Conjugated GLP-1 and Estradiol as a Novel Treatment for Age-related Cognitive Decline in Males and Females

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Abstract
Middle age represents a critical window for metabolic and cognitive health, particularly in the context of rising obesity and diabetes rates. Glucagon-like peptide-1 (GLP-1)-based therapies, which regulate blood glucose and body weight, show sex-specific effects, with estradiol potentiating their metabolic benefits. However, research on GLP-1's cognitive and neuroprotective roles has largely been conducted in males. Here, we investigated the effects of GLP-1 conjugated to estradiol (GE2) on metabolism, cognition, inflammation and neurogenesis in the hippocampus of middle-aged male and female rats fed a standard (SD) or Western (WD) diet. In both sexes, WD increased body weight, and plasma leptin levels. Furthermore, GE2 treatment led to weight loss, enhanced cued and contextual fear memory, reduced inflammation in the hippocampus in SD rats and increased neurogenesis in the dorsal hippocampus. Sex-specific differences were observed in fat distribution, glucose regulation, central inflammation and neuroplasticity after WD and GE2 treatment. In females only, GE2 reduced visceral (gonadal) fat, reduced inflammation in the dorsal hippocampus and improved basal blood glucose in response to a WD. In males only, GE2 normalised reduced hippocampal neurogenesis after a WD and reduced inflammation in the amygdala. These findings suggest that although WD increased body weight and GE2 improved associative learning in both sexes, both WD and GE2 had differential affects on metabolic hormones, insulin regulation, inflammation and neuroplasticity. Our findings underscore the importance of sex-specific approaches in metabolic and neuroprotective therapeutics in middle-age.

Keywords: middle age, neurogenesis, hippocampus, insulin regulation, metabolic challenge, western diet, sex differences, obesity, neuroplasticity, amygdala.

1. Introduction

Diets high in saturated fats and refined carbohydrates are linked to obesity and diabetes (1). Hyperglycemia, both in diabetic and prediabetic states, is associated with an increased risk of cognitive decline, mild cognitive impairment (MCI), and Alzheimer's disease (AD; 2–4). High fat diets also can increase inflammation, insulin resistance (5–10) and may also impair cognitive function (11), particularly during middle age (12–14). As the prevalence of obesity and diabetes have reached epidemic proportions in many regions throughout the world (15), such findings underscore the urgent need for targeted therapies addressing brain health in the face of high fat diets, especially during middle age - a period critical for metabolic and cognitive health.

With the growing rates of obesity and diabetes, glucagon-like peptide-1 (GLP-1)-based therapies have emerged as powerful tools to tackle these conditions (16). GLP-1 is an incretin hormone produced in the intestinal L-cells that promotes satiety, regulates blood glucose, activates brown fat metabolism and increases energy expenditure (17,18). Centrally, GLP-1, primarily produced by neurons in the nucleus of the solitary tract (NTS), modulates appetite and body weight through widespread neural projections (19,20). Intriguingly, the effects of GLP-1 on appetite reduction, glycemic control and body-weight reduction are more pronounced in females, at least in part due to estrogens (21–26). Sex differences are also observed in the neurological actions of GLP-1, where targeting certain brain areas produces more potent effects in females (e.g., the ventral tegmental area), whereas the opposite is true for other regions (e.g., the supramammillary nucleus, lateral hypothalamus) (27–29). However, the net outcome of central stimulation leads to more prominent reductions in appetite, blood glucose and body weight in adult females, suggesting that GLP-1 receptor treatments are more effective treatments for diabetes and obesity in females compared to males.

Beyond its role in metabolic regulation, GLP-1 can impact cognitive and emotional processes through GLP-1 receptors in the hippocampus, prefrontal cortex (PFC), and amygdala (30,31). Preclinical studies, primarily in males, demonstrate that GLP-1 receptor agonists improve hippocampus-dependent learning and memory under standard- and high-fat diet conditions, as well as in diabetes and AD models (32–37). Furthermore, GLP-1 receptor agonists have neuroprotective effects in the hippocampus, including enhanced neurogenesis and reduced apoptosis in studies primarily conducted in adult males (32,38–41). The integrity of the

hippocampus is important for spatial and episodic memory, and that it is one of the first brain regions affected in AD (42). GLP-1 receptor agonists are associated with lower dementia rates and improved cognitive outcomes in clinical studies, independent of metabolic improvements (37,43). Thus, evidence suggests that GLP-1 receptor agonists have a neuroprotective role and can enhance learning and memory, although there is limited research examining effects in females or in middle-aged adults.

Middle age is a critical period for diet-induced cognitive decline (12–14), coinciding with the menopausal transition in females. This transition is characterized by declining ovarian hormone levels, including estrogens, which play a neuroprotective role during certain circumstances (44). Estradiol (E2)-based hormone therapies can reduce cognitive decline in postmenopausal women (45–49). Estrogens, including E2, also promote hippocampal plasticity (50–53) through several possible actions (54), including through it's actions on spinogenesis (55) , and inflammation in the hippocampus (56) in female rodents. Moreover, E2 potentiates GLP-1's effects on food intake in female rats (57) and reward (58) in both sexes, but to a greater extent in females compared to males, suggesting a synergistic relationship between these hormones at least in young adult rats. But to date, no one has examined the effects of combined GLP and E2 treatment on hippocampus-dependent memory and inflammation in the hippocampus in both sexes at middle age.

To harness the cognitive and neuroprotective benefits of both hormones, we utilized GE2 (59), a conjugate combining GLP-1 and E2. This study investigated whether GE2 improves cognition, reduces neuroinflammation, and enhances synaptic plasticity in the hippocampus of middle-aged male and female rats fed a standard (SD) or high-fat, high-sugar (Western) diet (WD). We hypothesize that GE2 treatment will mitigate the cognitive and inflammatory impacts of a WD and enhance cognition, reduce inflammation, and increase neuroplasticity in limbic regions of the brain in middle-aged male and female rats. Furthermore, whereas sex-specific effects of GLP-1 have been reported for its metabolic outcomes and reward behaviors (28,29,58), research revolving GLP-1's effects on cognition and neuroprotection have been conducted mainly in males. This research addresses a significant gap by examining these outcomes in both male and female rats. We hypothesize that more pronounced effects will be observed in females due to the potentiating effects of estradiol conjugated to GLP-1.

2. Methods

2.1. Animals

Adult male (n=68) and female (n=68) Sprague–Dawley rats were bred in house from breeding pairs obtained from Charles River, Quebec, Canada, after weaning rats were double-housed in a 12-h light/dark cycle. Rats had *ad libitum* access to water and SD (Picolab®, rodent diet 5053) until 10 months of age in which they were divided into weight-matched groups within each sex, and fed a SD (Picolab®, rodent diet 5053) or WD (Research Diets (RD) Western Diet, D12079B, Research Diets Inc., New Brunswick, NJ, USA) for the following 8 weeks of the experiment as described below and demonstrated in Figure 1A. All protocols were approved by the Institutional Animal Care Committee at the University of British Columbia and conformed to the guidelines set out by the Canadian Counsel on Animal Care.

2.2. Drugs

Compound GE2 stable conjugate and GLP-1 (GLP-1-X) were provided by Brian Finan and Bin Yang (Novo Nordisk Research Center Indianapolis, Indianapolis, IN, USA). A dose of 20 µg/kg (4.5nmol/kg) was chosen based on (59,60) and under guidance from the Novo Nordisk team. This dose is 2-6 times lower than previous studies using the conjugate (59,61) to minimize the risk of side effects; incidences of which have been higher in females (27). GE2 was dissolved in saline (0.9% sodium chloride saline solution for injection, JB1323, Baxter); used as vehicle.

2.3. Experimental timeline

At 11 months of age (after one month of diet exposure) females and males received daily subcutaneous injections of vehicle (saline) or GE2 as described above (n = 6-7 (vehicle) and n = 6-8 (GE2); Fig. 1A). Rats were weighed weekly during the diet only period and daily prior to drug injection. Starting at 11 months, or 4 weeks after diet manipulation, male and female rats received daily subcutaneous injections of GE2, or vehicle (saline) for 1 month until tissue collection. Vaginal samples were collected via lavage during the diet and treatment periods and it was observed that 80% of females were irregular or acyclic during diet and treatment manipulations (data not shown).

2.4. Intraperitoneal glucose tolerance test (IGTT)

IGTT was performed after 20 days of hormone/vehicle treatment. Rats were fasted overnight (from 8 pm to 9 am), then weighed and the tip of the tail was scored using a fresh scalpel. Blood was collected from the tail vein and basal blood glucose was measured using a glucometer (OneTouch Ultra®2, Lifescan). Rats then received an intraperitoneal injection of glucose solution (2g/kg body weight with a volume of 1mL/kg). Blood was sampled by gently massaging the tail, 15, 30, 60, and 120 minutes after injection and blood glucose measured using the glucometer. Area of the curve (AOC) was calculated for each subject by subtracting the starting glucose value from the value at each time point as described in (62).

2.5. *Fear conditioning*

After 1 month of treatment (2 months of diet), rats were tested using the fear conditioning paradigm, which relies on the integrity of the amygdala (cued fear conditioning), and hippocampus (contextual fear conditioning) (63). The apparatus and testing paradigm was previously described in (64). Briefly, conditioning and testing were conducted in four identical observation chambers (30.5 × 24 × 21 cm; Med Associates, St Albans, VT), illuminated by a 100 mA house light and enclosed within sound-attenuating boxes. A speaker connected to a programmable audio generator (ANL-926, Med Associates) provided tones (4 kHz, 80 dB) and 19 stainless steel rods, spaced 1.5 cm apart, were connected to a shock generator and scrambler for the delivery of an unconditioned stimulus foot shock (1.0 mA). A video camera positioned above each chamber recorded behavior for video scoring. Ventilation fans in each box supplied background noise (70 dB, A scale). Locomotor activity was measured (Med Associates) and time spent freezing was scored manually using video footage with the experimenter blinded to group membership.

Training and testing were conducted over 2 days. On day 1 (conditioning day) rats were injected with GE2 or vehicle by one experimenter. Thirty minutes later, rats were individually transported to the testing room in a clean, unused, transparent cage, on a four-wheeled cart by a different experimenter. Rats were then placed in the Context A operant chamber. Context A consisted of bare aluminum and Plexiglass walls, and a lavender air freshener was used as an odor cue. Three minutes after placement into the chamber, rats were given three presentations of the tone conditioned stimulus (CS; 30 seconds), each co-terminating with a foot shock (2 seconds) with 60 second intershock intervals. Sixty seconds after the third and final

tone/shock pairing (after 3 minutes of exposure to Context A), rats were immediately returned to their home cage. To assess whether there were group differences in pain sensitivity, we recorded freezing behavior during the minute after each shock presentation.

On day 2 (24 h after conditioning), rats were again given hormone treatment and then individually transported, in a clean, unused, transparent cage, back to the testing room 30 minutes after injection. The same procedure was followed as the conditioning day to activate the representation of the context. Rats were placed back into Context A (same as described above) for 8 min to assess contextual fear conditioning (no tones or shocks were present). One hour later, rats were transported back to the testing room in an unused, transparent cage, but along a completely novel route. Rats were placed into the novel Context B, which had striped inserts covering the Plexiglass walls, and a tropical air freshener was used as an odor cue. Three minutes after being placed in the box, they were presented with three tones (4kHz, 80 dB, 30 seconds), 60 seconds apart. Time spent freezing to the tone (cue) was measured during each tone presentation to assess cued fear conditioning. Unfortunately, due to technical difficulties in two of the boxes, the tone did not play during conditioning for 14 animals, and they were therefore excluded from the analysis.

2.6. *Tissue and blood collection*

Trunk blood was collected following live decapitation and placed into cold EDTA-coated tubes. Tubes were centrifuged for 15 minutes, and plasma was stored at -80ºC. Brains were quickly extracted, and the hemispheres were divided. The left hemisphere was flash frozen on dry ice and stored at -80ºC. Brain areas of interest (amygdala, dorsal and ventral hippocampus, PFC) were punched from slices (50µm sections) using a cryostat (-20°C), and cytokine levels analyzed using electrochemiluminescence immunoassay kits. The right hemisphere was placed in paraformaldehyde (PFA) and stored at 4°C. After 24 hours the brains were transferred to a 30% sucrose/PBS solution for cryoprotection. Serial coronal sections (30 µm) were cut with a freezing microtome (SM2000R; Leica, Richmond Hill, ON) across the extent of the hippocampus (collected in 10 series) and stored in an antifreeze solution (containing 0.1M PBS, 30% ethylene glycol, and 20% glycerol) and stored at -20ºC for immunohistochemistry. Inguinal (IWAT) and gonadal (GWAT) white adipose tissue were collected and weighed, and a sample was collected and flash frozen on dry ice.

2.7. Hippocampal cytokine measurements

Electrochemiluminescence immunoassay kits (Meso-Scale Discovery; Rockville, MD) were used to measure cytokines (IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, CXCL-1, and TNF-α; V-PLEX Proinflammatory Panel 2 Rat Kit; Cat# K15059D) and postsynaptic density 95 (PSD95; PSD95 kit; Cat# K150QND) in homogenized amygdala, hippocampus (dorsal and ventral) and PFC samples. Each sample was homogenized using a TissueLyser III (Qiagen) with 5 homogenization beads in 200 µl of cold Tris lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X) containing a cocktail of protease (cOmplete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2 and 3, Sigma Aldrich), NaF and PMSF in DMSO. Homogenates were centrifuged at 100 rpm for 30 seconds at 4ºC and supernatants were aliquoted and stored at -80ºC. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Homogenates were diluted with Tris lysis buffer 1:10 for PSD95 and 1:4 for proinflammatory analysis. Samples were run in duplicate following the manufacturer's protocols, with the exception of longer sample and calibrator incubation for cytokine plates (overnight instead of 2 hours at 4°C). Plates were read with a MESO QuickPlex SQ 120 (Meso Scale Discovery) and data were analyzed using the Discovery Workbench 4.0 software (Meso Scale Discovery). Cytokine levels were obtained using a standard curve and expressed as pg/mg protein and PSD95 measured protein signal levels which were normalized to protein and expressed as signal/ pg mg^{-1}.

2.8. Plasma hormone levels

U-PLEX Metabolic Group 1 Rat Kits (Meso-Scale Discovery; Rockville, MD; Cat# K15200L) were used to measure metabolic hormones (c-peptide, ghrelin, glp-1, glucagon, insulin, leptin and PYY) in plasma. Plasma was diluted using a 2-fold dilution using the Metabolic Assay Working Solution. Samples were run in duplicate following the manufacturer's protocol. Plates were read with a MESO QuickPlex SQ 120 (Meso Scale Discovery) and data were analyzed using the Discovery Workbench 4.0 software (Meso Scale Discovery). Hormone levels were obtained using a standard curve and expressed as pg/ml.

2.9. Immunohistochemistry

A series of free-floating tissue was rinsed 3 times at room temperature with 0.1 M phosphate-buffered saline (PBS) for 10 minutes each. The tissue was then incubated in 10% Triton X for 30 minutes. Sections were added to the blocking solution (10% normal donkey serum (NDS) and 3% Triton X in PBS) for 1 hour. The tissue was immediately incubated in rabbit anti-doublecortin (DCX; 4604S, Cell Signal Technology Inc. Danvers) diluted 1:1000 in blocking solution (10% NDS and 3% Triton X in PBS) for ~48 hours at 4°C. Following PBS rinses (10 minutes X3), tissue was incubated for 4 hours in Alexa Fluor 488 conjugated donkey anti-rabbit (1:500; A-21206, Thermo Fisher Scientific) in a solution PBS containing 10% NDS, 3% Triton-X. Sections were then washed in PBS for 3x 10 minutes and then stained with 4,6-diamidino-2-phenylindole (DAPI; 1:500; D1306, Thermo Fisher Scientific) for 2.5 minutes at room temperature. The tissue was rinsed for 5 minutes (3X). Finally, tissue was mounted onto Superfrost/Plus slides (Fisher scientific) and cover slipped using PVA-DABCO mounting medium.

2.10. *DCX- expressing cells: quantification and microscopy*

DCX-expressing cells were imaged with Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy, Thornwood, NY, USA) with a 20x objective using fluorescent imaging. The area around the dentate gyrus was first identified and measured using DAPI only and all DCX-expressing cells within the marked area were then quantified by an investigator blinded to experimental conditions. Two dorsal and two ventral sections were quantified for each rat. The density was calculated by dividing the amount of DCX-expressing cells with the area of the dentate gyrus for each section. Photomicrographs were acquired using the Fluoview 4000 confocal microscope (Evident Scientific) with a 20x objective lens. DAPI was visualized using the 405nm laser (blue) and DCX was visualized using the 488nm laser (green). Contrast was adjusted to remove background signal and single cell images were deconvolved using cellSens imaging software.

2.11. *Statistical analyses*

All the data are presented as mean ± standard error of the mean. Data were analyzed using TIBCO Statistica software (v. 9, StatSoft, Inc., Tulsa, OK, USA) using analyses of variance (ANOVA) with sex (male, female), diet (SD, WD) and treatment (vehicle, GE2) as between subject factors on dependent variables of interest, and plotted using GraphPad Prism (GraphPad

Software, Inc., San Diego, CA). Effect sizes (partial eta squared (η_p^2) and Cohen's *d*) are reported for significant effects. Post hoc comparisons used Newman-Keul's and *a priori* comparisons were subjected to a Bonferroni correction. P- values ≤ 0.05 were considered statistically significant.

2.11.1. For z-stack and PERMANOVA

Data Preprocessing and Imputation: To address missing values in our dataset, we employed a multiple imputation approach using the random forest (RF) method implemented in the mice package (65). A predictor matrix was constructed establishing region-specific relationships, giving full weight to predictors within the same brain region (dHPC, vHPC, Amyg, PFC), partial weight (0.5) to predictors from other regions, and including demographic variables (Group, Treatment, Diet) as full predictors. The RF imputation was executed with 5 multiple datasets (m = 5), 20 iterations, and 200 trees per forest. The quality of imputation was assessed through density plots comparing original and imputed distributions for each variable within each brain region, ensuring the preservation of the original data structure (**Supplementary Fig. 5**).

2.11.2. *Regional Cytokine Profiling and Multivariate Analysis:*

The multivariate patterns of cytokine expression across brain regions were analyzed using a permutational multivariate analysis of variance (PERMANOVA) approach implemented in the R vegan package on imputed data (66,67). For each region, we extracted cytokine measurements and constructed Euclidean distance matrices after standardizing the data (z-scoring). Cytokines were categorized as either TH1 (pro-inflammatory: IL1β, TNFα, IFNγ) or TH2 (anti-inflammatory: IL4, IL5, IL10, IL13) to characterize distinct immune response patterns. The PERMANOVA was performed using 15,000 permutations and a three-way factorial design (Sex × Diet × Treatment). Non-metric multidimensional scaling (NMDS) was used to visualize multivariate patterns in cytokine profiles. Mean inflammatory profiles were calculated by averaging z-scored values within TH1 and TH2 categories for each region. Differences between inflammatory profiles within experimental groups were assessed using pairwise Wilcoxon tests with false discovery rate (FDR) correction.

Software and Packages: All analyses were conducted in R (version 4.3.2). The following packages were utilized: mice for multiple imputation; tidyverse for data manipulation and

visualization and vegan for multivariate analyses.

3. Results

3.1. WD increased, whereas GE2 reduced, body weight in both sexes

As expected, four weeks of WD consumption resulted in weight gain, compared to SD in both sexes ($F_{(1, 132)} = 18.895$, $\eta_p^2 = 0.125$, $p = 0.000$; males: SD vs WD: $p = 0.000$, Cohen's $d = 4.331$; and females: SD vs WD: $p = 0.000$, Cohen's $d = 2.590$; **Fig. 1B**). Females on the WD had a greater percentage body weight gain than males on the WD ($p = 0.0001$, Cohen's $d = 0.735$). Females on SD had a slight body weight reduction compared to their starting weight, which was greater than the body-weight change seen in males on SD ($p = 0.022$, Cohens $d = 0.950$).

After one month of the hormone treatment, GE2-treated subjects of both sexes lost significantly more weight their vehicle-treated counterparts (Treatment and diet interaction: $F_{(1,47)} = 9.936$, $\eta_p^2 = 0.175$, $p = 0.003$; SD: $p = 0.001$, Cohen's $d = 1.140$; WD: $p = 0.000$, Cohen's $d = 3.733$). Not surprisingly, GE2 treatment led to a larger body-weight reduction on WD than SD fed ($p = 0.000$, Cohen's $d = 1.238$). The main effects of Treatment ($F_{(1, 47)} = 72.550$, $\eta_p^2 = 0.607$, $p = 0.000$), Diet ($F_{(1, 47)} = 6.798$, $\eta_p^2 = 0.126$, $p = 0.012$) and sex ($F_{(1, 47)} = 5.451$, $\eta_p^2 = 0.104$, $p = 0.024$), were also significant, there were no other significant main or interaction effects ($p \geq 0.062$).

3.2. Diet and GE2 treatment affect GWAT in a sex-specific manner

GWAT was collected as a measure of visceral fat, a type of fat that is more detrimental to health, increasing the risk of metabolic disorders and cardiovascular disease, whereas IWAT was collected as a measure of subcutaneous fat, which is considered a metabolically healthy fat type (65). WD increased IWAT (main effect of diet: $F_{(1, 46)} = 13.735$, $\eta_p^2 = 0.230$, $p = 0.001$, **Fig. 1D**) compared to SD, with an especially large diet-induced increase in IWAT in males. Males also had more IWAT (main effect of sex: $F_{(1, 46)} = 30.170$, $\eta_p^2 = 0.396$, $p < 0.001$) than females overall.

WD consumption also led to a significant increase in GWAT (measure of visceral fat) in vehicle-treated groups compared to SD, but only in females ($p < 0.000$, Cohen's $d = 2.147$; Sex x Diet x Treatment interaction: $F_{(1, 47)} = 4.485$, $\eta_p^2 = 0.087$, $p = 0.040$, **Fig. 1E**). GE2 treatment reduced (strong trend) GWAT in WD-fed females compared to vehicle-treated females ($p = 0.057$) and significantly reduced GWAT in SD-fed males compared to vehicle-treated SD males ($p = 0.028$,

Cohen's $d = 1.848$). GE2-treated males had more GWAT on the WD than the SD ($p = 0.024$, Cohen's $d = 0.099$). In addition, vehicle-treated males had significantly more GWAT than vehicle-treated females on the SD ($p = 0.003$, Cohen's $d = 2.324$). There were no other significant main effects or interactions (all p's ≥ 0.071).

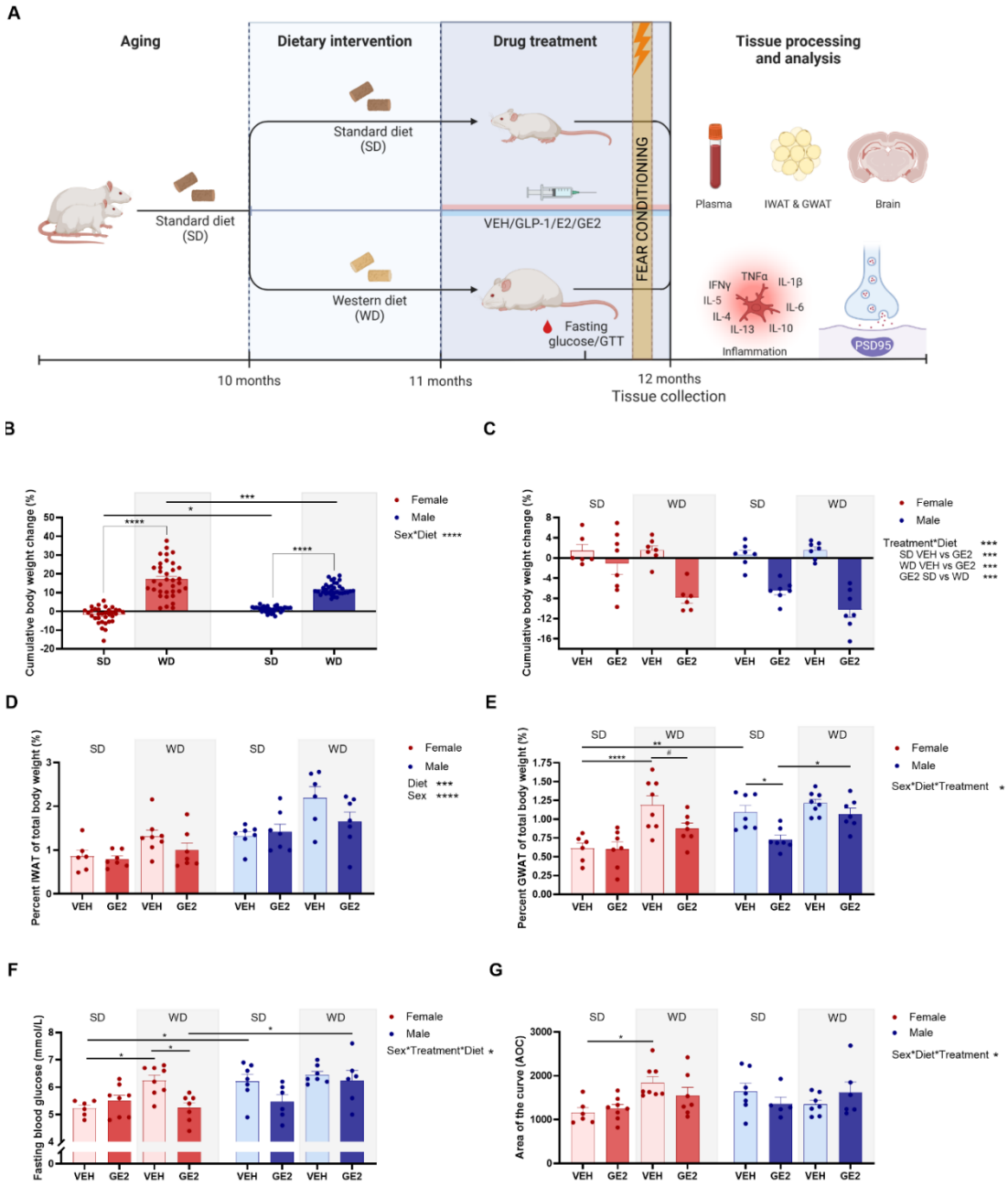

Figure 1. **Diet and treatment affect body weight, fat and blood glucose in a sex-specific manner.** Graphical illustration of experimental plan (A). Mean (±SEM) percent body weight (B), as well as mean (±SEM) percentage of inguinal white adipose tissue (IWAT; D) and gonadal white adipose tissue (GWAT; E) after 4 weeks of treatment. Mean (±SEM) Basal blood glucose levels after an overnight fast (F) and area of the curve (AOC) after being challenged by a glucose tolerance test (GTT; G). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and # $p < 0.100$. Diet only: n = 33-34 (SD) and n = 34-35 (WD); Treatment groups: n = 6-8 (VEH) and n = 5-8 (GE2) per diet and sex. VEH = vehicle; GE2 = GLP-1-E2; SD = standard diet; WD = western diet; DCX = doublecortin; PSD95 = postsynaptic protein 95; SEM = standard error of the mean. Timeline image created using BioRender.com.

3.3. Diet and GE2 treatment affect fasting blood glucose in a sex-specific manner

Next, we investigated the effects of diet and hormone treatment on blood glucose. WD consumption increased fasting blood glucose in vehicle-treated subjects compared to SD, only in females ($p = 0.035$, Cohen's $d = 2.342$; Sex x Diet x Treatment interaction: $F_{(1, 47)} = 6.624$, $\eta_p^2 = 0.124$, $p = 0.013$, **Fig. 1F**). GE2 treatment in WD females significantly reduced fasting blood glucose compared to WD females receiving vehicle ($p = 0.032$, Cohen's $d = 2.002$), but not in males ($p = 0.803$). Furthermore, GE2-treated WD females had lower fasting blood glucose levels than GE2-treated WD males ($p = 0.024$, Cohen's $d = 7.084$). Vehicle-treated males on SD had higher fasting glucose than vehicle-treated females on SD ($p = 0.022$, Cohen's $d = 1.869$). The main effects of Treatment ($F_{(1, 47)} = 5.720$, $\eta_p^2 = 0.109$, $p = 0.021$), Diet ($F_{(1, 47)} = 5.513$, $\eta_p^2 = 0.105$, $p = 0.023$) and sex ($F_{(1, 47)} = 13.599$, $\eta_p^2 = 0.224$, $p = 0.001$), were also significant, there were no other significant main or interaction effects ($p > 0.139$).

After a glucose challenge, we found an increase in the area under the curve in WD-fed vehicle-treated females compared to SD-fed vehicle-treated females ($p = 0.050$; Sex x Diet x Treatment interaction: $F_{(1, 46)} = 4.663$, $\eta_p^2 = 0.092$, $p = 0.036$, **Fig. 1G**), but not in males ($p = 0.650$). There was also a significant interaction between sex and diet ($F_{(1, 46)} = 5.516$, $\eta_p^2 = 0.107$, $p = 0.023$) and a significant main effect of diet ($F_{(1, 46)} = 4.618$, $\eta_p^2 = 0.091$, $p = 0.037$), but no other significant main or interaction effects ($p > 0.627$).

3.4. GE2 treatment increases plasma GLP-1 only in females and PYY in both sexes, WD increased leptin levels

To investigate if the rodents were metabolically affected by WD consumption after 8 weeks, we measured the levels of several plasma metabolic hormones. Firstly, we wanted to know if our treatment affected the levels of plasma GLP-1. We found that GE2 treatment significantly increased plasma GLP-1 compared to vehicle in females ($p = 0.001$; Cohen's $d = 0.157$; sex and treatment interaction: $F_{(1, 46)} = 7.146$, $\eta_p^2 = 0.134$, $p < 0.010$; **Fig. 2A**), but not in males ($p = 0.806$). The main effect of treatment ($F_{(1, 46)} = 9.137$, $\eta_p^2 = 0.166$, $p = 0.004$) and sex ($F_{(1, 46)} = 4.586$, $\eta_p^2 = 0.091$, $p = 0.038$) were also significant, but there were no other significant main effects or interactions (all $p \geq 0.105$).

Next, we looked at glucagon and insulin, hormones regulated by GLP-1. We saw no statistical

differences in glucagon levels by sex, treatment or diet, nor an interaction between these factors (all $p \geq 0.240$, **Fig. 2B**). Males demonstrated higher levels of insulin than females (main effect of sex: $F_{(1, 37)} = 5.576$, $\eta_p^2 = 0.131$, $p = 0.024$; **Fig. 2C)**, but there were no other significant main effects or interactions (all p's ≥ 0.155).

Leptin is directly affected by fat content. As expected, leptin levels were increased in WD compared to SD fed subjects (main effect of diet: ($F_{(1, 46)} = 21.606$, $\eta_p^2 = 0.320$, $p < 0.001$; **Fig. 2D**). Males had higher levels of leptin than females (main effect of sex: $F_{(1,46)} = 7.280$, $\eta_p^2 = 0.137$, $p = 0.010$). There were no other significant main effects or interactions (all p's ≥ 0.399).

Levels of PYY, an anorexigenic peptide, were increased in GE2 -treated subjects of both sexes (main effect of treatment: $F_{(1,34)} = 10.896$, $\eta_p^2 = 0.243$, $p = 0.002$; vehicle and GE2: $p = 0.002$; Cohen's $d = 1.131$; **Fig. 2E**). Plasma ghrelin and c-peptide were also measured but most values were below detection level (all ghrelin levels were below detection, as expected since the rodents did not undergo long-term fasting prior to blood collection to ensure that GLP-1 would be at detectable levels, and >80% of c-peptide levels were below detection), and thus we were unable to statistically analyze these hormones.

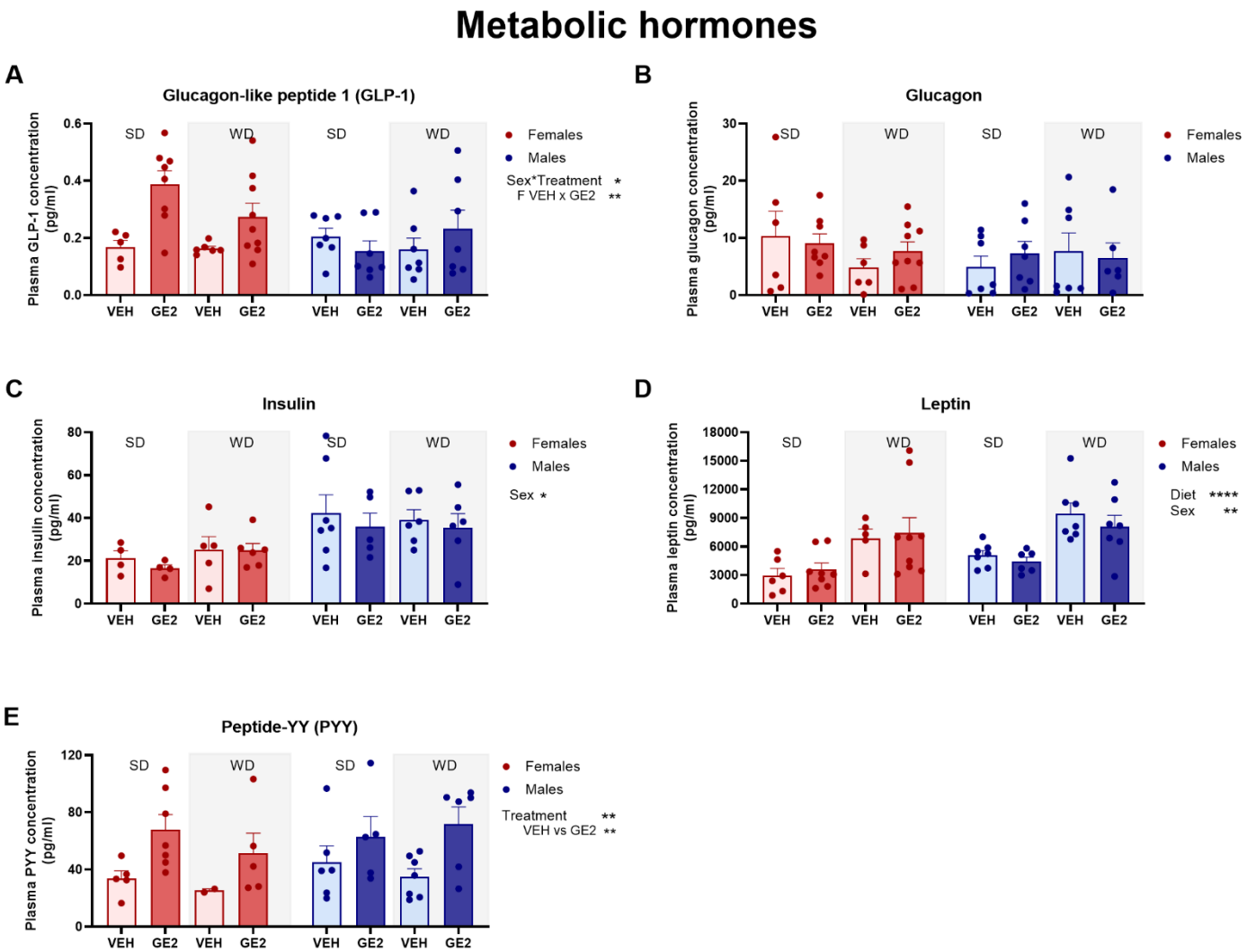

Figure 2. **GE2 treatment increases plasma peptide YY (PYY) levels in both sexes, and GLP-1 levels only in females, compared to Vehicle**. Mean (±SEM) levels of glucagon-like peptide-1 (GLP-1; A), glucagon (B), insulin (C), leptin (D) and PYY (E) in plasma. Main and interaction effects are listed with graphs* $p < 0.05$ ** $p < 0.01$, **** $p < 0.0001$. n = 2-7 (Vehicle) and n = 5-9 (GE2) per diet and sex. SEM = standard error of the mean.

3.5. GE2 treatment improved both cued and contextual fear memory in both sexes

For cued fear conditioning, GE2 treated rats displayed more time freezing than vehicle-treated rats in males ($p < 0.001$, Cohen's $d = 1.899$) and females ($p = 0.043$, Cohen's $d = 1.240$), despite the sex and treatment interaction ($F_{(1,33)} = 4.468$, $\eta_p^2 = 0.119$, $p = 0.042$; **Fig. 3C**). The main effects of treatment ($F_{(1,33)} = 30.065$, $\eta_p^2 = 0.477$, $p < 0.001$) and sex ($F_{(1,33)} = 35.776$, $\eta_p^2 = 0.149$, $p = 0.022$) were also significant, and there was a trend towards reduced freezing in WD-

fed rats, though this is more prominent in males (main effect of diet: $F_{(1,33)} = 4.037$, $\eta_p^2 = 0.109$, $p = 0.053$) . As for the contextual fear conditioning task, GE2-treated rats displayed more time freezing to the context than vehicle-treated rats regardless of sex (main effect of treatment: $F_{(1,48)} = 39.617$, $\eta_p^2 = 0.452$, $p < 0.001$; **Fig. 3B**. No other significant main effects or interactions for contextual fear conditioning were present (all $p \geq 0.353$).

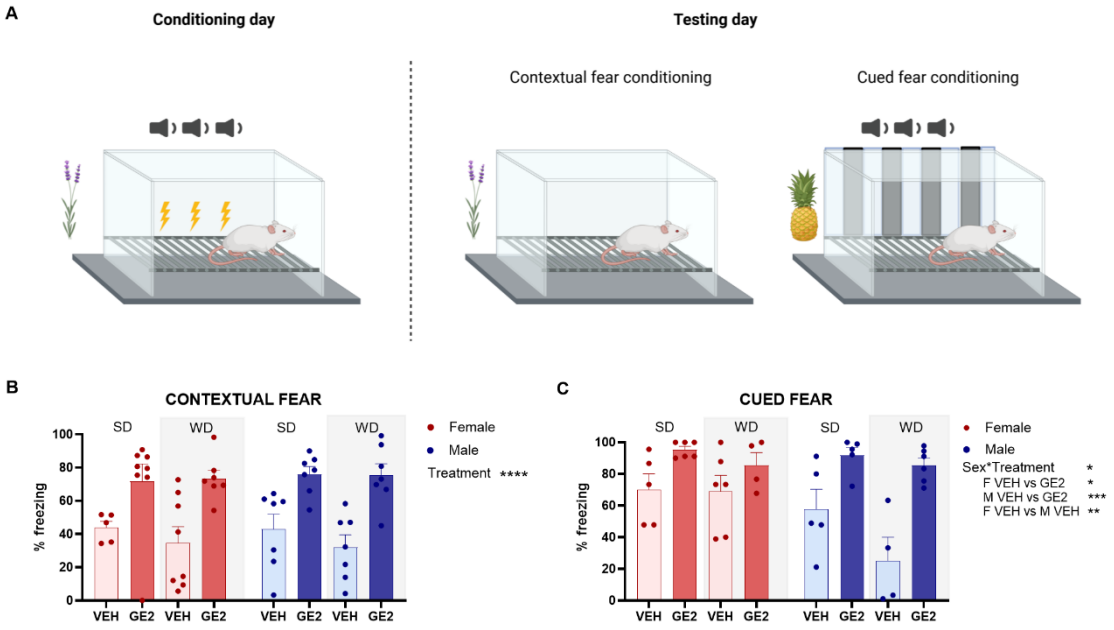

Figure 3. **GE2 increases freezing in both the contextual and cued fear conditioning tests.** Schematic representation of the 2-day fear conditioning training and testing (A). **Mean** percent freezing (±SEM) during the contextual fear conditioning task (B) and the cued fear conditioning task (C). * $p < 0.05$, ** $p = 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. n = 5-10 (VEH) and 4-7 (GE2) per diet and sex. F = female; M = male, VEH = vehicle; GE2 = GLP-1-E2, SD = standard diet; WD = western diet; SEM = standard error of the mean. Schematic representation of fear conditioning task created using BioRender.com.

3.6. GE2 treatment increases neurogenesis in the dorsal hippocampus depending on sex and diet

WD reduced the density of DCX-expressing cells compared to SD ($p = 0.024$, Cohen's $d = 1.309$), but only in males, and GE2 treatment normalized the density of DCX-expressing cells under a WD in males ($p = 0.008$, Cohen's $d = 4.023$; **Fig. 4C**). In females, GE2 treatment also increased the density of DCX-expressing cells compared to vehicle-treated females, but only under a SD (a priori comparison: $p = 0.036$, Cohen's $d = 0.725$; sex, diet and treatment

interaction: $F_{(1,46)} = 3.445$, $\eta_p^2 = 0.070$, $p = 0.070$). The main effect of treatment was also significant (main effect of treatment: $F_{(1,46)} = 8.883$, $\eta_p^2 = 0.162$, $p = 0.005$). There were no other significant main effects of interactions in the dorsal hippocampus (all $p \geq 0.092$). In the ventral hippocampus, we found no significant main effects or interactions (all $p \geq 0.334$; **Fig. 4D**).

3.7. *PSD95 levels were not significantly affected by sex, diet or GE2 treatment across brain regions*

Levels of the postsynaptic protein PSD95 were not affected by diet, sex or treatment in the dorsal hippocampus (all $p \geq 0.161$; **Fig. 4E**), ventral hippocampus (all $p \geq 0.182$; **Fig. 4F**), or PFC (all $p \geq 0.202$; **Fig. 4H**). In the amygdala, there was a trend towards lower levels of PSD95 in WD compared to SD subjects (main effect of diet: $F_{(1,38)} = 3.838$, $\eta_p^2 = 0.092$, $p = 0.057$; **Fig. 4G**). Furthermore, there was a trend towards an interaction between sex, diet and treatment in the amygdala (main effect of diet: $F_{(1,38)} = 3.263$, $\eta_p^2 = 0.079$, $p = 0.079$).

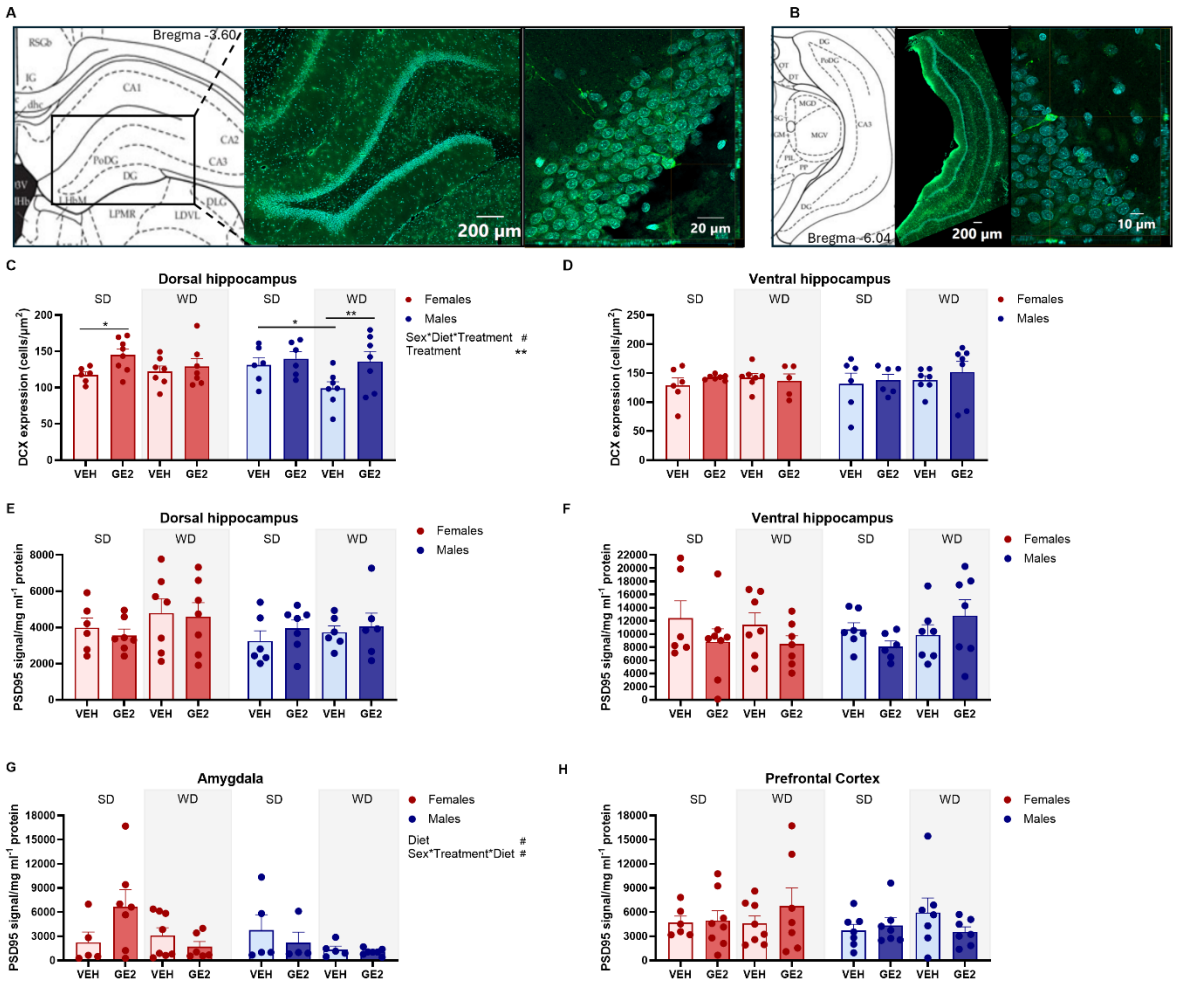

Figure 4. **WD reduces doublecortin (DCX) expression in the dorsal hippocampus and GE2 treatment increases the density of DCX in the dorsal hippocampus in SD females and WD males, whereas postsynaptic density 95 (PSD95) signal is unchanged. Representative image of DCX-expressing cells (green) in the dorsal (A) and ventral (B) hippocampus.** Mean DCX cells per µm^2 (±SEM) measured in the dorsal (C) and ventral (D) hippocampus. PSD95 signal in the dorsal hippocampus (E) ventral hippocampus (F), amygdala (G), and prefrontal cortex (H). DCX is depicted in green and DAPI in light blue. # $p < 0.100$, ** $p < 0.010$. n = 5-8 (vehicle) and 6-8 (GE2) per diet and sex. SD = standard diet; WD = western diet; SEM = standard error of the mean.

3.8. WD increased inflammation in a sex- and region-specific manner, and GE2 reduced inflammation in the hippocampus in SD-fed rats, as well as in the amygdala of males regardless of diet

Brain aging is associated with increased inflammation, however diets, such as the WD, may further affect inflammation. Eight weeks of exposure to WD at middle-age increased brain

cytokines in a sex-dependent manner, in which females demonstrated increased inflammation in the amygdala in response to WD, whereas males trended towards increased levels in the ventral hippocampus. GE2 treatment reduced inflammation in the dorsal and ventral hippocampus, however only in SD subjects, regardless of sex, as well as in the amygdala in males regardless of diet.

3.8.1. Dorsal hippocampus

Prominent effects of GE2 treatment to reduce cytokines were seen in the dorsal hippocampus (**Fig. 5**). Z-score analysis of the cytokine profiles in the dorsal hippocampus revealed GE2 reduced inflammation in SD rats ($F_{(1,25)} = 8.971$, $R^2 = 0.264$, $p < 0.001$; diet and treatment interaction: $F_{(1,54)} = 5.225$, $R^2 = 0.080$, $p = 0.007$; **Fig. 5A**) but not WD rats ($p = 0.499$). GE2 treatment reduced cytokine levels in females ($F_{(1,26)} = 6.050$, $R^2 = 0.189$, $p = 0.005$; Sex and Treatment interaction: $F_{(1,54)} = 3.763$, $R^2 = 0.057$, $p = 0.022$), but not males ($p = 0.414$). The main effect of treatment also significant ($F_{(1,54)} = 4.949$, $R^2 = 0.075$, $p = 0.008$) but there were no other significant main effects or interactions $p \geq 0.194$).

To assess whether inflammatory changes are pro- or anti-inflammatory we also assessed cytokines related to TH1 and TH2 cells. TH1 and TH2 are subsets of CD4+ T helper cells, with TH1 cells promoting pro-inflammatory responses against intracellular pathogens through IL-1β, IFN-γ and TNFα production, whereas TH2 cells drive anti-inflammatory responses by secreting IL-4, IL-5, IL-10 and IL-13.

We observed a reduction TH1-related cytokines after GE2 treatment in females ($p = 0.016$), but not in males ($p = 0.474$; sex and treatment interaction: $F_{(1,54)} = 4.859$, $R^2 = 0.076$, $p = 0.013$). Furthermore, we observed a reduction in TH1-related cytokines after GE2 treatment in SD-fed rats ($F_{(1,25)} = 5.527$, $R^2 = 0.101$, $p = 0.010$; diet and treatment interaction: $F_{(1,54)} = 5.709$, $R^2 = 0.089$, $p = 0.008$), but not WD ($p = 0.524$). We found no other significant main effects or interactions ($p \geq 0.117$).

A reduction in TH2-related cytokines was also observed after GE2 treatment in females ($p = 0.005$; sex and treatment interaction: $F_{(1,54)} = 4.518$, $R^2 = 0.065$, $p = 0.026$) but not males ($p = 0.654$). Reduced TH2-related cytokine levels were present in GE2-treated SD rats ($F_{(1,25)} = 14.580$, $R^2 = 0.368$, $p < 0.001$; diet and treatment interaction: $F_{(1,54)} = 5.824$, $R^2 = 0.084$, $p =$

0.010), but not WD ($p = 0.945$). The main effect of treatment also significant ($F_{(1,54)} = 2.791$, $R^2 = 0.040$, $p = 0.080$), and there was a trend towards a significant main effect of diet (main effect of diet: $F_{(1,54)} = 6.413$, $R^2 = 0.093$, $p = 0.008$). There were no other significant main effects or interactions ($p \geq 0.324$). Results for individual cytokines can be found in the Supplement.

3.8.2. Ventral hippocampus

In this area we observed that WD increased inflammation in males only, and GE2 treatment reduced inflammation in SD-fed males and females (**Fig. 5B**).

WD increased cytokine levels in males compared to SD ($F_{(1,12)} = 3.172$, $R^2 = 0.209$, $p = 0.010$). However, in females, SD-fed rats had higher inflammation than WD-fed rats ($F_{(1,11)} = 3.705$, $R^2 = 0.252$, $p = 0.007$), this was primarily driven by high levels of TH2-produced, or anti-inflammatory, cytokines ($F_{(1,11)} = 8.701$, $R^2 = 0.442$, $p = 0.002$). SD-fed females also had higher inflammation than SD-fed males ($F_{(1,10)} = 8.501$, $R^2 = 0.460$, $p = 0.001$).

GE2 treatment significantly reduced inflammation in SD-fed females ($F_{(1,11)} = 4.936$, $R^2 = 0.310$, $p < 0.001$; sex, diet and treatment interaction: $F_{(1,54)} = 3.844$, $R^2 = 0.051$, $p = 0.002$), but not in WD-fed females ($p = 0.781$). In males, GE2 treatment reduced inflammation in SD-fed rats ($F_{(1,12)} = 5.740$, $R^2 = 0.324$, $p < 0.001$), contrarily there was a trend towards increased inflammation after GE2 treatment in WD-fed rats ($F_{(1,12)} = 2.192$, $R^2 = 0.154$, $p = 0.073$). We also found a significant interaction between diet and treatment ($F_{(1,54)} = 5.225$, $R^2 = 0.080$, $p = 0.007$) and the main effect of treatment also significant ($F_{(1,54)} = 4.949$, $R^2 = 0.075$, $p = 0.008$). No other significant main effects or interactions $p \geq 0.194$.

Assessing TH1-related cytokines specifically we found a significant main effect of diet, with SD increasing compared to WD ($F_{(1,54)} = 4.805$, $R^2 = 0.079$, $p = 0.008$). As for TH2-related cytokines, we found higher levels of TH2-related cytokines in SD-fed females compared to WD-fed females ($F_{(1,11)} = 8.701$, $R^2 = 0.442$, $p = 0.002$). GE2 treatment reduced the levels of TH2-related cytokines in SD-fed rats of both sexes (females: $F_{(1,11)} = 9.253$, $R^2 = 0.457$, $p < 0.001$; males: $F_{(1,12)} = 51.496$, $R^2 = 0.811$, $p < 0.001$; sex, diet, treatment interaction: $F_{(1,54)} = 9.078$, $R^2 = 0.081$, $p < 0.001$). This effect was not present in WD-fed females ($p = 0.474$) and an opposite effect was seen in WD-fed males, in which GE2 treatment increased the levels of GE2-produced

cytokines ($F_{(1,12)} = 6.833$, $R^2 = 0.363$, $p = 0.003$). There was also a significant interaction between diet and treatment ($F_{(1,54)} = 12.950$, $R^2 = 0.116$, $p < 0.001$), sex and treatment ($F_{(1,54)} = 6.074$, $R^2 = 0.054$, $p < 0.001$), sex and diet ($F_{(1,54)} = 14.022$, $R^2 = 0.125$, $p < 0.001$), as well as significant main effects of treatment ($F_{(1,54)} = 6.377$, $R^2 = 0.057$, $p < 0.001$), diet ($F_{(1,54)} = 7.918$, $R^2 = 0.071$, $p < 0.001$) and sex ($F_{(1,54)} = 7.558$, $R^2 = 0.068$, $p < 0.001$). Results for individual cytokines can be found in the Supplement.

3.8.3. Amygdala

In the amygdala we observed elevated inflammation in response to a WD in females only and GE2 treatment reduced inflammation in males regardless of diet, but increased it in SD-fed females only (**Fig. 5C**). WD increased the cytokine levels in the amygdala compared to SD in females ($F_{(1,51)} = 3.958$, $R^2 = 0.265$, $p = 0.042$; sex, diet and treatment interaction: $F_{(1,54)} = 7.570$, $R^2 = 0.084$, $p = 0.002$), but not in males ($p = 0.849$).

GE2 reduced cytokine levels in the amygdala of WD-fed males ($F_{(1,12)} = 2.045$, $R^2 = 0.146$, $p = 0.018$), but not WD-fed females ($p = 0.157$). GE2 also reduced cytokine levels in SD-fed males ($F_{(1,12)} = 10.971$, $R^2 = 0.478$, $p < 0.001$), but increased cytokine levels in SD-fed females ($F_{(1,11)} = 11.371$, $R^2 = 0.508$, $p = 0.003$). There were trends for significant interactions between diet and treatment ($F_{(1,54)} = 10.746$, $R^2 = 0.036$, $p = 0.053$), sex and treatment ($F_{(1,54)} = 3.217$, $R^2 = 0.033$, $p = 0.053$) and sex and diet ($F_{(1,54)} = 2.970$, $R^2 = 0.033$, $p = 0.065$). The main effect of sex was significant ($F_{(1,54)} = 13.770$, $R^2 = 153$, $p < 0.001$) and there was a trend towards a significant main effect of diet ($F_{(1,54)} = 2.623$, $R^2 = 0.029$, $p = 0.086$), but no significant main effect of treatment

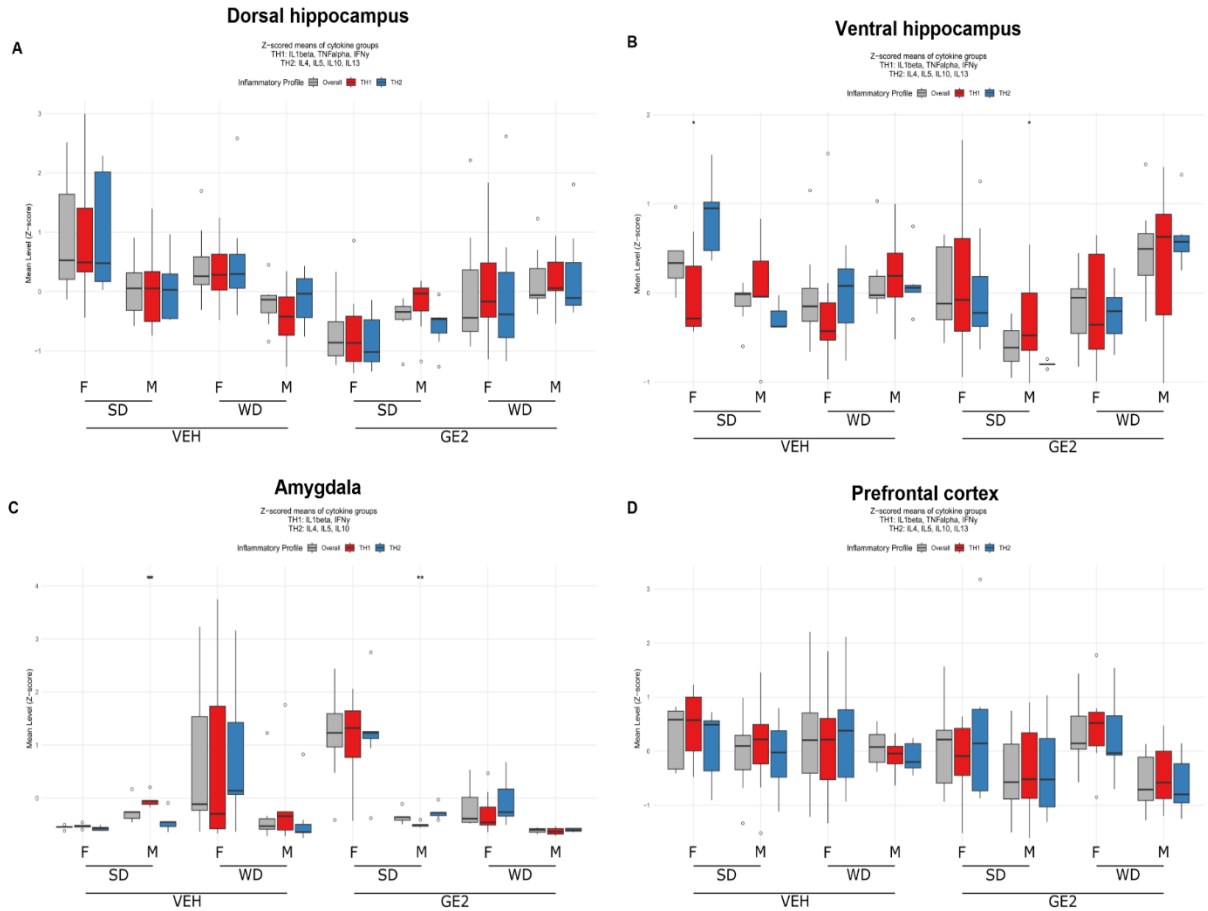

Figure 5. **Effects of sex, diet and GE2 treatment on inflammation in the dorsal hippocampus, ventral hippocampus, amygdala and prefrontal cortex.** Z-score composites for cytokines in the dorsal hippocampus (A) ventral hippocampus (B), amygdala (C) and prefrontal cortex (D). For Z-score composites the total cytokine score is presented in grey, TH1-produced cytokines in red and TH2-produced cytokines in blue. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and # $p < 0.100$. n = 6-8 (VEH) and 6-8 (GE2) per diet and sex. SD = standard diet; WD = western diet; VEH = vehicle; GE2 = GLP-1-E2; F = female, M = male. Data are presented as mean ± confidence intervals (CIs).

($p = 0.245$).

GE2 treatment decreased in males the levels of TH1- and TH2-related cytokines in SD males (TH1: $F_{(1,12)} = 77.656$, $R^2 = 0.866$, $p < 0.001$; TH2: $F_{(1,12)} = 13.013$, $R^2 = 0.520$, $p < 0.001$). However, we found an increase in TH1- and TH2-related cytokines in SD females (TH1: $F_{(1,11)} = 13.075$, $R^2 = 0.543$, $p = 0.004$; sex, diet and treatment interaction: $F_{(1,54)} = 7.970$, $R^2 = 0.100$, $p = 0.004$; TH2: $F_{(1,11)} = 15.013$, $R^2 = 0.577$, $p = 0.004$; sex, diet and treatment interaction: $F_{(1,54)} = 8.161$, $R^2 = 0.077$, $p < 0.001$). A reduction in TH1-related cytokines was also present in WD-fed males ($F_{(1,12)} = 2.424$, $R^2 = 0.168$, $p = 0.038$) but not females ($p = 0.188$), and no significant differences in TH2-related cytokines were present between treatment groups in WD-fed subjects ($p \geq 0.115$).

For TH1-related cytokines, we also found a significant interactions between diet and treatment (diet and treatment interaction: $F_{(1,54)} = 8.136$, $R^2 = 0.102$, $p = 0.003$) and the main effect of sex was also significant (main effect of sex: $F_{(1,54)} = 9.488$, $R^2 = 0.119$, $p = 0.001$). For TH2-related cytokines, we also found a significant interaction between diet and treatment (diet and treatment interaction: $F_{(1,54)} = 13.881$, $R^2 = 0.130$, $p < 0.002$), sex and treatment (sex and treatment interaction: $F_{(1,54)} = 4.068$, $R^2 = 0.038$, $p = 0.028$) and sex and diet (sex and diet interaction: $F_{(1,54)} = 6.159$, $R^2 = 0.058$, $p = 0.005$). Furthermore, the main effect of sex (main effect of sex: $F_{(1,54)} = 20.908$, $R^2 = 0.196$, $p < 0.001$) and diet (sex, diet and treatment interaction: $F_{(1,54)} = 4.155$, $R^2 = 0.196$, $p < 0.001$).

The levels of IL-6, IL-13 and TNFα were below detection in all of the samples in the amygdala. Furthermore, 59% of IL-5 and 50% of IFNγ samples were below detection. Differences in the levels of these cytokines could therefore not be analyzed. Results for individual cytokines above detection threshold can be found in the Supplement.

3.8.4. PFC

Females had significantly higher levels of inflammation in the PFC than male males (main effect of sex: $F_{(1,74)} = 3.597$, $p = 0.022$, $R^2 = 0.061$; **Fig. 5D**). When assessing TH1- and TH2-related cytokines separately, we observed that females had higher levels of TH2-related cytokines than males (main effect of sex for TH2: $F_{(1,55)} = 4.615$, $R^2 = 0.083$, $p = 0.018$), but not TH1-related

cytokines, nor were there any other significant main effects or interactions present, although there was a trend towards a diet effect (main effect of diet: $p = 0.060$; all other $p \geq 0.117$).

4. Discussion

The results from this study revealed prominent sex differences in metabolic and central aging biomarkers in response to a WD, as well as potent effects of GE2 to offset effects of a WD on cognition and aging biomarkers, in middle-aged rodents. WD increased weight gain and plasma leptin levels and reduced PSD95 in the amygdala in both sexes, whereas GE2 treatment induced weight loss, elevated plasma PYY levels, and improved associative learning in both sexes. However, there were distinct sex differences in patterns of fat distribution, blood glucose regulation, neurogenesis, inflammation and on PSD95 expression, though these effects were dependent on diet. Females on a WD accumulated more visceral fat, whereas males accumulated more subcutaneous fat. Blood glucose regulation also differed, with GE2 improving glucose levels only in females. Neurogenesis in the hippocampus was reduced with WD, but only in males, although GE2 increased neurogenesis in both sexes which depended on diet (under SD in females and WD in males). WD consumption in middle-age, increased inflammation in the amygdala of females, and in the ventral hippocampus of males, whereas GE2 treatment reduced inflammation in the hippocampus and amygdala, depending on diet and sex. These findings underscore the complex interplay between sex, diet, and GE2 efficacy in middle age, emphasizing the need for tailored therapeutic strategies.

4.1. Sex-specific increases in subcutaneous and visceral fat in response to the WD

Both sexes gained subcutaneous fat (IWAT) on a WD, but only females on the WD gained visceral fat (GWAT). This is consistent with changes in fat distribution at this age in humans, as females gain visceral fat due to the menopausal transition (44). Therefore, we may be capturing a susceptible period, in middle age, for weight gain in females, directed towards increased fat storage in their visceral depots.

Consistent with previous results, four weeks of GE2 treatment reduced body weight (59). GE2 treatment resulted in loss of visceral fat (GWAT), however, this was dependent on diet and sex. More specifically, GE2 reduced visceral fat in males on SD, and tended to reduce this fat depot

in WD-fed females. Of note, SD females had low levels of visceral fat, whereas males on SD had similar levels to their WD-fed counterparts. Collectively, these findings highlight sex-specific effects of diet and GE2 treatment on fat distribution and weight loss during middle age which may influence the effectiveness of GE2 treatment in different metabolic contexts.

4.2. Females were more sensitive to GE2-mediated blood-glucose regulation than males

In the current study, WD increased fasting glucose and impaired glucose tolerance in the GTT, but only in females. GE2 treatment reduced basal blood glucose in females but did not improve glucose tolerance in the GTT. These data indicate that females may be more sensitive to the blood-glucose regulating effects of the GE2 conjugate than males. Although this finding is inconsistent with previous results that showed a potent blood glucose regulating effect of GE2 in males but in those studies the mice (61) were younger and were given dose of GE2 6-20 times higher than used in the current study (59). Data collected in humans using GLP-1 receptor agonists do, however, indicate that females are more sensitive to the blood glucose regulating effects of these treatments, and report higher incidences of symptomatic hypoglycemia than males (66). Collectively these findings demonstrate that in middle age, although GE2 treatment effectively reduces body weight in both sexes, females may be more sensitive to its blood-glucose regulating effects.

4.3. Sex-dependent increases in plasma insulin and GLP-1 after treatment

Consistent with previous studies (67,68), we found that WD led to increases in plasma leptin compared to SD in both sexes. Interestingly, increases in plasma GLP-1 in GE2-treated rats were observed only in females. Females have previously been demonstrated to have substantially higher GLP-1 levels postprandially (69,70), and males have higher levels for Dipeptidyl Peptidase-4 (DPP-4), the main metabolizer of GLP-1 (71). Thus, that elevated plasma GLP-1 levels were found only in females, may be the result of higher endogenous GLP-1 remaining after their last meal or prolonged exposure following treatment due to differences in meal-stimulated GLP-1 secretion and GLP-1 metabolism. Of note, treatment and last meal occurred at the same time in both males and females. Furthermore, E2 increases GLP-1 secretion, and GLP-1 levels decrease in response to ovariectomy, an effect which can be rescued by E2 administration (72). It is therefore possible that GE2 increased GLP-1 secretion

in females at middle age; a mechanism which is not present in males. We also found that males had higher levels of plasma insulin than females. Females are more sensitive to insulin and males are more vulnerable to insulin resistance (73), which may explain this sex difference.

GE2 treatment also led to increased levels of plasma PYY, an appetite-suppressing hormone produced in the small intestine (74), in both sexes. Levels of PYY are body-weight dependent, with normal weight subjects presenting with higher levels of plasma PYY than obese humans, and elevations in PYY have also been seen after weight loss, especially in combination with exercise (75). Levels of PYY were increased after GE2 treatment regardless of diet or sex which is in line with the weight reductions seen in these rats.

4.4. GE2 improved associative memory in both sexes

Previous findings demonstrated that GLP-1 improves hippocampus-dependent learning and memory in both standard- (32) and high-fat diet fed male rodents (33). A recent meta-analysis found that E2 positively affects spatial memory in the Morris water maze in females, and E2 can potentiate the actions of GLP-1 on other centrally mediated behaviors, such as food intake and reward (57,58). Here we found increased contextual and cued fear memory in both sexes after GE2 treatment.

Surprisingly, WD reduced cued fear memory only in middle-aged males, but did not significantly influence contextual fear conditioning in either sex, which is consistent with a study assessing the impact of high-fat diet in middle-aged male and female mice on the fear conditioning task reported that only males were affected by the diet (76). High-fat or WD has equivocal effects on hippocampus-based memory tasks (77–84), however in tasks using amygdala-based memory task, male mice were found to have selectively impaired cued fear memory in response to diet exposure (85), consistent with our results, or finding no significant impact of WD on hippocampus-dependent memory tasks (83,84). Of note, most of these studies were conducted exclusively in younger adult male rodents. Collectively, this indicates that males may be more vulnerable to the detrimental effects of high-fat or WD in middle age, especially on amygdala-dependent associative conditioning.

4.5. WD increased inflammation in the amygdala and ventral hippocampus in a sex-specific manner and GE2 reduces inflammation in the hippocampus and amygdala dependent on diet and sex, respectively

In the present study, we found sex- and site-specific increases in inflammation in response to WD exposure, in which males presented with elevated inflammation in the ventral hippocampus, whereas females presented with increased inflammation in the amygdala. Hippocampal data is consistent with previous results in aged mice (8.5 months), that detected increased inflammatory genes in response to a high-fat diet in males only (86). Furthermore, one previous study conducted exclusively in females demonstrated that the amygdala, but not hippocampus, may be especially vulnerable to diet-induced changes in females, resulting in inflammatory dysregulation (87).

Treatment with GE2 was effective at reducing cytokine levels in the dorsal and ventral hippocampus, in rats fed a SD, but not WD. Furthermore, GE2 reduced inflammation in the amygdala, regardless of diet, but only in males The inflammatory reductions in these areas were found to encompass both TH1 and TH2 produced cytokines. Previous studies show that GLP-1 receptor agonist treatment reduced microglial activation and reverse lipopolysaccharide (LPS)-induced inflammation in the hippocampus (36,88,89), indicating that these properties are preserved in its conjugated form. Collectively, GE2 reduced inflammation in both the dorsal and ventral hippocampus, dependent on diet and sex, in addition to in the amygdala in males, regardless of diet.

4.6. WD reduced hippocampal neurogenesis in WD-fed males and GE2 treatment restored hippocampal neurogenesis in males. GE2 increased hippocampal neurogenesis in SD-fed females

In the present study, GE2 treatment increased neurogenesis in the dentate gyrus of middle-aged females fed a SD, consistent with a study examining effects of a GLP-1 receptor agonist treatment alone in middle-aged female mice (90). In males, the WD reduced neurogenesis in the dentate gyrus, was commensurate with impaired cued memory after WD consumption in males only, consistent with previous studies in young adult and middle-aged males (91–93). WD-reduced hippocampal neurogenesis was normalized by GE2 treatment consistent with effects

mediated by GLP-1 receptor agonist treatment in young adult male rodents (94–98) and adult and aged female mice (90). Collectively, GE2-treatment can increase neurogenesis in middle age, but this depended on diet and sex.

In the present study, we found that WD tended to reduce PSD95 in the amygdala only. This is in partial contrast to a study that found an increase in PSD95 in the hippocampus of male mice after GLP-1 receptor agonist treatment (99) in 5XFAD mice (a mouse model of Alzheimer's disease), but not young, male wild-type young male mice.

4.7. Limitations

Although our study demonstrated clear improvements in body weight with WD, we did not observe strong effects of the diet on fear conditioning after 8 weeks of WD exposure in middle-aged rats. It is possible stronger effects may be observed with WD in disease models for at risk for AD or diabetes or that a longer exposure to a WD could have led to more pronounced effects on behavior. However, we did see clear sustained differences in body weight, fat and plasma leptin, indicating the diet intervention was long enough to impact metabolic health. These metabolic changes may precede or contribute to neuroinflammatory and cognitive alterations over a longer duration, suggesting that extended WD exposure or pre-existing metabolic vulnerability could amplify these effects. Future studies with extended diet exposure and/or the use of rodent disease models are necessary to further explore the potential benefits of GE2 treatment.

In this study we selected a low dose of GE2, based on prior research in rats (60) and the original study using this conjugate (59), after guidance from the Novo Nordisk team (BF, JD, BY). This decision was influenced by the fact that we included female rats, which are more sensitive to the effects of GLP-1 receptor agonists (27). Research indicates that females report more adverse effects when treated with GLP-1-based therapies (100). Additionally, individuals with lower body mass, including females, are more likely to experience nausea and other gastrointestinal side effects from GLP-1 receptor agonists (101). Furthermore, because our study was conducted in normal aging rats, without diabetes or other pathological conditions such as Alzheimer's disease, we aimed to use the lowest effective dose to minimize potential adverse effects while still achieving beneficial effects on the brain. However, since some effects, such as those on

inflammation, were only observed in SD-fed subjects, it is possible that the dose was not high enough to counteract the effects of the WD, and a higher dose may have led to reductions in WD-fed rats.

4.8. Conclusions

In conclusion, the findings from this study underscore the potential of GE2 treatment as a promising therapeutic approach for reducing body weight, visceral fat, and inflammation in the dorsal and ventral hippocampus, as well as the amygdala, depending on sex and diet, while increasing neurogenesis and associative memory. The observed sex-specific effects highlight the necessity of designing treatments that account for sex differences in neurodegenerative and metabolic diseases, considering that females are disproportionately affected by both Alzheimer's disease and obesity. As obesity, diabetes and dementia rates continue to rise globally, the intersection between metabolic dysfunction and cognitive decline emphasizes the urgent need for tailored, sex-specific therapies to address these overlapping health crises, offering hope for improving the quality of life for individuals at risk.

Author contributions

Jennifer E Richard - Writing – original draft, review & editing, Methodology - in vivo experiments, inflammation, metabolic hormones and PSD95 quantification, immunohistochemistry (IHC), Study design and funding acquisition, Statistical analysis; **Ahmad Mohammad** – DCX expression quantification, aided in immunoluminescent experiments and IHC staining; **Kimberly A Go** – Aided in in vivo experiments, glucose tolerance tests and tissue collection; **Andrew J McGovern** – Statistical analysis of inflammatory cytokines (z-score and PERMANOVA and Regional Cytokine Profiling and Multivariate Analysis); **Rebecca K Rechlin** – Scoring of the fear conditioning task; **Tallinn FL Splinter** – Scoring of the fear conditioning task; **Stephanie E Lieblich** – Breeding and animal care; **Lara K Radovic** – Aided in drug delivery, weighing, behavioral testing and fear conditioning scoring; **Lydia Feng** – Aided in drug delivery, weighing, behavioral testing and fear conditioning scoring; **Samantha A Blankers** – Confocal microscopy and tissue collection; **Bin Yang** – Drug synthesis; **Jonathan D Douros** – provided the compound, and offered scientific guidance on the project; **Brian Finan** – provided the compound, and offered scientific guidance on the project; **Liisa AM**

Galea - Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest
B.F., J.D.D., and B.Y. are former employees of Novo Nordisk. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability
Data will be made available on request.

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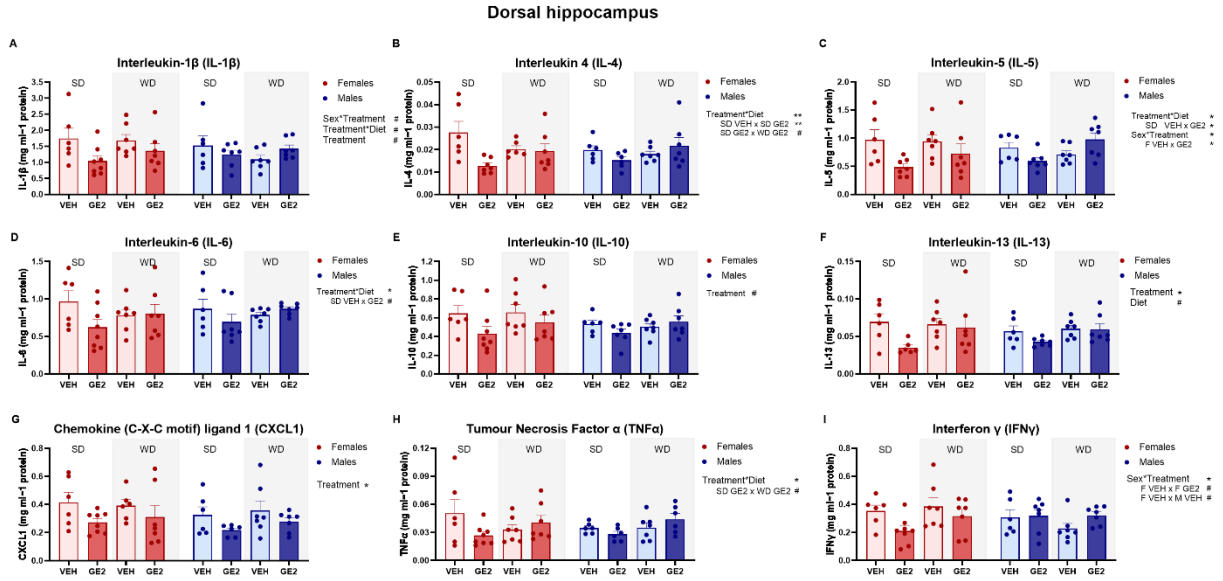
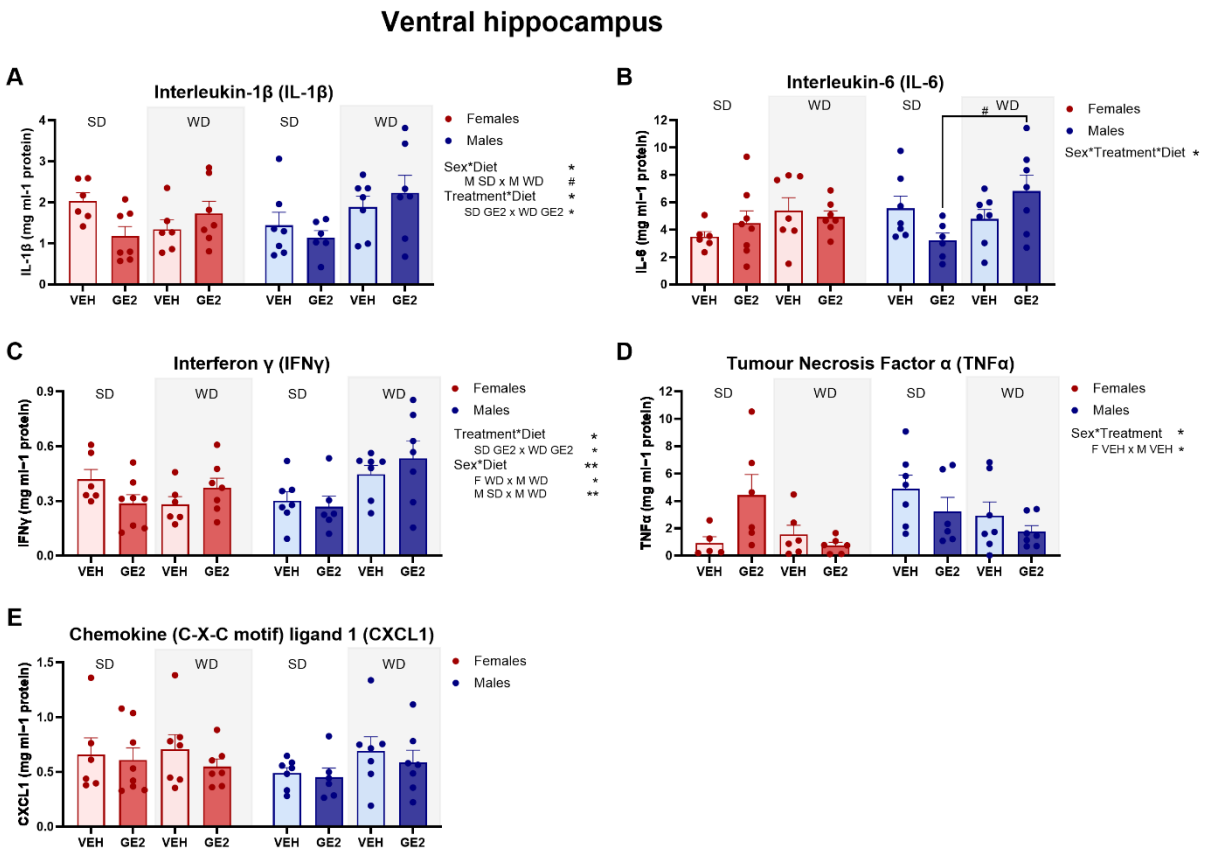
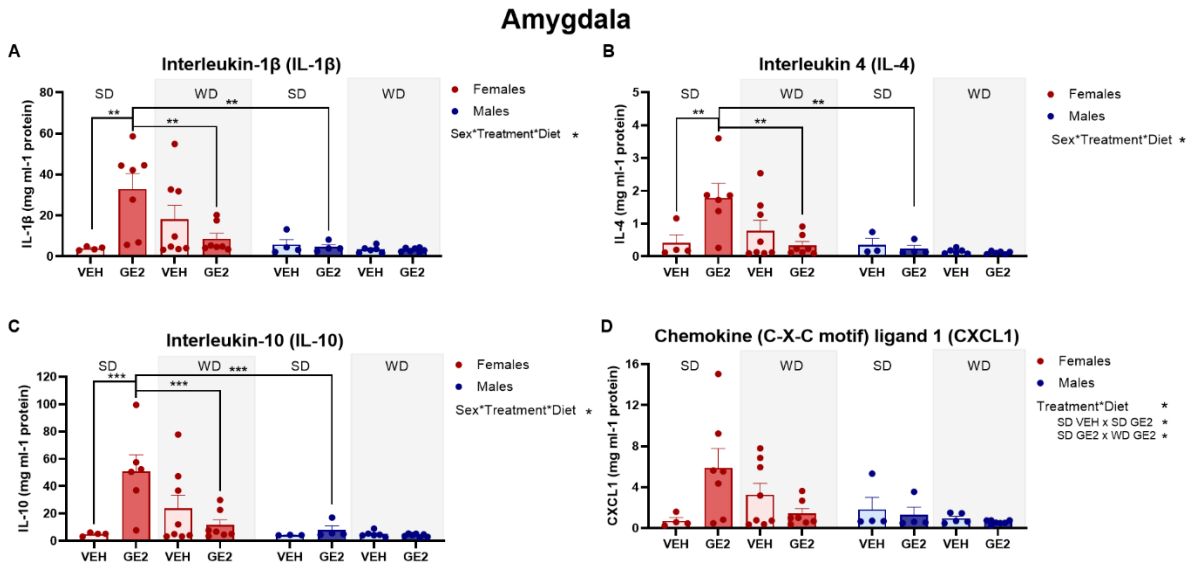
Supplementary Figures



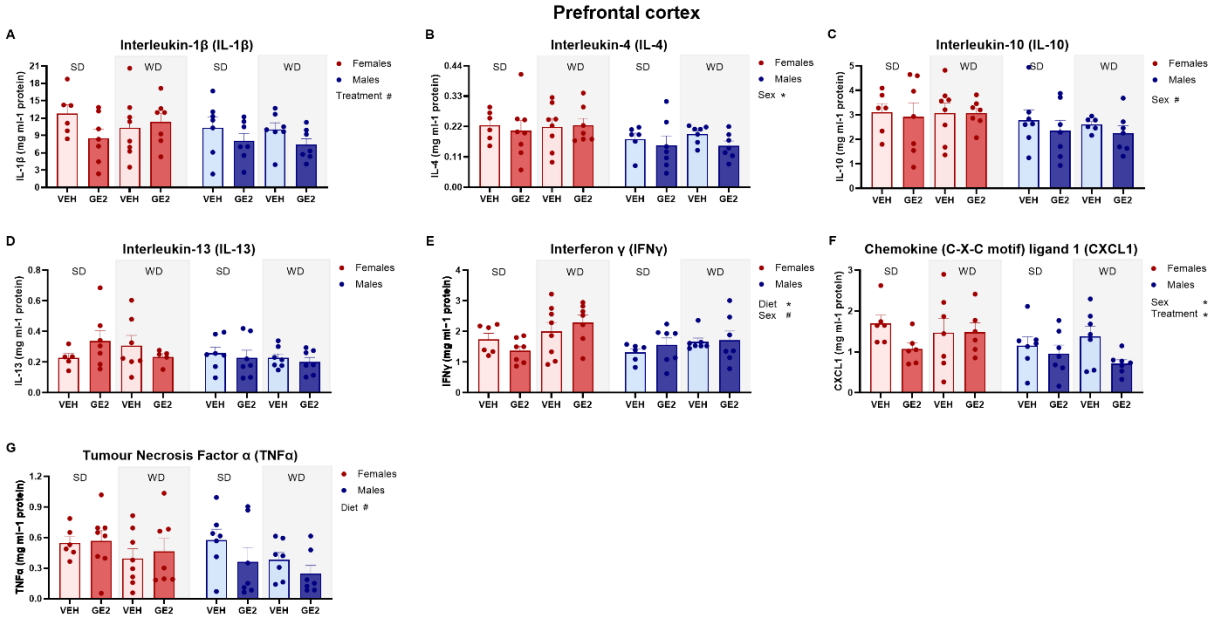
Supplementary Figure 1. **GE2 treatment reduced the levels of several cytokines in the dorsal hippocampus, with most prominent reductions in standard-diet fed females.** Levels of interleukin (IL)-1β (A), IL-4 (B), IL-5 (C), IL-6 (D), IL-10 (E), IL-13 (F), interferon (IFN) γ (G), C-X-C motif ligand 1 (CXCL1; H) and tumor necrosis factor (TNF) α (I). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and # $p < 0.100$. n = 6-8 (VEH) and 6-8 (GE2) per diet and sex. SD = standard diet; WD = western diet, VEH = vehicle, GE2 = GLP-1-E2, F = female, M = male. Cytokine levels measured in relation to protein level and expressed as mg ml^{-1}.



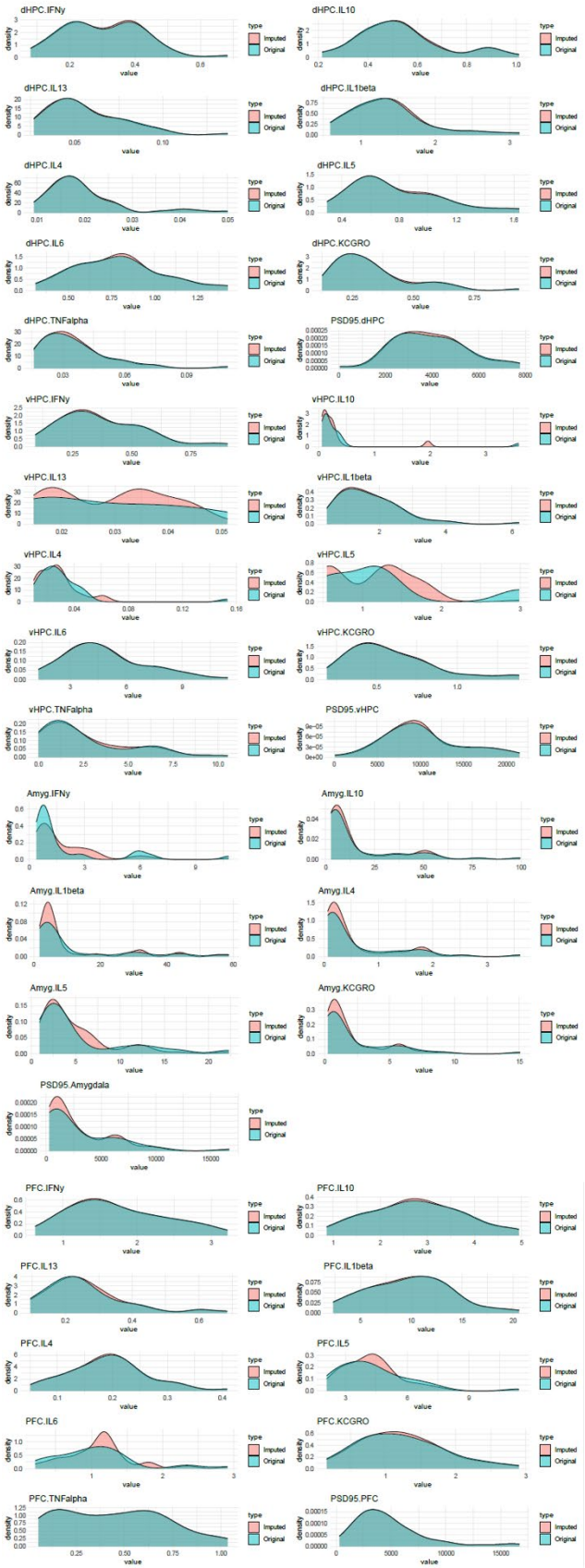
Supplementary Figure 2. **Cytokine levels in the ventral hippocampus.** Levels of IL-1β (A), IL-6 (B), IFNγ (C), TNFα (D) and CXCL1 (E) expressed as mg ml^{-1} protein. ** $p < 0.01$, *** $p < 0.001$ and # $p < 0.100$. n = 5-8 (VEH) and 6-8 (GE2) per diet and sex. SD = standard diet; WD = western diet, F = female, M = male, VEH = vehicle, GE2 = GLP-1-E2.



Supplementary Figure 3. **Cytokine levels in the Amygdala.** Levels of IL-1β (A), IL-4 (B), IL-10 (C), and CXCL1 (D) expressed as mg ml^{-1} protein. VEH = vehicle, GE2 = GLP-1-E2. * $p < 0.05$. n = 4-9 (VEH) and 4-8 (GE2) per diet and sex.



Supplementary Figure 4. **Cytokine levels in the prefrontal cortex (PFC).** Levels of IL-1β (A), IL-4 (B), IL-10 (C), IL-13 (D), IFNγ (E), CXCL1 (F) and TNFα (G) expressed as mg ml^{-1} protein. * $p < 0.05$, ** $p < 0.01$ and # $p < 0.100$. n = 5-8 (VEH) and 6-8 (GE2) per diet and sex. SD = standard diet; WD = western diet, F = female, M = male, VEH = vehicle, GE2 = GLP-1-E2.


dHPC.IFNy
dHPC.IL10
dHPC.IL13
dHPC.IL1beta
dHPC.IL4
dHPC.IL5
dHPC.IL6
dHPC.KCGRO
dHPC.TNFalpha
PSD95.dHPC
vHPC.IFNy
vHPC.IL10
vHPC.IL13
vHPC.IL1beta
vHPC.IL4
vHPC.IL5
vHPC.IL6
vHPC.KCGRO
vHPC.TNFalpha
PSD95.vHPC
Amyg.IFNy
Amyg.IL10
Amyg.IL1beta
Amyg.IL4
Amyg.IL5
Amyg.KCGRO
PSD95.Amygdala
PFC.IFNy
PFC.IL10
PFC.IL13
PFC.IL1beta
PFC.IL4
PFC.IL5
PFC.IL6
PFC.KCGRO
PFC.TNFalpha
PSD95.PFC
density
value
type
Imputed
Original

Supplementary Figure 5. Density Plots of Original and Imputed Cytokine Data. Distributions of original (teal) and imputed (pink) values for cytokines and PSD95 across four brain regions: dorsal hippocampus (dHPC), ventral hippocampus (vHPC), amygdala (Amyg), and prefrontal cortex (PFC), demonstrating the preservation of data structure following multiple imputation using the random forest method.

Individual cytokine results

Dorsal hippocampus: There was a trend towards lower levels of IL-1β in GE2-treated compared to vehicle-treated females ($p = 0.054$, Cohen's $d = 0.910$; treatment and sex interaction: $F_{(1,47)} = 3.589$, $\eta_p^2 = 0.071$, $p = 0.064$; **Supplementary Fig. 1A**), but not males ($p = 0.858$). There was also a trend towards lower levels of IL-1β specifically in SD-fed subjects ($p = 0.068$, Cohen's $d = 0.849$; treatment and diet interaction: $F_{(1,47)} = 3.124$, $\eta_p^2 = 0.062$, $p = 0.084$), but now WD ($p = 0.995$). There was also a trend towards a significant main effect of treatment (main effect of treatment: $F_{(1,47)} = 3.095$, $\eta_p^2 = 0.062$, $p = 0.085$), but no other significant main effects of interactions ($p \geq 0.342$).

GE2 treatment significant reduced levels of IL-4 in SD subjects compared to vehicle ($p = 0.005$, Cohen's $d = 1.336$; treatment and diet interaction: $F_{(1,44)} = 8.132$, $\eta_p^2 = 0.156$, $p = 0.007$; **Supplementary Fig. 1B**), but not in WD-fed subjects ($p = 0.598$). In addition, levels of IL-5 were reduced in GE2- treated subjects fed SD, but not WD, compared to vehicle-treated subjects ($p = 0.016$, Cohen's $d = 1.354$; treatment and diet interaction: $F_{(1,46)} = 5.646$, $\eta_p^2 = 0.109$, $p = 0.022$; **Supplementary Fig. 1C**). Furthermore, GE2 treatment reduced IL-5 levels in females, regardless of diet ($p = 0.020$, Cohen's $d = 0.980$; treatment and sex interaction: $F_{(1,46)} = 5.031$, $\eta_p^2 = 0.099$, $p = 0.030$); an effect not seen in males ($p = 0.863$). There was also a trend towards a significant reduction in IL-6 in GE2, compared to vehicle treated, but only on the SD diet ($p = 0.054$, Cohen's $d = 0.876$; treatment and diet interaction: $F_{(1,47)} = 4.860$, $\eta_p^2 = 0.094$, $p = 0.032$; **Supplementary Fig. 1D**).

For IL-10, GE2 tended to reduce levels compared to vehicle-treated subjects (main effect of treatment: $F_{(1,47)} = 3.744$, $\eta_p^2 = 0.074$, $p = 0.059$; **Supplementary Fig. 1E**). GE2 treatment significantly reduced IL-13 levels in the dorsal hippocampus compared to vehicle (main effect of treatment: $F_{(1,44)} = 5.346$, $\eta_p^2 = 0.106$, $p = 0.025$; **Supplementary Fig. 1F**). There was also a trend towards increased levels of IL-13 in WD compared to SD subjects (diet: $F_{(1,45)} = 3.601$, $\eta_p^2 = 0.074$, $p = 0.064$). GE2 treatment also led to a significant reduction in the levels of the chemokine CXCL1 compared to vehicle (main effect of treatment: $F_{(1,45)} = 7.319$, $\eta_p^2 = 0.140$, p

= 0.010; **Supplementary Fig. 1G**).

TNFα levels were higher in GE2-treated WD-fed subjects compared to GE2-treated SD subjects (p = 0.079, Cohen's d =1.067; treatment and sex interaction: $F_{(1,45)}$ = 6.122, η_p^2 = 0.120, p = 0.017; **Supplementary Fig. 1H**). Lastly, we found that GE2 treatment reduced levels of IFNγ compared to vehicle in females (p = 0.075, Cohen's d =0.855; treatment and sex interaction: $F_{(1,46)}$ = 6.349, η_p^2 = 0.119, p = 0.015; **Supplementary Fig. 1I**), but not males (p = 0.213).

Ventral hippocampus: WD-fed rats receiving GE2 treatment had higher levels of IL-1β than SD fed rats receiving GE2, regardless of sex (treatment and diet interaction: p = 0.030, Cohen's d =0.589; treatment and diet interaction: $F_{(1,45)}$ = 5.459, η_p^2 = 0.108, p = 0.024; **Supplementary Fig. 2A**). There was a trend towards an increase in IL-6 after GE2 treatment in WD fed compared to SD fed in males (p = 0.059, Cohen's d =1.509; sex, treatment and diet interaction: $F_{(1,47)}$ = 4.978, η_p^2 = 0.096, p = 0.030; **Supplementary Fig. 2B**), but not females (p = 0.922). Similarly, we saw in increase in IFNγ in WD-fed GE2-treated subjects compared to SD fed receiving this treatment (p = 0.006, Cohen's d =0.980; treatment and diet interaction: $F_{(1,46)}$ = 4.213, η_p^2 = 0.084, p = 0.046; **Supplementary Fig. 2C**). In addition, we found that WD consumption in males led to higher levels of IFNγ compared to WD fed females (p = 0.024, Cohen's d =0.605; sex and diet interaction: $F_{(1,46)}$ = 7.748, η_p^2 = 0.144, p = 0.008) and SD fed males (p = 0.006, Cohen's d =1.221). For TNFα we found that vehicle-treated females had lower levels of TNFα than vehicle-treated males (p = 0.018, Cohen's d =1.213; sex and treatment interaction: $F_{(1,43)}$ = 5.124, η_p^2 = 0.106, p = 0.029; **Supplementary Fig. 2D**). The main effect of sex was also significant (main effect of sex: $F_{(1,43)}$ = 4.530, η_p^2 = 0.095, p = 0.039), as well as the main effect of diet (main effect of diet: $F_{(1,43)}$ = 7.031, η_p^2 = 0.106, p = 0.011). Lastly, no significant main effects or interactions were found for CXCL1 in this area (all $p \geq 0.269$).

Amygdala: In the amygdala we found that GE2-treated females on the SD had higher levels of IL-1β than all other groups (all $p \leq 0.047$; sex, treatment and diet interaction: $F_{(1,39)}$ = 7.461, η_p^2 = 0.161, p = 0.009; **Supplementary Fig. 3A**). This effect was also present for IL-4 levels and IL-10, in which elevated levels of IL-4 (all $p \leq 0.014$; sex, treatment and diet interaction: $F_{(1,39)}$ = 6.082, η_p^2 = 0.145, p = 0.019; **Supplementary Fig. 3B**) and IL-10 (all $p \leq 0.017$ sex, treatment

and diet interaction: $F_{(1,40)} = 4.258$, $\eta_p^2 = 0.155$, $p = 0.046$; **Supplementary Fig. 3C**) in SD-fed GE2-treated females was observed, compared to all other groups. Furthermore found that GE2 increased the levels of chemokine CXCL1 compared to vehicles in SD-treated subjects ($p = 0.035$, Cohen's $d = 0.844$; treatment and diet interaction: $F_{(1,39)} = 3.580$, $\eta_p^2 = 0.125$, $p = 0.023$; **Supplementary Fig. 3D**) and that GE2 treatment in SD-fed subjects resulted in higher levels of CXCL1 than in WD-fed subjects ($p = 0.034$, Cohen's $d = 0.424$).

PFC: In the PFC, GE2 treatment led to a trend towards reduction in IL-1β levels in GE2 treated subjects compared to vehicle (main effect of treatment: $F_{(1,48)} = 3.528$, $\eta_p^2 = 0.068$, $p = 0.066$; **Supplementary Fig. 4A**). For IL-4, we found no effects of treatment, we did however find that the levels of this cytokine were higher in females than males (main effect of sex: $F_{(1,48)} = 6.954$, $\eta_p^2 = 0.127$, $p = 0.011$; **Supplementary Fig. 4B**). A similar trend was also seen for IL-10, although the sex effect did not reach significance (main effect of sex: $F_{(1,47)} = 3.847$, $\eta_p^2 = 0.076$, $p = 0.056$; **Supplementary Fig. 4C**). We found no significant effects or differences between treatment, diet groups and sex for IL-13 ($p \geq 0.163$; **Supplementary Fig. 4D**). We found higher levels of IFNγ in WD-fed subjects compared to SD fed (main effect of diet: $F_{(1,47)} = 6.785$, $\eta_p^2 = 0.126$, $p = 0.012$; **Supplementary Fig. 4E**). We also found a trend towards elevated levels of IFNγ in females compared to males (main effect of sex: $F_{(1,47)} = 3.211$, $\eta_p^2 = 0.064$, $p = 0.080$). We also found elevated levels of chemokine CXCL1 in females compared to males (main effect of sex: $F_{(1,45)} = 5.495$, $\eta_p^2 = 0.109$, $p = 0.024$; **Supplementary Fig. 4F**) . Furthermore, WD exposure led to increased levels of CXCL1 compared to SD (main effect of treatment: $F_{(1,45)} = 5.288$, $\eta_p^2 = 0.105$, $p = 0.026$; **Supplementary Fig. 4F**). No other main effects of significant interactions were present for CXCL1 ($p \geq 0.093$). In contrary to CXCL1, we found that WD led to reduced levels of TNFα compared to SD (main effect of diet: $F_{(1,49)} = 3.760$, $\eta_p^2 = 0.071$, $p = 0.058$; **Supplementary Fig. 4G**). There were no other significant main effects of interactions (all $p \geq 0.136$).