCOM-BC methodology controlling for age, gender, BMI and the years of education. In the IRONMET cohort, patients with higher attention had higher levels of several bacteria from the phyla Actinobacteria, Verrucomicrobia and Synergistetes, but lower levels of sulfate-reducing bacteria from the phylum Proteobacteria and the genera *Parabacteroides* (figure 1C, online supplemental table S3). In line with these findings, in a small sample (n=43) of community-dwelling older adults, those with impaired scores on ≥2 cognitive tests had higher proportions of Proteobacteria but lower proportions of Verrucomicrobia, which were particularly correlated with attention/executive function measures.[27] Similarly, the phylum Verrucomicrobia was positively correlated with the performance on measurements of executive function such as the Stroop Colour-Word.[28] Recently, results from a pilot study including older cognitively healthy adults (n=25) and elderly with mild cognitive impairment (MCI, n=23) found a consistent increase in Proteobacteria along with a decrease in the abundance of Synergistetes and Verrucomicrobia in the faeces of the latter group.[29] In both patients with obesity (figure 1D, online supplemental table S4) and without obesity (figure 1E, online supplemental table S5), we found that the digit forwards scores were negatively associated with species from the phylum Proteobacteria, but positively with Actinobacteria species. Importantly, we replicated these findings in the IRONMET-CGM cohort, where higher attention scores were again linked to lower amounts of sulfate-reducing species from the phylum Proteobacteria, and higher levels of Actinobacteria and Synergistetes, particularly among patients with obesity (figure 1F–H, online supplemental tables S6–S8). Notably, nearly all Actinobacteria species associated with attention belonged to the *Coriobacteriia* class, especially to the *Eggerthellales* and *Coriobacteriales* order. Unlike species from the *Actinomyces* genus that are mainly inflammatory, members of these groups are primarily non-pathogenic commensal bacteria. In particular, in patients with obesity we found that attention scores were associated with species such as *Adlercreutzia equolifaciens*, *Asaccharobacter celatus* or *Gordonibacter* sp, all from the *Coriobacteriia* class. Notably, both *A. equolifaciens* and *A. celatus* have been shown to produce equol,[30 31] which has antioxidant, anti-inflammatory and anticancer effects.[32] A recent study has linked *A. equolifaciens* to a healthy gut microbiome and is reduced in patients with non-alcoholic fatty liver disease.[33] Similarly, a comprehensive analysis across nine metagenomic cohorts of inflammatory bowel disease patients identified a consistent signature of commensal gut microbiota. Particularly, three bacteria, including *A. celatus*, were depleted in patients with IBD.[34] Furthermore, *Gordonibacter* species have been shown to metabolise polyphenols, especially ellagic acid to urolithins, which may also possess anti-inflammatory and anticancer properties.[35 36]

Microbial biosynthesis of aromatic amino acids is linked to attention

We next performed functional analyses by mapping reads to KEGG orthologs and using the ANCOM-BC methodology to identify microbial molecular functions associated with attention after controlling for age, BMI, gender and the years of education (figure 2A, online supplemental table S9). To gain further insights into the microbial pathways involved in attention, we then performed over-representation analyses using the KEGG orthologs significantly associated with attention (online supplemental tables S10 and S11). Using only those microbial molecular functions negatively associated with attention, we found that the biosynthesis of aromatic amino acids (AAA: phenylalanine, tyrosine and tryptophan) was one of the microbial pathways most over-represented (figure 2B, online supplemental tables S11). Notably, the vast majority of the microbial functions from this pathway that were significantly associated with attention (figure 2C) were involved in the tryptophan metabolism (figure 2D). In addition, the phenylalanine, tyrosine and tryptophan biosynthesis was over-represented in patients with obesity (figure 2E, online supplemental tables S12 and S13), but not in patients without obesity (online supplemental tables S14 and S15), and mainly included molecular functions participating in the synthesis of tryptophan from anthranilic acid (AA) (figure 2F). Finally, functional analyses in the IRONMET-CGM cohort also highlighted shikimate kinase, the molecular function involved in the first step of the synthesis of AAA, as one of the microbial genes most negatively associated with attention (figure 2G, online supplemental table S16).

The tryptophan catabolite 3-hydroxyanthranilic acid is strongly and positively associated with attention

To further explore the role of tryptophan metabolism on attention, we performed a targeted metabolomics approach in plasma samples from the IRONMET cohort by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) to comprehensively characterise tryptophan and downstream metabolites from the main catabolic pathways, that is, the kynurenine, the serotonin and the microbial-derived indole pathways.[37] The method also included markers of inflammation and gut health such as neopterin and citrulline. Then, we applied a machine learning feature selection strategy based on applying multiple random forests to identify tryptophan-related metabolites associated with the digit forward scores controlling for age, BMI, sex and education years (figure 3A, online supplemental table S17). In addition, to facilitate the interpretation of the machine learning models, we computed the SHAP scores (figure 3C, online supplemental table S18). We found that the microbial-derived indole-3-acetic acid (IAA) was the metabolite most strongly and positively associated with attention (figure 3A,C). Other tryptophan catabolites from the kynurenine pathway, such as 3-kynurenic acid or

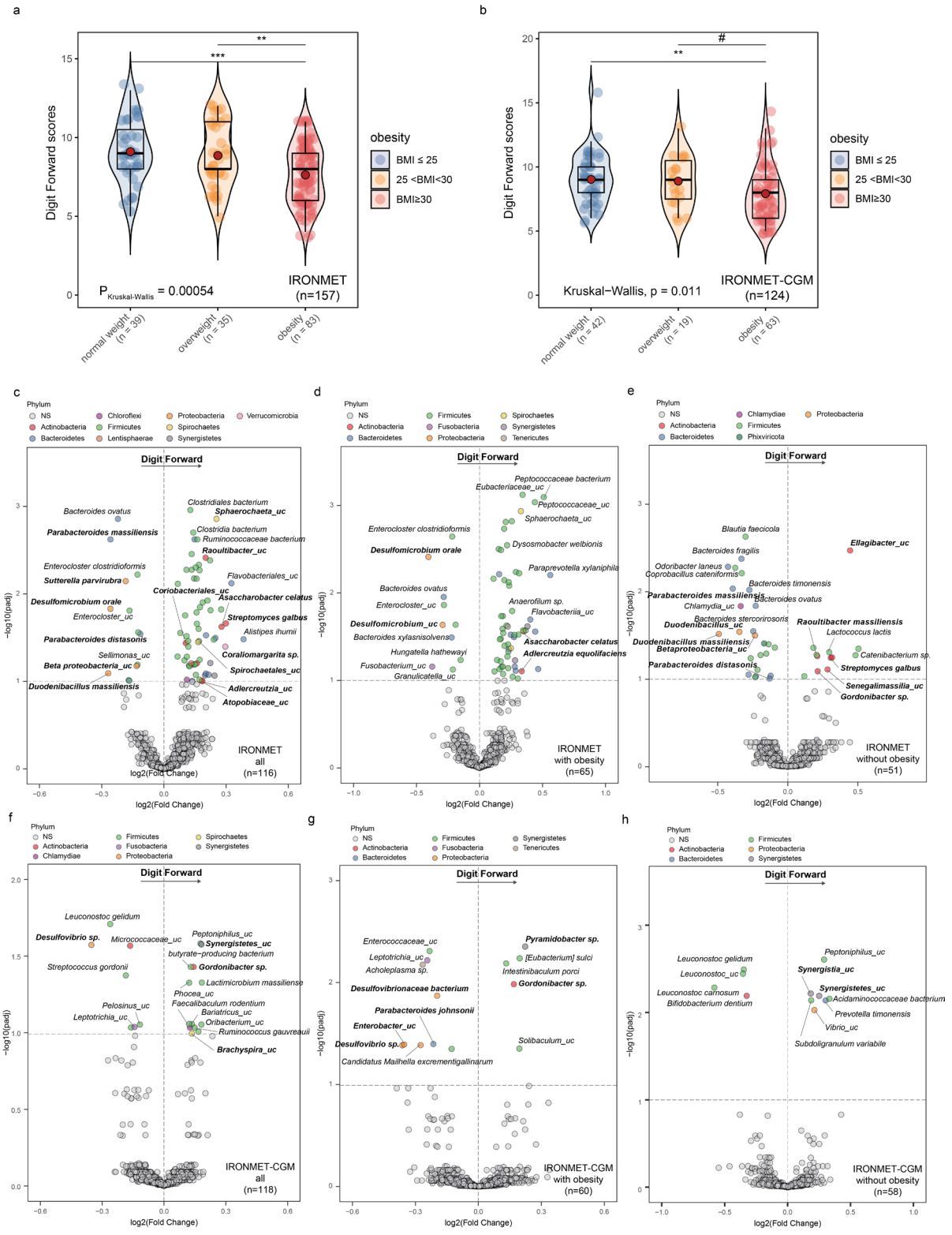

Figure 1 Gut microbiome profiles associated with attention in subjects with and without obesity. Violin plots of the digit forward scores in the (A) IRONMET cohort and (B) IRONMET-CGM cohort according to the obesity status. Overall significance was assessed using a Kruskal-Wallis test and between group significance using a Wilcoxon test (two-sided). Red dots represent the mean. #p<0.1, *p<0.05, **p<0.01; ***p<0.001. (C) Volcano plot of differential bacterial abundance associated with attention in the IRONMET cohort in all patients, (D) patients with obesity, and (E) patients without obesity. (F) Volcano plot of differential bacterial abundance associated with attention in the IRONMET-CGM cohort in all patients, (G) patients with obesity and (H) patients without obesity subjects. Significant species were identified using ANCOM-BC (two-sided Z-test using the statistic W = $\log_2$Fold Change/SE) from shotgun metagenomics data adjusted for age, gender, BMI and years of education. The log2 fold change associated with a unit change in the digit forward and the log10 p values adjusted for multiple testing (padj) are plotted for each taxon. Significantly different taxa are coloured according to phylum. Significance was set at padj<0.1. ACOM-BC, analysis of compositions of microbiomes with bias correction; BMI, body mass index; NS, not significant.

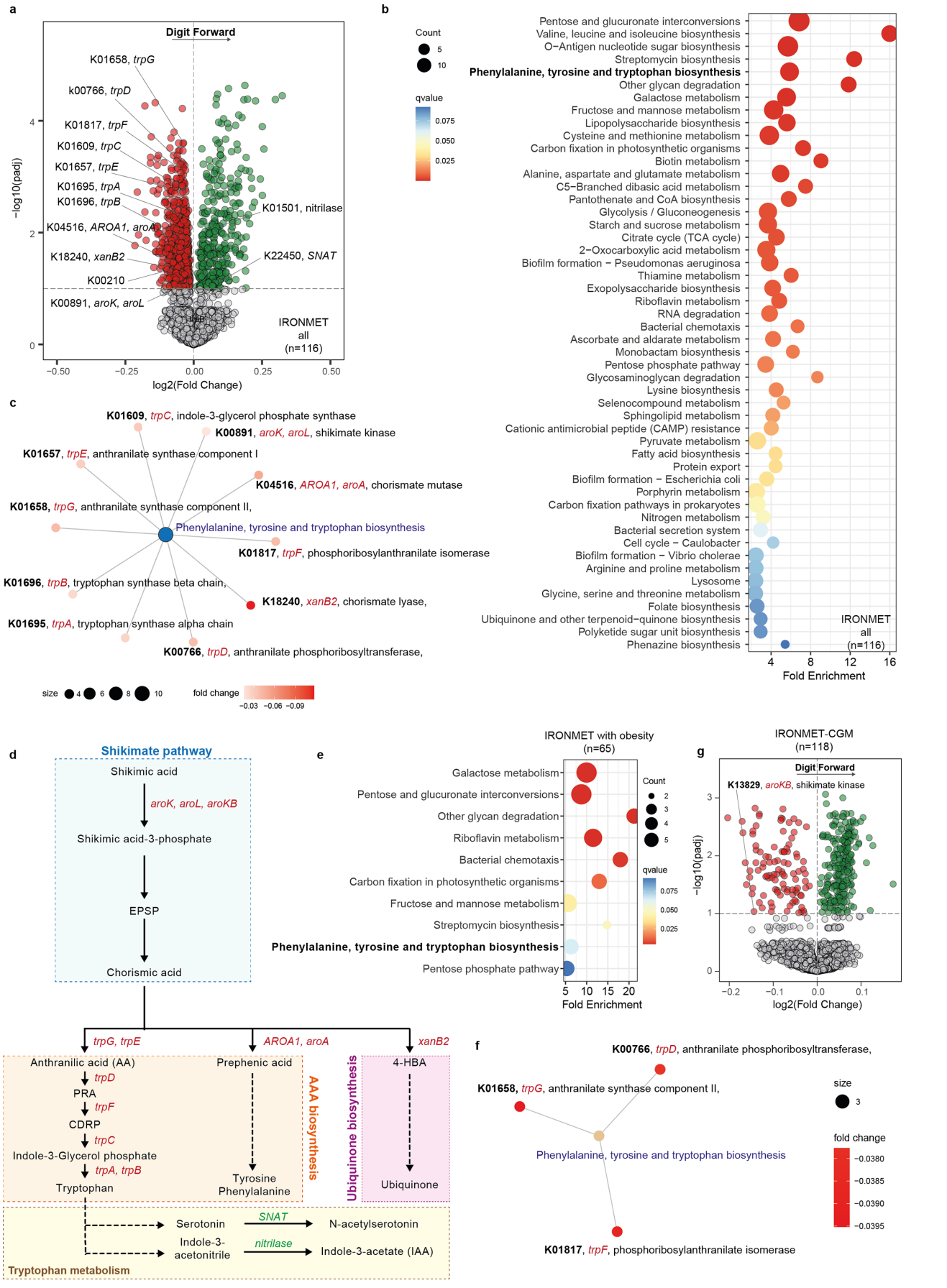

Figure 2 Continued

Figure 2 Microbial molecular functions involved in aromatic amino acid metabolism are associated with attention. (A) Volcano plot of differential microbial gene abundance associated with the digit forward scores in the IRONMET cohort calculated by ANCOM-BC (two-sided Z-test using the statistic W = $\log_2$ Fold Change/SE) from shotgun metagenomics data adjusted for age, gender, BMI and years of education. Microbial functions positively associated with attention are shown in green, whereas those negatively associated are shown in red. The log2 fold change associated with a unit change in the digit forward and the log10 p values adjusted for multiple testing (padj) are plotted for each taxon. Significance was set at padj<0.1. Genes involved in the phenylalanine, tyrosine and tryptophan metabolism are highlighted. (B) Dotplot of enriched KEGG pathways (*q*-value <0.1, one-sided hypergeometric test) from significantly differentially expressed microbial molecular functions associated with the digit forward scores in the IRONMET cohort. Dots are coloured according to *q*-value. (C) KEGG pathway enrichment network displaying the significant KEGG orthologues associated with attention involved in the phenylalanine, tyrosine and tryptophan metabolism, coloured according to the fold change. (D) Overview of the phenylalanine, tyrosine and tryptophan biosynthesis pathway (Shikimate pathway, ubiquinone biosynthesis and tryptophan catabolism are shown in blue, purple and yellow boxes, respectively). Microbial molecular functions associated with attention are displayed in red (negative association) or green (positive association). (E) Dotplot of enriched KEGG pathways (*q*-value <0.1, one-sided hypergeometric test) from significantly differentially expressed microbial molecular functions (padj<0.1) associated with the digit forward scores in patients with obesity in the IRONMET cohort. Dots are coloured according to *q*-value. (F) KEGG pathway enrichment network displaying the significant KEGG orthologues associated with attention in patients with obesity involved in the phenylalanine, tyrosine and tryptophan metabolism, coloured according to the fold change. (G) Volcano plot of differential microbial genes associated with the digit forward scores in the IRONMET-CGM cohort calculated by ANCOM-BC (two-sided Z-test using the statistic W = $\log_2$Fold Change/SE) from shotgun metagenomics data adjusted for age, gender, BMI and years of education. Microbial functions positively associated with attention are shown in green, whereas those negatively associated are shown in red. The log2 fold change associated with a unit change in the digit forward and the log10 p values adjusted for multiple testing (padj) are plotted for each taxon. Significance was set at padj<0.1. 4-HBA, 4-hydroxybenzoic acid; ANCOM-BC, analysis of compositions of microbiomes with bias correction; BMI, body mass index; CDRP, 1-(2-Carboxyphenylamino)-1'-deoxy-D-ribulose 5-phosphate; CoA, coenzyme A; EPSP, 5-enolpyruvylshikimate-3-phosphate; KEGG, Kyoto Encyclopedia of Genes and Genomes; PRA, N-(5-Phospho-β-D-ribosyl)-anthranilate.

3-hydroxyanthranilic acid (3-HAA), and also serotonin were also positively linked to attention. Kynurenic acid is a neuroprotective antagonist against N-methyl-D-aspartate (NMDA) receptors and its abundance can be affected by the gut microbiota to regulate neuroinflammation and behaviour,[38] whereas 3-HAA has recently been found to be an agonist ligand for transcription factor YY1,[39] which has an important role in the development and function of the central nervous system. Notably, the ADHD risk allele rs2271338 on *ADGRL3* has been shown to disrupt binding of YY1. Lower serum concentrations of kynurenic acid and 3-HAA have been previously associated with ADHD.[40] These two metabolites (kynurenic acid and 3-HAA) were also positively associated with attention in patients with obesity (figure 3B,D, online supplemental tables S19 and S20), but not in patients without obesity (figure 3E, online supplemental table S21), where only picolinic acid, IAA, 3-OH-kynurenine and 5-hydroxy-indole acetic acid (5-HIAA) were associated with attention.

Importantly, the inflammatory marker neopterin was the metabolite most strongly and negatively associated with attention in patients with obesity. This is consistent with chronic low-grade inflammation as a hallmark of obesity. Chronic inflammation activates both indoleamine 2,3-deoxygenase, the first and rate-limiting enzyme in the tryptophan degradation through the kynurenine pathway, and guanosine triphosphate (GTP)-cyclohydrolase 1, which converts GTP to neopterin at the expense of BH4, which plays a key role in the biosynthesis of serotonin and dopamine, fundamental for neurotransmission and cognitive function[41] (figure 3F). To further validate these findings, we also performed a targeted analysis of the tryptophan metabolism in the IRONMET-CGM cohort by chemical isotope labelling liquid chromatography-mass spectrometry. The most consistent finding was the strong (figure 3G, online supplemental table S22) and positive (figure 3H, online supplemental table S23) association between 3-HAA and attention in patients with obesity, also highlighted by the SHAP dependence plots between the digit forward and the 3-HAA ranked residuals (adjusted for age, gender, BMI and years of education) in subjects with obesity in both the IRONMET and IRONMET-CGM cohorts (figure 3I,J). Additionally, in both cohorts, individuals with obesity with digit forward scores higher than the median showed significantly higher 3-HAA levels compared with those with digit forward scores lower than the median (online supplemental figure S2A,B). In addition, digit forward scores showed a consistent positive association with 3-HAA levels (online supplemental figure S2C,D), reinforcing the link between 3-HAA and attentional capacity in the obese population. Notably, 3-HAA levels were positively associated with several species within the Actinobacteria phylum, whereas species from the Proteobacteria exhibited the most pronounced negative fold changes, underscoring the potential role of these bacterial species in modulating attention through 3-HAA (online supplemental figure S2E, online supplemental table S24).

Higher plasma 3-HAA and lower AA are linked to attention in the general population

We next sought to validate these findings in a large-scale cohort from the general population comprising community-dwelling participants (Health Imageomics, n=804, online supplemental table S25). Consistent with our previous findings, 3-HAA had the strongest (figure 4A, online supplemental table S26) and positive (figure 4B,G, online supplemental table S27) association with the digit forward scores, while serotonin was also positively linked to attention. AA, the precursor of 3-HAA along the kynurenine pathway, was negatively associated with attention in the whole cohort, particularly in patients without obesity (figure 4C,D,G, online supplemental tables S28 and S29). In patients with obesity, 3-HAA also had the strongest and positive association with attention, followed by its degradation product, the neurotoxic quinolinic acid (figure 4E–G, online supplemental tables S30 and S31). Notably, decreased plasma 3-HAA concentrations, along with increased AA levels, thereby leading to a lower 3-HAA/AA ratio, have been found in a wide range of neurological and other disorders with an inflammatory component (figure 4H). This suggests that the 3-HAA/AA ratio represents a marker for inflammation and progression,[42] which is consistent with our findings. Recently, the cerebrospinal fluid 3-HAA/AA ratio has also been shown to be strongly discriminatory for neuroinflammatory conditions.[43] Also in line with our

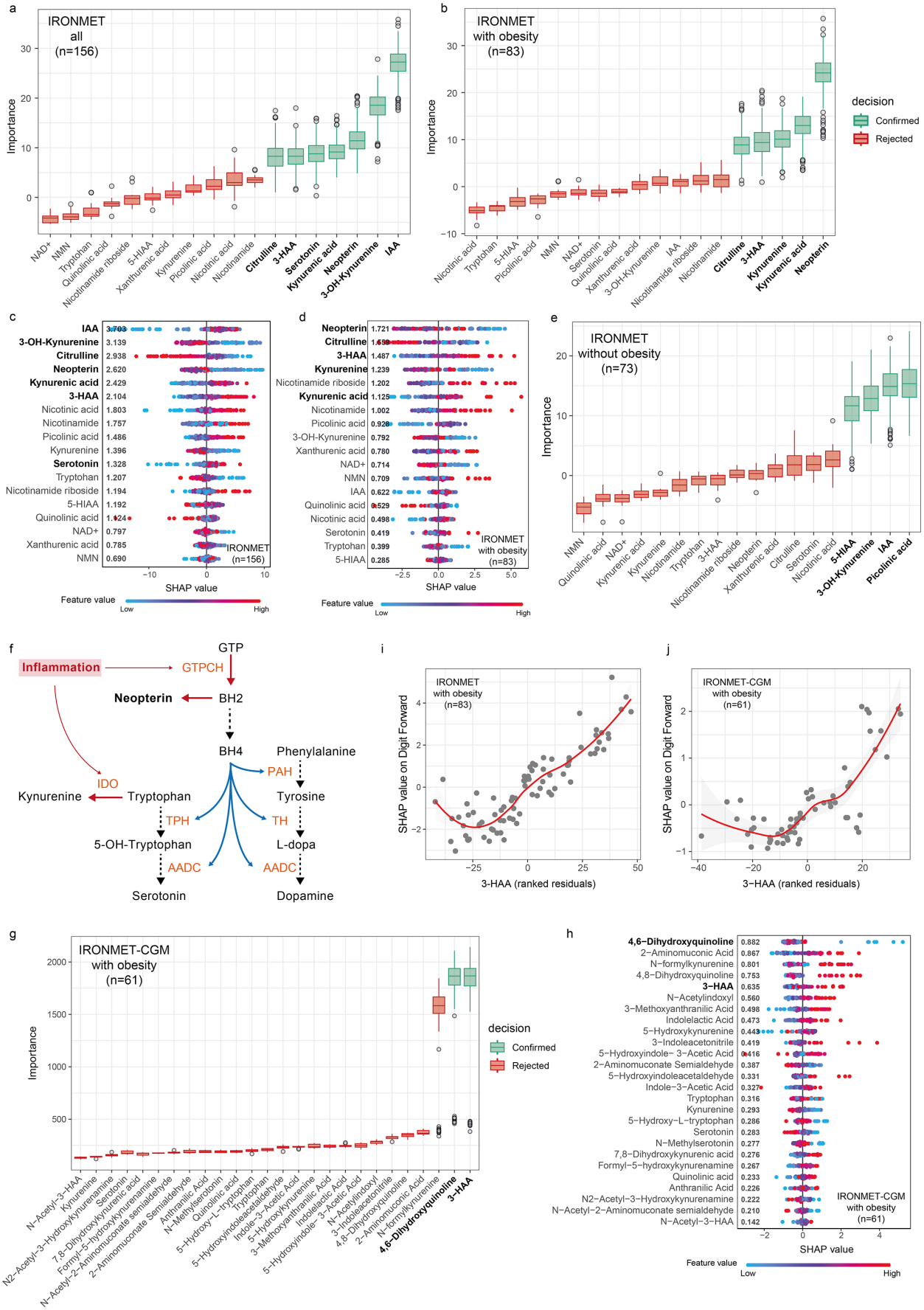

Figure 3 Continued

Figure 3 Targeted plasma tryptophan metabolomics signatures associated with attention in the IRONMET and IRONMET-CGM cohorts. Boxplots of the normalised variable importance measure for the tryptophan pathway-related metabolites associated with the digit forward scores in (A) all patients (n=156), (B) patients with obesity (n=83) and (E) patients without obesity (n=73) in the IRONMET cohort. Significant metabolites (confirmed) were identified using a machine learning variable selection strategy based on applying multiple random forests as implemented in the Boruta algorithm with 5000 trees, a confidence level cut-off of 0.005 for the Bonferroni adjusted p values, and a number of features randomly sampled at each split given by the rounded down number of features/3, and controlling for age, BMI, sex and education years. (C) SHAP summary of the tryptophan-related metabolites associated with the digit forward scores in all patients, and (D) patients with obesity. Significant metabolites identified by the machine learning approach are highlighted in bold. Each dot represents an individual sample. The X-axis represents the SHAP value, that is, the impact of a specific tryptophan-related metabolite on the prediction of the digit forward scores for a given individual. The overall importance for final prediction (average absolute SHAP values) is shown in bold. Colours represent the values of the metabolite levels, ranging from blue (low concentrations) to red (high concentrations). (F) Scheme of the synthesis of the neurotransmitter serotonin and dopamine from aromatic amino acids and the impact of inflammation. Pathways activated by inflammation are highlighted in red. The names of enzymes participating in each step are shown in orange. (G) Boxplots of the normalised variable importance measure for the tryptophan pathway-related metabolites associated with the digit forward scores in patients with obesity in the IRONMET-CGM cohort identified using the Boruta algorithm controlling for age, BMI, sex and education years. (H) SHAP summary of the tryptophan-related metabolites associated with the digit forward scores in patients with obesity in the IRONMET-CGM cohort. Significant metabolites identified by the machine learning approach are highlighted in bold. (I,J) SHAP dependence plot for the digit forward against the 3-HAA ranked residuals (adjusted for age, gender, BMI and years of education) in subjects with obesity in the IRONMET and IRONMET-CGM cohorts, respectively. 3-HAA, 3-hydroxyanthranilic acid; 5-HIAA, 5-hydroxy-indole acetic acid; BMI, body mass index; IAA, indole-3-acetic acid; NAD, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; SHAP, Shapley Additive exPlanations.

results, in participants with pre-diabetes and type 2 diabetes, which is also characterised by low-grade chronic inflammation, 3-HAA was associated with a lower odds of cognitive decline.[44] 3-HAA is an anti-inflammatory tryptophan catabolite capable of modulating the release of several cytokines by different immune cells.[45] Hence, it has been shown to modulate neuroinflammation by suppressing the proliferation of myelin-specific T cells and inhibiting the release of cytokines from T-helper (Th) 1 cells,[46 47] while it has also been implicated in the Th17/T regulatory balance, influencing the release of IL-17 and chronic inflammation.[48] It has also shown anti-inflammatory and neuroprotective roles in human brain cells under inflammatory conditions through the induction of hemeoxygenase-1 expression in astrocytes.[49] Moreover, 3-HAA is also a potent antioxidant, inhibiting low-density lipoprotein (LDL) and plasma lipid peroxidation[50] and protecting against oxidative stress in the brain.[51] However, it has also been proposed to mediate excitotoxic and neurodegenerative effects by inducing the apoptosis of neutrophils, natural killer and T and B cells.[45]

Bariatric surgery-induced weight loss increases attention and modulates the gut microbiota

Weight loss after bariatric surgery has been linked to improved cognitive function, although its impact remains controversial, as not all studies reported cognitive improvement[52] and the factors and mechanisms are still unknown. Therefore, we assessed the effects on attention 1 year after bariatric surgery in patients with severe obesity (BMI>35 kg/m^2) compared with patients with severe obesity who did not undergo bariatric surgery and a control group of patients without obesity (online supplemental table S32). Consistent with our other findings, patients without obesity had higher scores in the digit forward test compared with patients with obesity after controlling for age, gender, education years and the scores in the digit forward at baseline (online supplemental figure S3A). In addition, those patients with obesity who underwent bariatric surgery had higher attention than those patients who did not undergo surgery. Interestingly, we found that bariatric surgery increased levels of several species from the phylum Verrucomicrobia and Actinobacteria, including several species from the *Akkermansia* and *Bifidobacterium* genera, compared with patients with obesity (online supplemental figure S3B, online supplemental table S33), which is consistent with our findings associating species from these phyla with better attention (figure 1). High levels of Verrucomicrobia have been associated with better performance on several cognitive tests,[27 28] while administration of *Akkermansia muciniphila* has shown beneficial effects on cognitive function.[53] Importantly, evidences suggest that *Akkermansia muciniphila* plays a role in regulating the levels of tryptophan and gamma-aminobutyric acid (GABA).[54] Similarly, the abundances of *Bifidobacterium pseudocatenulatum*, *Bifidobacterium bifidum* and *Akkermansia muciniphila* have been recently found to be decreased in the faeces of subjects with MCI compared with cognitively healthy controls.[29] Also consistent with our findings, the aromatic amino acid biosynthesis was one of the microbial pathways significantly associated with the digit forward scores in patients with obesity that underwent bariatric surgery (online supplemental figure S3C, online supplemental table S34), with all genes involved upregulated (online supplemental figure S3D).

DIET-INDUCED OBESITY AND GUT MICROBIOTA DEPLETION DECREASE BRAIN 5-HIAA AND 3-HAA LEVELS IN MICE MPFC, WHILE FMT FROM DONORS WITH HIGH ATTENTION INCREASES THEIR LEVELS AND MODULATES THE EXPRESSION OF GENES PARTICIPATING IN THEIR DEGRADATION

To investigate the potential roles of obesity and the gut microbiota on tryptophan metabolism in the brain, we next fed C57BL/6J mice with either a standard-chow diet (SD) or a high-fat diet (HFD) for 9 weeks. In addition, two other groups of mice were fed an SD and treated with a combination of antibiotics for 2 weeks, followed by gavage with either vehicle or FMT from human donors with varying digit forward scores (online supplemental table S35) for 7 weeks. Afterwards, we performed a targeted analysis of the tryptophan metabolism to characterise 17 metabolites involved in the three main tryptophan catabolic routes in the mice mPFC (figure 5A), a brain region analogous to the dorsolateral PFC in humans, which plays an important role in attentional function.[55] Remarkably, 3-HAA was one of the only two tryptophan-related metabolites significantly decreased in the mPFC of HFD-fed mice compared with SD-fed mice (figure 5B,C and online supplemental table S36 and S37). Notably, aside from 5-HIAA (a serotonin degradation product), 3-HAA was the tryptophan catabolite with the highest discriminatory power distinguishing control mice from those with partially depleted microbiota due to antibiotics treatment

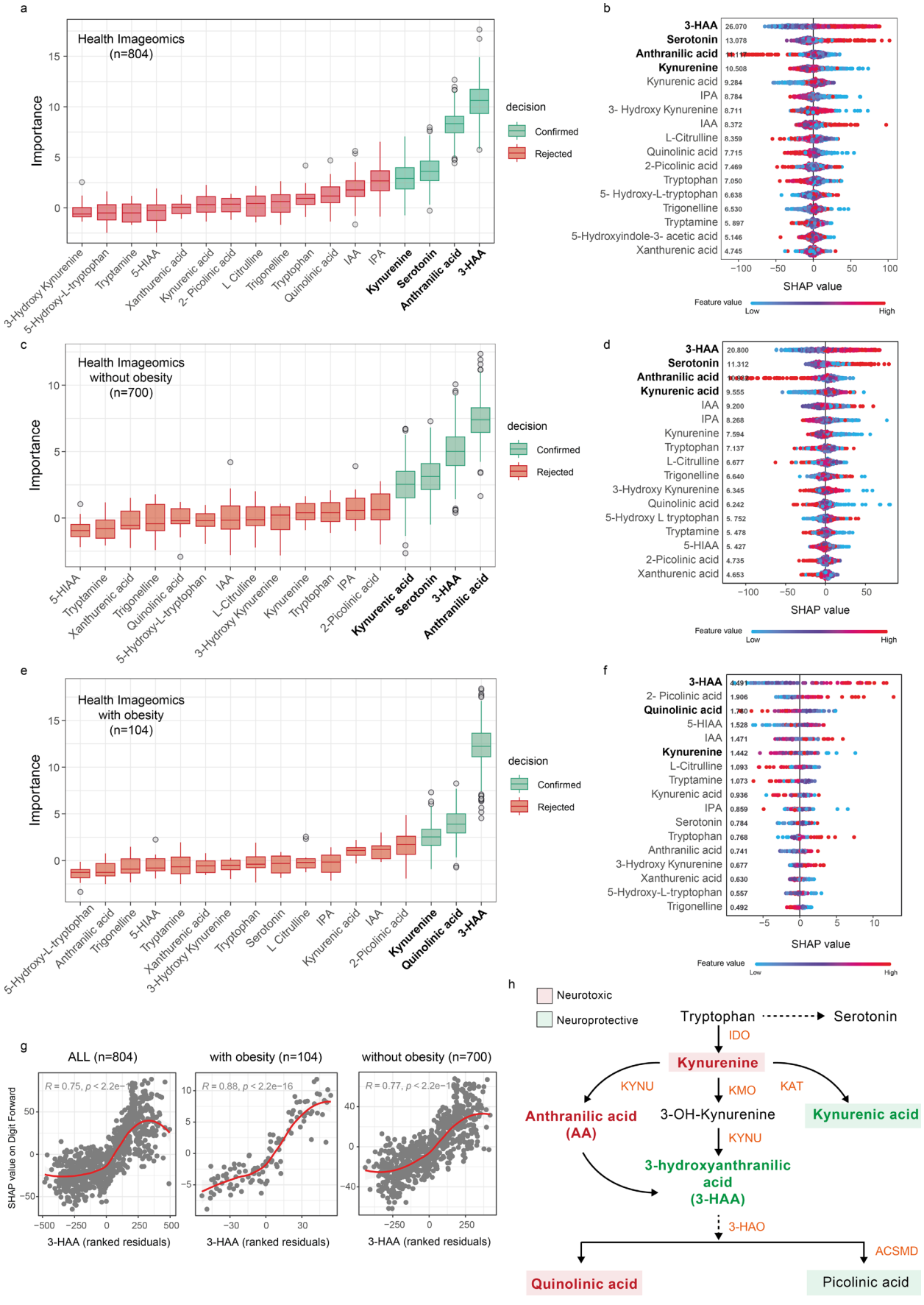

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Figure 4 Targeted plasma tryptophan metabolomics signatures associated with attention in the Health Imageomics cohort. Boxplots of the normalised variable importance measure for the tryptophan pathway-related metabolites associated with the digit forward scores in (A) all patients (n=804), (C) patients without obesity (n=700) and (E) patients with obesity (n=104) in the Health Imageomics cohort. Significant metabolites (confirmed) were identified using a machine learning variable selection strategy based on applying multiple random forests as implemented in the Boruta algorithm with 5000 trees, a confidence level cut-off of 0.005 for the Bonferroni adjusted p values and a number of features randomly sampled at each split given by the rounded down number of features/3, and controlling for age, BMI, sex and education years. (B) SHAP summary of the tryptophan-related metabolites associated with the digit forward scores in all patients (n=804), (D) patients without obesity (n=700), and (F) patients with obesity (n=104). Significant metabolites identified by the machine learning approach are highlighted in bold. Each dot represents an individual sample. The X-axis represents the SHAP value, that is, the impact of a specific tryptophan-related metabolite on the prediction of the digit forward scores for a given individual. The overall importance for final prediction (average absolute SHAP values) is shown in bold. Colours represent the values of the metabolite levels, ranging from blue (low concentrations) to red (high concentrations). (G) SHAP dependence plot for the digit forward against the 3-HAA ranked residuals (adjusted for age, gender, BMI and years of education) in subjects all patients, patients with obesity and patients without obesity from the Health Imageomics cohort (n=804). (H) Scheme of the kynurenine pathway. Metabolites with neuroprotective and neurotoxic effects are highlighted in green and red, respectively. Metabolites associated with attention are highlighted in bold. 3-HAA, 3-hydroxyanthranilic acid; 5-HIAA, 5-hydroxy-indole acetic acid; BMI, body mass index; IAA, indole-3-acetic acid; IPA, Indole-3-propionic acid; SHAP, Shapley Additive exPlanations.

(online supplemental figure S4A, figure 5D, online supplemental tables S38 and S39). Importantly, 5-HIAA and 3-HAA were the only metabolites restored in the mPFC of antibiotic-treated mice following microbiota recovery with FMT (online supplemental figure S4B, figure 5E, online supplemental tables S37 and S38), suggesting a direct role of the microbiota in regulating brain 3-HAA levels. Interestingly, both obesity and antibiotic treatment produced similar reductions in mPFC 3-HAA levels, which were restored only after FMT (figure 5F). These findings suggest that the gut microbiota plays a pivotal role in modulating the brain levels of 3-HAA.

To further elucidate the gut microbiota's role in attention, we performed a global metabolic profiling (online supplemental table S42) of the mPFC in mice from the FMT group (n=11) to identify differential metabolites between mice receiving microbiota from donors with low attention (digit forward scores≤6, n=6) and high attention (digit forward scores≥9, n=5). Remarkably, among all 631 identified metabolites, 3-HAA emerged as one of the most significantly (based on adjusted p value) and strongly (based on fold change) increased in the mPFC of mice receiving microbiota from high attention donors (figure 5G, online supplemental table S43). Conversely, quinolinic acid, a product of 3-HAA through 3-hydroxyanthranilic acid dioxygenase (*Haao*), was decreased in these mice compared with mice receiving microbiota from low attention donors (figure 5G,I,J). Additionally, 5-HIAA was the other tryptophan catabolite that increased after FMT in mice receiving microbiota of high attention donors. Interestingly, a KEGG-based pathway over-representation analysis revealed that aromatic amino acids metabolism, particularly tyrosine and tryptophan metabolism—pathways central to the production of serotonin and dopamine—were the two most enriched pathways distinguishing between mice receiving microbiota from donors with low and high attention (figure 5H, online supplemental table S44). Other significant pathways included the neuroactive ligand-receptor interaction, which contained several biogenic amines for class A rhodopsin-like receptors such as the monoamine neurotransmitters histamine, dopamine and norepinephrine, as well as the trace amines synephrine and octopamine, all of which were increased in the mPFC of mice receiving microbiota from low attention donors (figure 5G,I,J).

To deepen our understanding of how the gut microbiota influences attention, we conducted a second mouse FMT experiment from human donors (n=22) with different digit forward scores (figure 5K, online supplemental tables S45). This time, we performed RNA sequencing of the mPFC of recipient mice and identified genes associated with the donor's attention scores (online supplemental figure S4C, online supplemental table S46). Consistent with the metabolomics findings from the other FMT experiment, a KEGG-based enrichment analysis highlighted again tryptophan and tyrosine metabolism as the most significantly enriched (figure 5L, online supplemental figure S4D, online supplemental table S47). Remarkably, the significant genes involved in tryptophan metabolism included aldehyde oxidase 4, which oxidises 5-HIAA to the mono-hydroxylated product,[56] and *Haao*, the most active enzyme in the kynurenine pathways responsible for the conversion of 3-HAA to quinolinic acid (figure 5M, online supplemental figure S4D). These findings align with the metabolomics results of the first FMT experiment, where 5-HIAA and 3-HAA were decreased after antibiotic treatment and had the highest discriminant ability to differentiate control and antibiotic-treated mice (online supplemental figure S4A, figure 5D), whereas FMT from donors with high attention increased both tryptophan catabolites in the mPFC compared with mice receiving microbiota from low attention donors (figure 5G). Additionally, mice receiving microbiota from donors with high attention scores exhibited a downregulation of dopamine beta-monooxygenase gene, which encodes for a protein that catalyses the conversion of dopamine to norepinephrine and tyramine to octopamine in mammals (figure 5I,M, online supplemental figure S4D), aligning again with the metabolomics findings from the first FMT study. Dopaminergic activity in the PFC has been associated with attention in humans, primates and rodents, and administration of dopamine receptor agonist alleviates symptoms of attentional dysfunction.[57]

Other consistent findings included an enrichment of the neuroactive ligand-receptor interaction pathway (figure 5L, online supplemental table S47), which included alterations in several genes encoding for GABA (*Gabbr2, Gabra1*) and serotonin (*Htr5b*) receptors (online supplemental figure S4D). Consistent with these findings, several neurotransmitter systems innerving this region, including the dopaminergic, serotonergic and GABAergic systems, have been shown to modulate attentional processing.[57] GABA has been shown to modulate attention by inhibiting cortical acetylcholine release, while 5-HT1A receptor modulates serotonin neurotransmission through the inhibition of serotonin release from the presynaptic terminal.

FMT transmits attentional traits from humans to mice through serotonergic and dopaminergic alterations in the dorsal striatum

To further explore and uncover additional insights about the gut microbiota's influence on attention, we conducted a third FMT

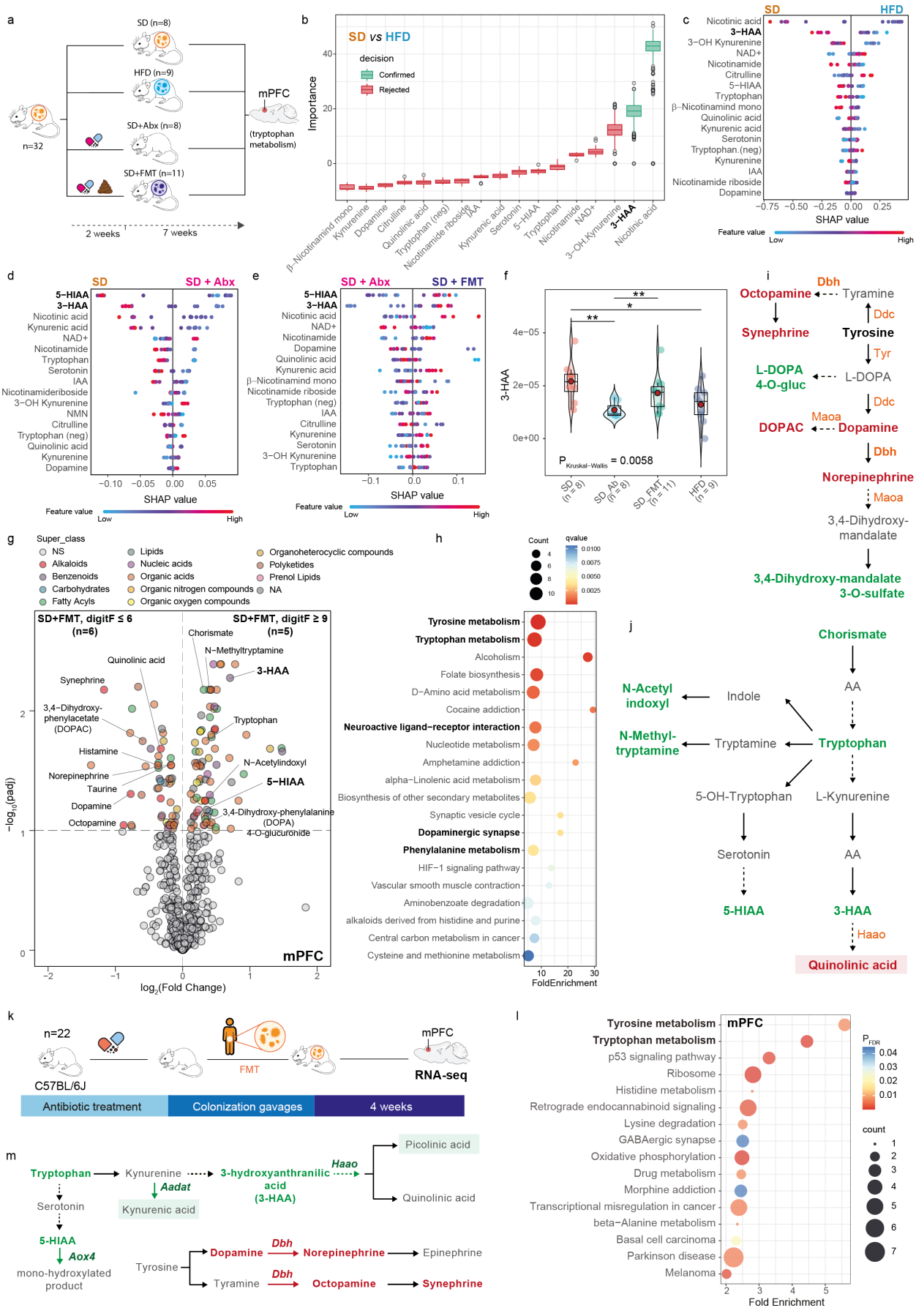

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Figure 5 Effect of obesity, microbiota depletion and the gut microbiota from donors with high and low attention on the recipient mice mPFC tryptophan metabolism, metabolome and transcriptome. (A) Study design for the first mice experiment. 16 C57BL/6J mice were fed a standard diet (SD, n=8) or a high fat diet (HFD, n=9) for 9 weeks. In addition, 16 mice were fed an SD and treated with a combination of antibiotics for 2 week and then treated with vehicle (SD + Abx, n=8) or oral gavage of donor microbiota (SD + FMT, n=11) for 7 weeks. At the end of the experiment, a targeted tryptophan analysis of the medial prefrontal cortex (mPFC) was performed. (B) Boxplots of the normalised variable importance measure for the tryptophan pathway-related metabolites discriminating between the SD and HFD groups. Significant metabolites (confirmed) were identified using a machine learning variable selection strategy based on applying multiple random forests as implemented in the Boruta algorithm with 5000 trees, a confidence level cut-off of 0.005 for the Bonferroni adjusted p values, and a number of features randomly sampled at each split given by the rounded down number of features/3. (C) SHAP summary plot of the tryptophan-related metabolites discriminating between the SD and HFD, (D) the SD and SD + Abx, and (E) SD + Abx and SD + FMT groups. Each dot represents an individual mouse. The X-axis represents the SHAP value, that is, the impact of a specific tryptophan-related metabolite on the prediction the group for a given mouse. Colours represent the values of the metabolites levels, ranging from blue (low concentrations) to red (high concentrations). (F) Violin plot of the normalised 3-HAA levels in the mouse mPFC according to the different groups (Kruskal-Wallis test). (G) Volcano plot of differential metabolites in the mPFC of mice receiving microbiota from donors with low attention (digit forward scores ≤ 6) and high attention (digit forward scores ≥ 9) identified using robust linear regression models (*t*-statistic based on M-estimation with Huber weighting solved using iteratively reweighted least squares). Fold change and log10 p values adjusted for multiple comparisons are plotted. (H) Dot-plot of KEGG enriched pathways in the mPFC of recipient mice from significant metabolites in the comparison between mice receiving microbiota donors with low and high attention (one-sided hypergeometric test). P values adjusted for multiple comparisons are plotted (*q*-value ≤0.1). Pathways involving precursors and neuroactive compounds are highlighted in bold. (I,J) Schematic representation of the metabolism of tyrosine and tryptophan. Metabolites highlighted in green and red are significantly increased and decreased in the mPFC of mice receiving microbiota from high attention donors compared with low attention donors, respectively. Key enzymes are shown in orange. (K) Study design for the second faecal microbiota transplantation experiment in mice. Microbiota from n=22 human donors with different digit forward scores was delivered to n=22 recipient mice pretreated with antibiotics for 14 days. After 4 weeks, an RNA sequencing from the mPFC was performed. (L) Dotplot of enriched KEGG pathways (*q*-value <0.1, one-sided hypergeometric test) from significantly differentially expressed mPFC genes associated with the donor digit forward scores. Dots are coloured according to *q*-value. Tyrosine and tryptophan pathways are highlighted in bold. (M) Scheme of tryptophan and tyrosine degradation pathways highlighting those significant genes positively (green) and negatively (red) associated with the donor digit forward scores. Neuroprotective compounds are displayed in green boxes. 3-HAA, 3-hydroxyanthranilic acid; 5-HIAA, 5-hydroxyindole acetic acid; Abx, antibiotic; FMT, faecal microbiota transplantation; IAA, indole-3-acetic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; NA, not available; NAD, nicotinamide adenine dinucleotide; NS, not significant; RNA-seq, RNA sequencing; SHAP, Shapley Additive exPlanations.

experiment from human donors with low (digit forward ≤6, n=5) and high (digit forward ≥8, n=5) attention (figure 6A, online supplemental table S48). After 2 weeks of colonisation gavages, mice underwent an operant behaviour task for the next 18 days, where mice received a pellet as a reward for pressing the active lever, while no reward was provided for pressing the inactive lever. In the last 2 days, mice underwent two sessions of reversal learning, where the active and inactive levers were switched. Reversal learning performance is determined by several cognitive processes, mainly cognitive flexibility, inhibitory control and attention. While reversal learning does not directly measure only attention, attentional processes are critical for successful performance. Specifically, attention allocation to the reward during the task is essential for initial discrimination and for adapting attentional focus following reversals.[58] Selective attention is required to prioritise task-relevant stimuli, that is, rewards, over irrelevant ones, while sustained attention is crucial to maintaining throughout the course of the task to detect the reversal and adapt behaviour accordingly. In addition, previous learnt reward value has been shown to strongly modulate attentional prioritisation.[59] Notably, mice colonised with microbiota from donors with higher attention pressed the active lever (previously inactive) more frequently during the reversal test than those receiving microbiota from lower attention group (figure 6B and online supplemental figure S5A), indicating better cognitive flexibility and attention. Remarkably, we found a really strong positive correlation (r=0.85, p=0.002) between the donors' attentional scores and the number of correct lever presses in the final reversal session (figure 6C and online supplemental figure S5A).

The prelimbic area of the mPFC projects to the striatum, which is a central component of reversal learning through striatal dopamine release in humans.[60] In addition, the dorsal striatum has been shown to control attention in the five-choice serial reaction time in rats.[61] Lesions in the striatum result in deficits in attentional performance without impairing Pavlovian approach behaviour.[62 63] Based on these findings, we performed a global metabolic profiling of the dorsal striatum of recipient mice (online supplemental table S49) to identify metabolites associated with donor's attention scores using robust linear regression models controlling for donors' age, sex and BMI (figure 6D, online supplemental table S50). Consistent with the other FMT experiments, the most significantly enriched pathways were again tryptophan and tyrosine metabolism (figure 6E, online supplemental table S51). Notably, aligning again with our previous findings, two of the tryptophan catabolites that decreased in recipient mice with higher donor attention scores included 5-HIAA and N-acetyl-cinnavalininate, a non-enzymatic oxidation product of 3-HAA (undetected in the dorsal striatum) (figure 6D).[64] Furthermore, also in agreement with previous FMT experiments, we found a significant enrichment of the neuroactive ligand-receptor interaction (figure 6E), that included again class A Rhodopsin-like biogenic amines derived from tryptophan and tyrosine, such as serotonin and tyramine (figure 6F).

We next performed RNA sequencing on the mPFC of recipient mice to identify differentially expressed genes between mice receiving microbiota from low- and high-attention donors (figure 6H, online supplemental table S52). Consistent with the metabolomics findings in the dorsal striatum and other FMT experiments, over-representation analyses based on Reactome and KEGG databases highlighted the class A Rhodopsin-like receptors and the neuroactive ligand-receptor interaction as the most enriched pathways, respectively (figure 6I, online supplemental figure S6A, online supplemental table S53). Notably, we identified several pathways that were downregulated in mice receiving microbiota from high attention donors compared with

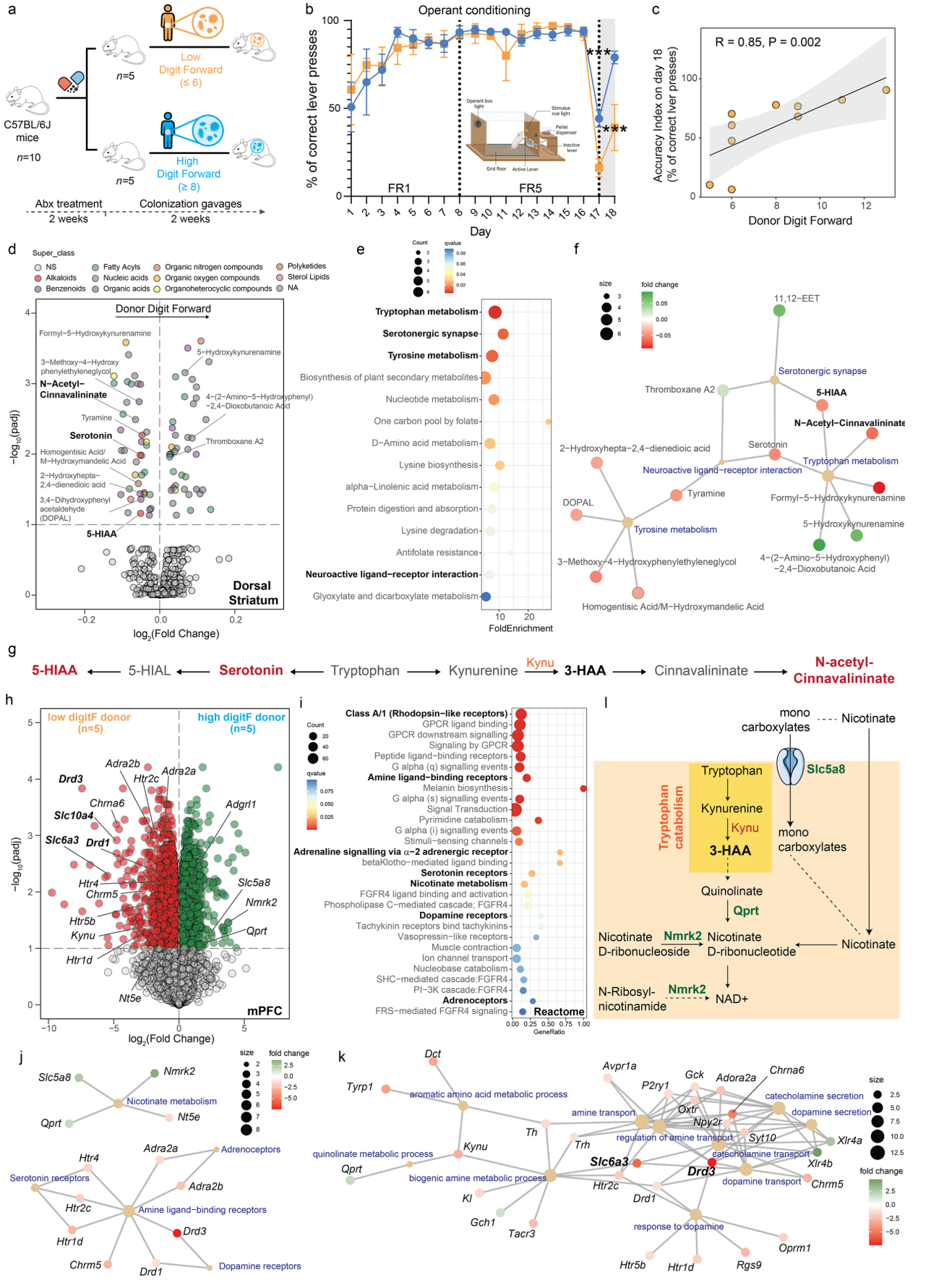

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Figure 6 Effects of microbiota transplantation from donors with high and low attention on reversal learning, the dorsal striatum metabolome and the mPFC transcriptome. (A) Study design for the third faecal microbiota transplantation experiment in mice. Microbiota from n=10 human donors with low attention (n=5, digit forward scores ≤ 6) and high attention (n=5, digit forward scores ≥8) was delivered to n=10 recipient mice treated with antibiotics for 2 weeks. After 10 days, mice were subjected to an operant behaviour procedure for the next 18 days. (B) Accuracy index measured by the percentage of correct lever presses (active/(active+inactive)). FR1: fixed ratio 1 (one active lever-press resulted in a delivery of one pellet); FR5: fixed ratio 5 (five lever-presses in order to obtain one reward). (C) Scatter plot and Spearman correlation between the human donor digit forwards scores and the recipient mice accuracy index on day 18. (D) Volcano plot of differential metabolites in the dorsal striatum of recipient mice associated with the human donor digit forward scores identified using robust linear regression models (t-statistic based on M-estimation with Huber weighting solved using iteratively reweighted least squares). Fold change and log10 p values adjusted for multiple comparisons are plotted. (E) Dot-plot of KEGG enriched pathways in the dorsal striatum of recipient mice from significant metabolites associated with the donor digit forward scores (one-sided hypergeometric test). P values adjusted for multiple comparisons are plotted (q-value ≤0.1). Pathways involving precursors and neuroactive compounds are highlighted in bold. (F) Metabolite concept network depicting significant metabolites (from d) involved in the selected pathways coloured according to the fold change. (G) Schematic representation of the tryptophan metabolism. Metabolites highlighted in red are significantly associated with lower donors' attention scores in the dorsal striatum of recipient mice. Key enzymes are shown in orange. (H) Volcano plot of differentially expressed mPFC genes in recipient mice receiving microbiota from donors with low and high attention identified using robust linear regression models (*t*-statistic based on M-estimation with Huber weighting solved using iteratively reweighted least squares). Fold change and log10 p values adjusted for multiple comparisons are plotted. (I) Dotplot of enriched KEGG pathways (q-value <0.1, one-sided hypergeometric test) from significantly differentially expressed (padj<0.1, logFC>1.25) recipient mice mPFC genes between donors with high and low attention. Dots are coloured according to q-value. (J) Network of differentially expressed genes participating in the selected Reactome pathways and (K) Gene Ontology Biological Processes from coloured according to the fold change. (L) Schematic representation of the connection between the tryptophan and nicotinate metabolism. The expression of genes highlighted in green and orange is increased and decreased in the mPFC of mice receiving microbiota from donors with high attention compared to those receiving microbiota from donors with low attention, respectively. Abx, antibiotic; KEGG, Kyoto Encyclopedia of Genes and Genomes; NA, not available; NAD, icotinamide adenine dinucleotide ;NS, not significant; mPFC, medial prefrontal cortex; 3-hydroxyanthranilic acid; 5-HIAA, 5-hydroxy-indole acetic acid; 5-HIAL, 5-Hydroxyindoleacetaldehyde.

low attention donors involving amine ligand-binding receptors, in particular, serotonin and dopamine receptors (figure 6I,J). The involvement of the dopaminergic systems was further highlighted by gene ontology enrichment analyses, where the dopaminergic synapse and the catecholamine transport were among the most over-represented cellular components and biological processes (online supplemental figure S6B,C, online supplemental tables S55 and S56). Indeed, genes related to dopamine synapse and transport were among the most significantly and strongly downregulated, in particular the dopamine receptor D3 (*Drd3*), the dopamine transporter (*Slc6a3*), the cholinergic receptor nicotinic alpha 6 subunit (*Chrna6*), and *Slc10a4* (figure 6H,J,K). Notably, dopaminergic signalling dysregulation has been linked to attention-related disorders such as ADHD, a neurodevelopmental disease characterised by impaired attention, hyperactivity and impulsivity.[65 66] *SLC6A3* (also known as *DAT1*) has been consistently associated with ADHD in genetic and molecular studies.[66 67] Additionally, *DRD3* has been linked to ADHD in pharmaco-behavioural studies,[68 69] while genetic variants in *CHRNA6* have been reported to interact with ADHD symptoms and nicotine addiction.[70] Notably, 19 of the genes found to be downregulated in the mPFC of mice receiving microbiota from low-attention donors overlap with the 83 genes associated with the biological term 'ADHD' in literature-supported statements describing functions of genes from the GeneRIF Biological Term Annotations dataset (Harmonizome.3)[71] (online supplemental table S56). This represents a 2.4-fold enrichment compared with what would be expected by chance (p=0.0007).

Other over-represented pathways and biological processes included the nicotinate metabolism (figure 6I, online supplemental figure S6A, online supplemental table S53), and the quinolinate metabolic process (online supplemental table S55). Interestingly, these pathways are closely connected to the tryptophan metabolism through the kynurenine pathway, which eventually leads to the production of quinolinic acid, a degradation product of 3-HAA and a precursor for nicotinamide adenine dinucleotide (NAD)+biosynthesis (figure 6L). Notably, we found that mice receiving microbiota from high attention donors exhibited an upregulation of quinolinate phosphoribosyltransferase (*Qprt*), a key in the catabolism of quinolinate, but a downregulation of kynureninase (*Kynu*), which converts kynurenine to 3-HAA (figure 6L).

Mono-colonisation with *E. cloacae* induces attention-deficit/hyperactivity-related traits in *Drosophila* and is exacerbated by diet-induced obesity

To gain deeper insights into the complex interplay between microbiota, attention and obesity, we employed the model organism *Drosophila melanogaster* (fruit fly). In both humans and animal models, attention deficits and hyperactivity often co-occur, as observed in individuals with ADHD,[65 66] and common molecular mechanisms have been proposed to regulate both processes.[72] A locomotor endophenotype, exhibiting increased total activity and a reduction in total sleep, has been described for genetic *Drosophila* models of ADHD,[73] which is often accompanied by increased wakefulness-related activity.[74] Notably, these phenotypes have been successfully rescued by administering methylphenidate, a drug commonly used to treat ADHD, which modulates dopaminergic signalling in the brain.[73] Consequently, assessing overall locomotor activity and sleep parameters in *Drosophila* may serve as a valuable readout for assessing attention-like defects in this organism.[73–75]

To further investigate the role of *Proteobacteria* in modulating attention-related behaviours, we conducted mono-colonisation experiments in *Drosophila melanogaster*. *Enterobacter cloacae* was selected as a representative member of the Proteobacteria phylum, while *Lactobacillus plantarum (L. plantarum)*, a member of the Firmicutes phylum, was used as an unrelated control bacterium (figure 7A, online supplemental table S57). Both species are commensal organisms previously identified in *Drosophila*.[76 77] Following mono-colonisation, we assessed locomotor activity and sleep in individual male flies using activity monitors (Trikinetics) (figure 7B, online supplemental

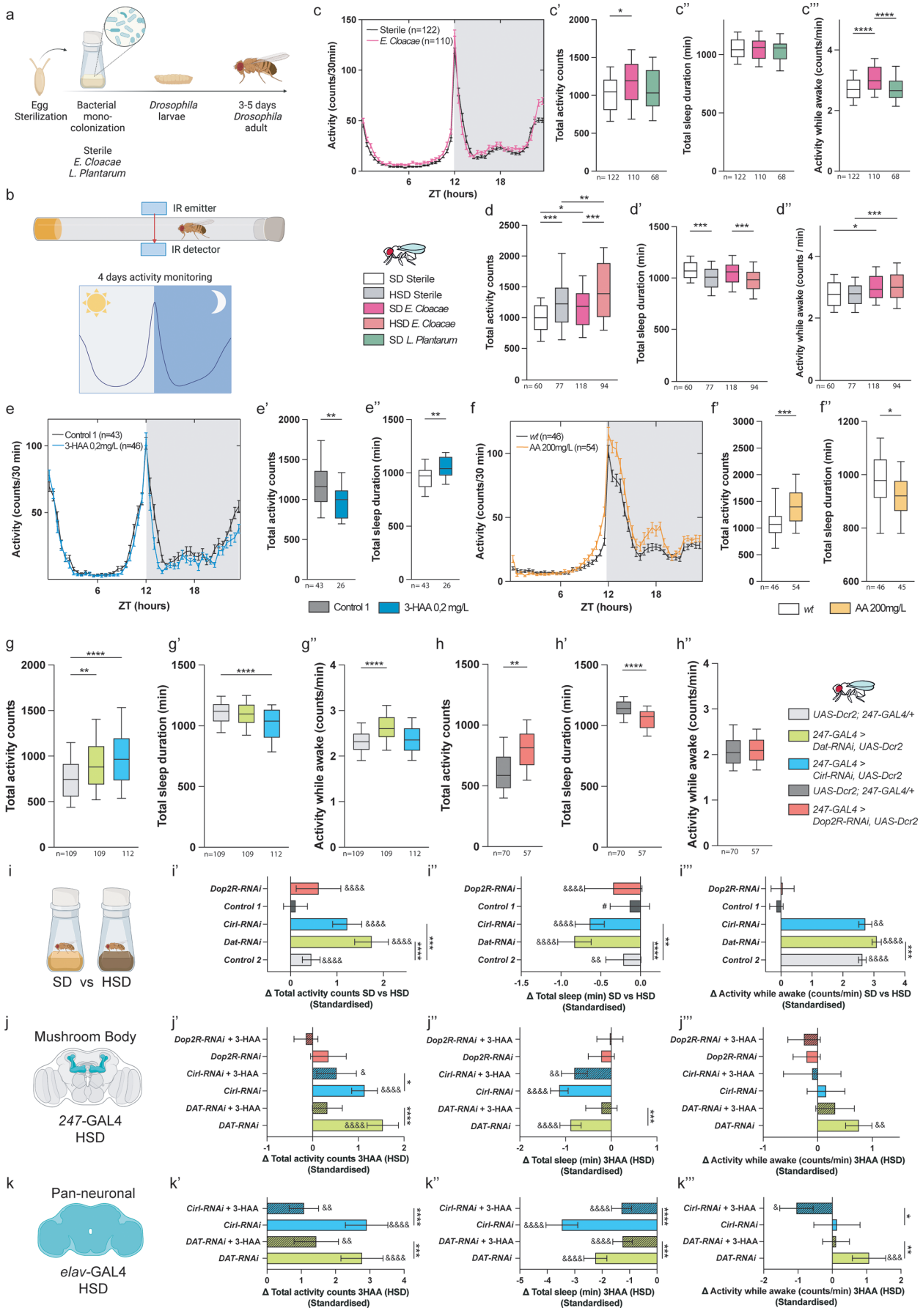

Figure 7 Continued

Figure 7 Mono-colonisation with *E. cloacae* and administration of high-sugar diet induce ADHD-like phenotypes in *Drosophila melanogaster* and 3-HAA supplementation reverses these effects (A) Schematic representation of the experimental design used to generate *Drosophila* wild-type flies under axenic (germ-free) conditions or mono-colonised with *E. cloacae* or *L. plantarum*. After egg sterilisation, embryos were transferred to sterile fly food supplemented with the bacteria of interest. 3–5 day-old adult flies were then collected for (B) activity and sleep monitoring over 4 days using single-beam activity monitors (Trikinetics). *Drosophila* activity patterns exhibit two main peaks at dawn and dusk, while activity levels decrease during the central hours of both day and night, when flies predominantly engage in sleep. (c) 24-hour activity profile within a 12-hour light-dark cycle, (C′) total activity, (C″) total sleep, and (C‴) Activity while awake in axenic flies and mono-colonised with *E. cloacae*, and *L. plantarum*, maintained on SD. (D) Total activity, (D′) total sleep, and (D‴) activity while awake in axenic flies and flies mono-colonised with *E. cloacae*, fed either with SD or HSD. Effects of dietary supplementation of 3-hydroxyanthranilic acid (3-HAA, 0.2 mg/L) or anthranilic acid (AA, 200 mg/L) on (E, F) activity profiles, (E′, F′) total activity, and (E″, F″) total sleep compared with vehicle-treated controls. (G, H) Role of mushroom body-specific knockdown of (G) DAT (247-GAL4 > DAT RNAi, UAS-Dcr2) and Cirl (247-GAL4 > Cirl RNAi, UAS-Dcr2), and (H) Dop2R (247-GAL4 > Dop2R-RNAi, UAS-Dcr2), compared with its corresponding genetic background controls (UAS-Dcr2/+; 247-GAL4/+) in (G, H) total activity, (G′, H′) total sleep, and (G″, H″) activity while awake. (I) Effect of HSD administration on flies with mushroom body-specific RNAi mediated knockdown of DAT, Cirl and Dop2R, standardised to their respective genotype fed on SD, Control 2 (n=100), DAT-RNAi (n=100), Cirl-RNAi (n=106), Control 1 (n=75), Dop2R-RNAi (n=45). (J) Effect of 3-HAA supplementation (0.2 mg/L) on flies maintained on a HSD with mushroom body-specific downregulation of DAT, Cirl and Dop2R, standardised to their respective genetic background controls, DAT-RNAi (n=100), DAT-RNAi + 3 HAA (n=74), Cirl-RNAi (n=106), DAT-RNAi + 3HAA (n=76), Dop2R-RNAi (n=45), Dop2R -RNAi+3HAA (n=31). (K) Effect of 3-HAA supplementation (0.2 mg/L) on flies maintained on HSD with pan-neuronal downregulation of DAT (elav-GAL4>DAT-RNAi, UAS-Dcr2) and Cirl (elav-GAL4>Cirl-RNAi, UAS-Dcr2), standardised to their respective genetic background controls (elav-GAL4/+, UAS-Dcr2/+), DAT-RNAi (n=65), DAT-RNAi+3HAA (n=45), Cirl-RNAi (n=36), DAT-RNAi+3 HAA (n=45). (I′, J′, K′) Increase in total activity, (I″, J″, K″) Increase in total sleep, and (I‴, J‴, K‴) Increase in activity while awake. Boxplots represent the median with the 25th–75th percentile and whiskers extending from the 10th to 90th percentile. Bar graphs represent the mean with a 95% CI, while activity plots show the mean with SEM. Statistical significance was calculated using a two-sided unpaired t-test, Welch's test, or one-way Brown-Forsythe analysis of variance with Dunnett's correction for multiple comparisons. P values: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 or #p<0.1, &p<0.05, &&p<0.01, &&&p<0.001, &&&&p<0.0001 indicate significant differences between the dataset representing the condition and the dataset used for standardisation. ADHD, attention-deficit hyperactivity disorder; *E. cloacae, Enterobacter cloacae;* HSD, high-sugar diet; IR, infrared detector; *L. plantarum, Lactobacillus plantarum;* SD, standard-chow diet; wt, wild-type; ZT, Zeitgeber time.

table S57). Flies mono-colonised with *E. cloacae* exhibited a significant increase in total activity compared with axenic flies (p=0.01) (figure 7C–C'', online supplemental table S57). Notably, while total sleep duration (defined as periods of inactivity ≥5 min) remained unaffected, flies mono-colonised with *E. cloacae* exhibited significantly increased activity levels during wakefulness (activity while awake) compared with both sterile (p<0.0001) and *L. plantarum* mono-colonised flies (p<0.0001), indicative of hyperactivity (figure 7C'''', online supplemental table S57). This effect was specific to *E. cloacae*, as *L. plantarum* colonisation did not produce similar phenotypic changes, highlighting the distinct contribution of *E. cloacae* to hyperactive behaviours in *Drosophila*.

Given our human data linking obesity with reduced attention, we next examined whether diet-induced obesity influenced locomotor activity and sleep. To this end, we generated *Drosophila* obesity models by administering either a high-sugar diet (HSD) or a HFD. HFD increased overall activity during the central hours of day and night (online supplemental figure S7A), but led to reduced peak activity, possibly due to locomotor impairments from the oily food affecting locomotor efficiency. In contrast, HSD administration resulted in a robust and consistent hyperactivity endo-phenotype, characterised by a significant increase in total activity, alongside a reduction in sleep in both axenic (p_{Act}=0.0004, p_{Sleep}=0.0001) and flies mono-colonised with *E. cloacae* (p_{Act}=0.0003, p_{Sleep}=0.0009) (figure 7D,D'', online supplemental figure S7B, online supplemental table S57).

Dietary supplementation of 3-hydroxyanthranilic reduces attention-deficit/hyperactivity-related phenotypes in *Drosophila melanogaster*

To further evaluate the effects of 3-HAA and AA on attention-deficit/hyperactivity-related traits, we examined how these metabolites influence daily activity and sleep patterns through dietary supplementation in SD-reared flies. Supplementation with 3-HAA (0.2 mg/L) led to a slight but significant decrease in total activity (p=0.0065) and increase in total sleep (p=0.0091). In contrast, AA supplementation (200 mg/L) produced the opposite effect, significantly increasing total activity (p=0.0006), reducing sleep duration (p=0.0223), and elevating wakefulness-related activity (p=0.0094) (figure 7E–F', online supplemental figure S7C,D, online supplemental table S58). Consistent with our previous findings in humans and mice, 3-HAA and AA seem to exhibit opposing effects in *Drosophila*, with AA enhancing attention-deficit/hyperactivity-like traits, while 3-HAA appeared to mitigate these behaviours.

RNA sequencing of the mPFC in mice receiving microbiota from individuals with low attention revealed an enrichment of genes involved in dopaminergic signalling (figure 6H–K). Among the most significantly downregulated genes were the dopamine receptor D3 (*Drd3*) and the dopamine transporter (*Slc6a3*). Notably, their *Drosophila* orthologs, *Dop2R* and *DAT*, have already been implicated in ADHD-associated locomotor phenotypes in flies.[73 78] Similarly, *Cirl*, the *Drosophila* ortholog of *Adgrl1*, upregulated in our RNAseq dataset (figure 6H), has also been linked to attention-deficit/hyperactivity-like phenotypes in flies.[73 79] For these reasons, we selected *Dop2R*, *DAT* and *Cirl* for targeted downregulation in the *Drosophila* brain. Using the UAS-GAL4 system, we specifically knocked down these genes via RNA interference (RNAi) in the mushroom body (MB), a key brain region implicated in the regulation of locomotor activity and sleep in *Drosophila*.[79 80] As expected, RNAi-mediated downregulation of *Dop2R*, *DAT* and *Cirl* resulted in a significant reduction in total sleep time (p_{Dop2R}<0.0001, p_{Cirl}<0.0001) accompanied by a corresponding increase in total activity (p_{Dop2R}=0.0038, p_{DAT}=0.0069, p_{Cirl}<0.0001). *DAT* knockdown also led to a significant increase in wakefulness-related activity (p_{DAT}<0.0001) compared with their respective genetic background controls (figure 7G–H'',E,F, online supplemental tables S59).

To assess the impact of obesity on these phenotypes, we repeated the experiment under HSD conditions. Consistent with our previous findings, HSD alone increased total activity and reduced sleep duration in control flies compared with those fed SD. However, the increase of activity and reduction in sleep were significantly more pronounced when *Dop2R*, *DAT* or *Cirl* were downregulated in the MB and flies were reared in HSD, suggesting that diet-induced obesity exacerbates attention-deficit/hyperactivity-like phenotypes in *Drosophila* genetic models of ADHD (figure 7I–I'', online supplemental figure S7G–J, online supplemental table S60).

Given that 3-HAA was primarily associated with improved attention in obese individuals, we investigated its effects by supplementing 0.2 mg/L of 3-HAA in the *Drosophila* diet under different dietary conditions. While 3-HAA supplementation did not significantly alter attention-deficit/hyperactivity-like phenotypes in flies fed a standard diet (SD) (online supplemental figure S7K–K''), it notably mitigated these effects in flies reared on HSD. 3-HAA supplementation of flies with MB-specific downregulation of *DAT*, *Cirl* and *Dop2R* reared in HSD significantly reduced the increment of activity and partially rescued the decrease of sleep with respect to its genetic background controls (online supplemental figure S7J–J''). Finally, when *DAT* and *Cirl* were pan-neuronally downregulated, 3-HAA supplementation had even more pronounced effects, attenuating the increase in activity, mitigating sleep loss and reducing wakefulness-related activity compared with respective genetic background controls under HSD conditions (figure 7K–K''', online supplemental table S61).

These findings suggest that obesity models in *Drosophila* generated via HSD exhibit hyperactivity and sleep disruptions reminiscent of attention-deficit/hyperactivity phenotypes, similar to those observed in obese individuals with reduced attention. Additionally, the exacerbation of hyperactivity in flies monocolonised with *E. cloacae* supports the hypothesis that specific *Proteobacteria*, particularly *E. cloacae*, contribute to attention-deficit/hyperactivity-like traits, both under SD and HSD. Finally, we show that *Drosophila* orthologs of ADHD genes dysregulated after FMT contribute to attention-deficit/hyperactivity-like phenotypes in *Drosophila* when downregulated in the mushroom body and overall neurons, and these phenotypes can be partially rescued through dietary 3-HAA supplementation under HSD-induced obesity conditions.

CONCLUSIONS

In summary, we have identified a robust positive association between 3-HAA and attention, particularly in individuals with obesity. Our findings suggest that the gut microbiota may play a crucial role in modulating 3-HAA levels. Microbial genes responsible for synthesising tryptophan from AA were negatively associated with attention, supporting the role of microbial metabolism in this behavioural domain. In preclinical mouse models, depletion of the gut microbiota led to reduced brain levels of 3-HAA, while FMT from high-attention-score donors influenced the expression of *Haao*, a key enzyme involved in 3-HAA regulation. Consistently, diet-induced obesity reduced 3-HAA levels in the mPFC of mice.

Microbiota depletion via antibiotics reduced brain 3-HAA concentrations, while FMT restored them, underscoring the microbiota's role in regulating this metabolite. Moreover, FMT experiments from human donors with high attention scores led to significant enrichment of tryptophan and tyrosine metabolism pathways, at both the metabolic and transcriptional levels, compared with FMT from low-attention donors. Additionally, mice receiving microbiota from donors with high-attention scores also showed increased expression of *Haao*, a key enzyme involved in the regulation of 3-HAA.

In a subsequent FMT experiment, attentional traits were transferred to mice correlating with the donor's attention score. RNA sequencing of the mPFC revealed downregulation of several pathways in mice receiving microbiota from high-attention donors compared with those from low-attention donors, with the most pronounced changes observed in serotonin and dopamine receptors. At the metabolic level, alterations in serotonergic and dopaminergic pathways were observed in the brains of recipient mice. In fact, both pathways have been previously linked to attention deficits in animal models and attention-related disorders such as ADHD.[57 72]

In *Drosophila*, diet-induced obesity triggered attention-deficit/hyperactivity-like phenotypes. Importantly, dietary supplementation with 3-HAA mitigated these effects in *Drosophila* models of ADHD, whereas supplementation with AA induced attention-deficit/hyperactivity traits.

Together, our findings reinforce the critical role of the microbiota-gut-brain axis in modulating cognitive performance, particularly attention. We have identified 3-HAA as a promising candidate for innovative strategies aimed at enhancing attention, particularly in the context of obesity and related conditions. Furthermore, we also identified the enzyme *Haao* as a key regulator of 3-HAA levels, offering promising therapeutic targets for future interventions focused on enhancing attention and cognitive function.

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Contributors JM-P and JMF-R carried out the conception and coordination of the study. AC-N, AF and AdC-I researched the data, performed the statistical analysis, prepared the figures and wrote the manuscript. AM-A and CC are trained neuropsychologists who performed the neurophysiological assessments of the human cohorts and researched the data. MA-R contributed to collect data for both discovery cohorts. JM-P performed the RNA-seq analysis of mice prefrontal cortex. JM-P and JMF-R contributed to the discussion and reviewed the manuscript. All authors participated in final approval of the version to be published. JM-P and JMF-R are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data.

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