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Full-length Article

Maternal high-fat diet-induced microbiota changes are associated with alterations in embryonic brain metabolites and adolescent behaviour

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ABSTRACT

The developing central nervous system is highly sensitive to nutrient changes during the perinatal period, emphasising the potential impact of alterations of maternal diet on offspring brain development and behaviour. A growing body of research implicates the gut microbiota in neurodevelopment and behaviour. Maternal overweight and obesity during the perinatal period has been linked to changes in neurodevelopment, plasticity and affective disorders in the offspring, with implications for microbial signals from the maternal gut. Here we investigate the impact of maternal high-fat diet (mHFD)-induced changes in microbial signals on offspring brain development, and neuroimmune signals, and the enduring effects on behaviour into adolescence. We first demonstrate that maternal caecal microbiota composition at term pregnancy (embryonic day 18: E18) differs significantly in response to maternal diet. Moreover, mHFD resulted in the upregulation of microbial genes in the maternal intestinal tissue linked to alterations in quinolinic acid synthesis and elevated kynurenine levels in the maternal plasma, both neuronal plasticity mediators related to glutamate metabolism. Metabolomics of mHFD embryonic brains at E18 also detected molecules linked to glutamate-glutamine cycle, including glutamic acid, glutathione disulphide, and kynurenine. During adolescence, the mHFD offspring exhibited increased locomotor activity and anxiety-like behaviour in a sex-dependent manner, along with upregulation of glutamate-related genes compared to controls. Overall, our results demonstrate that maternal exposure to high-fat diet results in microbiota changes, behavioural imprinting, altered brain metabolism, and glutamate signalling during critical developmental windows during the perinatal period.

1. Introduction

Overweight and obesity is a global phenomenon that increasingly affects Western societies and is prevalent among women of reproducing age (Aubry et al., 2019). The main factors contributing to obesity include genetics, environment and a diet high in sugar and fat, generally consumed in Western societies. Epidemiological studies have shown that maternal obesity before and during pregnancy is associated with increased prevalence of neuropsychiatric conditions in the offspring, including attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and cognitive impairments (Edlow, 2017; Kong et al., 2020; Mehta et al., 2014).

Apart from being a critical factor linked with obesity, diet shapes the composition and subsequently, the function of the microbes present in

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our intestine, termed the gut microbiota (Cryan et al., 2019). The gut microbiota communicates with the brain via different pathways including the immune system, metabolites, neurotransmitters, and the vagus nerve (Cryan et al., 2019). Moreover, alterations in these systems can, in turn, influence the composition and function of the gut microbiota (Serino et al., 2011; Sgritta et al., 2019; Zheng et al., 2020). During pregnancy, various physiological changes, including metabolic, hormonal, and immunological shifts in the maternal body influence the gut microbiota. For instance, rising levels of progesterone during pregnancy lead to an increase in *Bifidobacteria* abundance in the maternal gut (Nuriel-Ohayon et al., 2019). Additionally, metabolic adaptations, including weight gain and increased adiposity, along with low-grade inflammation are essential to support the developing fetus and prepare the maternal body for lactation. In response to these adaptive changes perinatally, the composition of gut microbiota undergoes alterations, with elevated Bacillota:Bacteroidetes ratio in pregnant women compared with non-pregnant controls (Collado et al., 2008; Santacruz et al., 2010). Interestingly, similar changes in weight gain, increased adiposity, low-grade inflammation and microbiota composition are also observed in non-pregnant individuals with obesity (Cani et al., 2012). When pregnancy and obesity coincide, these parameters are amplified, with short- and long-term health implications for the mother and the offspring (Gohir et al., 2015; Josefson et al., 2013; Kimura et al., 2020).

The impact of perinatal obesity and related maternal microbial signals on offspring health has been studied using diets high in fat in preclinical rodent models (Buffington et al., 2016; Di Gesù et al., 2021; Jašarević and Bale, 2019). Indeed, it is well appreciated that the composition and function of the microbiota is sensitive to nutrient changes, and signals from maternal microbes influence neurodevelopmental outcomes in offspring (Urbonaite et al., 2022; Vuong et al., 2020). Recent studies present evidence that microbes residing in the maternal gut mediate perinatal neurodevelopment and prime offspring behaviour (Jašarević and Bale, 2019; Vuong et al., 2020). Furthermore, studies have found that maternal-induced obesity during pregnancy and lactation results in hyperactivity and anxiety-like behaviour in adolescent and adult offspring, along with changes in glutamatergic signalling (Glendining et al., 2018). Moreover, maternal high-fat diet (mHFD)-induced shifts in microbiota composition contribute to changes in brain synaptic plasticity, resulting in social deficits in the offspring (Buffington et al., 2016; Di Gesù et al., 2022).

Synaptic plasticity, an essential function of neuronal synapses, is vital for maintaining proper neuronal communication. The disruption of the balance between excitatory and inhibitory synapses is relevant to neurodevelopmental disorders, including ASD (Rubenstein and Merzenich, 2003; Tyzio et al., 2014; Yizhar et al., 2011). Rodent studies have explored the possibility of input from factors such as maternal infection and maternal microbiota disruption by HFD on the offspring synaptic function and behaviour (Andoh et al., 2019; Buffington et al., 2016; Sgritta et al., 2019), while others link HFD with glutamate metabolism and clearance in the mouse hippocampus (Valladolid-Acebes et al., 2012).

Critical developmental processes in the brain, including myelination and glial maturation, are time-dependent and can be regulated by microbiota-derived signals. For instance, microbiota colonisation before weaning rescued the cortical hypermyelination phenotype observed in germ-free animals (Hoban et al., 2016). The gut microbiota is also essential for microglia maturation and function (Erny et al., 2015, 2021; Needham et al., 2022) and signals from the maternal microbiota during pregnancy influence the fate of foetal microglia development and maturation (Thion et al., 2018). Since aberrant myelination and glial function have been linked with ASD and ADHD (Demontis et al., 2019; Khanbabaei et al., 2019), investigating these time-dependent processes in areas linked to cognition, social behaviour and decision-making is of utmost importance.

Although the maternal microbiota has emerged as a critical mediator of offspring brain development, the mechanism underlying the onset of neurodevelopmental disorders and the intricate relationship among maternal diet, intestinal microbiota, and offspring brain health remain elusive. In this study, we aim to investigate the impact of maternal diet and microbiota signals on the fetal brain transcriptome, metabolome, and subsequent enduring effects on offspring behaviour in adolescence in a sex-dependent manner at two key timepoints relevant to neurodevelopment. At E18, we were able to interrogate the impact of altered maternal microbiota composition on whole fetal brain metabolites and microglia gene expression. Alterations in both have been implicated in neurodevelopmental process (Gandal et al., 2018; Vuong et al., 2020). In particular, we were interested in kynurenine pathway metabolites given the role of glutamate in modulating brain development and plasticity (Potter et al., 2010; Schwarcz, 2016). Next, we turned to adolescence because of its critical window in the ontogeny of neurodevelopmental disorders (Cowan et al., 2020) and characterised the effect of mHFD on social, anxiety-like, and locomotor behaviour and finally, focused on amygdala glutamatergic gene expression. We hypothesise that identifying a link among maternal diet during pregnancy, maternal gut microbiota composition and fetal brain priming could lead to novel, early interventions for altered neurodevelopment.

2. Methods

2.1. Animals

All procedures were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) at University College Cork and the Health Products Regulatory Authority (HPRA), under project authorization number AE1930/P039 in accordance with the recommendations of the Directive 2010/63/EU.

C57/Bl6 female and male mice were obtained from Envigo UK and kept on a 12-hour light/dark cycle with *ad libitum* access to food and water. Females were randomly assigned to either a control diet (Control) consisting of 10 % kcal from fat, 20 % kcal from protein, 70 % kcal from carbohydrates, and 5 % kcal from fibre (ssniff Spezialdiäten GmbH, E157453-04), or a high-fat diet (HFD) consisting of 60 % kcal from fat, 20 % kcal from protein, 20 % kcal from carbohydrates and 6 % kcal from fibre (ssniff Spezialdiäten GmbH, E15742-34), starting 8 weeks pre-mating until end of lactation/weaning. Maternal weight was measured weekly during pregnancy. After 8 weeks on intervention diets before mating, mice were housed in breeding pairs and females were checked daily for vaginal plugs. Females were removed upon plug detection or visible signs of pregnancy and subsequently single-housed. On embryonic day 18 (E18) a group of 6 Control and 6 mHFD pregnant females were sacrificed by decapitation between 10 am and 12 pm (noon). Trunk blood was collected in EDTA tubes, centrifuged for 10 min at 3000xg at 4 °C, and plasma was collected. Caecum content, maternal plasma, fetal brains, and placentas were snap-frozen and stored at −80 °C until further analysis. Another group of pregnant dams remained with their offspring until the end of lactation at postnatal day 21 (PND21) on the intervention diets. Offspring were then weaned to a chow diet (Teklad Global Rodent Diets) regardless of maternal diet (control or HFD). Maximum 2 males and 2 females per litter per experimental group were randomly assigned to behavioural tests. After completion of behavioural tests in adolescence, 1 male and 1 female mouse per litter was sacrificed by decapitation between 10 am and 12 pm. Plasma was collected as described above. Biological samples, including gut and brain regions were rapidly dissected, snap-frozen, and stored at −80 °C until further analysis. Up to 1 male and 1 female mouse per litter per experimental group was perfused with 4 % paraformaldehyde. Maternal microbiota composition analysis and maternal plasma kynurenine metabolites were measured on pregnant females sacrificed at E18. Whole brain metabolomics and whole brain RT-PCR were performed on male and female fetal brains and microglia isolation fetal E18 male brain samples. Amygdala RNA extraction and behavioural evaluation was performed on male and female adolescent offspring of mHFD and Control. All of these methods are described below in detail.

2.2. Microbiota composition analysis

Caecal microbiota composition was analysed via shotgun sequencing for samples that were collected at gestational day 18 from pregnant dams. Briefly, the QIAmp Fast DNA Stool Mini Kit DNA (Qiagen) was used for caecal DNA extraction, coupled with an initial bead-beating step. Briefly, 200 mg of each caecal sample was homogenised in 2 ml screw-cap tubes containing 250 mg of a 1:1 mix of 0.1 mm and 1.5 mm sterile zirconia beads plus a single 2.5 mm diameter bead (BioSpec Products) with 1 ml of Qiagen InhibitEx® buffer. The remaining extraction steps followed the manufacturer's instructions. DNA was quantified using the QubitTM 3.0 Fluorometer (BioSciences) and the Qubit® dsDNA HS Assay Kit (Life Technologies).

Samples were prepared for shotgun metagenomic sequencing according to Illumina Nextera XT library preparation kit guidelines, with the use of unique dual indexes for multiplexing with the Nextera XT index kit (Illumina). Briefly, Illumina libraries were constructed from total genomic DNA isolated from each sample. The DNA was sheared into approximately 400–600 bp fragments followed by ligation of Illumina adaptors containing molecular barcodes for downstream demultiplexing. These tagmentation products were then amplified using Illumina index primers following bead purification with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). The quality of the library was then evaluated by running it on a Bioanalyzer using the Agilent High Sensitivity DNA chip. The DNA was quantified using Qubit, normalised, pooled, and outsourced to the Teagasc sequencing facility for sequencing on a NextSeq 500 using high output 300 cycle V2.5 chemistry according to manufacturer's instructions. Sequencing files were demultiplexed with CASAVA version 1.8.3 (Illumina).

Quality filtering, trimming and de-multiplexing was carried out by a custom pipeline containing Trim Galore and cutadapt (Martin, 2011) for adaptor and quality trimming, and PRINSEQ (Schmieder and Edwards, 2011) for low-complexity filtering and sequence deduplication. In addition, Bowtie2 v2.2.1 was used to map reads to MetaPhlAn markers for the classification of bacterial species (Segata et al., 2012). We performed quality checks on raw sequences from all caecal samples using FastQC. Shotgun metagenomic sequencing data were then processed through analysis workflow that utilises Huttenhower Biobakery pipeline (Beghini et al., 2021), including Kneaddata, MetaPhlAn3 and HUMAnN3 to obtain species, genes, and pathways abundance matrix. Briefly, quality filtering and host genome decontamination (HUMAnN3) (Beghini et al., 2021) was performed using Trimmomatic and Bowtie2 (Langmead and Salzberg, 2012) via Kneaddata wrapper programme with following parameters: ILLUMINACLIP:/NexteraPE-PE.fa:2:30:10, SLIDINGWINDOW:5:25, MINLEN:60, LEADING:3, TRAILING:3. Taxonomic and functional profiling of the microbial community was performed using MetaPhlan3 and HUMAnN3 using default parameters. Next, gene abundance matrix was further collapsed by KEGG Orthology (KO) term and Gene Ontology (GO) term mapping via "humann_regroup_table" function provided within HUMAnN3.

Further microbiome data-handling was done in R (version 4.1.2) with the Rstudio GUI (version 1.4.1717). The iNEXT library (Hsieh et al., 2016) was used to compute alpha diversity for the first three hill numbers (Chao1, Shannon entropy and Simpson Index). Differences in alpha diversity were assessed using linear models (Bates et al., 2015). Principal component analysis (PCA) was performed on centred log-ratio (CLR) transformed values as a visual companion to the beta diversity analysis. Zeroes were imputed using the "unif" method (Lubbe et al., 2021). Beta diversity was computed in terms of Aitchison distance (Aitchison et al., 2000) (Euclidean distance of CLR-transformed counts) and differences in beta diversity were assessed using the PERMANOVA implementation from the vegan library using 10,000 permutations. Gut-Brain Modules (GBMs) were calculated using the R version of the Gomixer tool (Valles-Colomer et al., 2019). Differential abundance of taxa and functional modules was assessed by fitting linear mixed effects models on the CLR-transformed count tables. To correct for multiple testing in tests involving microbiome features, the Benjamini-Hochberg stepwise Bonferroni procedure was performed with a false discovery rate (FDR) q- value of 0.1 as a cutoff (Benjamini and Hochberg, 1995). R scripts are available online at https://github.com/thomazbastiaanssen/Tjazi. Plotting was handled using ggplot2.

2.3. Maternal plasma kynurenine metabolites

Tryptophan metabolites quantified included L-kynurenine (L-Kyn), 3-hydroxy-anthranilic acid (3-HANA) and were determined in plasma using HPLC with UV and fluorescence detector as previously described (Clarke et al., 2009; Kennedy et al., 2015). Briefly, the samples were spiked with internal standard (3-Nitro-L-Tyrosine) prior to being deproteinised with the addition of 20 μL of 4 M perchloric acid to 200 μL of plasma. The samples were centrifuged at 21,000 g for 15 min at 4 °C on a Hettich Mikro 22R centrifuge (AGB, Dublin, Ireland), and 100 μL of supernatant was then transferred to a HPLC vial for analysis on the HPLC system (consisting of a CBM-20A system controller, a LC-20AD pump, a CTO-20AC column oven at 30 °C, a SIL-20AC HT autosampler, a Prominence DGU-20A5R degasser, Shimadzu SPD-10 VP UV–Vis detector and Prominence RF-20A fluorescence detector).

All samples were injected onto a reversed phase Luna 3 μm C18 (2) 150 × 2 mm column (Phenomenex), which was protected by Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50 mM acetic acid, 100 mM zinc acetate with 3 % (v/v) acetonitrile and was filtered through Millipore 0.45 μm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30-min runtime at a flow rate of 0.3 mL/min after a 20 μl injection. The column was maintained at a temperature of 30 °C and samples/standards were kept at 8 °C in the cooled autoinjector prior to injection. The fluorescent detector was set at an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The UV detector was set to 330 nm. Tryptophan metabolites were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Internal standard peak height rations were measured and compared with standard injections using the software LabSolutions (Shimadzu) and results were expressed as ng/ml of plasma.

2.4. Whole brain metabolomics and integrated pathway analysis

Whole brains from 1 male and 1 female embryo per litter per experimental group were analysed for metabolomics at E18. The analysis was carried out by MS-Omics using a Thermo Scientific Vanquish LC coupled to Thermo Q Exactive HF MS. An electrospray ionization interface was used as ionization source. Analysis was performed in negative and positive ionization mode. The UPLC was performed using a slightly modified version of the protocol described by (Doneanu et al., 2011). Peak areas were extracted using Compound Discoverer 3.1 (Thermo Scientific). Identification of compounds were performed at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra, Level 2a: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm). Level 2b:identification by accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra, Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm). We considered only Level 1 data for PCA and integrated pathway analysis as they are measured more accurately against internal standards. SCFA detection was not possible using the UPLC/MS method and Compound Discoverer 3.1. Biostatistics were run in R (version 4.1.2) with the Rstudio GUI (version 1.4.1717). PCA was performed in CLR-transformed values (Aitchison et al., 2000) using the Tzazi library (Bastiaanssen et al., 2023a; Bastiaanssen et al., 2023b). PERMANOVA followed by a pairwise PERMANOVA was used to find structural

differences between treatments on a compositional level. The number of permutations as set to 1,000. To correct for multiple testing, Benjamin-Hochberg q-value post hoc procedure was performed with a q-value of 0.1 as a cutoff.

Integrated pathway analysis was performed incorporating the lists of differentially abundant metabolites and differentially expressed genes in the fetal brains at E18 using MetaboAnalyst software V6.0 with default parameter settings on all KEGG pathways (Pang et al., 2021) to identify potential pathways of interest that were modulated in the fetal brains by maternal diet. Integrated analysis allows for knowledge- and data-driven multi-omics investigation (Ewald et al., 2024). This analysis takes into account the enrichment ratio of components of the pathway, as well as their centrality in that pathway to calculate a p-value and pathway impact score (Pang et al., 2020). Data obtained from male and female brain metabolomics from each group were analysed altogether.

2.5. Microglia isolation, RNA extraction and qPCR

Microglia CD11b+ cells were isolated with CD11B (Microglia Microbeads Cat: 130-093-634, Miltenyi Biotec Inc, CA, USA) from 1 male embryo per litter per experimental group. Briefly, neural tissue from E18 brains was dissociated with Neural Tissue Dissociation Kit (Cat: 130-092-628 Miltenyi Biotec Inc, CA, USA). The magnetic labelling and separation of microglia was done in 10^5 cells from each brain via columns in a MACS Separator. Whole RNA was isolated from E18 brain CD11b+ cells with TRIzol Reagent (Invitrogen). Chloroform and isopropanol were added according to the manufacturer's protocol and the RNA pellet was solubilised in RNase-free water and stored in −80C until further use. RNA was reversed transcribed using the high-capacity cDNA kit (Thermo Fisher Scientific) in a G-storm thermocycler. Real-time PCR amplification was performed with Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step (Cat: 600825, Agilent Technologies) and analysed in a Lightcycler 480 II (Roche). Expression levels were calculated as the average of X replicated relative to a stably expressed housekeeper gene, *Gapdh*. Relative mRNA expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Predesigned primers were purchased from Eurofins Genomics (Table 2 Supplementary Fig. 2).

2.6. Amygdala RNA extraction and qPCR

Amygdala were isolated from animals at P50 (3–5 males and females per experimental group) and frozen immediately on dry ice. Whole RNA was isolated with the mirVana RNA Isolation Kit (Invitrogen). RNA was reversed transcribed using the high-capacity cDNA kit (Thermo Fisher Scientific) in a G-storm thermocycler. Real-time PCR amplification was performed with Brilliant II SYBR® Green qRT-PCR Master Mix Kit, 1-Step (Cat: 600825, Agilent Technologies) and analysed in a Lightcycler 480 II (Roche). Expression levels were calculated as the average of X replicated relative to a stably expressed housekeeper gene, *Gapdh*. Relative mRNA expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Predesigned primers were purchased from Eurofins Genomics (Table 2 Supplementary Fig. 2).

2.7. Behavioural testing

Behavioural testing was performed in adolescent male and female offspring between PND35 and PND45. All behaviour was performed between 9 am and 2 pm. All experiments were recorded using a ceiling-mounted camera. The behavioural apparatus was cleaned with 70 % ethanol and allowed to dry in between test runs.

2.7.1. Open field

The open-field (OF) test is used to assess locomotor activity and anxiety response to a novel stressful environment. Light was set at 60 lx. The test mouse was placed in the centre of an OF arena (Perspex box with grey base: 45 × 45 × 45 cm) and was allowed to explore the arena for 10 min. The distance moved and time in peripheral or central (0.5 of peripheral zone) zones were recorded using Ethovision video tracking system (Noldus Information Technology).

2.7.2. Elevated plus maze

The elevated plus maze (EPM) test is used to evaluate anxiety-like behaviour and was performed as previously described (Burokas et al., 2017). Briefly, an elevated grey plastic cross-shaped maze 1 m elevated from the floor, comprising two open (fearful) and two closed (safe) arms (50 × 5 × 15 cm walls). Mice were individually placed into the centre of the maze facing an open arm (to avoid direct entrance into a closed one) and were allowed 5-min free exploration under red light (~5 lx). The number of transitions in each arm were measured, for anxiety-like behaviour (entrance into an arm was defined as all four paws inside the arm) using Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016).

2.7.3. Light-dark box

The light–dark box (LDB) test is a widely used and well-validated assay for assessing anxiety-like behaviour (Cryan et al., 2003; Finger et al., 2010). The apparatus consisted of a plexiglass enclosure (44 cm × 21 cm × 21 cm) divided into two compartments (one light and one dark) by a partition, in which there was a small opening (12 cm × 5 cm) at the floor level. The light compartment was open roofed, with walls of transparent plexiglass and was brightly illuminated by a 60 W desk lamp overhead (approximately 1000 lx). The smaller, dark compartment (14 cm width) was closed-roofed and was constructed of black plexiglass. The mice were initially placed in the light compartment. Each animal received a single 10-min-trial, the number of transitions between the two parts (all four paws), latency to enter the dark part and the time spent in the light part were manually scored post-test using BORIS (Friard and Gamba, 2016).

2.7.4. Three-chambers social interaction test

The three-chambers social interaction test is widely used to evaluate sociability and social novelty recognition as previously described (Desbonnet et al., 2014). In brief, animals were placed in a rectangular apparatus (20 × 40 × 22 cm, L × W × H) divided into three connected chambers (left and right, and a smaller centre chamber), small circular openings allowed easy access to all compartments. The test was composed of three sequential 10 min trials:

(1) Habituation: the test animal was allowed to explore the three chambers containing only an inner mesh wire cage in either left or right chamber
(2) Sociability: an unfamiliar con-specific animal was placed in an inner mesh wire cage in either the left or right chamber, the alternative chamber had an empty inner cage
(3) Social novelty recognition: a novel conspecific mouse was placed into the previously empty inner mesh cage.

All mice were age- and sex-matched; each chamber was cleaned and lined with fresh bedding between trials. Time spent in active exploration of inner cages (t) was measured using BORIS (Friard and Gamba, 2016).

2.8. Statistical analysis

All statistical analysis (apart from the maternal caecal microbiota and foetal brain metabolomics analysis) was performed using IBM SPSS Statistics (IBM, SPSS Statistics V28). Before statistical analysis, data was analysed for normality using the Shapiro-Wilk test and homogeneity of variances using Levene's test. For statistical analysis of parametric data, we used an independent sample Student *t*-test, while for non-parametric data we used the Kolmogorov-Smirnov test. For the behavioural results from OF, EPM, and LDB we used two-way between-subjects ANOVAs

(with Tukey's post-hoc comparisons). For the three-chambers social interaction test we used repeated-measures three-way ANOVAs (with Bonferroni-adjusted post-hoc comparisons). Data is shown as mean ± SEM. Statistical significance was set at $p < 0.05$.

3. Results

3.1. mHFD led to altered intestinal length and weight parameters at term pregnancy

mHFD led to increased body weight in the dams from the first week

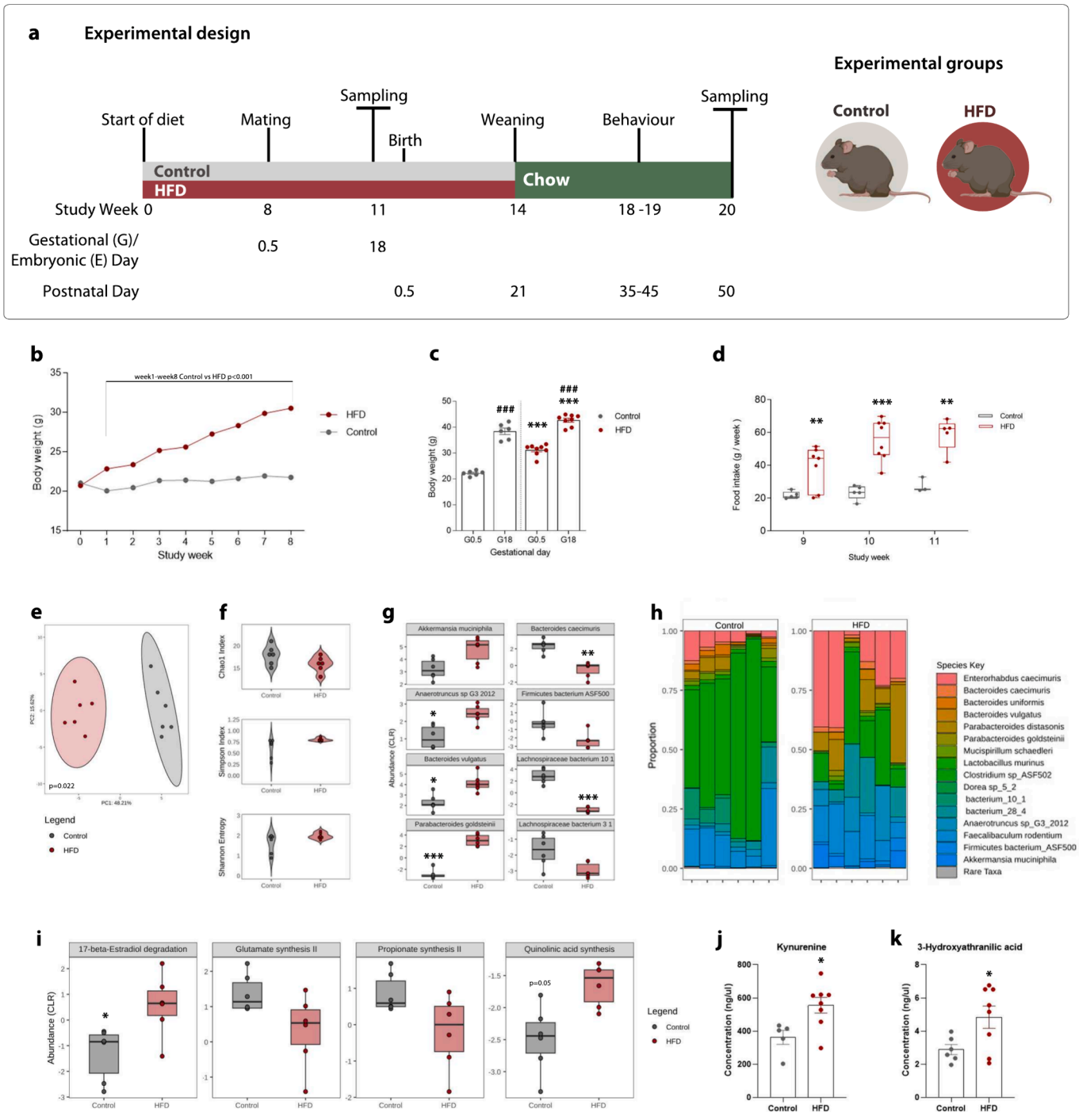

Fig. 1. Changes in maternal weight, caecal microbiota composition and function and plasma metabolites at term pregnancy induced by mHFD. a. Experimental design and experimental groups, b. Maternal weight from the beginning of the dietary intervention (week 0) until mating (week 8) (n = 8 animals/group), c. Body weight at G0.5 and G18 (n = 6–8 animals/group), d. Food intake during pregnancy week9-week11 (n = 5–8 animals/group), e. PCA plot of beta diversity of maternal caecal microbiota composition at G18 (n = 6 animals/group), f. Alpha diversity indices of maternal caecal microbiota composition at G18 (n = 6 animals/group), g. Differentially abundant species present in the maternal caecum at G18 (n = 6 animals/group), h. Mean relative abundance of major taxa in the maternal caecum at G18 (n = 6 animals/group), i. Differentially abundant gut-brain modules (GBMs) from the maternal caecal microbiota at G18 (n = 6 animals/group), j. Kynurenine levels in the maternal plasma at G18, k. 3-Hydroxy-anthralic acid levels in the maternal plasma at G18. *$p < 0.05$ mHFD vs Control, **$p < 0.01$ mHFD vs Control, ***$p < 0.01$ mHFD vs Control, ###$p < 0.01$ G0.5 vs G18. Values presented as mean ± SEM.

of the intervention and all the way throughout pregnancy and lactation compared with controls (Fig. 1b week1-week8 Control vs HFD $p < 0.001$ see Table 1 in Supplementary Fig. 2, and Fig. 1c G0.5 control vs HFD: $t[12] = -9.463$, $p < 0.001$, G18 control vs HFD: $t[12] = -3.064$, $p = 0.005$). Food intake was significantly higher in the mHFD dams compared to controls for the whole length of pregnancy (Fig. 1d, week9: control vs HFD $t[10] = -3.464$, $p = 0.006$, week10: control vs HFD $t[11] = -6.710$, $p < 0.001$, week11: control vs HFD $t[6] = -4.989$, $p = 0.001$). Caecum weight was significantly lower in HFD-fed dams (Supplementary Fig. 1i $t[12] = 2.269$, $p = 0.021$). Additionally, the length of the small intestine (Supplementary Fig. 1k $t[12] = 3.606$, $p = 0.002$) and colon (Supplementary Fig. 1j $t[12] = 1.870$, $p = 0.043$) was significantly decreased in response to HFD at term pregnancy (G18), indicating that HFD has a general effect on the intestinal compartment. However, the number of fetuses (Supplementary Fig. 1l $t[12] = 1.225$, $p = 0.122$) and the sex-ratio per litter (Supplementary Fig. 1m $t[10] = -0.507$, $p = 0.312$) was not affected by the maternal diet. Overall, maternal HFD induced an obesogenic effect on the dam from preconception to term pregnancy with significant effects on the maternal intestinal length and caecum weight.

3.2. Maternal caecal microbiota composition and function is altered in response to mHFD

Microbiota composition of the maternal caecum was significantly different in response to maternal diet at G18 (Fig. 1e $F[1,10] = 7.897$, $p = 0.022$), while for the **α**-diversity, indicating the richness and evenness of the microbiota composition, there was only a trend for the three indices (Fig. 1f Chao: $t[10] = -1.785$, $p = 0.106$, Simpson: $t[5] = 2.132$, $p = 0.083$, Shannon: $t[6] = 1.795$, $p = 0.122$). On a species level, the abundance of *Akkermansia muciniphila*, *Anaerotruncuc spp*, *Bacteroides vulgatus,* and *Parabacteroides goldsteinii* was increased in the caecum of HFD dams compared to controls, while *Bacteroides caecimuris*, *Firmicutes bacterium ASF500*, *Lachnospiraceae bacterium 101*, and *Lachnospiraceae bacterium 31* were decreased in the caecum of HFD dams compared with controls (Fig. 1g, for statistics see Table 1 in Supplementary Fig. 2). Notably, *Akkermansia muciniphila* and *Parabacteroides goldsteiini* are known commensals inversely correlated with maternal obesity during pregnancy (Everard et al., 2013; Wu et al., 2019). *Firmicutes ASF500 and Lachnospiraceae bacterium 101* and *31*, which are beneficial SCFA-producing bacteria, exhibited significantly lower abundance in the mHFD caecum compared to controls.

Using *in silico* predictive modelling we investigated GBMs, allowing us to identify pathways important for brain function that may be influenced by the maternal intestinal microbes in response to mHFD at term pregnancy (Bastiaanssen et al., 2021; Valles-Colomer et al., 2019). Pathways that were significantly upregulated were associated with quinolinic acid synthesis ($t[9.67] = 2.224$, $p = 0.05$) and 17-beta estradiol degradation ($t[9.87] = 2.517$, $p = 0.03$), while the pathways that were downregulated in response to mHFD were glutamate synthesis ($t[7.71] = -2.03$, $p = 0.078$) and propionate synthesis ($t[6.83] = -1.979$, $p = 0.08$) (Fig. 1i). Quinolinic acid (QA), a known neurotoxic agent with strong excitatory potential for the brain (Guillemin, 2012) and a metabolite within the kynurenine pathway, showed increased levels in the mHFD GBMs. To validate the potential gut-brain communication involving quinolinic acid, we assessed the levels of kynurenine pathway metabolites in the maternal plasma. As anticipated, we observed significantly elevated levels of kynurenine (Fig. 1j $t[11] = 2.814$, $p = 0.008$) and 3-hydroxyanthralic acid (3-HANA) (Fig. 1k $t[12] = 2.658$, $p = 0.012$) in the plasma of mHFD dams compared to controls. These findings suggest that mHFD induces alterations in the production of microbially-derived metabolites, particularly promoting the synthesis of kynurenine metabolites in the gut and subsequently increasing its levels in the maternal circulation. As this shift could potentially alter embryonic brain development we investigated if metabolites linked to this pathway could be detected in embryonic brains at E18.

3.3. mHFD alters genes and metabolites linked to excitatory neurotransmission in fetal brains

Analysis of the whole fetal brain metabolome at E18 revealed a trend toward an effect of mHFD on the overall composition of brain metabolites (Fig. 2a $F[1,20] = 1.653$, $p = 0.06$). Targeting specific metabolites of interest revealed significant differences in the fetal brains of mHFD group compared with controls (Fig. 2b–f). Glutamate was present in significantly lower levels in the brains of embryos whose mother had received a mHFD compared with controls (Fig. 2b $t[22] = 1.531$, $p = 0.01$), confirming the maternal GBMs (Fig. 1i). Glutamate is the major excitatory neurotransmitter, an imbalance of which might prime neuronal excitation, glial cell function with implications for behaviour later in life. It has been suggested previously that the glutathione cycle acts as a glutamate reserve for the brain (Sedlak et al., 2019). Glutathione dissulphide (GSSG), which is the reduced form of glutathione, is decreased in the mHFD fetal brains compared with controls (Fig. 2c $t[21] = 5.000$, $p = 0.041$). GSSG is an endogenous neuromodulator as it binds on the NMDA and AMPA glutamatergic receptors in the brain (Steullet et al., 2006; Varga et al., 1997). Collectively, glutamate and GSSG which both bind glutamatergic receptors, are present in lower levels in the fetal brains originating from mHFD dams compared with controls. We therefore hypothesise that glutamatergic signalling in the fetal brains is altered in response to mHFD. Similarly, complementing our maternal plasma results we observed significantly higher levels of kynurenine in the fetal brains of mice originating from mHFD-fed dams compared with controls (Fig. 2e $t[22] = -1.898$, $p = 0.035$). While tryptophan (Fig. 2f $t[22] = -0.129$, $p = 0.449$) and glutathione (Fig. 2d $t[22] = -0.314$, $p = 0.378$) levels were unchanged.

In order to investigate the regulation of glutamate in embryonic brains, we chose to interrogate the gene expression of glutamate receptors (*mGlur2*, *mGlur5*, *mGlur7*, *iGrin2a*), glutamate transporter EAAT1 (*Glt1*), scaffold protein *Shank2*, enzyme glutamine synthase (*GS*) important for the conversion of glutamate to glutamine and transcription factor *Tbr1*, which is selectively expressed in glutamatergic neurons and linked to neuronal plasticity.

The expression of receptors *GluR7* and *Grin2a* was downregulated (Fig. 2g $t[15] = 2.286$, $p = 0.019$ and $t[15] = -2.437$, $p = 0.014$ respectively) in the mHFD embryo brains compared with controls. Both of these receptors have been implicated in hyperactivity disorder in children (Dorval et al., 2007; Park et al., 2013; Turic et al., 2004). Additionally, the expression of *Shank2*, a scaffold protein that links metabotropic and ionotropic glutamate receptors was downregulated in the mHFD embryo brains compared with controls (Fig. 2g $t[15] = 2.557$, $p = 0.011$). The gene expression of metabotropic glutamate receptors *Glur2* and *Glur5* was not significantly altered at E18 brains (Fig. 2g $t[15] = 0.756$, $p = 0.231$ and $t[15] = 0.878$, $p = 0.210$ respectively). The mRNA expression of *GS* (Fig. 2g $t[15] = 0.241$, $p = 0.815$), glutamate transporter *Glt1* (Fig. 2g $t[14] = 1.373$, $p = 0.104$) and transcription factor *Tbr1* (Fig. 2g $t[15] = 0.825$, $p = 0.211$) were not altered in the embryonic brains at E18 in response to maternal diet.

In order to identify pathways of interest for brain function, we performed an integrated pathway analysis (Pang et al., 2020, 2021) using metabolites and genes that were significantly affected by mHFD at E18. Results demonstrated that glutamatergic synapse was the most affected pathways ($p_{adj} < 0.001$, impact = 0.274) in response to mHFD in the embryonic brains (Fig. 2h). Other pathways of interest include long-term potentiation ($p_{adj} = 0.03$, impact = 0.225), neuroactive ligand-receptor interaction (p_{adj}:0.03, impact: 0.045), glutathione metabolism ($p_{adj} = 0.04$, impact = 0.116), and glutamate and glutamine metabolism ($p_{adj} = 0.211$, impact = 0.188) (Fig. 2h). Taken together, the imbalance of glutamate levels and glutamatergic receptor expression in embryonic brains in response to mHFD may be associated with alterations in glutamatergic function in the fetal brains with potential implications for behaviour later in life.

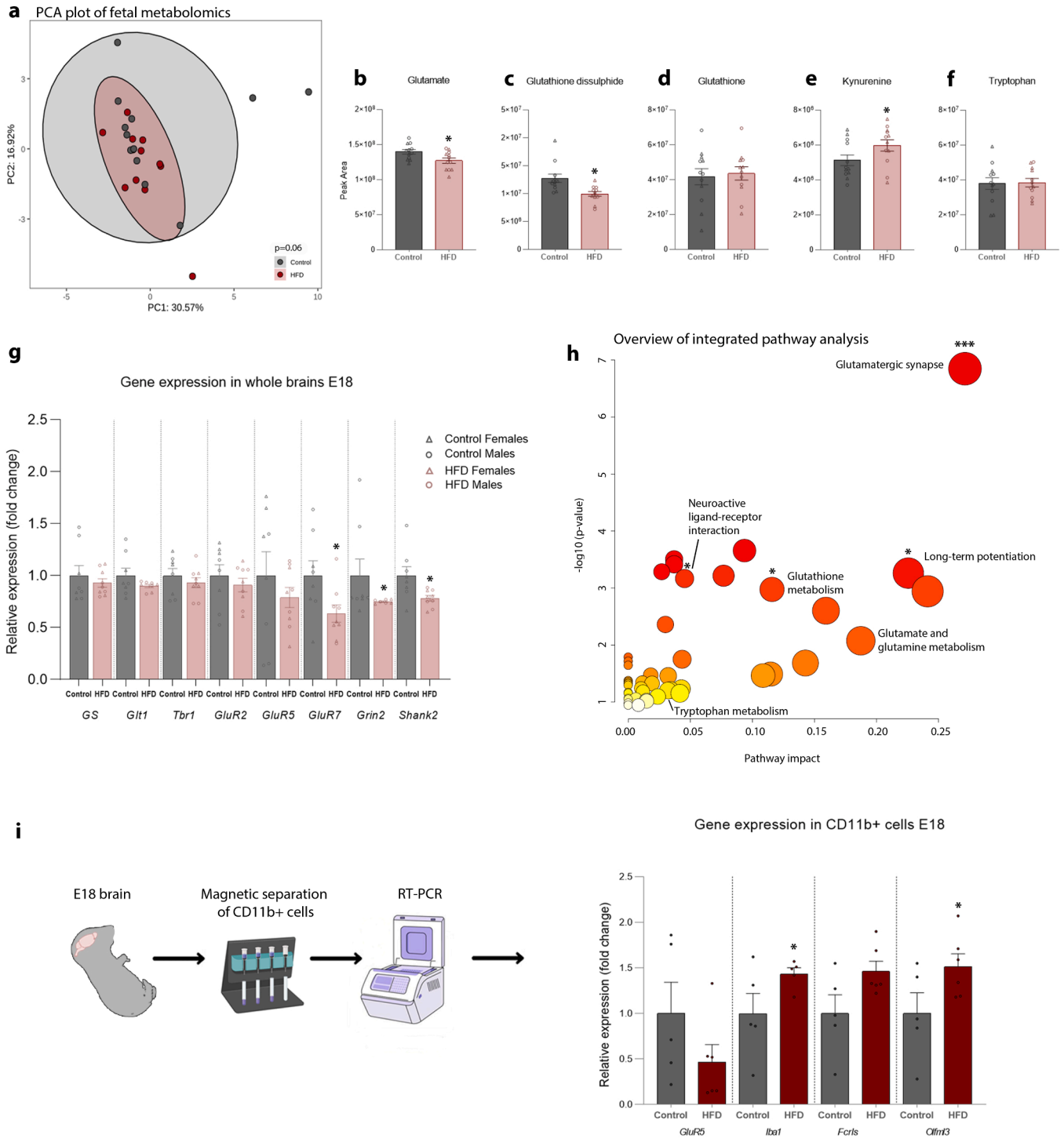

Fig. 2. mHFD changed fetal brain metabolome, transcriptome, and microglia maturation genes at term pregnancy. a. PCA plot of fetal whole brain metabolome at E18 (n = 12 per group), b-f. Metabolites present in the fetal brains at E18 (n = 12 per group), g. Gene expression in E18 whole brains (n = 7–9 per group), h. Overview of integrated pathway analysis in mHFD samples based on the significantly altered metabolites and gene expression in E18 brains: pathway impact represents a combination of the centrality of the gene/metabolite and pathway enrichment results; higher impact values represent the relative importance of the gene/metabolite within that pathway. The size of the circle indicates the impact of the pathway while the colour represents the significance (the more intense the red colour, the lower the p value). i. Schematic representation of CD11b+ cell isolation from whole embryo brains and gene expression in CD11b+ cells at E18 (only males: n = 5–6 per group). *p < 0.05 mHFD vs Control, **p < 0.01 mHFD vs Control, ***p < 0.01 mHFD vs Control. Values presented as mean ± SEM.

3.4. mHFD led to alterations in glutamate-related genes in E18 microglia

Microglia, the predominant immune cells within the brain, play a crucial role in clearing debris throughout life and shapes neuronal connections during development (Borst et al., 2021). We observed significantly increased expression of *Iba1* in microglia isolated from mHFD embryonic male brains compared with controls (Fig. 2i $t[8] = -1.886$, $p = 0.048$), and this upregulation has been previously linked with microglia activation (Ito et al., 1998). Additionally, there was a trend toward increased expression of *Fcrls* in response to mHFD (Fig. 2i *t*

[9] = 1.826, p = 0.082), a maturation marker that is uniformly expressed in microglia (Hammond et al., 2019). An increase in *Fcrls* expression could indicate alterations in the microglia maturation process, suggesting that microglia from mHFD embryonic brains are less mature when compared to controls at E18. It is widely known that CD11b is a general marker for monocytes, macrophages and microglia. To investigate if the CD11b + cells that we isolated were indeed mature microglia, we screened for the *Olfml3* gene which is selectively expressed in microglia and not in other CD11b + cells. Indeed, the gene was expressed in both groups, but significantly higher in the mHFD group when compared to controls (Fig. 2i t[9] = −1.996, p = 0.038). Moreover, we observed a trend for lower expression of *Glur5* in mHFD microglia compared with controls (Fig. 2i t[9] = 1.441, p = 0.092). Collectively, isolated CD11b + cells derived from embryonic brains of offspring exposed to a mHFD exhibit elevated levels of maturation markers and genes associated with microglia activation compared to controls. This implies that maternal diet and the corresponding microbial signals during the perinatal period could potentially influence the

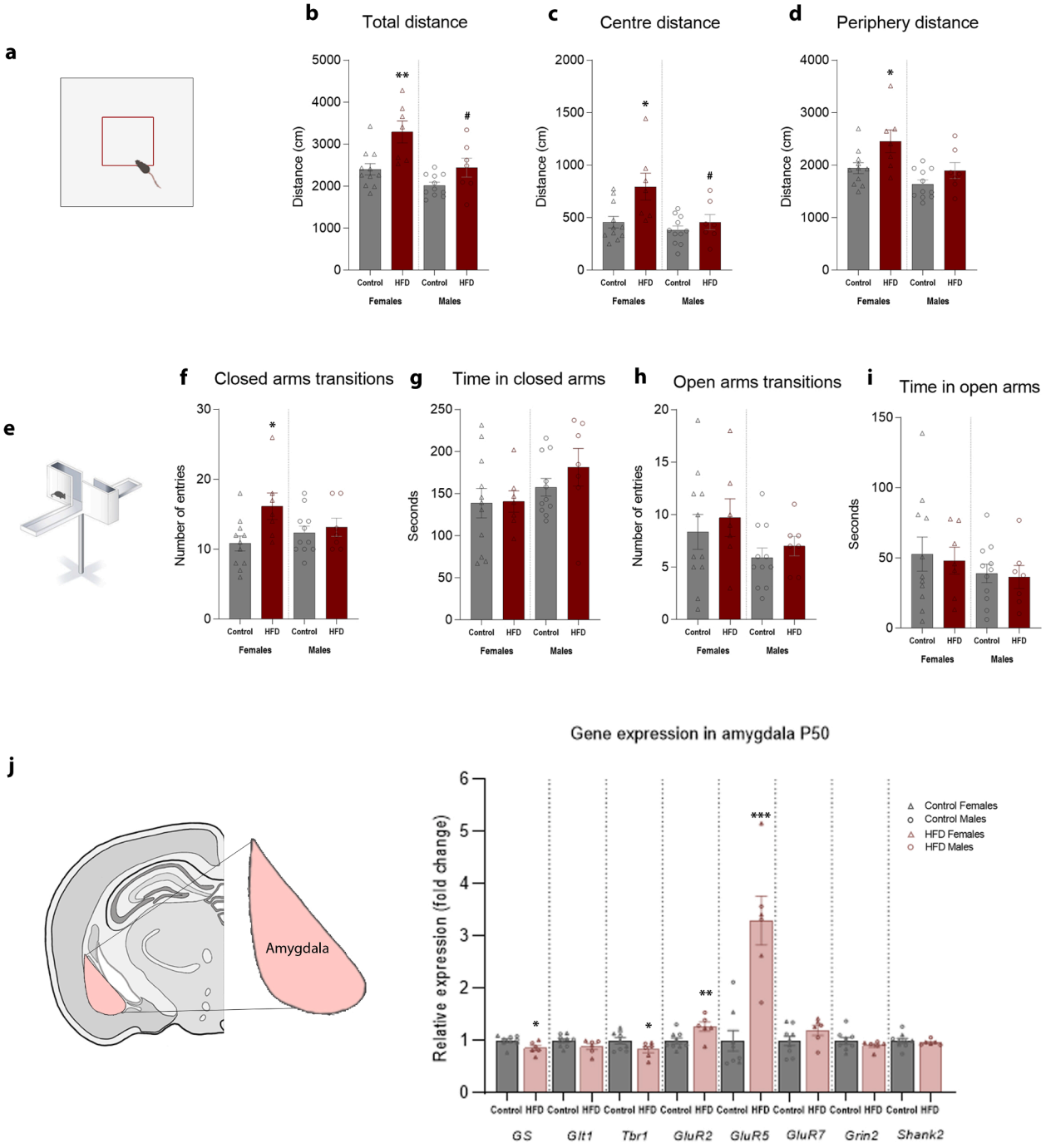

Fig. 3. mHFD increased hyperactivity and anxiety-like behaviour in the female adolescent offspring, along with glutamatergic genes in the amygdala. a. Schematic of open field arena, b. Total distance travelled in the whole open field arena, c. Distance travelled in the centre of the open field arena, d. Distance travelled in the periphery of the open field arena, e. Schematic of the elevated plus maze (EPM), f. Transitions in the closed arms of the EPM, g. Time spent in the closed arms of the EPM, h. Transitions in the open arms of the EPM, i. Time spent in the open arms of the EPM, j. Schematic representation of amygdala and gene expression of glutamate-related genes in amygdala at P50. n = 7–11 per group, *p < 0.05 mHFD vs Control, **p < 0.01 mHFD vs Control, ***p < 0.01 mHFD vs Control, #p < 0.05 male vs female. Values presented as mean ± SEM.

function of embryonic microglia.

3.5. mHFD led to increased locomotion, anxiety-like behaviour and alterations in glutamatergic gene expression in the amygdala of adolescent offspring

We next analysed the behaviour of adolescent male and female offspring in the open field (OF) test. Regarding the total distance travelled, there was no significant interaction between maternal diet and sex (Fig. 3b diet*sex: $F[1,32] = 1.96$, $p = 0.171$), but there was a significant effect of diet and sex alone (Fig. 3b diet: $F[1,32] = 1.96$, $p < 0.001$ and sex: $F[1,32] = 1.96$, $p < 0.001$). Specifically, mHFD females travelled significantly more distance in the OF compared to female controls (Fig. 3b $p = 0.004$), and when compared to mHFD males (Fig. 3b $p = 0.014$). There was no significant interaction between maternal diet and sex in relation to centre distance travelled (Fig. 3c diet*sex $F[1,32] = 3.309$, $p < 0.078$). However, there was an effect of diet (Fig. 3c F[1,32] $= 3.309$, $p = 0.007$) and sex alone (Fig. 3c F[1,32] $= 3.309$, $p = 0.007$), where mHFD females travelled significantly more distance in the centre compared to female controls ($p = 0.012$) and also mHFD males ($p = 0.026$). There was no diet*sex interaction in the peripheral distance travelled (Fig. 3d diet*sex: $F[1,32] = 0.881$, $p = 0.355$). However, there was a significant effect of diet (Fig. 3d F[1,32] $= 0.881$, $p = 0.007$) and an effect of sex (Fig. 3d F[1,32] $= 0.881$, $p = 0.003$) where mHFD females had a greater distance travelled compared to female controls ($p = 0.049$) and a trend compared with males ($p = 0.055$). Collectively, these results suggest that mHFD results in sex-specific effects where mHFD females demonstrate increased locomotor activity, indicative of a hyperactive phenotype.

Next, we assessed anxiety-like behaviour in the EPM. There was a trend towards an interaction between maternal diet and sex in the number of entries to the closed arms of the EPM (Fig. 3f diet*sex: $F[1,32] = 3.171$, $p = 0.084$). There was a significant effect of diet (Fig. 3f diet: $F[1,32] = 3.171$, $p = 0.029$) were mHFD had a significantly increased number of entries in the closed arms compared to control females. Interestingly, there was a trend for male offspring to spend more time in the closed arms compared with females (Fig. 3g sex: $F[1,32] = 0.446$, $p = 0.078$), but that was independent of maternal diet (Fig. 3g diet: $F[1,32] = 0.446$, $p = 0.439$). However, there was no interaction between offspring sex and maternal diet in the number of transitions to the open arms (diet*sex: $F[1,32] = 0.008$, p = 0.929), the time spent in closed (diet*sex: $F[1,32] = 0.446$, p = 0.509) and open arms (diet*sex: $F[1,32] = 0.011$, p = 0.918) of the EPM (Fig. 3h-i).

The LDB is a complementary test to assess anxiety-like behaviour which also taps into the rodent's natural approach avoidance behaviour to brightly lit areas (Hascoët et al., 2001). There was no significant interaction between maternal diet and offspring sex in the latency to enter the dark compartment (diet*sex: $F[1,29] = 1.139$, $p = 0.295$) nor the time spent in light (diet*sex: $F[1,29] = 1.984$, $p = 0.170$), but there was a trend for an effect of offspring sex, with females displaying higher latency to enter the dark compartment (sex: $F[1,29] = 1.139$, $p = 0.051$) and spending more time in light (sex: $F[1,29] = 1.984$, $p = 0.05$) when compared to males (Supplementary Fig. 1f-h).

Next, we assessed the social behaviour of male and female adolescent mice. There was a significant preference for the mouse over the object in sociability phase of the test in both diets and sexes (Supplementary Fig. 1a OvsM: $F[1,31] = 87.178$, $p < 0.001$). A significant interaction was detected between the social stimulus and offspring sex (OvsM*sex: $F[1,31] = 87.178$, $p = 0.008$), and a trend between the social stimulus and diet (OvsM*diet: $F[1,31] = 87.178$, $p = 0.071$) but the interaction of the three variables was not significant (Supplementary Fig. 1a OvsM*-diet*sex: $F[1,31] = 87.178$, $p = 0.362$). In the novelty phase there was no significant preference for the novel versus the familiar conspecific (Supplementary Fig. 1b FvsN: $F[1,31] = 2.645$, $p = 0.114$). The interactions between offspring sex, maternal diet and novelty preference were not significantly different between groups (Supplementary Fig. 1b FvsN*diet*sex: $F[1,31] = 0.044$, $p = 0.715$). Taken together, mHFD did not have enduring effects on social behaviour in adolescent offspring.

Furthermore, we assessed the enduring effects of mHFD on the expression of glutamatergic genes in adolescent offspring at P50. To complement our behavioural outputs, we assessed these genes in the amygdala, a brain region that is implicated in hyperactivity and anxiety-like behaviour. *Tbr1* is a gene expressed by excitatory glutamatergic neurons within the amygdala and modulates the expression of neuronal activation markers (Chuang et al., 2015; Glendining et al., 2018). In our study, the expression of *Tbr1* was significantly decreased in response to maternal diet (Fig. 3j $t[13] = 1.953$, $p = 0.036$). Interestingly, we found that the expression of two metabotropic glutamatergic receptors *GluR2* and *Glur5* was significantly increased in mHFD offspring compared with controls (Fig. 3j $t[13] = -2.794$, $p = 0.008$ and $t[12] = -5.036$, $p < 0.001$ respectively). Additionally, there was a trend toward increased expression of *GluR7* (Fig. 3j $t[13] = -1.453$, $p = 0.085$) in mHFD offspring. We also interrogated the expression of the enzyme *GS* and glutamate transporter *Glt1*, since glutamate and GSSG are downregulated in mHFD offspring compared with controls in brain metabolomics at E18. *GS* was significantly downregulated in the mHFD group compared with controls (Fig. 3j $t[13] = 2.460$, $p = 0.014$), while there was a trend towards downregulation of *Glt1* in mHFD offspring compared with controls (Fig. 3j $t[13] = 1.712$, $p = 0.055$) in adolescence. Collectively, mHFD during the perinatal period had enduring effects on the expression of glutamatergic genes in the amygdala, which may contribute to offspring behaviour.

4. Discussion

Investigating the influence of maternal diet on offspring brain and behaviour is crucial, given the emerging recognition of maternal overweight and obesity as significant risk factors for offspring health (Bilbo and Tsang, 2010; Edlow, 2017; Godfrey et al., 2017; Kimura et al., 2020; Kong et al., 2020). Recent studies indicate a growing role for maternal microbiota composition and its metabolites in priming offspring neurodevelopment and behaviour (Di Gesù et al., 2021; Pronovost et al., 2023; Vuong et al., 2020). Here, we report the maternal HFD widespread effects on the diversity and function of the maternal caecal microbiota, resulting in alterations in metabolic pathways and metabolite levels in the maternal plasma, fetal brain transcriptome, metabolome, and subsequent adolescent brain function and behaviour.

Changes is the maternal microbiota observed in our study are in line with previous data in rodent and in human models of metabolic dysfunction (Serino et al., 2011; Turnbaugh et al., 2006). The expansion of *Akkermansia* that we observed in mHFD has been previously linked with increasing energy storage (De Siena et al., 2021). *Bacteroides vulgatus* is increased in the caeca of mHFD dams, which is a controversial result as it is known for its anti-obesogenic effects. However, we speculate that this might be an adaptation to long-term dietary intervention and crosstalk with hosts' metabolism to reduce the metabolic effect of mHFD. The negative feedback of this microbe as a response to high levels of lipids present in the diet has been reported in another preclinical study (Xu et al., 2023). Additionally, in a clinical study of obese pregnant individuals, the levels of *Bacteroides* were increased compared with lean controls (Collado et al., 2008). A higher abundance of *Anaerotroncus sp G32012* in the gut has been associated with increased levels of triglycerides in circulation (Guo et al., 2023) generally observed in obese subjects. In another study, the enrichment of *Anaerotruncus* in the third trimester has been linked with gestational diabetes, a comorbidity of obesity during pregnancy (Crusell et al., 2018; Hasan et al., 2018). Furthermore, microbiota analysis revealed significantly lower abundance of SCFA-producing bacteria (*Firmicutes ASF500 and Lachnospiraceae bacterium 101* and *31*) in mHFD caeca compared with controls. Decreased levels of SCFA-producing bacteria in the maternal intestines might translate to decreased levels in circulation and therefore, impact the microbiota-brain communication during pregnancy. Although the

mechanism via which SCFAs impact the brain is still unresolved and may involve direct effects or indirect via the immune system or vagus nerve (O'Riordan et al., 2022). Interestingly, lower maternal SCFA serum levels have been associated with worse neurodevelopmental outcomes in the offspring (Hernández-Martínez et al., 2022), as well as disruption of energy metabolism and programming the differentiation of neural, intestinal, and pancreatic cells in the offspring (Kimura et al., 2020). Furthermore, lower levels of SCFAs during pregnancy can be detrimental for brain development and behaviour later in life (Erny et al., 2015; Yu et al., 2020). Our study is limited by the lack of temporal dynamics of maternal microbiota composition during the intervention and maternal serum and fetal brain SCFAs levels at term pregnancy. Even though microbiota composition results agree with the literature, microbiota adaptation to dietary components during this period as well as the crosstalk among diet, hormonal and metabolic remodelling perinatally might explain the differences between our study compared with others.

Apart from microbiota composition, we found that mHFD altered GBMs, a proxy for maternal microbiota function (Bastiaanssen et al., 2021; Valles-Colomer et al., 2019), with increased levels of excitotoxic mediators compared with controls. Additionally, we found that maternal plasma metabolites with neurotoxic potential, such as kynurenine and 3HA, are upregulated in the circulation of mHFD dams at term pregnancy. Kynurenine pathway metabolites play a key role in pregnancy, including the regulation of vascular tone in the mother's placenta and immune tolerance (Van Zundert et al., 2024). Moreover, levels of kynurenine pathway metabolites in the maternal plasma are associated with depressive symptoms during pregnancy and post-partum (Nazzari et al., 2020). Upregulation of the kynurenine pathway in the brain is linked to increased neuronal excitotoxicity, cognitive decline, and neurodegenerative disorders (Gheorghe et al., 2019; O'Mahony et al., 2015; Potter et al., 2010; Schwarcz et al., 2012). It has been suggested in the past that kynurenine and downstream metabolites alter the regulation of glutamate signalling in the brain directly, by binding to glutamatergic receptors and indirectly, by causing over-release of glutamate in the extracellular space. We therefore hypothesised that these metabolites could reach the fetus via the placenta, impact the fetal brain transcriptome and metabolome and prime brain development and adolescent behaviour. Further studies are needed to dissect the exact temporality of such potential changes. The increased presence of the neurotoxic metabolite kynurenine was confirmed in fetal brains from mHFD by metabolomics analysis, along with lower levels of GSSG and glutamic acid at E18. Therefore, exploring glutamatergic gene expression was of great importance. The downregulation of glutamate-related genes in whole brains of mHFD embryos compared with controls includes the metabotropic glutamate receptor *Glur7*, the subunit of the NMDA receptor *Grin2a* as well as the scaffold protein *Shank2* that is involved in bridging metabotropic and ionotropic receptors (Sheng and Kim, 2000). *Shank2* has also been implicated in hyperactivity and ASD phenotype in humans and animals (Schmeisser et al., 2012; Zaslavsky et al., 2019). Mice lacking *Shank2* display hyperactivity and ASD-like behaviour mediated by changes in synaptic plasticity, NMDA receptor expression and glutamatergic transmission (Schmeisser et al., 2012). Integration of gene expression and metabolomics data for pathway analysis revealed that mHFD during pregnancy led to significant changes in brain neurochemistry, with high impact on the glutamatergic synapse pathway in the mHFD embryo brains at E18. The levels of glutamate present in fetal brains were decreased in response to mHFD which might explain the downregulation of glutamate-receptor genes in E18 mHFD brains compared to controls and suggests that there is a potential protective mechanism for embryonic brains to avoid the neurotoxic effects of kynurenine and its metabolites on glutamate-induced excitotoxicity.

During early life development, the maturation of microglia is linked with their ability to effectively prune developing neuronal synapses (Paolicelli et al., 2011). Alterations in microglia activation and maturation lead to changes in neuronal pruning and brain function (Pasciuto et al., 2020). In the current study, we provide evidence of upregulation of genes relevant for microglia maturation and activation at E18. It has been demonstrated that mHFD is detrimental for offspring microglia function and morphology (Bordeleau et al., 2021, 2022; Musillo et al., 2023) with long-term implications for key homeostatic microglia function in adolescence (Musillo et al., 2023). Given that microbiota composition regulates microglia maturation, and specifically SCFA supplementation in animals lacking microbes rescued microglia maturation (Erny et al., 2015), we suggest that changes seen in fetal microglia could be related to functional changes in maternal microbiota composition imposed by the diet. Even though we did not measure SCFAs in maternal serum, we reported a lower abundance of SCFA-producing bacteria in mHFD caeca compared with controls. We suggest that fetal exposure to mHFD and the associated microbiota and circulating maternal metabolites alter the maturation and function of fetal microglia. Aberrant microglia could ultimately induce over-pruning of excitatory glutamatergic neurons, leading to an imbalance of glutamate and glutamatergic receptors in mHFD brains at term pregnancy. However, the implication of such microglia alterations and its enduring effects on behaviour merit further analysis. Although our whole-brain gene expression analysis was performed on both sexes, our analysis on the role of maternal diet on microglia in the prenatal environment is limited to males at E18 and future studies should also focus on analysis in females.

In the current work, we reported that mHFD led to increased anxiety and hyperactivity specifically in the female adolescent offspring. Others have reported similar results in female offspring originating from mHFD litters (Glendining et al., 2018; Kang et al., 2014; Sullivan et al., 2010). Supporting data from gene expression in the amygdala, an area implicated in anxiety-related behaviour in rodents (Felix-Ortiz et al., 2013) and humans (Etkin and Wager, 2007; Parsaei et al., 2024), showed that genes relevant for glutamatergic neuronal activity are altered in response to mHFD. Increased attention has been attributed to glutamate receptors (Huang et al., 2019) and, particularly, to GluR5 in relation to locomotor hyperactivity in rodents (Halberstadt et al., 2011). In our study we have demonstrated that the regulation of glutamate receptors by maternal diet is altered at embryonic and adolescent stages, and we have reported locomotor hyperactivity in mHFD female adolescent offspring. Our results indicate sexual dimorphism on the effect of mHFD on adolescent offspring behaviour, that is in line with recent literature findings on increased emotionality in female offspring (Musillo et al., 2023). We suggest that the behavioural changes we observed might be partly attributed to altered regulation of glutamatergic transmission throughout development in response to maternal diet and relevant microbiota signals. However, we were unable to reproduce the social deficits seen in the adolescent offspring of mHFD model (Buffington et al., 2016; Di Gesù et al., 2022). Recently, another similar study suggested that the effect of mHFD could be driven by fibre content (Morrison et al., 2020). Although our mHFD was very similar to Buffington et al., 2016 and Di Gesù et al., 2022, we used a refined diet as a control while they used a regular chow. In our case, both the control and mHFD have comparable amounts of fibre content. Differences in microbiota composition and behavioural readouts with Buffington et al.,2016 and others could incentivise the notion of fibre content being the main driver of those changes. Furthermore, the fact that the dams originated from different animal facilities (Mushegian, 2017), and we did not use automated analysis or evaluated reciprocal social interaction, could account for the differences seen in our behavioural output compared with others. The data provided in the current work discuss the mHFD programming of anxiety and hyperactivity as well as glutamatergic regulation in adolescence. Conclusively, maternal diet perinatally had a long-lasting, enduring impact on the offspring brain and behaviour with maternal microbiota holding a central role in mediating these changes in the offspring.

One of the limitations of the present study is that we did not evaluate

the temporal effects of maternal diet during pregnancy on maternal microbiota, fetal brain metabolomics, and microglia analysis. Temporal dynamics would be more informative regarding the overall pregnancy-related changes in the gut microbiota (Koren et al., 2012) and the fetal brain, yet choosing E18 as our unique timepoint during pregnancy allowed us to explore in detail the perinatal period, which is a critical time window for offspring brain development and neurobehavioural outcomes (Morais et al., 2020). It is important to note here that in the current study, the fetal brains were still actively impacted by maternal diet, and transplacental signals from the maternal microbes probably facilitated the priming of pathways linked to microglia activation and glutamatergic signalling. In contrast, during adolescence the animals have been weaned to a chow diet, therefore the effects seen at that timepoint are attributed to the mHFD impact on the offspring during the perinatal and lactation period. Such signals during critical developmental windows of opportunity like pregnancy and lactation, have long-lasting impacts on the brain and behaviour of the offspring during adolescence (Lynch et al., 2023). Our findings validate the perinatal period as a critical timepoint for microbiota-targeted interventions, aimed at reducing the risk for adverse neurodevelopmental outcomes in the offspring. It is hypothesised that shifting the microbiota composition would prevent kynurenine pathway metabolites from interacting with the fetal brain. Whether a short-term antibiotic-induced microbiota depletion, selective probiotic, prebiotic, or symbiotic formulations would counter the effects of mHFD-induced offspring brain priming is yet to be determined. Although more experiments are required to confirm microglial cells as valid targets, SCFAs intervention could be a potential strategy to compensate for the decreased abundance of SCFA-producing bacteria in the maternal gut during pregnancy and associated signals to the fetal brain. As the prevalence of maternal obesity rises, there is an increasing need to unravel the mechanisms via which maternal diet affects the offspring's brain and behaviour. Our data suggest that more research is essential to unmask the role of microbiota on mediating the effects of HFD on offspring brain and behaviour. Moreover, large-scale epidemiological studies are crucial in order to understand and establish a causative role of the impact of maternal diet and obesity on the gut microbiota signalling to the foetal brain *in utero*.

CRediT authorship contribution statement

Anna Ratsika: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Formal analysis, Conceptualization. **Martin G. Codagnone:** Writing – review & editing, Visualization, Supervision, Software, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Thomaz F.S. Bastiaanssen:** Visualization, Software, Investigation, Data curation. **Fabiana A. Hoffmann Sarda:** Writing – review & editing, Investigation. **Caoimhe M.K. Lynch:** Writing – review & editing, Investigation. **Ana Paula Ventura-Silva:** Writing – review & editing, Investigation. **Cristina Rosell-Cardona:** Writing – review & editing, Investigation. **Valentina Caputi:** Writing – review & editing, Investigation. **Catherine Stanton:** Writing – review & editing, Investigation. **Christine Fülling:** Writing – review & editing, Investigation. **Gerard Clarke:** Conceptualization, Methodology, Supervision. **John F. Cryan:** Conceptualization, Methodology, Formal analysis, Supervision, Funding acquisition.

Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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