OBESOGENIC ENVIRONMENTAL CONDITIONS AFFECT NEURODEVELOPMENT AND NEURODEGENERATION

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EDITORIAL
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Editorial: Obesogenic Environmental Conditions Affect Neurodevelopment and Neurodegeneration

Gustavo Pacheco-López[1]*, *Marcel Pérez-Morales*[1]*, *Kioko Rubí Guzmán-Ramos*[1], *Johnny Davis Figueroa*[2], *Ute Krügel*[3] and *Javier A. Bravo*[4]

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Keywords: obesogenic environments, neurodevelopmental diseases, neurodegenerative diseases, obesity, energy homeostasis

Editorial on the Research Topic

Obesogenic Environmental Conditions Affect Neurodevelopment and Neurodegeneration

The classical debate between nativism (nature, genes) and empiricism (environment, nurture) originated in ancient Greece and developed in the Renaissance, including analogies of Aristotle and metaphors of Gottfried Wilhelm Leibniz about the contents of the mind, persists to our days in neurobiology. The subject of this Research Topic is an example of this controversy. From a methodological perspective, an obesogenic environment is an experimental approach in which environmental manipulations, such as exposure to hypercaloric diets, drinking sugar-sweetened beverages (i.e., diet-induced obesity), and reduced physical activity, separately or in combination, may lead to metabolic imbalances, weight gain, and finally to adiposity. The scientific field continues making strides to determine the contribution of the myriad of obesogenic factors modulating energy homeostasis.

In this Research Topic, six original papers, two reviews, and one mini-review summarize the impact of several obesogenic factors upon food intake regulation, obesity-associated biomarkers, and describe several behavioral and molecular approaches that enhance our understanding of obesity. In the context of the sedentary lifestyle and the constant availability of junk food that has dramatically increased in the last 30 years all over the world, Flores-Dorantes et al. provide an integrative view on the relationship between obesogenic environments and obesity, with both neurodegenerative and neurodevelopmental diseases. The authors emphasize the involvement of genes traditionally associated with food intake regulation, such as those that code for the leptin-melanocortin pathway proteins and their incidence on neurodegenerative and neurodevelopmental diseases. They call our attention to the gene-environment interaction that promotes an imbalance between caloric intake and energy expenditure. They provide further evidence for common neurobiological pathways between obesity and neurodegenerative diseases, suggesting that metabolic syndrome and obesity are risk factors for Parkinson's disease and Alzheimer's disease (AD).

Whereas a relationship between obesity and the development of neurodegenerative diseases is widely accepted, we are just beginning to understand how environmental factors and the microbiome contribute to these conditions. Bello-Medina et al. demonstrate evidence for spatial memory deficits, differences in beta diversity, and lower relative abundances of *Actinobacteria* and *TM7* in the gut microbiota of 3 months-old 3xTg-AD mice, one of the most used transgenic models of AD, compared to non-transgenic mice. Their results suggest that microbiota alterations could be

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related causally to cognitive decline and may be employed as non-invasive biomarkers of AD, even at a presymptomatic stage.

Peleg-Raibstein focuses on the impact of maternal overnutrition by highly palatable diets, high-fat diets, a combination of high-fat-high-sugar diets, or cafeteria diets on the development of obesity and cardio-metabolic dysfunction and cognitive aging in the progeny. The article emphasizes the risk of unhealthy food environments provided by parents and the consequent exposure of their children. The author points to difficulties to discern between genetic and environmental risk factors to develop obesity and brain pathologies in the offspring. Related to these concepts, Cruz-Carrillo et al. employed a 9-week maternal overnutrition protocol. The authors report that the offspring from cafeteria diet-fed mothers exhibit an increased motivation to obtain pellets in operant tasks and reinforce their orexigenic response following subcutaneous injection of ghrelin. This behavior was associated with a higher global methylation in the shell region of the nucleus accumbens (NAcSh) and correlated with changes in the expression of pro-inflammatory cytokines. These results propose that high caloric nutritional intake during pregnancy and lactation increases the susceptibility to develop addictive-like behavior patterns in the progeny.

Apart from influencing obesity and metabolic dysfunction, Tsan et al. review literature in which overconsumption of highly palatable and dense foods leads to long-lasting multiple neurocognitive impairments in diverse rodent models. They conclude that overconsumption of *Western diets* during critical neurodevelopment stages is associated with detrimental cognitive performance across the lifespan. An example of this intricated relationship is given by Vega-Torres et al. In their study, the consumption of a *Western-like* high-saturated diet by adolescent rats for only 1 week is sufficient to impair emotional reactivity and anxiety. Cued fear extinction memory and neuronal activation of the basomedial amygdala attenuated, and corticosterone mRNA levels in the medial prefrontal cortex increased, altogether reveal the harmful impact of short-term exposure to obesogenic environments on the corticolimbic circuitry and emotional regulation during specific stages of neurodevelopment. Another study provided by Han et al. investigated the effect of a much more chronic exposure of young C57BL/6J mice to a high-fat diet. In their study, body weight, anxiety-like behavior, and, at the molecular level, the midbrain content of dopamine and D2 dopamine receptors increased after chronic diet exposure. From the pharmacological perspective and in line with food intake-associated dopaminergic activity, Kalyanasundar et al. evaluated the effect of D-norpseudoephedrine (NPE, also Cathine), an amphetamine-like sympathomimetic and anorexigenic drug, on food intake, body weight loss, and locomotion. Their results suggest that NPE-induced anorexia and body weight loss modulates neuronal spiking activity in the NAcSh. Though NPE is an African lifestyle drug with addiction risk, as an experimental tool, it elucidates feeding regulation associated with NAcSh neurons' activity in part controlled by midbrain dopaminergic neurons. Regarding the genetic aspects of metabolic diseases, Amaya et al. performed a translational study in $AdKO_{2.0}$ transgenic mice, an animal model of Cushing's syndrome (CS), which is characterized by hypercorticosteronemia and metabolic abnormalities predisposing for obesity and metabolic syndrome, due to an inactivation of the gene encoding the regulatory subunit 1 alpha of the PKA, targeted to the adrenal cortex. Long-term exposure to high plasma glucocorticoids is strongly related to the gain of visceral tissue and stimulation of lipogenic pathways triggered by chronic stress. Their study shows overall parallels between imaging data from CS patients and $AdKO_{2.0}$ transgenic mice as there is volume reduction in several brain areas and lower astrocytic and microglial markers, providing the opportunity to study the molecular basis and consequences of hypercorticosteronemia in a preclinical model.

The articles included in this Research Topic contribute to a more profound view of the complex relationship and potential causality between various obesogenic environmental conditions with the etiology of relevant neurodevelopmental and neurodegenerative diseases. The editors of this topic want to thank all the authors for their important contributions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Fetal Programming by Methyl Donors Modulates Central Inflammation and Prevents Food Addiction-Like Behavior in Rats

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Fetal programming by hypercaloric intake leads to food addiction-like behavior and brain pro-inflammatory gene expression in offspring. The role of methylome modulation during programming on central immune activation and addiction-like behavior has not been characterized. We employed a nutritional programming model exposing female Wistar rats to chow diet, cafeteria (CAF), or CAF-methyl donor's diet from pre-pregnancy to weaning. Addiction-like behavior in offspring was characterized by the operant training response using Skinner boxes. Food intake in offspring was determined after fasting–refeeding schedule and subcutaneous injection of ghrelin. Genome-wide DNA methylation in the nucleus accumbens (NAc) shell was performed by fluorescence polarization, and brain immune activation was evaluated using real-time PCR for pro-inflammatory cytokines (IL-1β, TNF-1α, and IL-6). Molecular effects of methyl modulators [S-adenosylmethionine (SAM) or 5-azatidine (5-AZA)] on pro-inflammatory cytokine expression and phagocytosis were identified in the cultures of immortalized SIM-A9 microglia cells following palmitic acid (100 μM) or LPS (100 nM) stimulation for 6 or 24 h. Our results show that fetal programming by CAF exposure increases the number of offspring subjects and reinforcers under the operant training response schedule, which correlates with an increase in the NAc shell global methylation. Notably, methyl donor's diet selectively decreases lever-pressing responses for reinforcers and unexpectedly decreases the NAc shell global methylation. Also, programmed offspring by CAF diet shows a selective IL-6 gene expression in the NAc shell, which is reverted to control values by methyl diet exposure. *In vitro* analysis identified that LPS and palmitic acid activate IL-1β, TNF-1α, and IL-6 gene expression, which is repressed by the methyl donor SAM. Finally, methylation actively represses phagocytosis activity of SIM-A9 microglia cells induced by LPS and palmitic acid stimulation. Our *in vivo* and *in vitro* data suggest that fetal programming by methyl donors actively decreases addiction-like

behavior to palatable food in the offspring, which correlates with a decrease in NAc shell methylome, expression of pro-inflammatory cytokine genes, and activity of phagocytic microglia. These results support the role of fetal programming in brain methylome on immune activation and food addiction-like behavior in the offspring.

Keywords: programming, methylome, addiction, nucleus accumbens, inflammation

INTRODUCTION

Maternal obesity or maternal hypercaloric intake in humans and murine models is associated with an increased risk of systemic and central pathologies early in life. We and others have published that maternal overnutrition programs negative metabolic and immune profiles (Cardenas-Perez et al., 2018; Maldonado-Ruiz et al., 2019), and addiction (Camacho, 2017; Sarker et al., 2018), as well as depression-like behavior phenotypes in the offspring (de la Garza et al., 2019). Excessive high-energy food consumption seems to modulate positively or negatively a hedonic phenotype in humans and animal models. Several neurotransmitter-related hypotheses were proposed to explain unhealthy eating; for instance, the hypothesis of the reward deficiency states that in the context of high caloric overfeeding or obesity, uncontrolled food intake is activated in order to compensate for a deficient reward effect of food consumption due to failure in the dopaminergic activity (Kenny, 2011; Land and DiLeone, 2012; Barry et al., 2018; Courtney et al., 2018; Gold et al., 2018; van Galen et al., 2018; DiFeliceantonio and Small, 2019; Ducrocq et al., 2019). Under this scenario, impulsiveness for unhealthy eating and caloric overconsumption as a reward is developed (Volkow et al., 2011; Dietrich et al., 2014; Bongers et al., 2015). Classically, reward and incentive salience are integrated to the nucleus accumbens (NAc), whereas preoccupation/anticipation including craving, impulsivity, and executive function involve the prefrontal cortex (PFC) (Volkow and Wise, 2005; Volkow et al., 2013; Koob and Volkow, 2016).

Potential molecular or cellular mechanism leading to addiction-like behavior during maternal hypercaloric exposure is not totally understood. Hypercaloric diets themselves stimulate central and peripheral inflammatory nodes. For instance, exposure to saturated fatty acids during pregnancy activate the Toll-like type 4 receptor signaling (TLR4) in adipocytes and macrophages (Shi et al., 2006) and promotes substantial activation of microglia in the hypothalamus (Maldonado-Ruiz et al., 2019). Systemic and central immune activation pathways regulate abnormal behaviors including depression (Wohleb et al., 2016), schizophrenia (Yuan et al., 2019), and addiction (Alfonso-Loeches et al., 2010; Pascual et al., 2011; Hofford et al., 2018). Conversely, systemic treatment of the anti-inflammatory drug minocycline to methamphetamine-addicted rats reverted addiction-like behavior (Snider et al., 2013; Attarzadeh-Yazdi et al., 2014). Of note, morphine binds to the TLR4 directly, which increases the risk of drug-induced reinstatement (Schwarz and Bilbo, 2013), and blocking the TLR4 pathway in the VTA of rats reduces cocaine and morphine-primed drug seeking (Tanda et al., 2016; Chen et al., 2017; Brown et al., 2018), which seems to depend on microglia activation (Northcutt et al., 2015). Physiologically, TLR4 signaling regulates glutamatergic stimulation in the NAc shell (Kashima and Grueter, 2017); however, repeated cocaine administration activates striatal microglia, leading to TNF-α production and disrupting glutamatergic synaptic strength in the NAc shell (Lewitus et al., 2016). These data suggest that microglia activation and TLR4 signaling positively modulate reinstatement for drug-seeking behavior. It is unknown if addiction-like behavior primed by maternal hypercaloric programming sets a TLR4-like inflammatory profile in NAc capable of regulating addiction-like behavior in the offspring.

Exposure to hypercaloric diet in mice sets a higher proliferation and immune response of myeloid progenitor cells by activating an epigenetic reprogramming mechanism (Christ et al., 2018), known as trained immunity (Netea, 2013). Physiologically, epigenetic modulation, such as DNA methylation, positively activates neuronal differentiation in mammals (Mohn et al., 2008), regulates synaptic plasticity in the hippocampus (Levenson et al., 2006), and, by its own neuronal activity induced by external cues, closely modulates DNA methylation (Maag et al., 2017). For instance, chronic exposure to amphetamine, ecstasy, or MDMA favors a positive global DNA methylation and pro-inflammatory profile in the NAc shell of rats (Mychasiuk et al., 2013), and shows an increase in tri-methylation of lysine 4 in histone H3 (H3K4me3) on the pronociceptin, prodynorphin, and neuropsin promoters, while decreasing the acetylation of lysine 9 in Histone H3 (H3K9ac) in the nociceptin/orphaninFQ (pN/OFQ) (Caputi et al., 2016). Also, maternal care of rat offspring blocks drug-induced reinstatement of morphine by decreasing the methylation of the IL-10 promoter in NAc shell (Schwarz et al., 2011). These evidences support a potential role of the brain proinflammatory profile and drug addiction susceptibility actively modulated by methylation and/or acetylation of histones and/or DNA. Overall, we hypothesized that maternal programming by caloric diet exposure primes DNA methylation changes and a pro-inflammatory profile in NAc favoring addiction-like behavior for natural rewards in the offspring.

The present study aimed to explore the role of maternal nutritional programming and its effects on DNA methylation, pro-inflammatory profile, and susceptibility to addiction-like behavior in the offspring.

MATERIALS AND METHODS

Animals and Housing

All the experiments were performed using 2-month-old wild-type female Wistar rats (initial body weight, 200–250 g). Animals

were handled according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised in 1996). We followed the Basel Declaration to implement the ethical principles of Replacement, Reduction, and Refinement of experimental animal models. Our study was approved by the local Animal Care Committee (BI0002) at the Universidad Autónoma de Nuevo León, Mexico. Rats were housed individually in Plexiglas-style cages, maintained at 20–23°C in a temperature-controlled room with a 12-h light/dark cycle. Water was available *ad libitum* in the home cage. Food availability is described below.

Diets

- The standard chow diet formula contained 57% carbohydrates, 13% lipids, and 30% proteins, and 290 mg of sodium, caloric density = 3.35 kcal/g (LabDiet, St. Louis, MO 63144, 5001, United States). Cafeteria (CAF) diet was made of liquid chocolate, biscuits, bacon, fried potatoes, standard chow diet, and pork paté at a 1:1:1:1:1:1:2 ratio, including 39% carbohydrates, 49% lipids, 12% proteins, and 290 mg of sodium, caloric density = 3.72 kcal/g, as we reported (Camacho, 2017; Cardenas-Perez et al., 2018; Maldonado-Ruiz et al., 2019). CAF diet with methyl donors consisted of CAF formula enriched with betaine (5 g/kg of diet), choline (5,37 g/kg of diet), folic acid (5,5 mg/kg of diet), and vitamin B12 (0.5 mg/kg of diet). Total fiber available for CAF diet and standard chow diet is similar and can be found in **Supplementary Table S1** in **Supplementary Material**.

Maternal Programming Model

Animals were acclimated to the animal facility 7 days before being exposed to the diet. A total of 27 10-week-old female Wistar rats (initial body weight, 200–250 g) were housed in standard conditions as described above with *ad libitum* access to food and water. Females were randomized into three different dietary groups: standard chow diet (C, $n = 8$), CAF diet ($n = 14$), and CAF + methyl donor supplemented (CAF + Met, $n = 5$) and were exposed *ad libitum* to them for 9 weeks, including 3 weeks before mating, 3 weeks during gestation, and 3 weeks during lactation. Rats were mated with age-matched Wistar males for 2 days, after which males were removed from the home cage. Pregnancy diagnosis was performed after mating by vaginal plug. Female rats lacking plugs were returned to the home cage for a second mating. Pregnant rats were transferred to individual cages and were kept on the same diet until birth and lactation. Pregnant females might have uneven litter load during gestation; however, litters were adjusted to 10 pups per mother after birth. After 21 days of lactation, male offspring were exposed to a control diet until 2 months of age before the operant training test protocol (see below for details and **Figure 1A** for experimental design). We chose male offspring based on the potential hormone sensitive-behavioral effects in females. In any case, in order to follow the ethical principles of Replacement, Reduction, and Refinement of experimental animal models, we allocated all female offspring to a second experimental behavioral protocol, which is currently under investigation.

Operant Training Test

We followed the operant training protocol using the Skinner box as reported before (Camacho, 2017), with slight modifications.

Rat Acclimation and Food Restriction

Upon arrival, offspring rats from the three dietary groups: chow diet, CAF, and CAF + Met were group-housed for at least 7 days to acclimatize with *ad libitum* access to standard chow diet and water. After acclimation, rats were subsequently single-housed, and food was restricted by lowering to 70% their daily chow food intake until they lost 5–10% of total body weight at 7 days. This manipulation allowed acquisition of efficient lever-press responding during training. Upon reaching desired weight, we adjusted daily food intake to stabilize their weights for the remainder of the training period.

Fixed Ratio (FR)-1 Schedule

Rats were trained to press the lever on a fixed ratio (FR)-1 reinforcement schedule where a single lever press delivers a food pellet to the receptacle. Only one lever is designated as "active," showing a 5-s timeout (TO) to the FR1 schedule (FR1/TO-5), during which additional lever pressing does not result in the delivery of a food pellet. Each FR training session lasts 1 h or when 100 pellets have been delivered. Training on the FR1 schedule lasted 3 days.

Fixed Ratio (FR)-5 Schedule

We established the FR5/TO-5 seconds schedule where five active lever presses triggered the delivery of the food pellet. Training on the FR5 schedule lasted 4 days. As for FR1/TO-5, schedule rats were exposed to *ad libitum* access to standard chow and water.

Progressive Ratio (PR) Schedule

The PR testing was calculated as per Richardson and Roberts (1996) using the following formula (rounded to the nearest integer): = [5e (R × 0.2)]−5, where R is equal to the number of food rewards already earned plus 1 (i.e., next reinforcer). Thus, the number of responses required to earn a food reward follow the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, and so on. The PR session lasts a maximum of 1 h per day. Failure to press the lever in any 10-min period results in termination of the session. We also verified performance on the PR schedule by documenting the stable reinforcement for food when the number of rewards earned in a 1-h session deviates by ≤10% for at least 3 consecutive days. Like in the FR1/TO-5 and FR5/TO-5 schedule, rats were exposed to *ad libitum* access to standard chow and water. Training on the PR schedule lasted 5 days.

Calculation of Motivation and Intake Scores

Addiction-like and non-addiction-like behavior in the offspring subjects was diagnosed by integrating lever presses during the FR5 and PR schedules, as reported (Bock et al., 2013; Le et al., 2017). We use the $\times = \frac{X_i - \overline{X}}{s.d}$, where X_i is the behavior value for each rat, $\overline{X}$ is the mean behavior value for all the rats, and *s.d.* is the standard deviation of the population behavior value. We identified addiction-like and non-addiction-like behavior in the offspring by the FR5/PR ratio by setting a +0.5 or −0.5 score at

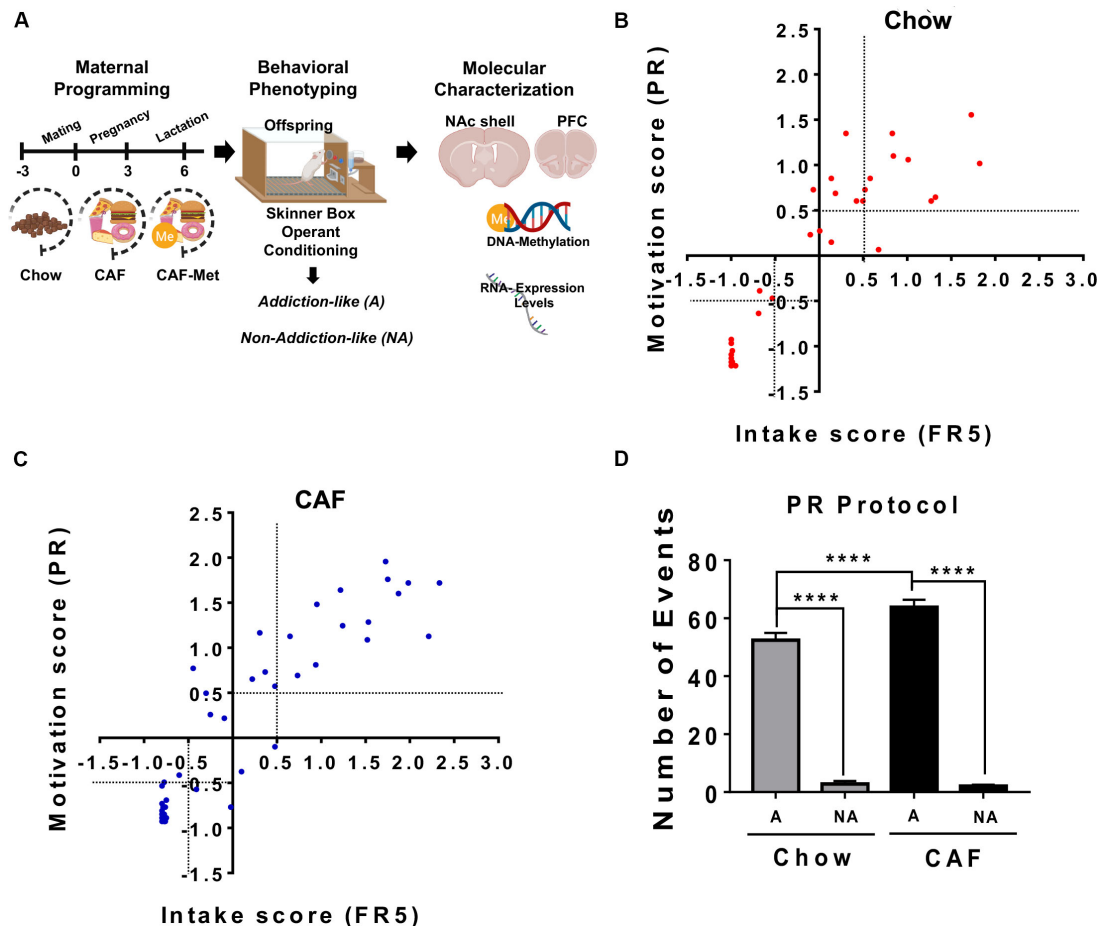

FIGURE 1 | Maternal programming by CAF diet primes addiction-like behavior in offspring. **(A)** Maternal programming model. We fed female Wistar rats for 9 weeks including pre-pregnancy, pregnancy, and lactation with standard chow diet (C, $n = 8$) or CAF diet ($n = 14$). Offspring was fed with control diet after weaning until 2 months of age and was trained in the operant training test protocol to diagnosed non-addiction and addiction-like behavior. Molecular characterization of pro-inflammatory expression and global DNA methylome in the NAc shell were performed in non-addiction and addiction-like behavior subjects. **(B,C)** To diagnose addiction-like and non-addiction-like behavior phenotypes in the offspring subjects, we calculated the intake and motivation scores by integrating lever presses during the FR5 and PR schedules, respectively, as reported (Bock et al., 2013; Le et al., 2017). We identified addiction-like and non-addiction-like behavior in the offspring by the FR5/PR ratio by setting a +0.5 or –0.5 score at "*x*" and "*y*" axis to characterize the addiction or non-addiction-like behavior subjects, respectively. Motivation (based on PR) or intake (based on FR5) scores in the offspring programmed by chow or CAF, respectively. Graphs show mean ± SEM. Chow, $n = 53$; CAF, $n = 36$. **(D)** Number of events during PR protocol of subjects exposed to chow or CAF diet during programming. Graphs show mean ± SEM. Chow diet programmed, addict = 10, non-addict = 14; CAF diet programmed, addict = 10, non-addict = 27. Statistical significance after using one-way ANOVA, followed by a Bonferroni's *post hoc* test. The differences between the groups of operant training test were tested by two-way repeated measures ANOVA. $^{****}p < 0.0001$. A, Addiction-like; NA, Non-addiction-like.

"*x*" (intake score) and "*y*" (motivation score) to characterize the addiction- or non-addiction-like behavior subjects, respectively. This analysis allows one to set the intake and motivation scores for each subject to classify them as high and low responses (addiction- or non-addiction-like behavior) to operant training for natural rewards during the FR5 and PR schedules.

Dams after lactation and offspring after addiction-like and non-addiction-like behavior characterization were sacrificed using decapitation according to the NORMA Oficial Mexicana NOM-062-ZOO-1999. We used decapitation to preserve DNA/RNA integrity. Also, we allocated all female offspring to a second experimental behavioral protocol, which is currently under investigation.

Microglia Cell Culture and Treatments

SIM-A9 (CRL-3265) mouse microglia cells were purchased from ATCC (Manassas, VA, United States) and were expanded in Corning® T75 cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM high glucose 4.5 g/L, Caisson Labs, EE.UU, Utah) supplemented with 10% (vol/vol) 5% heat-inactivated horse serum and heat-inactivated fetal bovine serum 10% (Sigma Aldrich, EE.UU, Missouri), 50 units/ml penicillin, and 50 μg/ml streptomycin (Penicillin/Streptomycin, Sigma Aldrich, EE.UU, Missouri) in a 5% CO_2 incubator at 37°C. The cells were split when they reached 70–90% confluence (plate ratio of 1:4) using a PBS/EDTA/EGTA/GLUCOSE solution

(1 × /1 mM/1 mM/1 mg/ml) at 37°C for 1–5 min, followed by washing/resuspension in growth medium.

After confluence, cells were plated in 6-well plates and 96-well plates, at 200,000 and 5000 cells per well accordingly and pre-incubated with 5-Aza-2′-deoxycytidine (5-AZA) (Sigma Aldrich, A3656) or S-(5′-adenosyl)-L-methionine (SAM) (Sigma-Aldrich, A2408) for 24 h and cell viability was evaluated as described below. Based on cell viability assay in some experiments, we used preincubation of 250 μM SAM or 75 nM 5-AZA or 0.1% DMSO (Control) for 24 h followed by 500 ng lipopolysaccharide (LPS) (stock 1 μg/ml) (Sigma-Aldrich, L3023) or 100 μM palmitic acid (PAL) (Sigma-Aldrich, P9767) stimulation for the next 6 and 24 h. PAL was first solubilized in DMEM containing 10% of free fatty acid bovine serum albumin and then administered in each well.

Cell Viability Analysis

Cell survival was determined using the MTT (Cell proliferation kit I, Roche Diagnostics, Mannheim, Germany) following manufacturer instructions by adding MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) for 1 h at 37°C in a CO_2 chamber. Cell viability was quantified at 570 nm wavelength. Results are expressed as percentage of MTT reduction relative to control cells treated with 0.1% DMSO.

Quantitative Phagocytosis Assay in Microglia Cells

Phagocytosis in microglia cells was determined using the green fluorescent latex beads (Sigma, L1030-1ML), which were pre-opsonized in fetal bovine serum (FBS) (1:5 ratio) for 1 h at 37°C. Subsequently, the FBS with the beads was added to the wells to obtain a final concentration of beads = 0.1% (v/v) as previously reported in Lian et al. (2016). After the cell treatment described previously, cells were incubated with the beads during 6 h at 37°C. We also incubated the experiment at 4°C for 6 h as a negative control.

The percentage of phagocytosis of the microglia was analyzed using a flow cytometer (BD Acuri C6 Plus). After the 6-h incubation ended, the culture medium containing the beads was discarded and the cells were washed twice with sterile 1 × PBS at 37°C. Cells were detached using 1 ml of the PBS/EDTA/EGTA/GLUCOSE solution (1 × /1 mM/1 mM/1 mg/ml) and resuspended in 100 μl of 1 × PBS for analysis in the BD Acuri C6 Plus flow cytometer in the channel FL1-A.

Qualitative Phagocytosis Assay in Microglia Cells

To visualize phagocytosis under conditions of proinflammatory LPS and PAL stimulation and its modulation by 5-AZA and SAM methylation modulators, we used confocal microscopy. SIM-A9 cells were seeded on sterile coverslips in six-well plates covered with 0.01% poly-L-lysine solution (Sigma, p4707). After treatment, cells were fixed by adding 4% paraformaldehyde (PAF) (Sigma, 158127) for 10 min, washed three times with 1 × PBS, and were permeabilized with PBS solution 1 × + 0.1% Triton X-100 (PBST) (Sigma, × 100) for 10 min. Next, the cells were blocked in PBST + 10% goat serum (GIBCO, 16210064) for 30 min and washed three times with 1 × PBS. Microglia immunodetection was performed by overnight primary antibody anti-mouse Iba-1 (1:200) (Abcam, ab178847) incubation at 4°C followed by goat anti-rabbit IgG coupled to Alexa Fluor 546 (1:1000) (Invitrogen, A-11035). Finally, cells were washed 3 × with 1 × PBS and mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories, H-1000-10). Fluorescent signals were detected by confocal-laser microscopy using an Olympus BX61W1 microscope with an FV1000 module with diode laser. Finally, the images were processed with ImageJ software.

NAc Shell and PFC Isolation and DNA/RNA Extraction

Brains were isolated from addiction-like and non-addiction-like behavior of subjects from the three dietary groups: chow diet, CAF, and CAF + Met and were frozen on dry ice and stored at −80°C until the collection of tissue. NAc shell and *PFC* of each brain were surgically isolated following stereotaxic coordinates according to Paxinos and Watson (2007) and total DNA/RNA extraction was performed by utilizing a purification kit (Qiagen) according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR

We used 200 ng of total RNA for cDNA retro transcription using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. Quantitative real-time RT-PCR was performed on a Lightcycler 480 system (Roche) using a iQ™ SYBR® Green SuperMix (Bio-Rad) with cDNA and primers (0.5 μM). The sequence primers used for analysis were as follows: IL-6 forward, 5′-TAGTCCTTCCTACCCCAATTTCC-3′. IL-6 reverse, 5′-TTGGTCAGCCACTCCTTC-3′. IL-1β forward, 5′-GCAACTGTTCCTGAACTCAAC-3′. IL-1β reverse, 5′-ATCTTTTGGGGTCCGTCAACT-3′. IL-1α-forward, 5′-GCACCTTACACCTACCAGAGT-3′. IL-1α reverse, 5′-TGCAGGTCATTTAACCAAGTGG-3′. TNFα forward, 5′-CAGGCGGTGCCTATGTCTC-3′. TNFα reverse, 5′-CGATCACCCCGAAGTCAGTAG-3′. 36B4 forward, 5′-TCCAGGCTTTGGGCATCA-3′. 36B4 reverse, 5′-CTTTATCAGCTGCACATCACTCAGA-3′. Changes in gene expression were evaluated using the $-\Delta\Delta$Ct method.

Global DNA Methylation Analysis

Genomic DNA (50 ng/μl) was obtained as described before and quantified using the SYBR Green I protocol (Gragene, DNA Genotek Inc., Canada). For the global methylation analysis (Shiratori et al., 2016), 800 ng of DNA was digested with *Hpa*II (H) and with *Msp*I (M) restriction enzymes and incubated at 37°C for 2 h. Afterward, samples were incubated with the final extension reaction (1.7 × PCR buffer, 0.75 U Taq DNA polymerase) (Thermo Fisher Scientific) and 17 nM of TAMRA-dCTP (Jena Bioscience) for 1 h at 58°C in complete darkness. Aliquots of each final extension reaction were placed in 384-well black plates (Greiner Bio-One). The incorporation of TAMRA-dCTP fluorescence was directly measured using the

Infinite M1000-Tecan microplate reader (excitation/emission 535/590 nm). Fluorescence polarization values (FP) were computed by the i-control^{TM} software (Tecan). The average of the FP values was calculated for each condition: FPSD, FPH, and FPM. The FPH and FPM values were normalized by subtraction of the FPSD value. Once the FP values of each sample were normalized, the 5 mC content of the genomic DNA was obtained with the following formula:% 5 mC ADN = (1-(FPH/FPM)) × 100.

Statistical Analyses

All statistical tests were performed using the GraphPad Prism Version 7. For the *in vivo* data including the real-time RT-PCR analysis, we used one-way ANOVA, followed by a Bonferroni's *post hoc* test. The differences between the groups of operant training test were tested by two-way repeated measures ANOVA. For the *in vitro* data including the real time RT-PCR and phagocytosis analysis, we used one-way ANOVA, followed by a Tukey's *post hoc* test from one independent experiment of a total of four. Data are presented as mean ± SEM. The significance levels displayed on figures are as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Maternal Programming by Cafeteria Diet Favors Addiction-Like Behavior in Offspring

We initially evaluated the effect of cafeteria nutritional programming on addiction-like behavior in the offspring using the operant training response to get a cafeteria-like precision pellet. We identified responders to operant training based on number of lever presses during the FR1 and FR5 protocols. Analysis of self-administration during the FR5 schedule did not show effect on the total population of the first filial generation of offspring (F1) subjects from the cafeteria diet exposure during programming when compared with F1 control diet subjects (two-way repeated measures ANOVA, $p < 0.001$) (**Supplementary Figure S1A**). Data generated from the PR schedule also confirms that cafeteria programming does not affect lever presses responses in the F1 subjects (**Supplementary Figure S1B**). We previously reported that nutritional programming with CAF diet sets a low and high response to operant training for natural rewards during FR5 and PR schedules (Camacho, 2017). To diagnose addiction-like and non-addiction-like behavior phenotypes in the offspring subjects, we calculated the intake and motivation scores by integrating lever presses during the FR5 and PR schedules, respectively, as reported (Bock et al., 2013; Le et al., 2017). We use the $\times = \frac{X_i - \overline{X}}{s.d}$, where X_i is the behavior value for each rat, $\overline{X}$ is the mean behavior value for all the rats, and *s.d.* is the standard deviation of the population behavior value. We identified addiction-like and non-addiction-like behavior in the offspring by the FR5/PR ratio by setting a +0.5 or −0.5 score at "*x*" and "*y*" axis to characterize the addiction or non-addiction-like behavior subjects, respectively. Initially, we found that maternal programming by cafeteria diet exposure increases the number of F1 subjects diagnosed as addiction-like behavior when compared with control chow diet (chow = 11 vs. CAF = 14) (**Figures 1B,C**). Characterizing the F1 offspring showing major addiction-like behavior phenotype, we found that subjects of mothers exposed to CAF diet ($n = 10$) showed significant response to operant training schedule displaying high lever responses when compared to high responders ($n = 10$) of chow-exposed mothers (**Figure 1D**). No changes in lever responses were found in the non-addiction-like behavior subjects programmed by CAF or chow diets. This evidence confirms that caloric nutritional intake during pregnancy seems to modulate susceptibility to maximize operant responses to palatable food in offspring.

Methyl-Donor Diet Reverts Addiction-Like Behavior in Offspring

Maternal programming by external stimuli actively modulates epigenetic landscape, including the DNA methylome. We tested the effect of CAF diet exposure during programming on global DNA methylation and its modulation by methyl-donor supplementation [betaine (5 g/kg of diet), choline (5.37 g/kg of diet), folic acid (5.5 mg/kg of diet), and vitamin B12 (0.5 mg/kg of diet)] on addiction-like behavior. We found that programming by CAF diet actively increases global 5-methylcytosine DNA levels in the NAc shell and no changes were found in the PFC of offspring (**Figures 2A,B**). Unexpectedly, methyl-donor supplementation decreases 5-methylcytosine DNA levels similar to control values in the offspring (**Figure 2A**); however, it efficiently decreases the number of subjects displaying lever presses response on the FR5 and PR schedule (**Supplementary Figures S2A,B**). As before, we diagnosed the number of subjects showing addiction-like behavior diagnosed by the FR5/PR ratio by setting a +0.5 or −0.5 score at "*x*" and "*y*" axis to characterize the addiction- or non-addiction-like behavior. We identified that methyl donors decrease the addiction-like behavior displaying 14 offspring of CAF vs. 4 subjects included into the CAF-methyl donors (**Figure 2C**). Also, methyl donors efficiently decrease the number of events during the PR protocol (**Figure 2D**). These data propose that exposure to methyl groups during CAF programming decreases the 5-methylcytosine DNA levels in NAc shell and efficiently revert addiction-like behavior in the offspring.

Methyl Supplementation to CAF Diet Increases Feed, Fasting, and Ghrelin-Sensitive Food Intake in Offspring

Next, we identified the effect of maternal CAF and/or methyl-donor supplementation on physiological fast-feeding and ghrelin-sensitive food intake. We have recently reported that maternal programming by CAF diet primes ghrelin response to food intake in the offspring (Maldonado-Ruiz et al., 2019). Ghrelin has also been reported to modulate addiction-like behavior by its positive effects on the reward circuit. Our data initially showed that offspring from CAF diet-fed mothers showed increase in food intake of chow diet during feed and fasting scenarios, which is exacerbated when exposed to CAF

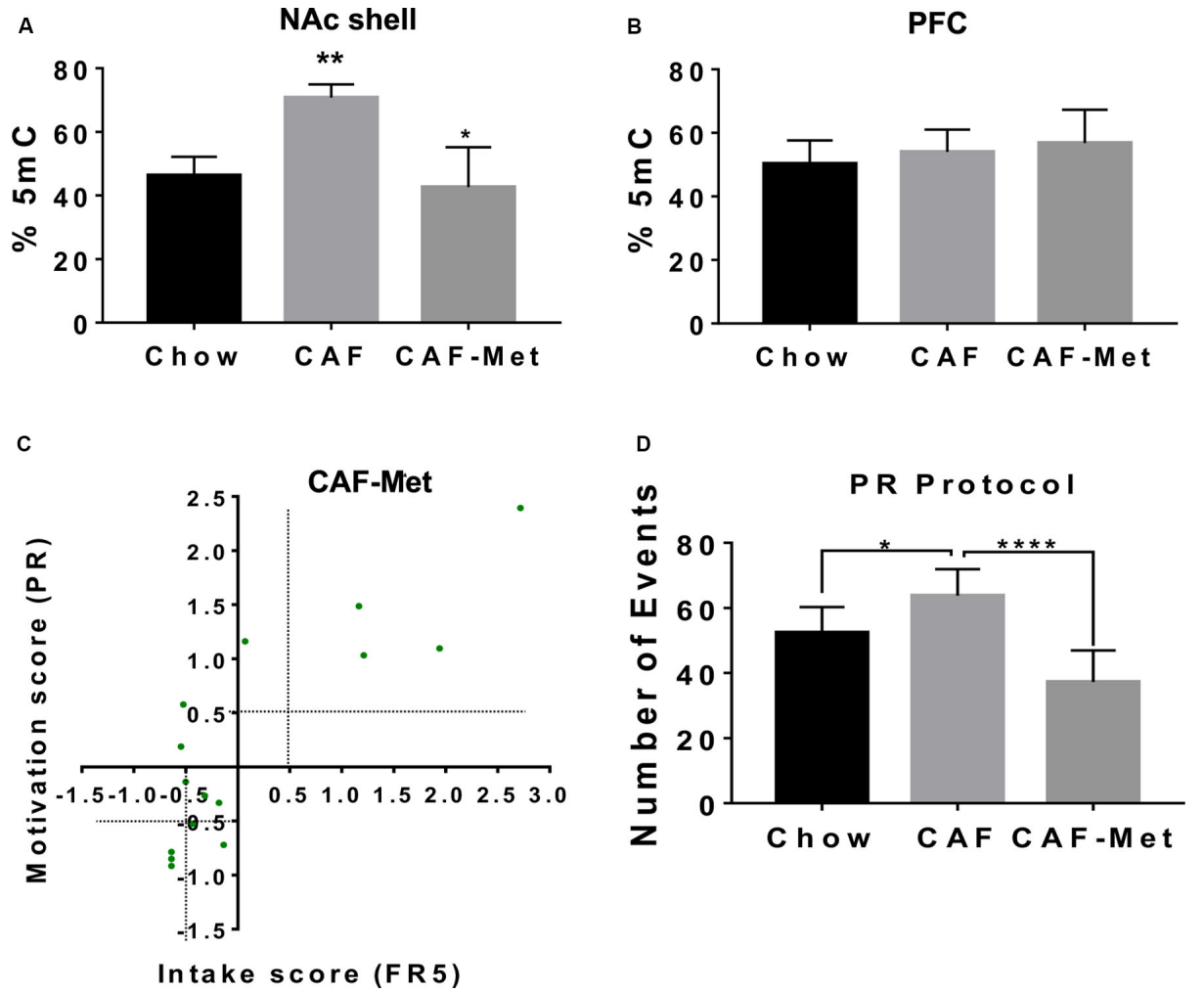

FIGURE 2 | Methyl donors revert addiction-like behavior in the offspring. We fed female Wistar rats for 9 weeks as described with CAF + methyl donors (CAF + Met, $n = 5$). Offspring was fed with control diet and was trained in the operant training test protocol. **(A,B)** Global DNA methylome in NAc shell and PFC were identified using fluorescence polarization. Molecular characterization of pro-inflammatory expression and global DNA methylome in the NAc shell were performed in non-addiction and addiction-like behavior subjects. Graphs show mean ± SEM. Statistical significance after using one-way ANOVA, followed by a Bonferroni's *post hoc* test. The differences between the groups of operant training test were tested by two-way repeated measures ANOVA. $^{*}p < 0.05$ for CAF vs. CAF-Met; $^{**}p < 0.01$ for CAF vs. chow. **(C)** Intake/motivation score for individual offspring subjects from mothers exposed to chow or CAF + Met diet followed the $\times = (\times i - \times^{--})/(s.d)$ analysis (chow, $n = 10$; CAF, $n = 14$; CAF-Met, $n = 4$). **(D)** Number of events during PR protocol of subjects exposed to chow or CAF or CAF + Met diet during programming. Graphs show mean ± SEM. Statistical significance after using one-way ANOVA, followed by a Bonferroni's *post hoc* test. The differences between the groups of operant training test were tested by two-way repeated measures ANOVA. $^{*}p < 0.05$ for CAF vs. chow; $^{****}p < 0.0001$ for CAF vs. CAF-Met.

diet (**Figure 3A**). Notably, programming by methyl donors leads to a substantial increase in CAF diet intake during the feed state, and a significant chow and CAF diet intake after fasting (**Figure 3A**). Also, methyl donors increase chow and CAF diet intake, up to twice and three times higher after intraperitoneal saline administration (**Figure 3B**). Remarkably, methyl donors promote a substantial increase up to three to five times of chow and CAF diet intake in the offspring following ghrelin systemic administration (**Figure 3B**). These results suggest that maternal programming by CAF diet actively promotes food intake by potentially priming ghrelin sensitivity in offspring, which is substantially exacerbated when exposed to methyl donors.

Maternal Programming by CAF Diet Promotes Pro-inflammatory Expression in the NAc Shell of Addiction-Like Behavior Subjects

We compared mRNA expression of proinflammatory cytokines involved in metabolic inflammation in subjects exposed to chow or CAF diets during programming. We found an increase in the IL-6 cytokine mRNA expression in the NAc shell of addiction-like behavior subjects programmed by CAF exposure when compared with non-addiction-like subjects or with chow-programmed F1 subjects (**Figure 4A**). Also, we found a decrease in the expression of IL-1β and no changes in TNF-α mRNA expression in the

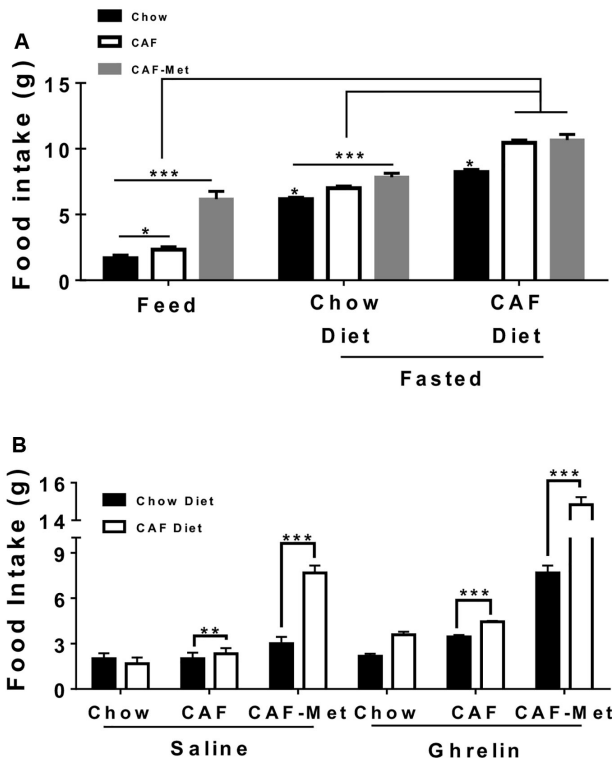

FIGURE 3 | Effect of maternal nutritional programming on fed-fasting and ghrelin-sensitive food intake in offspring. **(A)** Maternal programming was performed by exposing chow or CAF or CAF + Met diet and daily food intake was quantified for each group (Feed state). Chow and CAF diet consumption during 4 h in offspring was registered after fasting for 16 h and refeeding. **(B)** Chow and/or CAF diet intake for 2 h after subcutaneous 0.2 μg/kg ghrelin administration (n = 10–12 per group). Graphs show normalized data of the mean ± SEM. Two-way ANOVA followed by Bonferroni multiple comparison test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

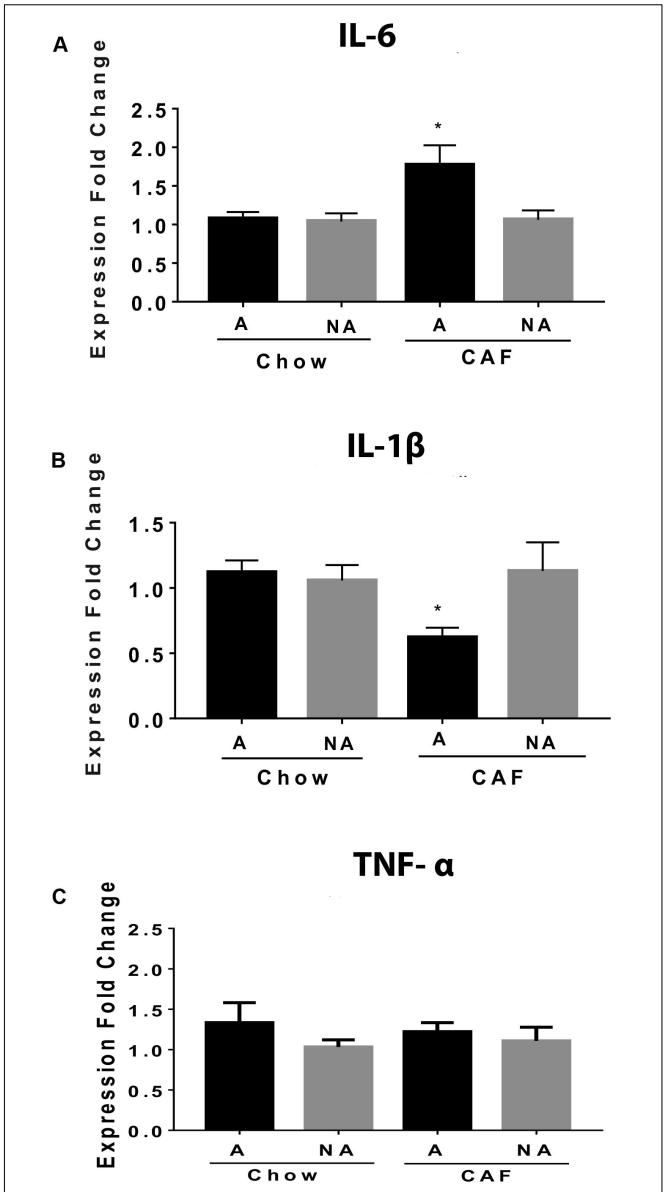

FIGURE 4 | Maternal programming by CAF diet promotes IL-6 expression in the NAc shell of addiction-like behavior subjects. Maternal programming by chow or CAF diet exposure and addiction-like and non-addiction-like behavior diagnosis was performed as described. NAc shell was isolated following stereotaxic coordinates, total DNA/RNA was isolated by purification kit (QIAGEN), and RT-PCR analysis was performed by standardized methods. IL-6 **(A)** and IL-1β **(B)**, and TNF-α **(C)** mRNA expression was performed by selective primers and standardized methods. Graphs show normalized data of the mean ± SEM (n = 10–12 per group). Statistical significance after using one-way ANOVA, followed by a Bonferroni's *post hoc* test. For IL-6, $^{*}p < 0.05$ for A chow vs. A CAF; for IL-1β, $^{*}p < 0.05$ for A CAF vs. A chow and NA CAF. A, Addiction-like; NA, Non-addiction-like.

same addiction-like behavior of subjects programmed by CAF exposure when compared with non-addiction-like subjects or with chow-programmed F1 subjects (**Figures 4B,C**).

Methylation Modulates Pro-inflammatory Cytokines Expression in Microglia Cells

Maternal programming by CAF exposure activates global DNA methylation, which correlates with an IL-6 increase and an IL-1β decrease in the NAc shell of addiction-like behavior subjects, we tested whether methylation/demethylation pharmacologic modulation promotes/reverts to a pro-inflammatory profile expression in the SIM-A9 microglia cells. Initially, we found a dose-dependent effect of 5-AZA or SAM on the MTT reduction showing no changes in cell survival at 75 nM 5-AZA and 250 μM SAM (**Figure 5A**). We did not find changes in IL-6, IL-1β, and TNF-1α gene expression followed by 5-AZA or SAM stimulation (data not shown). Real-time PCR analysis identified that favoring global DNA methylation by SAM incubation increases up to 292% and 200% IL-6 and IL-1β gene expression, respectively, following 6 h LPS incubation compared to LPS or LPS + 5-AZA treatments (**Figures 5B,C**). Conversely, active TNF-1α gene expression during LPS stimulation does not seem to depend on methylation/demethylation, given that SAM or 5-AZA incubation substantially downregulate TNF-1α gene activation

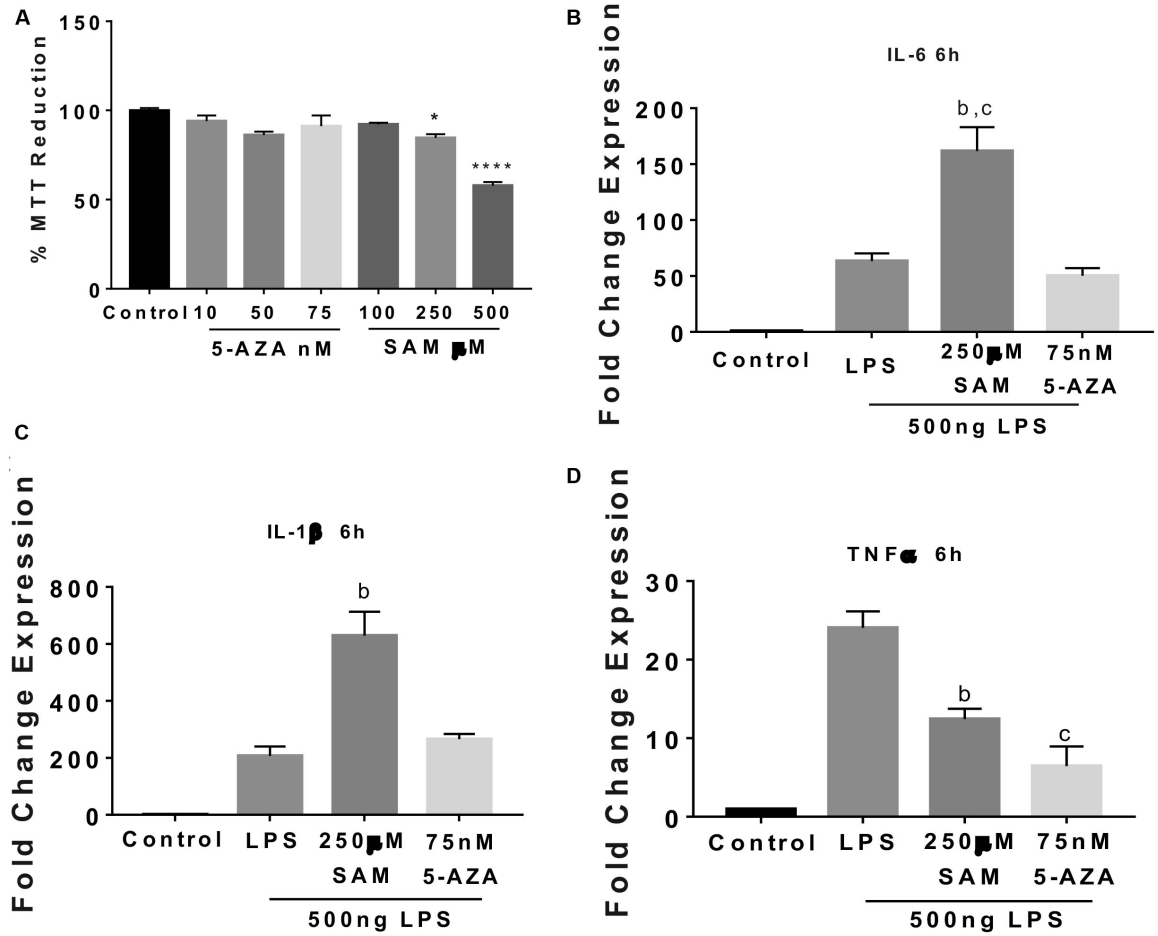

FIGURE 5 | Methylation activates IL-6 and IL-1β gene expression followed by LPS stimulation in microglia cells. **(A)** Dose-dependent microglia cell viability during 24 h 5-AZA or SAM incubation was registered using standardized MTT protocols. Graphs show normalized data of the mean ± SEM for $n = 4$ and statistical significance after using one-way ANOVA with *post hoc* Tukey's test. $^{*}p < 0.05$, $^{****}p < 0.0001$. IL-6 **(B)**, IL-1β **(C)**, and TNF-1α **(D)** RT-PCR gene expression after 24 h 75 nM 5-AZA or 250 μM SAM pre-incubation followed by 6 h 500 ng of LPS (stock 1 μg/ml). Graphs show normalized data of the mean ± SEM relative quantification using 36B4 as a normalizing gene from one independent experiment of a total of four. Statistical significance after using one-way ANOVA with Tukey's multiple comparisons test. For IL-6, $^{b}p < 0.001$ vs. LPS or vs. $^{c}p < 0.0001$ vs. 5-AZA; for IL-1β, $^{b}p < 0.001$ vs. 5-AZA, and for TNF-1α, $^{b}p < 0.001$ vs. LPS or vs. $^{c}p < 0.0001$ vs. LPS. 5-AZA, 5-Aza-2′-deoxycytidine; SAM, S-(5′-adenosyl)-L-methionine; PAL, palmitic acid.

up to 48% and 28%, respectively, when compared with LPS effect (**Figure 5D**).

We also evaluated the effect of methylation/demethylation on IL-6, IL-1β, and TNF-1α gene expression following PAL stimulation. We have reported that intraventricular PAL injection in rats or incubation in microglia cells promotes IL-6, IL-1β, and TNF-1α cytokine release in microglia cells (Maldonado-Ruiz et al., 2019). Also, we have found that maternal programming by CAF diet sets a hypothalamic microglia activation in the offspring (Maldonado-Ruiz et al., 2019). We found that 5-AZA-induced demethylation actively promotes IL-6 and IL-1β gene expression following 6 h of 100 μM PAL stimulation and no changes by 24 h when compared with PAL itself (**Figures 6A–D**). Of note, replicating the results found during LPS stimulation, IL-1 alpha and TNF-1α gene expression during 5-AZA or SAM and 100 μM PAL incubation do not seem to be mediated by methyl donors given that 5-AZA or SAM actively promotes a downregulation of gene activation by 6 h (**Figures 6E,G**). Again, these effects are reverted to 48% and 28% of the control values after 5-AZA or SAM pre-incubation, respectively, followed by 24 h of PAL stimulation (**Figures 6F,H**). These results confirm that demethylation primes IL-6 and IL-1β gene activation following PAL or LPS stimulation.

Methylation Modulates Microglia Phagocytosis in Culture

Finally, we tested if methylation/demethylation potentially coordinates microglia phagocytosis following LPS or PAL incubation. Initially, we characterized a significant reduction of basal microglia phagocytosis followed by 5-AZA incubation when compared with basal microglia phagocytosis, LPS, or following SAM-induced methylation (**Figures 7A,B**).

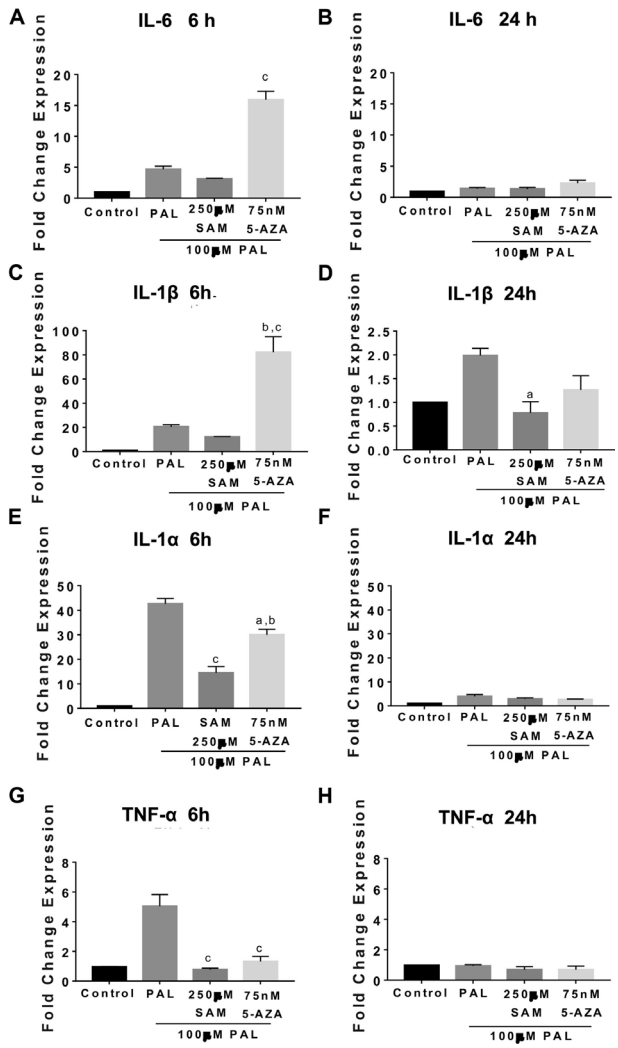

FIGURE 6 | Demethylation activates IL-6 and IL-1β gene expression followed palmitic acid stimulation in microglia cells. Microglia cells were pre-incubated with 75 nM 5-AZA or 250 μM SAM for 24 h followed by 6 or 24 h stimulation of 100 μM PAL. IL-6 **(A,B)**, IL-1β **(C,D)**, IL-1α **(E,F)**, and TNF-1α **(G,H)** RT-PCR gene expression. Graphs show normalized data of the mean ± SEM relative quantification using 36B4 as a normalizing gene from one independent experiment of a total of four. Statistical significance after using one-way ANOVA with Tukey's multiple comparisons test. For IL-6, $^{c}p < 0.0001$ vs. PAL and SAM; TNF-1α, $^{c}p < 0.0001$ vs. PAL; IL-1α $^{a}p < 0.05$ vs. PAL, $^{b}p < 0.001$ vs. SAM, and $^{c}p < 0.0001$ vs. PAL. 5-AZA. 5-AZA: 5-Aza-2′-deoxycytidine, SAM: S-(5′-adenosyl)-L-methionine, PAL, palmitic acid. IL-1β, $^{a}p < 0.05$ vs. PAL, $^{b}p < 0.001$ vs. PAL, and $^{c}p < 0.0001$ vs. SAM.

As expected, LPS stimulation efficiently activates microglia phagocytosis, which is blocked by 5-AZA demethylation induced at 6 and 24 h (**Figures 7C,D**). Also, methyl donors by SAM incubation are capable of preventing LPS-induced phagocytosis at least by 6 h and no effect at 24 h (**Figures 7C,D**). On the other hand, PAL shows a significant phagocytosis activation at 24 h, which is also reverted by 5-AZA pre incubation (**Figures 7E,F**). No changes in microglia phagocytosis were identified by SAM incubation followed by PAL stimulation (**Figures 7E,F**).

DISCUSSION

We report that maternal programming by exposure to hypercaloric diet elicits significant motivation to work for food evidenced by positive lever press responses to caloric pellets in the offspring. Notably, we found that hypercaloric diet intake promotes global DNA methylation in the NAc shell of subjects with addiction-like behavior, which correlates with an upregulation of IL-6 gene expression. We confirm that methyl donor's exposure during programming efficiently decreases addiction-like behavior for caloric pellets in offspring, but instead, they showed a substantial ghrelin-sensitive response for food intake. These results suggest that maternal programming by hypercaloric diet selectively modulates the NAc shell methylome, favoring IL-6 gene expression, which is associated with incentive motivation for natural rewards and ghrelin-sensitive food intake response in the offspring.

In this study, we contribute to characterize the detrimental effect of maternal programming by caloric diets on incentive motivation behavior for food in the offspring. We identified that caloric overnutrition during pregnancy and lactation increases the number of offspring subjects that show addiction-like behavior for caloric pellets when compared with the offspring from mothers exposed to chow diet. Our results agree with previous reports confirming a hedonic phenotype to natural and non-natural rewards in the programmed offspring (Ong and Muhlhausler, 2011; Peleg-Raibstein et al., 2016; Sarker et al., 2018). Of interest, under FR1, FR5, and PR schedules, we identified two responder groups of subjects that displayed low and high motivation for lever presses, which are also identified under cocaine response (Verheij and Cools, 2011; Yamamoto et al., 2013), suggesting that susceptibility among individuals to addiction or external choices depends on genetic, epigenetic, and environmental factors (Pierce et al., 2018). Of note, motivation to work for hypercaloric food during the PR schedule showed a higher scale-up response in the offspring programmed by CAF diet, such as that reported for cocaine, alcohol, nicotine, heroin, and methamphetamine in rats (Peleg-Raibstein et al., 2016). It is important to mention that chronic food restriction in rats actively increases dopamine levels in NAc, leading to augmentation of hedonic behavior (D'Cunha et al., 2017). We used a food restriction protocol in order to enhance the lever presses response during the FR1 schedule; however, we evaluated the hedonic phenotype for natural rewards in the FR5 and PR schedules (4 and 7 days after the *ad libitum* feeding schedules). This protocol allows us to selectively characterize the effect of maternal programming on hedonic rather than metabolic parameters. In line with this evidence, humans have shown increased caloric food liking and/or increased hunger ratings in subjects classified as food addicts (Loxton and Tipman, 2017; Ruddock et al., 2017). Mechanistically, it has been proposed that excessive high-energy food consumption seems to negatively modulate a hedonic phenotype in animal models (Kenny, 2011; Barry et al., 2018; Ducrocq et al., 2019), in part, by downregulating the D2 dopamine receptors (Johnson and Kenny, 2010; Winterdahl et al., 2019), and/or reduced dopamine release (Avena and Bocarsly, 2012). In our context, it seems

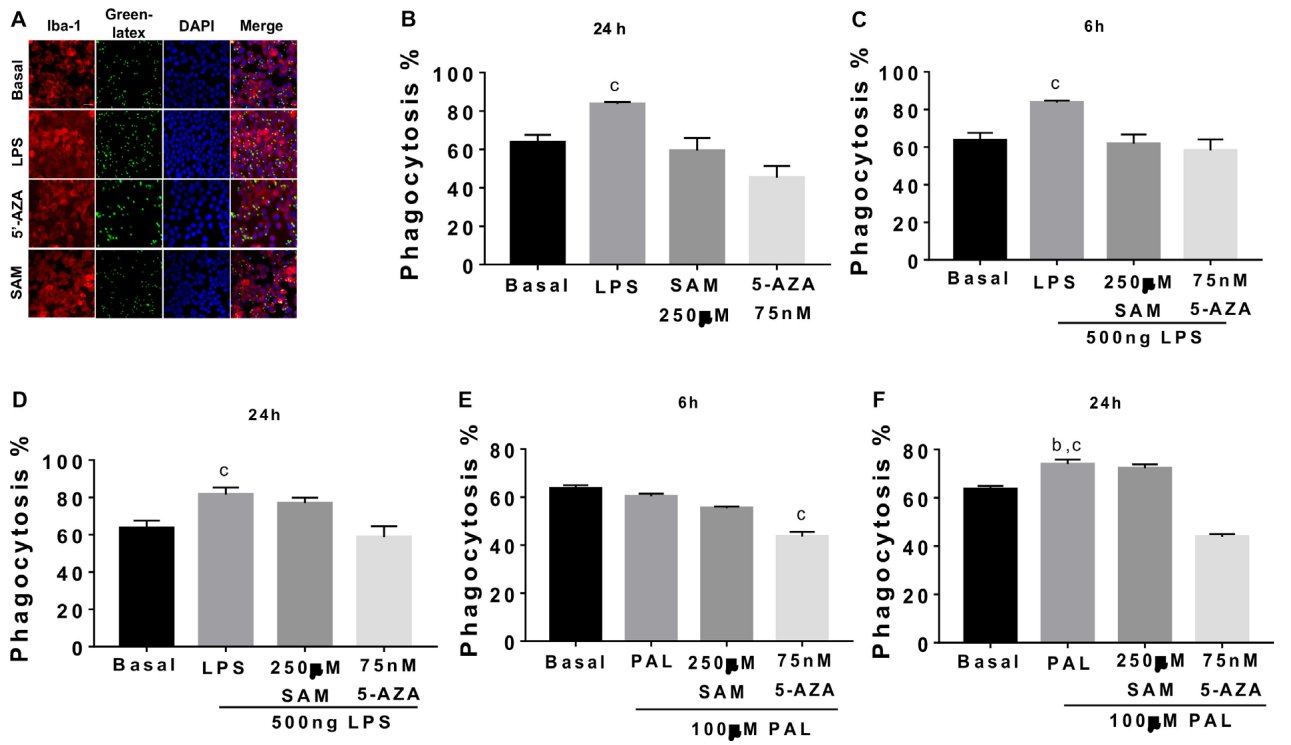

FIGURE 7 | Demethylation inhibits microglia phagocytosis in microglia cells. **(A,B)** Microglia cells were pre-incubated with 75 nM 5-AZA or 250 μM SAM for 24 h, or LPS followed by pre-opsonized green fluorescent latex beads incubation for 1 h at 37°C. Qualitative phagocytosis was evaluated using immunofluorescence against anti Iba-1 (1: 200) followed by goat anti-rabbit IgG coupled to Alexa Fluor 546 (1:1000) and VECTASHIELD mounting medium with DAPI. Fluorescent signals were detected by confocal-laser microscopy using an Olympus BX61W1 microscope with an FV1000 module with diode laser and ImageJ software. $^{c}p < 0.0001$ for LPS vs. Basal, SAM and 5-AZA. **(C,D)** After 5-AZA or SAM incubation, microglia cells were treated with 500 ng of LPS for 6 or 24 h, respectively, and pre-opsonized green fluorescent latex beads were incubated as before.$^{c}p < 0.0001$ for LPS vs. Basal, SAM + LPS and 5-AZA + LPS and $^{c}p < 0.0001$ for LPS vs. Basal and 5-AZA + LPS, respectively. **(E,F)** After 5-AZA or SAM incubation, microglia cells were treated with 100 μM PAL for 6 or 24 h, respectively, and pre-opsonized green fluorescent latex beads were incubated as before. Graphs show normalized data of the mean ± SEM relative to % phagocytosis of microglia cells from one independent experiment of a total of four. Statistical significance after using one-way ANOVA with Tukey's multiple comparisons test. $^{c}p < 0.0001$ for PAL vs. 5-AZA + PAL; $^{b}p < 0.001$ for PAL vs. Basal and $^{c}p < 0.0001$ for PAL vs. 5-AZA + PAL.

that maternal programming by caloric exposure and/or food restriction primes a new state of reward deficiency integrating lower availability dopamine receptors and dopamine release, which might favor impulsiveness for unhealthy eating in order to cope against deficient dopaminergic neurotransmission (Dietrich et al., 2014; Bongers et al., 2015). Taken together, our work reaffirms the proposal that fetal programming by hypercaloric diet in mothers favors motivating behavior toward natural high caloric value rewards in the offspring (Claycombe et al., 2015; Edlow, 2017), which might replicate major hedonic value for food in subjects classified as food addicts (Loxton and Tipman, 2017; Ruddock et al., 2017).

Here, we reported for the first time that CAF diet exposure during pregnancy and lactation leads to selective global DNA methylation in the NAc shell and no changes in the PFC of addict offspring subjects. DNA methylation is a deep coordinator of fetal programming by assisting selective developmental states capable of modulating long-lasting changes in brain plasticity related to motivation addiction (Honegger and de Bivort, 2018). In our maternal programming model, pregnant females are expected to have uneven litter load during gestation and might be exposed to differential fetal programming, which denotes a potential limitation of the murine model. However, litters were adjusted to the same number in the chow and CAF experimental groups. Molecular epigenetic signatures for setting food addiction in the offspring are not totally understood because food is a natural and necessary reward for animals and humans when compared with drugs or alcohol. For example, changes in DNA methylation could be responsible for neuroplasticity changes induced by addictive drugs, such as repeated cocaine administration reduced global levels of H3K9 dimethylation in the NAc, which correlates with changes in dendritic spines (Maze et al., 2010). Also, changes of the histone dimethyltransferase G9a in the NAc shell actively affect cocaine self-administration in rats (Anderson et al., 2018, 2019). Of importance, DNA methylation in NAc during drug addiction seems to respond to a time-dependent regulation, enhancing DNA methylation in the NAc after 30 days of cocaine withdrawal (Massart et al., 2015), suggesting that an increase in DNA methylation is responsible for cocaine craving. In agreement with these data, we found that programming by CAF diet substantially increases global DNA methylation in the NAc shell of addict offspring that are

no longer exposed to CAF diet. Recent evidence have identified that CAF programming promotes D2 dopamine receptor DNA methylation in NAc of the offspring (Rossetti et al., 2020), which potentially might explain the impulsiveness for unhealthy eating. Notably, methyl donors revert the percentage of global DNA methylation and block addiction-like behavior in the offspring. Our data agree with the fact that chronic treatment with the methyl-donor methionine inhibits cocaine-induced conditioned place preference in rats (Tian et al., 2012), which seems to depend on cocaine-induced c-Fos expression in the NAc (Wright et al., 2015). Also, it has been reported that fetal programming by high-fat diet promotes global DNA hypomethylation in the offspring reward circuit (Skinner et al., 2011; Carlin et al., 2013), and in fact, it is also detected in response to fetal programming by environmental exposure to toxins (Baccarelli and Bollati, 2009), during restriction of nutrients *in utero* or prenatal exposure to cocaine (Novikova et al., 2008). In addition, global hypomethylation correlates with an increased expression of opioid receptors and the dopamine transporter in the NAc of the high-fat diet-programmed offspring (Carlin et al., 2013). Conversely, maternal diet supplementation with methyl donors seems to be beneficial by restoring weight gain and food intake in animals exposed to caloric diet (Carlin et al., 2013). Alternatively, methyl-donor supplementation during programming might also contribute to additional metabolic or systemic molecular pathways away to the effects in DNA methylation during neonatal development. For instance, methyl-donor infusion in piglets increases creatine, whereas it decreases phosphatidylcholine levels in liver (Robinson et al., 2016). Creatine synthesis itself requires glycine and an amidino group (provided by arginine) and a methyl group (provided by SAM) (Brosnan et al., 2009), supporting the notion that methyl donors modulates amino acids metabolism in energy-demand tissues including brain. Finally, in a remarkable report, Rizzardi et al. (2019) identified 12 Mb of differentially methylated CG regions in flash-frozen postmortem human tissues including dorsolateral PFC, hippocampus, NAc, and anterior cingulate gyrus implicated in neuropsychiatric disorders, including addiction (Rizzardi et al., 2019). Our data suggest a differential and opposite effect of drugs and food on DNA methylation in NAc; however, in both scenarios, NAc methylation favors addiction-like behavior.

One of the major results from our study proposes that maternal programming by methyl donors decreases the incentive to work for natural rewards; however, it does prime a metabolic aberrant phenotype promoting overfeeding during fasting and ghrelin-sensitive schedules. We have previously reported that CAF diet primes a fasting and ghrelin-sensitive food intake response in the offspring when compared with the offspring of mothers exposed to chow diet (Maldonado-Ruiz et al., 2019). Overfeeding in the programmed offspring might be explained by two scenarios, firstly, postnatal high caloric diet exposure has been identified to induce hypermethylation of the Pomc promoter, blocking leptin signaling and favoring food consumption and weight gain (Marco et al., 2013; Zhang et al., 2014). In fact, higher methylation levels at the proopiomelanocortin (POMC) CpG sites +136 bp, and +138 bp and lower methylation of the neuropeptide Y promoter in human leukocytes have been associated to weight gain (Crujeiras et al., 2013). A potential second scenario might be related with hypomethylation in the neuropeptide Y promoter (Lazzarino et al., 2017) favoring overfeeding after fasting or ghrelin administration in the offspring. We have reported that fetal programming by CAF diet exposure exacerbates hypothalamic c-fos neuronal response to ghrelin in the offspring (Maldonado-Ruiz et al., 2019), suggesting that DNA methylation during maternal CAF exposure might disrupt the expression of food intake neuropeptides favoring ghrelin-sensitive orexigenic signaling.

Next, we characterized the molecular immune signatures of fetal programming in the offspring that favor or correlate with addiction-like behavior. We have reported that maternal programming by CAF exposure leads to hypothalamic microglia activation in the offspring (Maldonado-Ruiz et al., 2019). Our *in vivo* data show a higher expression of IL-6 in the NAc shell of subjects that exhibited addiction-like behavior. Our *in vitro* experiments indicate that LPS or PAL incubation efficiently activates TNF-α, IL-6, and IL-1α gene expression. Also, methyl-donor incubation activates or decreases IL-6 and IL-1α gene expression following LPS or PAL stimulation, respectively. Our results agree with recent reports demonstrating that 5-AZA-induced demethylation significantly increases IL-6 and IL-8 gene expression in human dental pulp cells (Mo et al., 2019) and in polymorphonuclear cells (Shen et al., 2016) followed by LPS stimulation. Potentially, we propose that DNA methylation might coordinate a pro- and anti-inflammatory profile in macrophages toward both M1 and M2 phenotypes. For example, LPS efficiently leads to SOCS1 gene promoter methylation in macrophages favoring secretion of TNF-α and IL-6 cytokines (Cheng et al., 2014). Global DNA hypermethylation has also been reported in inflamed tissues of obesogenic-diabetic mellitus mice models, showing an M1 phenotype in macrophages (Babu et al., 2015; Kapellos and Iqbal, 2016). In our context, positive energy balance such as the obesogenic-diabetic mellitus mice models are closely related to the metabolic failure found in our maternal programming model (Cardenas-Perez et al., 2018), suggesting that global DNA methylation in NAc might potentially be associated to positive energy balance. Finally, our *in vitro* experiments identified that demethylation selectively blocks phagocytosis in microglia during LPS or PAL stimulation. There are no available studies regarding the effect of DNA methylation/demethylation on phagocytosis in microglia, which allow us to integrate this mechanism on addiction behavior. A previous study reported 5-AZA-induced demethylation and significantly increased efferocytosis in alveolar macrophages from COPD patients (Barnawi et al., 2017), which potentially reflects a pathological scenario linked to immunity.

We propose that maternal programming increases susceptibility to addiction-like behavior in the offspring integrating global DNA methylation in the NAc shell, which correlates with IL-6 gene expression. CAF diet made of lipids such as PAL might imitate foreign compounds and initiate a proinflammatory immune signaling in the brain to create its rewarding and/or overfeeding effects.

DATA AVAILABILITY STATEMENT

All relevant data are contained within the manuscript.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Animal Care Committee (BI0002) at the Universidad Autónoma de Nuevo León, Mexico.

AUTHOR CONTRIBUTIONS

GC-C, LM-M, RM-R, MC-T, SB-V, and AC-M: conceptualization. GC-C, LM-M, MC-T, SB-V, RM-R, DR-P, DR-R, GL, LG-O, and AC-M: investigation. GC-C, LM-M, RM-R, MC-T, SB-V, DR-R, GL, and AC-M: methodology. DR-P, GL, LG-O, and AC-M: supervision and visualization. GC-C, LM-M, RM-R, MC-T, DR-P, DR-R, GL, LG-O, and AC-M: writing – review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00452/full#supplementary-material

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Adolescent Vulnerability to Heightened Emotional Reactivity and Anxiety After Brief Exposure to an Obesogenic Diet

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Background: Emerging evidence demonstrates that diet-induced obesity disrupts corticolimbic circuits underlying emotional regulation. Studies directed at understanding how obesity alters brain and behavior are easily confounded by a myriad of complications related to obesity. This study investigated the early neurobiological stress response triggered by an obesogenic diet. Furthermore, this study directly determined the combined impact of a short-term obesogenic diet and adolescence on critical behavioral and molecular substrates implicated in emotion regulation and stress.

Methods: Adolescent (postnatal day 31) or adult (postnatal day 81) Lewis rats were fed for 1 week with an experimental Western-like high-saturated fat diet (*WD*, 41% kcal from fat) or a matched control diet (*CD*, 13% kcal from fat). We used the acoustic fear-potentiated startle (FPS) paradigm to determine the effects of the WD on cued fear conditioning and fear extinction. We used c-Fos mapping to determine the functional influence of the diet and stress on corticolimbic circuits.

Results: We report that 1-week WD consumption was sufficient to induce fear extinction deficits in adolescent rats, but not in adult rats. We identify fear-induced alterations in corticolimbic neuronal activation and demonstrate increased prefrontal cortex CRHR1 messenger RNA (mRNA) levels in the rats that consumed the WD.

Conclusion: Our findings demonstrate that short-term consumption of an obesogenic diet during adolescence heightens behavioral and molecular vulnerabilities associated with risk for anxiety and stress-related disorders. Given that fear extinction promotes resilience and that fear extinction principles are the foundation of psychological treatments for posttraumatic stress disorder (PTSD), understanding how obesogenic environments interact with the adolescent period to affect the acquisition and expression of fear extinction memories is of tremendous clinical relevance.

Keywords: PTSD, diet-induced obesity, adolescence, anxiety, fear-potentiated startle, CRHR1

HIGHLIGHTS

- Short-term WD consumption during adolescence impairs cued fear extinction memory retention in a fear-potentiated startle paradigm.
- Short-term WD consumption during adolescence attenuates neuronal activation to electric footshock stress in the basomedial nuclei of the amygdala.
- Short-term WD consumption increases CRHR1 mRNA levels in the medial prefrontal cortex.
- Adult LEW rats exhibit increased basal HPA axis tone and heightened emotional reactivity to footshock stress relative to adolescent rats.

INTRODUCTION

Early-life trauma is linked to obesity and the consumption of obesogenic diets (Perkonigg et al., 2009; Pagoto et al., 2012; Roenholt et al., 2012; Ehlert, 2013; Duncan et al., 2015; Farr et al., 2015; Masodkar et al., 2016; Mason et al., 2017; Wolf et al., 2017). The high comorbidity between obesity and posttraumatic stress disorders (PTSDs) suggest that adaptations to trauma may increase the risk for the consumption of obesogenic diets as a result of the traumatic experience (Kalyan-Masih et al., 2016; Michopoulos et al., 2016; Godfrey et al., 2018). There is also mounting evidence that exposure to obesogenic diets rich in saturated fat foods and sugars have a direct adverse effect on emotional regulation, anxiety-like behaviors, and neural substrates implicated with stress (Ortolani et al., 2011; Boitard et al., 2015; Reichelt et al., 2015; Sivanathan et al., 2015; Baker and Reichelt, 2016; Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Therefore, early-life exposure to obesogenic diets may predispose individuals to maladaptive stress responses, resulting in increased PTSD risk.

Several lines of evidence suggest that alterations in attention, memory, and learning contribute to the etiology and maintenance of PTSD symptoms (Lissek and van Meurs, 2015; Liberzon and Abelson, 2016). Interestingly, while fear learning emerges early in life, fear memories undergo dynamic changes during adolescence (King et al., 2014; Baker and Richardson, 2015; Ganella et al., 2017). Studies indicate that extinction learning is blunted during adolescence (Pattwell et al., 2012), which has important implications for PTSD treatment. The fear-potentiated startle (FPS) represents a proven and reliable method for examining conditioned fear responses (Davis et al., 1993). This method shows notable face validity, construct validity, and predictive validity in the assessment of behaviors and circuits implicated in PTSD. The highly conserved corticolimbic circuit is critical for cue-elicited fear responses and safety learning (Likhtik et al., 2014; Likhtik and Paz, 2015). In particular, the corticolimbic pathway connecting the medial prefrontal cortex (mPFC) and the amygdala undergoes dramatic structural reorganization during adolescence (Casey, 2015; Arruda-Carvalho et al., 2017; Silvers et al., 2017) and remains the focus of our recent investigations. Our studies indicate that this corticolimbic pathway is highly vulnerable to the disruptive effects of an obesogenic diet when exposure to the diet starts during adolescence (Vega-Torres et al., 2018). In that study, we demonstrated that Western-like high-saturated fat diet (WD) consumption during the critical period of adolescence leads to increased anxiety-like behaviors. Using the fear-potentiated startle paradigm, we found significant deficits in fear learning and fear extinction learning in the rats that consumed the WD during adolescence. Furthermore, we identified new structural impairments induced by the WD, particularly in brain regions related to fear and anxiety processing. Notably, we found that WD consumption for 4 weeks during adolescence was sufficient to disrupt brain structure and behavior. However, without a cohort of animals exposed to the obesogenic diet only during adulthood in our previous studies, it is difficult to determine if adolescence represents a distinct period of vulnerability to the detrimental effects of an obesogenic diet on learned fear and emotional reactivity. Furthermore, few studies have tested the hypothesis that short-term consumption of an obesogenic diet is sufficient to alter fear responses.

This study tested several hypotheses. First, we predicted impaired fear extinction in adolescent rats that consumed an obesogenic diet for 1 week. Not only would this represent a replication of our previous report in adult rats (Vega-Torres et al., 2018), but it would also extend that finding to the effects of diet that are independent of obesity-related processes. Second, we reasoned that short-term exposure to an obesogenic diet would reduce the neuronal activation in corticolimbic regions implicated in fear extinction and anxiolytic effects. Finally, given the robust effects of obesogenic diets on the hypothalamic–pituitary–adrenal (HPA) axis and dopamine systems (Reyes, 2012; Sharma et al., 2013; Boitard et al., 2015; Khazen et al., 2019) and the modulatory actions of these factors on mPFC–amygdala circuit function (Fadok et al., 2009b; Jovanovic et al., 2010, 2020; Veer et al., 2012; Whittle et al., 2016), we hypothesized that the obesogenic diet would increase the HPA tone while reducing dopamine receptor expression in the mPFC.

This study demonstrates that short-term exposure to an obesogenic diet is sufficient to impair retention of fear extinction training while altering neurobiological substrates implicated with emotional reactivity. These findings reveal a unique interplay between high-saturated fat/high-sugar foods and fear extinction during adolescence, which may prove informative for understanding risk factors implicated in stress-related disorders. More importantly, this study suggests that obesity and the consumption of obesogenic diets may represent a mediator of differential anxiety and stress-related disorders psychotherapy treatment outcomes.

MATERIALS AND METHODS

Animals

All the experiments were performed following protocols approved by the Institutional Animal Care and Use Committee

(IACUC) at the Loma Linda University School of Medicine. Adolescent [ADOL: postnatal day (PND), 24] and adult (ADUL: PND, 74) male Lewis rats were purchased from Charles River Laboratories (Portage, MI, United States). Although the precise correlation between age of rats and humans is still controversial, it has been proposed that adolescence occurs between PND 28 and 42 in rats (Spear, 2000; Sengupta, 2013). Thus, a human year corresponds to approximately 3 days in the life of a rat (Sengupta, 2013). The rationale for the use of Lewis rats is based on the relevant vulnerabilities of this strain to posttraumatic stress (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Immediately upon arrival, the rats were housed in groups (two per cage) with free access to food and water. Dietary manipulations commenced at PND 31 for adolescent rats and at PND 81 for adult rats. Animals were kept in customary housing conditions (21 ± 2°C, relative humidity of 45%, and 12-h light/dark cycle with lights on at 7:00 AM). The body weights were recorded once a week or daily during the week of behavioral testing. Food consumption was quantified at least twice per week. The rats were never food or water restricted.

Diets

The standard chow diet (CHOW, 4 g% fat, product no. 7001) was obtained from Teklad Diets (Madison, WI, United States), while the matched low-fat purified control diet (CD, 5 g% fat, product no. F7463) and Western-like high-saturated fat diet (WD, 20 g% fat, product no. F7462) were obtained from Bio-Serv (Frenchtown, NJ, United States). There is an increasing awareness of the impact of diet changes on stress reactivity (Sharma et al., 2013; Vega-Torres et al., 2018) and a need for using adequate matched diets in diet-induced obesity research (Pellizzon and Ricci, 2018). Thus, we decided to incorporate a matched low-fat control diet group along with the standard chow diet group. Research WD used to generate obesogenic phenotypes are generally high in fat and refined carbohydrates. More importantly, these diets are typically manufactured with purified ingredients. Unfortunately, several diet-induced obesity studies still report the use of grain-based chow diets as the control diet. Grain-based chow diets contain unrefined ingredients and have marked differences in the content and composition of various nutrients (e.g., fiber). Control diet choice can thus conceivably confound data interpretation and affect reproducibility. Given that initially both the CHOW and CD groups had identical biometric and behavioral outcomes, we opted to use the more appropriate CD group as control for the WD group (Vega-Torres et al., 2019). The macronutrient composition and fatty acid profiles are detailed in **Table 1**.

TABLE 1 | Detailed composition of the purified diets.

	Control diet (CD) #F7463	Western diet (WD) #F7462
Macronutrient (main source)	**% kcal**	**% kcal**
Carbohydrates (corn starch)	68	43
Proteins (casein)	19	16
Fats (milk fat)	13	41
Total kcal	3.7	4.6
Sugars	**g/kg**	**g/kg**
Sucrose	341	340
Fatty acid class	**g/kg**	**g/kg**
Saturated	20.8	121
Monosaturated	12.7	492
Polyunsaturated	11.3	11.5
Ratio omega-3 to omega-6	0.02	0.01

Dietary fat percentage was ∼5% in the CD and ∼20% in the WD. The WD contained higher levels of total saturated fatty acids (ninefold difference) and total monounsaturated fatty acids (threefold difference) when compared to the CD. The levels of total polyunsaturated fatty acids were similar between CD and WD.

Study Design

Behavior testing sessions involved a 20- to 30-min acclimation period to the testing facility. Following room acclimation, the rats were placed for 5 min inside the acoustic startle reflex (ASR) enclosure and testing chamber and then returned to their cages. The next day (ADOL: PND, 30; ADUL: PND, 80), we measured baseline ASR responses, which were used to generate balanced experimental groups. The ASR-based group matching resulted in an even distribution of rats with similar startle responses in all groups. The rats were divided in the following groups: ADOL + CD (n = 12), ADOL + WD (n = 12), ADUL + CD (n = 12), ADUL + WD (n = 12). We conducted a sensitivity power analysis (Cohen, 1992) (two-way ANOVA: diet type and age as factors) using *G*Power* (Faul et al., 2007). Analyses revealed that 12 rats per group are sufficient to detect medium effect sizes (d = 0.41) with power (1 - β) set at 0.80, and α = 0.05. The fear-potentiated startle (FPS) paradigm was performed to assess the short-term diet effects on cued fear conditioning and fear extinction learning at PND 38–41 (ADOL group) and PND 88–91 (ADUL group). We measured anxiety-like responses in the elevated plus maze (EPM) at PND 42 (ADOL) and PND 92 (ADUL). All the rats were euthanized 48 h following EPM testing. The rats were allowed to consume the custom diets until completion of the study at PND 44 (ADOL) and PND 94 (ADUL). **Figure 1** summarizes the timeline of experimental procedures and behavioral tests.

Acoustic Startle Reflex

The ASR experiments were performed using the SR-Lab acoustic chambers (San Diego Instruments, San Diego, CA, United States). ASR magnitudes were measured by placing animals in startle enclosures with sensors (piezoelectric transducers) that convert small movements to voltage. Thus, the magnitude of the change in voltage represents the size of the ASR. Acoustic stimuli intensities and response sensitivities were calibrated before commencing the experiments. The ASR protocol has been previously described by our group (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Briefly, experimental sessions were 22 min long and started with a 5-min habituation period (background noise = 60 dB). The rats

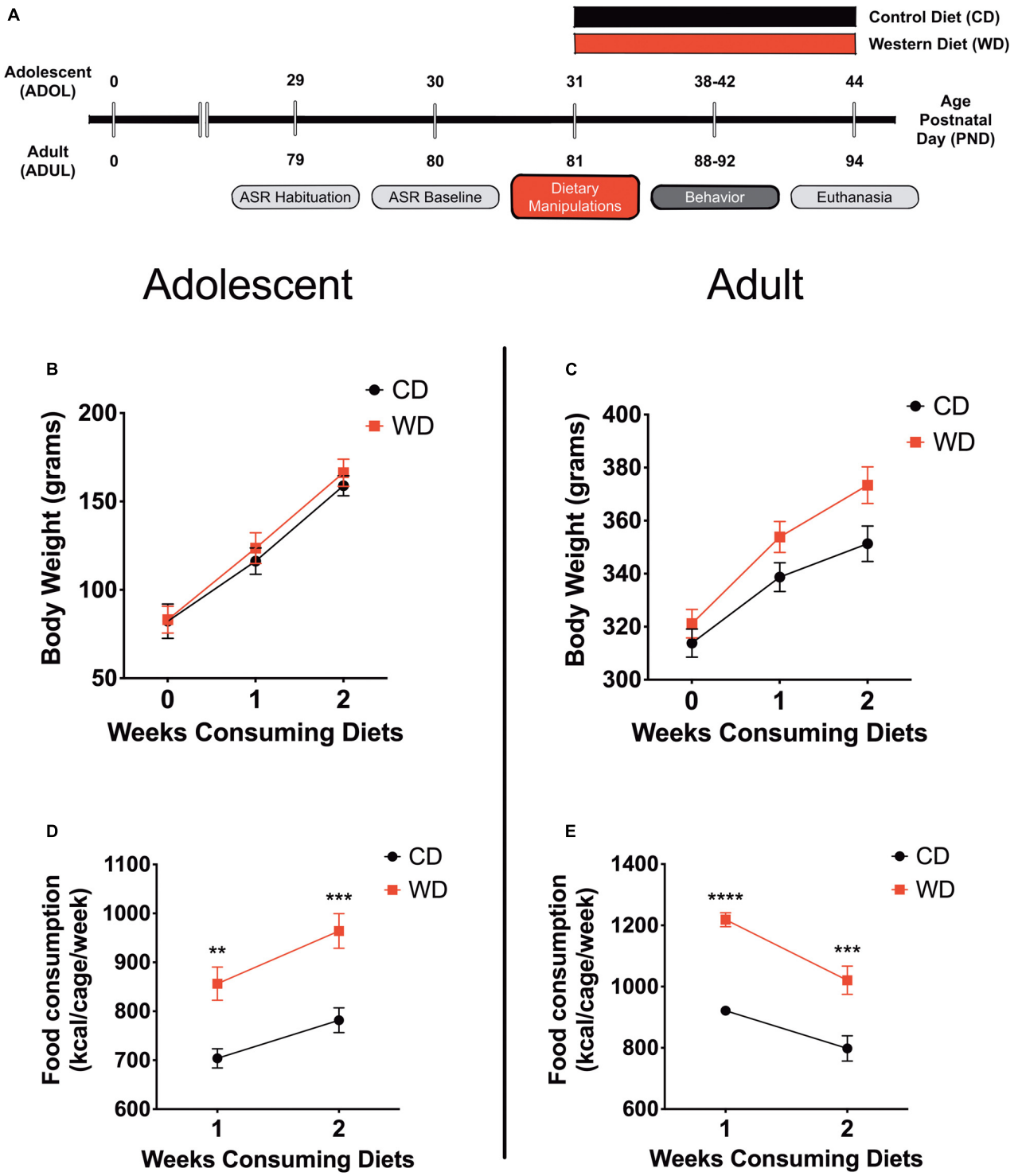

FIGURE 1 | (A) Timeline of experimental procedures. Adolescent (*ADOL*) and adult (*ADUL*) rats were matched based on their acoustic startle reflex (ASR) responses and allocated to one of two diets: control diet (CD) or Western high-saturated fat diet (WD). The rats were exposed to the diets for 1 week before behavioral testing. A 4-day fear-potentiated startle (FPS) paradigm was used to determine the effects of the WD on cued fear conditioning and fear extinction learning. Additional anxiety-like behaviors were investigated in the elevated plus maze (EPM). All the rats were euthanized 1 day after the completion of the EPM and brains dissected for RNA extraction. Please refer to the study design in *Methods* for more specific technical details on procedures and behavioral tests performed in this study. Average weekly body weight in grams for **(B)** adolescent and **(C)** adult groups (diet effect $p > 0.05$ for both age groups; $n = 11$–12 rats/group). Average caloric intake in kilocalories per cage per week for **(D)** adolescent and **(E)** adult groups. WD groups consumed more calories than CD rats, regardless of age (ADOL: diet effect $p = 0.002$; ADUL: diet effect $p < 0.0001$; for both groups: $n = 6$ cages/group). Error bars are SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$, respectively.

were then presented with a series of 30 tones (10 tones at each 105 dB) using a 30-s intertrial interval (ITI). The acoustic stimuli had a 20-ms duration. Subsequently, the rats were returned to their home cages. Enclosures were thoroughly cleaned and dried following each session. Averaged ASR magnitudes were normalized by weight to eliminate confounding factors associated with body weight (weight-corrected ASR = maximum startle magnitude in mV divided by body weight at testing day) (Gogos et al., 1999; Elkin et al., 2006; Grimsley et al., 2015; Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Baseline ASR responses were measured before commencing the dietary manipulations (PND 30, ADOL group; PND 80, ADUL group).

Fear Potentiated Startle

The fear potentiated startle (FPS) protocol was adapted from Davis (2001) and detailed in our previous studies (Vega-Torres et al., 2018). Each FPS session started with a 5-min acclimation period (background noise = 60 dB). During the first session of the paradigm (fear training), the rats were trained to associate a light stimulus [conditioned stimulus (CS)] with a 0.6-mA footshock [unconditioned stimulus (US)]. The conditioning session involved 10 CS + US pairing presentations. During each CS + US presentation, the light (3,200 ms duration) was paired with a coterminating footshock (500 ms duration). Light-shock pairings were presented in a quasi-random manner (ITI = 3–5 min). Cued fear acquisition was measured 24 h later. During the second session (fear learning testing; pre-extinction FPS), the rats were first presented with 15 startle-inducing tones (*leaders*; 5 each at 90, 95, and 105 dB) delivered alone at 30 s ITI. Subsequently, the rats were presented with 60 test trials. For half of these test trials, a 20-ms tone was presented alone (tone-alone trials; 10 trials for each tone: 90, 95, and 105 dB). For the other half, the tone was paired with a 3,200-ms light (light + tone trials; 10 trials for each pairing: 90, 95, and 105 dB). The 60 trials were divided into 10 blocks of 6 test trials each which included three tone-alone trials and three light + tone trials. To conclude the testing session, the rats were presented with 15 startle-inducing tones (*trailers*; 5 each at 90, 95, and 105 dB) delivered at 30 s ITI. Trials in this session were presented in a quasi-random order (ITI = 30 s). The startle-eliciting tones had a 20-ms duration. One day after fear conditioning testing, the rats were exposed to a single extinction-training session. The extinction training session consisted of 30 CS alone presentations (light without shock or noise bursts) with a duration of 3,700 ms (ITI = 30 s). One day after fear extinction training, we determined fear extinction acquisition using the same FPS session that was used to measure fear acquisition. It is noteworthy that, in this study, we shortened the fear extinction training protocol to a single session as opposed to three sessions in our previous report in adult rats (Vega-Torres et al., 2018). A single extinction training session enables accurate and precise fear extinction measurements without flooring effects in adolescent Lewis rats. We assessed fear learning and fear extinction learning by comparing the startle amplitude from light + tone trials [conditioned + unconditioned stimulus (CS + US)] relative to tone alone trials (US). FPS data were reported as the proportional change between US and CS + US {%FPS = [(light + tone startle) - (tone-alone startle)]/(tone-alone startle) × 100} (Walker and Davis, 2002). Fear recovery, a proxy for fear extinction memory retention, was scored as the ratio between the FPS value from fear extinction and fear learning testing sessions [%fear recovery = (FPS extinction/FPS learning) × 100].

Elevated Plus Maze

The near-infrared (NIR)-backlit EPM consisted of two opposite open arms (50.8 × 10.2 × 0.5 cm) and two opposite enclosed arms (50.8 × 10.2 × 40.6 cm) (Med Associates Inc., Fairfax, VT, United States). The arms were connected by a central 10 × 10 cm square-shaped area. The maze was elevated 72.4 cm from the floor. Behaviors were recorded in a completely dark room. The rats were placed on the central platform facing an open arm and allowed to explore the EPM for 5 min. The apparatus was cleaned after each trial (70% ethanol, rinsed with water, and then dried with paper towels). The behaviors were monitored via a monochrome video camera equipped with an NIR filter and recorded and tracked using Ethovision XT (Noldus Information Technology, Leesburg, VA). In rats, changes in the percentage of time spent on the open arms (OAs) indicate changes in anxiety (Pellow et al., 1985), and the number of closed arm (CA) entries is the best measure of locomotor activity (File, 1992). These data are used to calculate the anxiety index (Cohen et al., 2012; Contreras et al., 2014; Kalyan-Masih et al., 2016):

$$\begin{aligned}\text{Anxiety Index} &= 1 - \{[(\text{OA cumulative duration}/\text{total test duration}) \\ &\quad + (\text{OA entries}/\text{total number of entries to CA} + \text{OA})]/2\}\end{aligned}$$

c-Fos Free-Floating Immunofluorescence

A separate cohort of adolescent and adult rats was exposed to electric footshock stress using the same paradigm employed during fear conditioning. This session consisted of 10 CS + US pairing presentations with a light (3,200 ms duration) paired with a coterminating footshock (0.6 mA footshock; 500 ms duration). Light-shock pairings were presented in a quasi-random manner (ITI = 30 s–2 min). One hour after the session, the rats were sacrificed via transcardiac perfusion with 4% paraformaldehyde (PFA) using the Perfusion Two™ Automated Pressure Perfusion System (Leica Biosystems, Buffalo Grove, IL) (n = 12; 3 rats per group). The brains were removed from the cranial vault 4 h after fixation and postfixed in 4% PFA for 24 h. The brains were then washed with phosphate-buffered saline (PBS) and cryoprotected with sucrose (30%) for 12–16 h at 4°C prior to embedding in Tissue-Tek® O.C.T.™ compound (Sakura, Torrance, CA, United States).

Tissue Sampling

Brain tissue was cut coronally at 25 μm thickness. All immunohistochemical techniques were performed on free-floating sections. Based on the Paxinos and Watson rat brain atlas (from bregma: + 3.20 mm to + 2.20 mm), 10 sections

were chosen at a 75-μm interval (one of every four sections) covering a total 1,000 μm of area containing medial prefrontal cortex (Paxinos and Watson, 2006). An additional 13 sections were cut and chosen in the same manner (from bregma: −2.30 to −3.60 mm) covering a total 1,300 μm of area containing the amygdala.

c-Fos Immunofluorescence

The free-floating tissue sections were washed with PBS and then incubated for blocking with permeable buffer (0.3% Triton-X 100 in PBS) containing 10% normal goat serum for 50 min. The sections were incubated overnight at 4°C in the primary antibodies against c-Fos (1:6,400; catalog no. 2250S; Cell Signaling Technology, Danvers, MA) and then incubated with the secondary Alexa Fluor® 448 (1:800; catalog no. 4412; Cell Signaling Technology). The slices were then rinsed with PBS, mounted on microscope slides with ProLong™ Gold Antifade mountant containing 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) and cover-slipped.

Analyses

The mPFC and amygdala were identified as region of interest (ROI) based on common histological landmarks (Paxinos and Watson, 2006). We used randomization of location and orientation within navigation windows to sample within the mPFC and amygdala. We sampled from infralimbic and prelimbic cortices of the mPFC. Within the amygdala, the anterior basolateral amygdaloid nucleus (BL), the posterior basomedial amygdaloid nucleus (BM), and the ventromedial lateral amygdaloid nucleus (L) were analyzed. c-Fos-positive cells were counted within an unbiased counting frame using a cell count software (Keyence Corp. of America, Itasca, IL, United States). A Keyence Biorevo BZ-9000 All-In-One Fluorescence Microscope (Keyence) equipped with a Nikon CFI Plan Apo λ20X objective (Nikon, Melville, NY, United States) was used to collect *z*-stacks (numerical aperture, 0.75; working distance, 1.0 mm). Digitalization and image quantification were carried out by blinded observers. Cells identified as c-Fos positive after threshold correction were quantified automatically using the Macro Cell Count Software (batch image analysis tool from Keyence). For immunofluorescence analyses, a minimum of five images per area per animal were used (depending on the size of the ROI). Image analyses were averaged per ROI, and the total sample number used for statistical analysis equaled the number of animals used.

Real-Time Quantitative Polymerase Chain Reaction

Rats were euthanized with Euthasol (150 mg/kg, i.p.; Virbac, Fort Worth, TX, United States) and quickly perfused transcardially with PBS to remove residual blood from the brain capillaries. Following the perfusion procedure, the prefrontal cortex was isolated from a subcohort of rats that underwent behavioral testing (n = 6–8/group). Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA, United States). The only change to the recommended protocol was using 1-bromo-3-chloropropane (BCP, 0.1 ml per 1 ml of Trizol) instead of chloroform. BCP was obtained from the Molecular Research Center (Cincinnati, OH, United States). RNA concentration was determined on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). In our hands, this protocol results in an average RNA purity between 1.9 and 2.0, 260/280 ratio. We used 1 μg of the total RNA for complementary DNA (cDNA) synthesis (iScript cDNA Synthesis Kit, catalog no. 170-8891, Bio-Rad Laboratories, Hercules, CA, United States). cDNA synthesis protocol was performed according to the manufacturer's instructions. The total volume of the cDNA synthesis reaction mixture was 20 μl (4 μl, iScript reaction mix; 1 μl, iScript reverse transcriptase; 15 μl nuclease-free water, and 1 μg of RNA). After completion of cDNA synthesis, 80 μl of nuclease-free water was added to dilute the 20 μl of synthesized cDNA. The cDNA was amplified by PCR using the primer sets described in **Table 2**. Real-time PCR amplification and analyses were carried out on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). Real-time quantitative PCR (qPCR) conditions were optimized, and 25-μl reactions were prepared. The PCR reactions contained: 12.5 μl of iQ SYBR Green Supermix (catalog no. 170-8882, Bio-Rad Laboratories, Hercules, CA, United States), 1 μl of a mixture of 10 μM forward/reverse primer, 6.5 μl of water, and 5 μl of the previously synthesized cDNA. The PCR protocol started with 5 min at 95°C. This was followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing/extension. The relative levels of mRNA were calculated using the comparative *Ct* (crossing threshold). Each sample was normalized to its glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA content. Relative

TABLE 2 | Primer sequences used in the study.

Gene (accession number)	Forward (5′–3′)	Reverse (5′–3′)
Crhr1 (NM_030999)	CCAATCCAGCTTTCTGTCACTTA	CCGACCCGATCTTCCCAC
Drd1 (NM_010076)	ATCGTCACTTACACCAGTATCTACAGGA	GTGGTCTGGCAGTTCTTGGC
Drd2 (NM_012547)	AGACGATGAGCCGCAGAAAG	GCAGCCAGCAGATGATGAAC
Fkbp51 (NM_001012174)	TGGTTGAGCAGGGAGAAGAT	GCCAAGGCTAAAGACAAACG
Fkbp52 (NM_001191863)	ACACTGGCTGGCTGCTAGAT	ATTTGGGGGAATCTTTGGAG
Nr3c1 (M14053)	CTTGAGAAACTTACACCTCGATGACC	AGCAGTAGGTAAGGAGATTCTCAACC
Gapdh (NM_017008)	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA

gene expression levels were normalized to the adolescent CD group and expressed as fold change.

Glucose Measurements

Blood glucose levels were measured in a subcohort of rats that underwent behavioral testing. The rats' tail tip was cut under anesthesia before inducing euthanasia (n = 6–8/group; same animals used for qRT-PCR analyses). Blood glucose levels were measured using a glucometer (OneTouch UltraMini, LifeScan).

Corticosterone Measurements

We induced euthanasia in the same subcohort of rats used to collect molecular data (Euthasol; 150 mg/kg, i.p.). Blood collected through cardiac puncture. Blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-coated tubes. Subsequently, the samples were centrifuged at 1,000 rpm for plasma fraction collection. Measurement of circulating levels of corticosterone (CORT) was done using an ELISA kit (no. ADI-900-097) from Enzo Life Sciences (Farmingdale, NY) according to the manufacturer's instructions. Samples were diluted with kit assay buffer (1:4 dilutions) and ran in triplicates. Absorbance was measured at 405 nm with 570 nm correction using SpectraMax i3X detection platform (Molecular Devices, Sunnyvale, CA, United States). Concentrations for each sample were determined as the percentage bound using a standard curve with detection range between 20,000 and 31.25 pg/ml. Calculated values are reported as picograms of corticosterone per milliliter.

Statistical Analysis

We analyzed the data using GraphPad Prism version 8.0. Shapiro–Wilk statistical analyses were used to determine sample distribution. The Brown–Forsythe test was used to test for the equality of group variances. When appropriate, two-way analysis of variance (ANOVA) was used to examine the effect of the diet type, age, and interaction between factors on outcomes measures. Multiple comparisons were made using Dunnett's (following Welch's ANOVA) or Sidak's (repeated measures two-way ANOVA) tests. The ROUT method was used to investigate outliers. We considered differences to be significant if $p < 0.05$. The data are shown as the mean ± standard error of the mean (SEM). We also conducted a *post hoc* power analysis with the *G*Power* program (Faul et al., 2007). For two-way ANOVA behavioral analyses, the statistical power (1 - β) for data including the four study groups was 0.79 for detecting a medium size effect (d = 0.41), whereas the power exceeded 0.99 for the detection of a large effect size (d = 0.8). This indicates that this study is adequately powered at or above a moderate size level (d = 0.4). Therefore, if chosen at random, the probability that adolescent rats that consumed the WD described here will exhibit alterations in fear-related behaviors relative to controls is 0.61. With an alpha = 0.05 and power = 0.80, *post hoc* sensitivity power analyses revealed a medium size effect (d = 0.55) for molecular experiments (glucose, corticosterone, and mRNA level measurement). With similar alpha and power, sensitivity analyses demonstrated a large size effect (d = 0.92) for cFos histological experiments. These results indicate that the molecular studies were sensitive for effect sizes between 0.55 and 0.92, for a level of power equal to 0.80.

RESULTS

Bodyweight and Food Consumption

This study investigated early brain and behavior responses triggered by a short-term exposure to an obesogenic diet (**Figure 1A**). Body weight and caloric intake were measured weekly. We found that short-term exposure to the obesogenic WD did not alter body weights in adolescent or adult rats. Repeated measures two-way ANOVA revealed a significant effect of week [$F_{(1.21,25.35)} = 395.9$, $p < 0.0001$] but no diet [$F_{(1,21)} = 0.24$, $p = 0.63$] or interaction [$F_{(2,42)} = 0.88$, $p = 0.42$] effects on body weight for adolescent rats (**Figure 1B**). Analyses showed a significant effect of week [$F_{(1.42,31.14)} = 677.8$, $p < 0.0001$] but no diet [$F_{(1,22)} = 3.2$, $p = 0.09$] effects on body weight in adult rats. Interestingly, we found significant interaction between diet and week in adult rats [$F_{(2,44)} = 17.91$, $p < 0.0001$] (**Figure 1C**). No significant interaction [$F_{(1,36)} = 1.15$, $p = 0.29$] or main effects of the diet [$F_{(1,36)} = 0.04$, $p = 0.83$] and age [$F_{(1,36)} = 1.76$, $p = 0.19$] were observed for circulating blood glucose levels at endpoint.

In agreement with previous findings, we found significant changes in caloric intake in the rats that consumed the obesogenic diet. In adolescent rats, we found a significant effect of week [$F_{(1,10)} = 58.66$, $p < 0.0001$] and diet [$F_{(1,10)} = 17.92$, $p = 0.002$], while no significant interactions [$F_{(1,10)} = 1.52$, $p = 0.25$] on caloric intake (**Figure 1D**). Sidak's *post hoc* analyses showed that differences in caloric intake were statistically significant at week 1 (22% increase; $p = 0.003$) and week 2 (23% increase; $p = 0.0005$) when comparing CD and WD adolescent groups. Similarly, in adult rats, we found a significant effect of week [$F_{(1,10)} = 21.29$, $p = 0.001$] and diet [$F_{(1,10)} = 62.28$, $p < 0.0001$], while no significant interactions [$F_{(1,10)} = 1.15$, $p = 0.31$] on caloric intake (**Figure 1E**). *Post hoc* analyses showed that differences in caloric intake were statistically significant at week 1 (32% increase; $p < 0.0001$) and week 2 (28% increase; $p = 0.0003$) when comparing CD and WD adult groups. The caloric intake of adolescent rats exposed to the WD was similar to that of adult rats (∼900 kcal/cage/week). Notably, while adolescent rats increased caloric intake during week 2 (relative to week 1), the adult rats reduced their caloric intake. While differences in caloric needs or the period required to adjust to the novel diets may explain this effect, this finding also suggests an opposite effect of footshock stress on food consumption in adolescent and adult rats undergoing a cued fear paradigm.

Acute WD Exposure Attenuates Fear Extinction Learning in Adolescent, but Not in Adult Rats

We recently reported that chronic consumption of an obesogenic WD during adolescence leads to impairments in fear-related associative learning and extinction in adult rats

(Vega-Torres et al., 2018). However, it remains unclear from our studies whether adolescence and the obesogenic diet are predisposing and precipitating factors for the reported fear impairments. This follow-up study was designed to investigate the effects of short-term exposure to a WD on cued fear conditioning and fear extinction learning before the onset of an obesogenic phenotype in adolescent and adult rats. We used the ASR baseline values to assign and match the rats in each group based on their unconditioned responses to acoustic stimulation. Welch's ANOVA demonstrated no significant differences in baseline weight-corrected ASR responses between groups [$F_{(3,24.4)} = 0.21$, $p = 0.88$] (figure not shown). Following the ASR-based matching, the rats were exposed to either the control or the obesogenic custom diet and fed *ad libitum* for 1 week before behavioral testing. Anxiety and fear-related responses were investigated using the fear-potentiated startle (FPS) paradigm (**Figure 2A**). The rats were conditioned to learn an aversive association between a light (*CS*) and a foot shock (*US*) during the first day of the FPS paradigm. ANOVA revealed significantly reduced startle responses to the footshocks in adult rats when compared to adolescent rats [$F_{(3,24.24)} = 21.13$, $p < 0.0001$; CD rats, $p = 0.0001$; WD rats, $p < 0.0001$] (**Figure 2B**). Acquired fear was tested 24 h after conditioning and defined as significant differences in startle amplitudes between the US (tone alone) and the CS + US (light + tone). All groups showed significant differences between amplitudes of US and CS + US ($p < 0.05$; data not shown). We calculated the potentiation of the startle as a proxy for fear conditioning and found that 1-week WD consumption or age did not alter fear learning [$F_{(3,19.7)} = 1.52$, $p = 0.24$] (**Figure 2C**). Fear extinction training and fear extinction testing were performed on days 3 and 4 of the FPS paradigm, respectively. Successful fear extinction is defined as similar startle responses when comparing the US trials to CS + US trials (Vega-Torres et al., 2018). Although FPS responses following extinction testing were similar between groups [$F_{(3,19.7)} = 1.78$, $p = 0.18$] (figure not shown), we found heightened fear recovery in the adolescent rats that consumed the WD relative to CD ADOL rats [Welch's $F_{(3,18)} = 6.27$, $p = 0.004$; CD, 14.9 ± 17.92 vs. WD, 123 ± 45.21; $t_{(14.37)} = 2.22$, $p = 0.04$] (**Figure 2D**). This finding indicates that a short-term exposure to a WD is

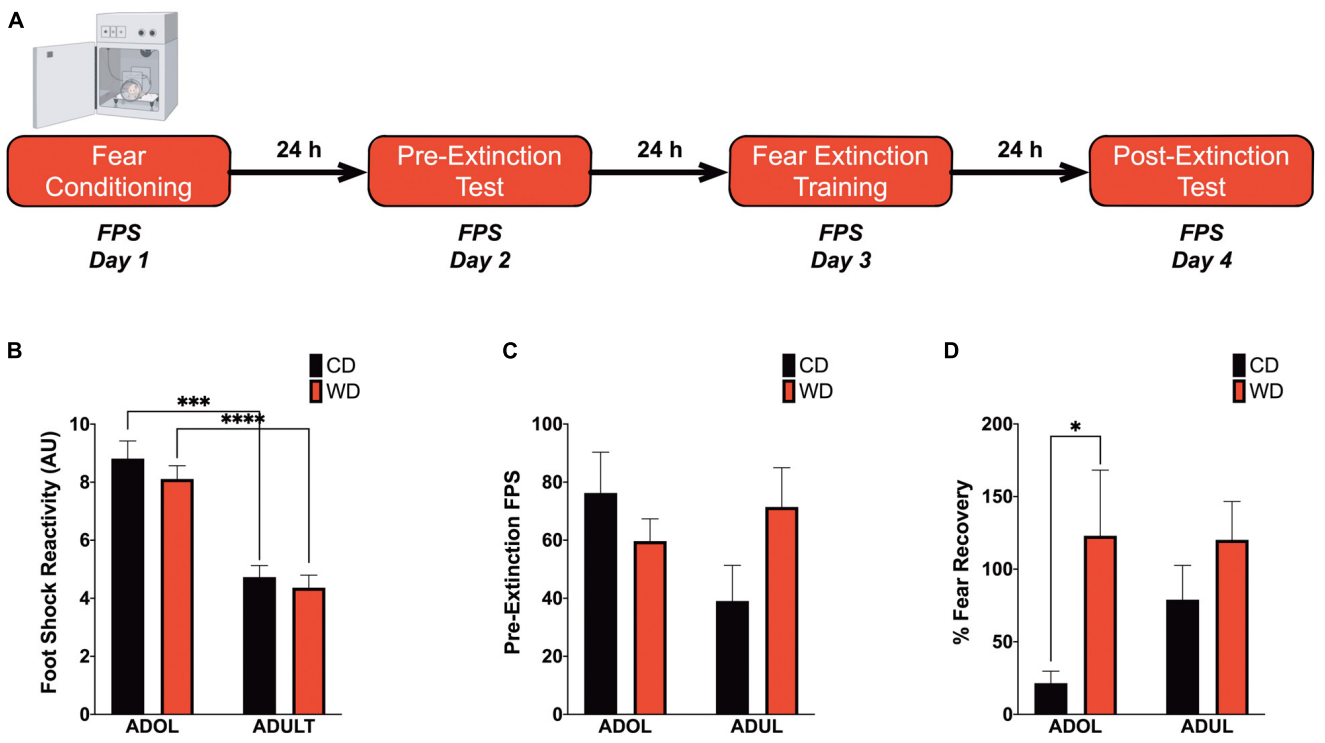

FIGURE 2 | Short-term Western high-saturated fat diet (WD) exposure attenuates fear extinction in adolescent rats. **(A)** Fear-potentiated startle (FPS) paradigm. Following habituation and acclimation periods, the rats received a series of light–shock pairings (*FPS day 1, fear conditioning*). Twenty-four hours later (*FPS day 2, pre-extinction testing*), the rats were returned to the startle chambers and enclosures and presented with startle stimuli (leaders). Subsequently, rats received startle stimuli presented alone (noise-alone trial) and startle stimuli after onset of the conditioning light (light–noise trials). The rats were presented with startle-eliciting stimuli again at the end of the session (trailers). The two trial types were presented in a balanced mixed order. Twenty-four hours later (*FPS day 3, fear extinction training*), the rats were returned to the startle chambers and enclosures and presented with trials of light without shock or noise bursts. One day after fear extinction training (*FPS day 4, postextinction FPS*), we determined fear extinction acquisition and retention using the same FPS session that was used to measure fear learning. **(B)** Average pre-extinction FPS responses are similar between groups ($p > 0.05$; n = 9–12 rats/group). **(C)** Average post-extinction FPS responses ($p > 0.05$; n = 9–12 rats/group). **(D)** Average percent change in FPS responses between testing sessions as an index of fear recovery following fear extinction training. Adolescent (*ADOL*) WD rats exhibited significantly more FPS than ADOL CD rats, indicating disrupted fear extinction retention (*$p < 0.05$; n = 9–12 rats/group). Error bars are SEM. ***$p \leq 0.001$ and ****$p \leq 0.0001$, respectively.

sufficient to impair fear extinction acquisition and/or retrieval in adolescent rats.

Acute WD Exposure Enhances Startle Sensitization in Adolescent Rats

We reported that chronic consumption of an obesogenic WD during adolescence enhances both pre- and postextinction background anxiety (BA), as evaluated by changes in ASR responses following cued fear conditioning in adult rats (Vega-Torres et al., 2018). Here, we demonstrate that adult rats exhibited greater background anxiety before extinction training [Welch's $F_{(3,23.3)} = 9.84$, $p = 0.0002$; Dunnett's *post hoc* showed an age effect for both the CD ($p = 0.02$) and WD ($p = 0.04$) rats] (**Figure 3A**). Notably, we found higher background anxiety in the adolescent rats that consumed the WD relative to CD adolescent rats following fear extinction training [$F_{(3,21.2)} = 7.16$, $p = 0.002$; CD ADOL vs. WD ADOL *post hoc* $p = 0.02$; CD ADOL vs. CD ADUL *post hoc* $p = 0.04$] (**Figure 3B**), supporting an anxiogenic effect of the WD. Short-term habituation of the ASR measures non-associative learning and is determined using the ratio percentage from leader trials to trailer trails within

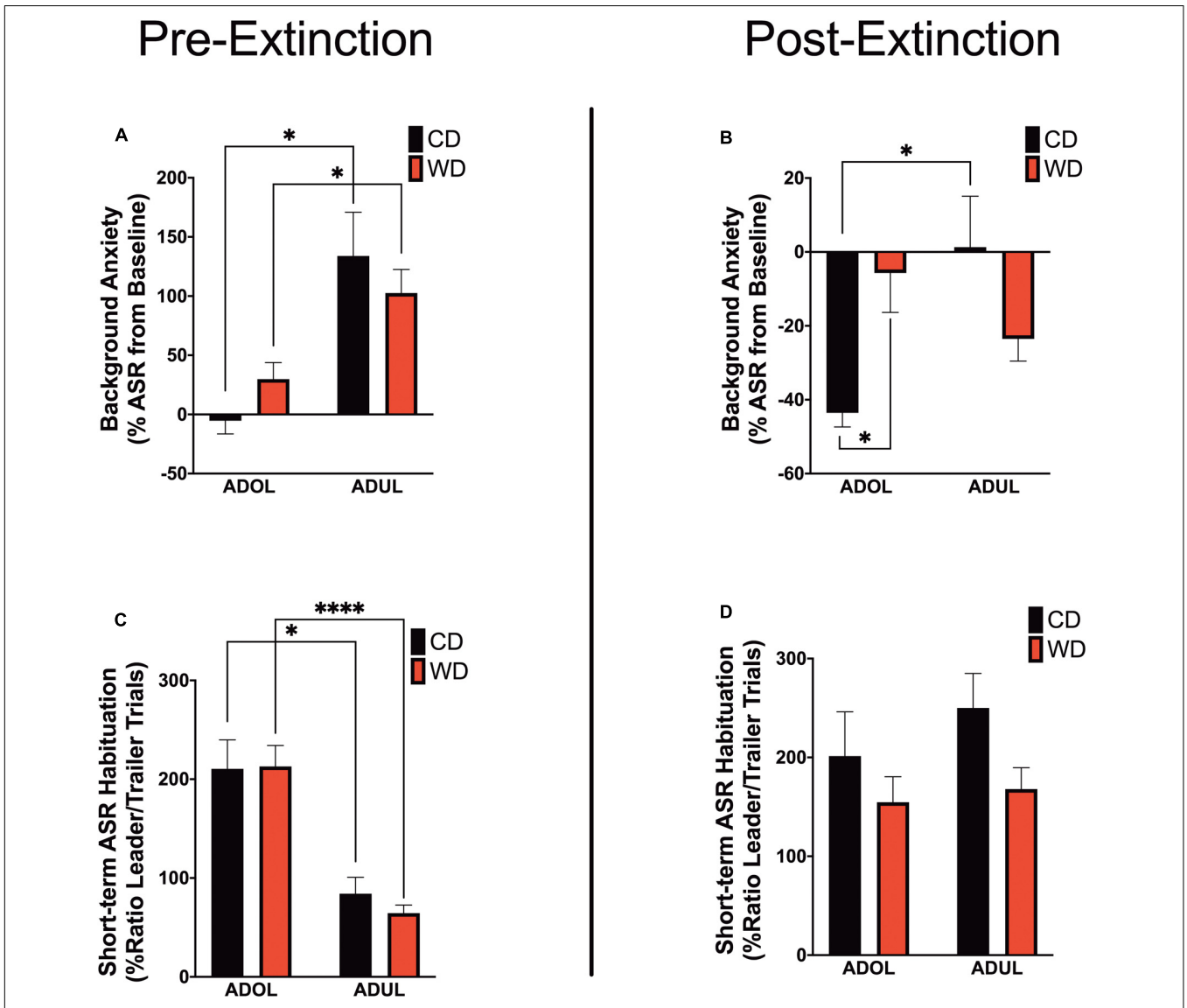

FIGURE 3 | Age and diet influence acoustic startle reflex (ASR) reactivity and plasticity. **(A)** Average percent change in acoustic startle reflex (ASR) responses following fear conditioning relative to baseline. ADUL rats exhibited higher ASR responses to cued fear conditioning (*$p < 0.05$; $n = 12$ rats/group). **(B)** Average percent change in ASR reactivity following fear extinction training. The ADOL rats that consumed the Western-like high-saturated fat diet exhibited increased indices of anxiety relative to ADOL CD rats (*$p < 0.05$; $n = 12$ rats/group). Postextinction background anxiety in ADOL WD rats was similar to that observed in adult rats ($p > 0.05$). **(C)** Average pre-extinction short-term habituation of the ASR. ADUL rats exhibited higher within-session ASR responses relative ADOL rats (*$p < 0.05$; ****$p \leq 0.0001$; $n = 11$–12 rats/group). **(D)** All groups exhibited similar short-term habituation of the ASR following fear extinction ($p > 0.05$; $n = 10$–12 rats/group). Error bars are SEM.

each FPS testing session. We found reduced habituation of the ASR in the adult rats when compared to adolescents before fear extinction training [$F_{(3,19.7)} = 18.87$, $p < 0.0001$; Dunnett's test revealed an age effect for both diet groups CD ($p = 0.05$) and WD ($p < 0.0001$)] (**Figure 3C**), suggesting heightened sensitization to footshocks in adult rats. The effect of age in the short-term habituation of the ASR was absent following extinction training [$F_{(3,22.4)} = 1.74$, $p = 0.18$] (**Figure 3D**). The long-term habituation of the ASR was determined using the ratio percentage from trailer trials to trailer trials between FPS testing sessions. Analyses revealed that adult rats exhibited reduced long-term habituation of the ASR [$F_{(3,20.8)} = 9.58$, $p = 0.0004$; Dunnett's test revealed an age effect for both diet groups CD ($p = 0.02$) and WD ($p < 0.037$)] (figure not shown).

Age Increases Locomotor Activity in the Elevated Plus Maze

Given that footshock stress and obesogenic diet consumption modulate anxiety-like behavior, we carried out analyses of behavioral responses in the EPM 1 day following fear extinction testing (**Figure 4A**). There was no difference in the duration in the open arms [$F_{(3,21.6)} = 1.6$, $p = 0.21$] (**Figure 4B**). The locomotor activity, reflected by the number of crossings between

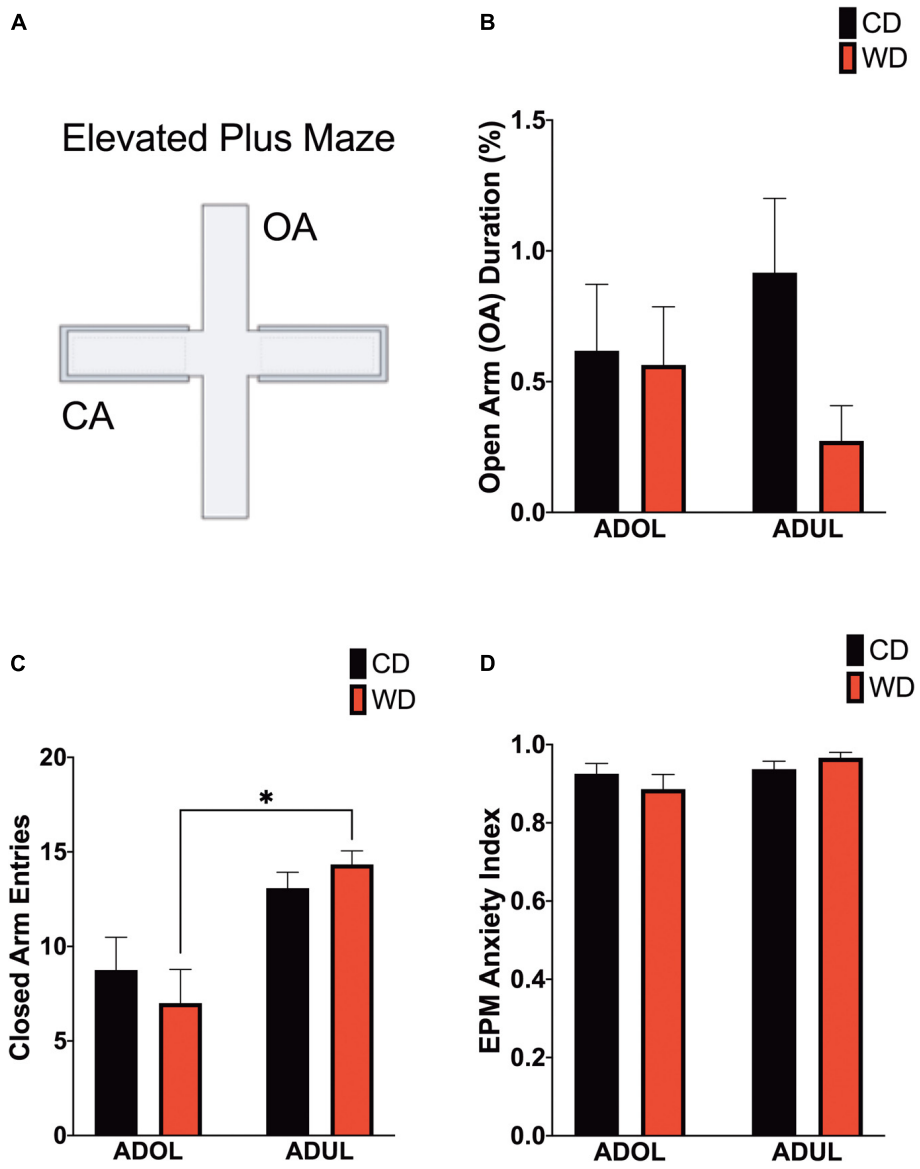

FIGURE 4 | Age influences locomotor activity in the elevated plus maze (EPM). **(A)** Schematic representation of the EPM. **(B)** Average time spent in open arms (expressed as percentage of total time in maze) was similar between groups ($p > 0.05$; $n = 10$–12 rats/group). **(C)** Average entries to closed arms. ADUL Western-like high-saturated fat diet (WD) rats exhibited higher ambulation when compared to ADOL WD rats (*$p < 0.05$; $n = 10$–12 rats/group). **(D)** There was no significant difference in the anxiety index ($p > 0.05$; $n = 10$–12 rats/group). Error bars are SEM.

closed arms, was different among groups [$F_{(3,23.19)} = 6.51$, $p = 0.002$] (**Figure 4C**). Ambulation was higher in WD ADUL rats than in WD ADOL rats (adjusted $p = 0.001$). It is noteworthy to mention that we found very robust avoidance responses and anxiety-like behaviors relative to previous studies, suggesting that the fear conditioning paradigm used in this study is anxiogenic. The analysis of the anxiety index, which incorporates several behavioral measures in the maze, revealed no significant differences between groups [$F_{(3,22.98)} = 1.72$, $p > 0.05$] (**Figure 4D**), possibly reflecting a ceiling effect.

Acute WD Exposure During Adolescence Attenuates Footshock Stress-Induced c-Fos Expression in the Basomedial Amygdala

We reported that the consumption of an obesogenic diet has detrimental consequences for the structural integrity of mPFC–amygdala circuits implicated in emotion regulation and fear (Vega-Torres et al., 2018). To further characterize neural substrates impacted by obesogenic diets, we evaluated neuronal activation in mPFC and amygdala regions implicated in fear. We mapped c-Fos expression 1 h after the rats were exposed to a cued fear conditioning session. We found no significant main effects of diet [$F_{(1,8)} = 0.61$, $p = 0.45$], age [$F_{(1,8)} = 0.33$, $p = 0.58$], or interactions [$F_{(1,8)} = 1.74$, $p = 0.22$] on footshock-induced c-Fos expression in the prelimbic region of the mPFC (**Figure 5A**). Similarly, the diet type [$F_{(1,8)} = 0.06$, $p = 0.81$], age [$F_{(1,8)} = 2.06$, $p = 0.18$], and interactions between factors [$F_{(1,8)} = 4.55$, $p = 0.06$] did not have a significant effect on c-Fos protein levels in the infralimbic region of the mPFC (**Figure 5B**). The basolateral, basomedial, and lateral amygdaloid nuclei were also assessed for cued fear conditioning-induced c-Fos expression. While analyses revealed no significant effects of the diet, age, and interactions in c-Fos expression in the basolateral [diet: $F_{(1,8)} = 1.20$, $p = 0.31$; age: $F_{(1,8)} = 0.11$, $p = 0.76$; interaction: $F_{(1,8)} = 0.23$, $p = 0.65$] (figure not shown) and lateral amygdaloid nuclei [diet: $F_{(1,8)} = 1.97$, $p = 0.20$; age: $F_{(1,8)} = 3.78$, $p = 0.09$; interaction: $F_{(1,8)} = 2.40$, $p = 0.16$] (**Figure 5C**), we found that the obesogenic diet reduced c-Fos expression levels in the basomedial nucleus of the amygdala [diet: $F_{(1,8)} = 10.15$, $p = 0.01$; age: $F_{(1,8)} = 0.4$, $p = 0.55$; interaction: $F_{(1,8)} = 3.21$, $p = 0.11$] (**Figure 5D**). Sidak's *post hoc* analysis revealed that acute exposure to the WD during adolescence led to a significant reduction in basomedial amygdala c-Fos expression relative to control rats (adjusted $p = 0.02$).

Age and Short-Term Obesogenic Diet Exposure Modulate the Levels of Critical Fear-Modulating Biomarkers

We evaluated the levels of critical fear-associated biomarkers to gain insights on putative early (mal)adaptive mechanisms impacted by a short-term exposure to the obesogenic diet. We found a significant effect of age on plasma corticosterone (CORT) levels [diet: $F_{(1,24)} = 0.007$, $p = 0.93$; age: $F_{(1,24)} = 13.22$, $p = 0.001$; interaction: $F_{(1,24)} = 0.51$, $p = 0.48$] (**Figure 6A**). The adult rats exposed to the short-term WD had significantly higher CORT levels when compared to the adolescent rats that consumed the same diet ($p = 0.01$). Consistent with this finding, age had a significant impact on the mRNA expression levels of the glucocorticoid receptor gene (*Nr3c1*) in the medial prefrontal cortex, with adult rats exhibiting higher Nr3c1 mRNA levels relative to adolescent rats [diet: $F_{(1,20)} = 0.0002$, $p = 0.98$; age: $F_{(1,20)} = 5.14$, $p = 0.03$; interaction: $F_{(1,20)} = 0.01$, $p = 0.9$] (**Figure 6B**). Although the adult rats showed a trend for higher mRNA levels of the glucocorticoid receptor chaperones, *Fkbp51* (figure not shown) and *Fkbp52* (**Figure 6C**), analyses showed no significant effects of the diet type, age, and interactions between factors [for FKBP5-1, diet: $F_{(1,20)} = 0.34$, $p = 0.56$; age: $F_{(1,20)} = 1.97$, $p = 0.17$; interaction: $F_{(1,20)} = 0.05$, $p = 0.82$; for FKBP5-2, diet: $F_{(1,19)} = 0.65$, $p = 0.42$; age: $F_{(1,19)} = 3.60$, $p = 0.07$; interaction: $F_{(1,19)} = 2.32$, $p = 0.14$].

The corticotropin-releasing hormone receptor 1 (*Crhr1*) is abundantly expressed in the mPFC and serves crucial roles in the regulation of stress and fear responses. We found that the rats that were exposed to the short-term obesogenic diet had higher Crhr1 mRNA levels in the mPFC relative to controls [diet: $F_{(1,20)} = 8.28$, $p = 0.009$] (**Figure 6D**). Adult rats showed increased Crhr1 mRNA levels in the mPFC relative to adolescent rats [age: $F_{(1,20)} = 21.58$, $p = 0.0002$; interaction: $F_{(1,20)} = 0.07$, $p = 0.79$]. Sidak's *post hoc* analysis revealed that adult rats in both CD ($p = 0.01$) and WD ($p = 0.004$) groups had significantly higher Crhr1 mRNA levels in the PFC when compared to adolescent rats. Previous studies indicate the active involvement of dopaminergic receptors in diet-induced obesity and fear-related behaviors. Of interest, evidence indicates that dopamine receptors are under regulatory actions of corticotropin-releasing hormone (CRH) and dictate fear conditioning and fear extinction responses. Thus, we aimed to determine the effects the obesogenic diet, age, and interactions on the mRNA levels of dopamine receptors 1 and 2. While diet [$F_{(1,19)} = 0.05$, $p = 0.82$] and age [$F_{(1,19)} = 0.11$, $p = 0.73$] did not have significant effects on the mRNA expression levels of the dopamine receptor 1 (*Drd1*), we found a significant interaction between these factors [$F_{(1,19)} = 4.39$, $p = 0.04$] (**Figure 6E**). While the two-way ANOVA revealed a significant global interaction effect for Drd1, *post hoc* analysis did not show significant differences between groups. It is likely that this discrepancy between the ANOVA and *post hoc* analysis may be related to the weakly global effect found ($p = 0.04$) and/or associated with the conservative Sidak's multiple comparison test used in this study. We found no significant effects of the diet [$F_{(1,18)} = 1.71$, $p = 0.20$], age [$F_{(1,18)} = 0.13$, $p = 0.72$], or interactions [$F_{(1,18)} = 2.38$, $p = 0.14$] on the expression of Drd2 mRNA levels in the mPFC (**Figure 6F**). Given the similar pattern of decreased mRNA expression of dopamine receptors in adult rats, we decided to collapse the data into a within-subjects analysis (with diet and receptor gene type as factors) to add power and determine the effects of the obesogenic diet. Interestingly, analyses revealed a significant global effect of the short-term exposure to the obesogenic diet in reducing Drd1 and Drd2 mRNA levels in the mPFC of adult rats [diet: $F_{(1,18)} = 5.40$, $p = 0.03$; receptor gene type: $F_{(1,18)} = 0.011$, $p = 0.92$; interaction: $F_{(1,18)} = 0.41$, $p = 0.53$]. On the other hand, there was no

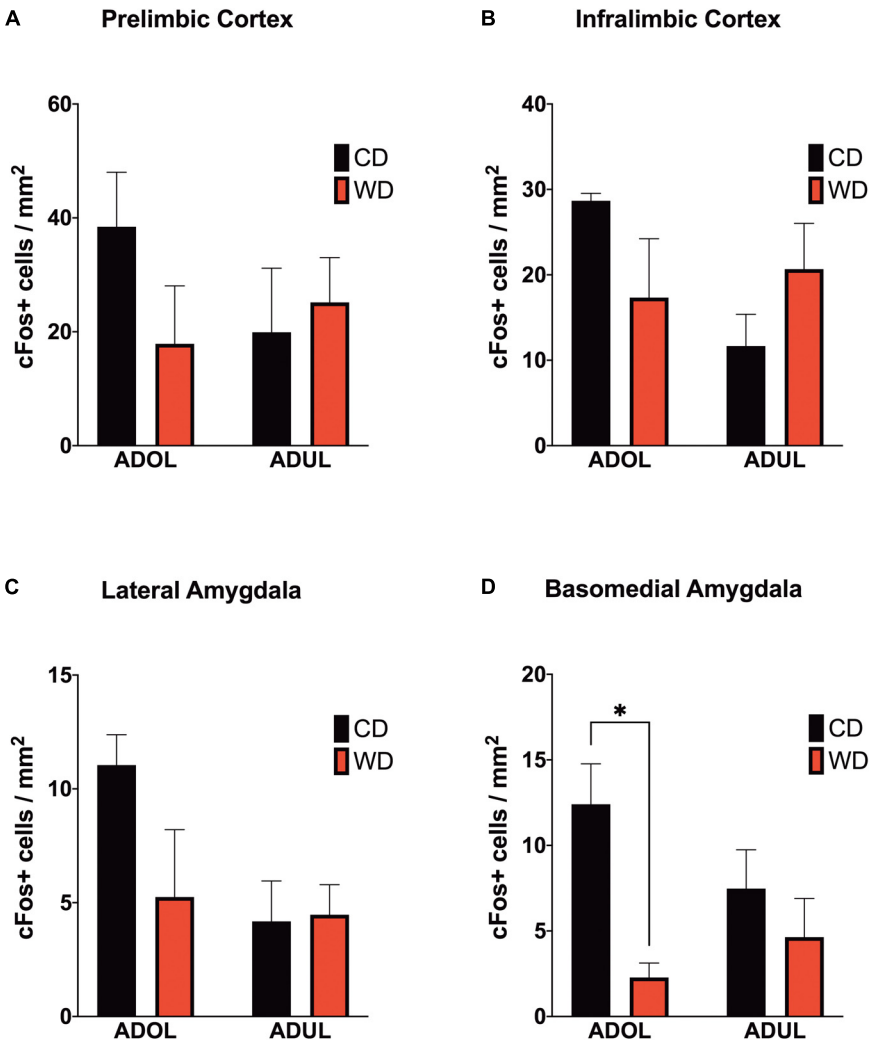

FIGURE 5 | Western-like high-saturated fat diet (WD) consumption during adolescence attenuates neuronal activation to footshock stress in the basomedial nuclei of the amygdala. Average expression of the immediate-early gene c-Fos in **(A)** prelimbic and **(B)** infralimbic medial prefrontal cortex, and in the **(C)** lateral and **(D)** basomedial nuclei of the amygdala. ADOL WD rats showed attenuated footshock-induced c-Fos expression in BMA neurons relative to ADOL CD rats (*$p < 0.05$; $n = 3$ rats/group). Error bars are SEM.

significant effect of the diet [$F_{(1,19)} = 1.54$, $p = 0.23$], dopamine receptor gene type [$F_{(1,19)} = 0.69$, $p = 0.43$], or interactions [$F_{(1,19)} = 0.96$, $p = 0.34$] in the adolescent rats.

DISCUSSION

Childhood trauma survivors who suffer from PTSD are at high risk for developing obesity and metabolic disorders (Llabre and Hadi, 2009; Roenholt et al., 2012; Assari et al., 2016; Brewerton and ONeil, 2016; Ramirez and Milan, 2016). We reported evidence in support of these observations while demonstrating a novel directionality in the relationship between early-life posttraumatic stress reactivity and diet-induced obesity (DIO) (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Our findings indicate that the consumption of an obesogenic Western-like high-saturated fat/high-sugar diet (WD) during adolescence heightens stress reactivity while altering key substrates implicated in PTSD (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018).

Although the long-term impact of DIO on emotion regulation is becoming clear, mechanistic studies are confounded by the multiple complications associated with obesity. In this study, we expanded our previous observations and investigated the impact of a short exposure to a WD, including groups exposed to the WD during adolescence and adulthood. Importantly, we tested the acute WD effects, relative to an ingredient-matched low-fat semipurified control diet, on cued fear conditioning and fear extinction using the FPS paradigm. The primary findings of this study are the following: (a) short exposure to a WD impairs cued fear extinction memory retention in adolescent rats; (b) short-term WD consumption attenuates basomedial amygdala activation to a fear conditioning paradigm; (c) short-term WD

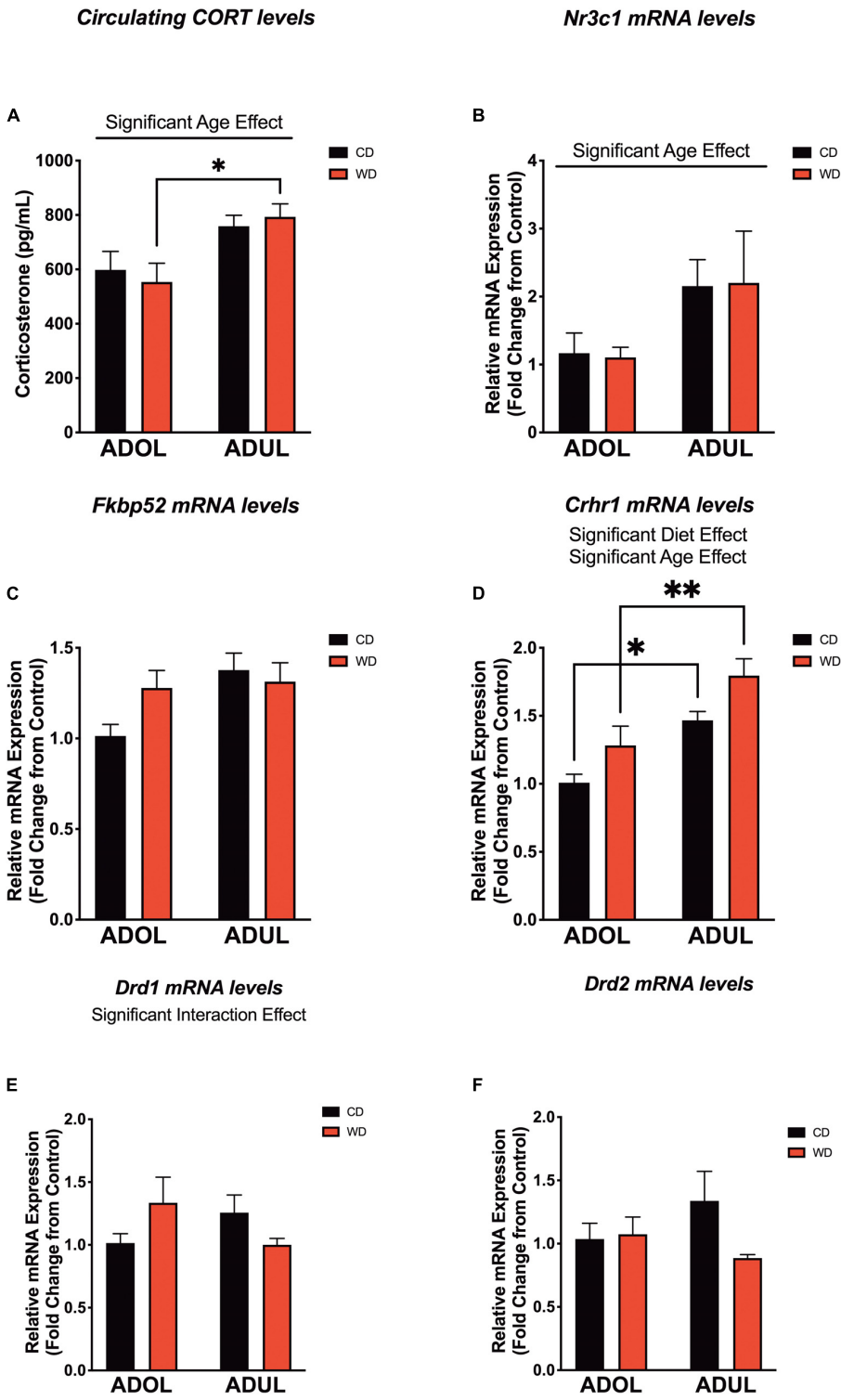

FIGURE 6 | Age and short-term exposure to obesogenic diet increase stress and fear-related biomarkers in the medial prefrontal cortex. **(A)** Age increased plasma corticosterone levels in Western-like high-saturated fat diet (WD) rats (*$p = 0.01$; $n = 6$–8 rats/group). **(B)** Similarly, age had a significant impact on the messenger RNA levels of glucocorticoid receptor gene (*Nr3c1*) (*$p = 0.03$; $n = 8$ rats/group). **(C)** Age and diet had no effects on messenger RNA (mRNA) levels of FK506-binding protein 2 (*Fkpb52*) ($p > 0.05$; $n = 8$ rats/group). **(D)** Both age ($p = 0.0002$) and diet ($p = 0.0093$) had robust effects in the expression of the corticotropin-releasing hormone receptor 1 (*Crhr1*) mRNA. **(E)** There was a significant interaction between age and the obesogenic diet on the expression of the dopamine receptor D1 (*Drd1*) ($p < 0.05$; $n = 7$–8 rats/group). **(F)** There were no significant differences in the mRNA levels of the dopamine receptor D2 (*Drd2*) ($p > 0.05$; $n = 7$–8 rats/group). *$p < 0.05$; **$p < 0.01$. Data represents mean ± SEM.

consumption increases CRHR1 mRNA expression levels in the medial prefrontal cortex; and (d) adult rats exhibit markers of heightened HPA axis tone and emotional reactivity relative to adolescent rats. Altogether, this follow-up study provides supportive evidence that behavioral and molecular substrates implicated in fear are selectively vulnerable to the consumption of an obesogenic WD during adolescence. Our findings also serve to emphasize the caution that must be exercised when interpreting experimental outcomes when unmatched diets are used as controls in DIO studies.

Short-Term WD Consumption Attenuates Fear Extinction Learning

An important finding of this study is that short-term WD consumption was sufficient to attenuate extinction learning of cued fear. Fear extinction has been characterized as an active form of inhibitory learning that allows for the adaptive regulation of conditioned fear responses (Myers and Davis, 2002). The inability to consolidate extinction memory and inhibit conditioned fear under safe conditions underlies some of the hallmark symptoms of anxiety and stress-related disorders (Milad et al., 2009; McGuire et al., 2016; Waters and Pine, 2016). It is now recognized that cognition, attention, mood, and anxiety disorders have a nutritional component or are promoted by poor dietary habits. Our results are consistent with evidence showing that the consumption of obesogenic high-fat diets can attenuate cognitive functions in humans and rodents in as short as 3–9 days (Kanoski and Davidson, 2010; Edwards et al., 2011; Holloway et al., 2011; Beilharz et al., 2014, 2016; Sobesky et al., 2016; Khazen et al., 2019). The findings of this study indicate that the adverse effects of consuming obesogenic foods expand to additional cognitive domains regulating emotional memories and fear. This is in agreement with new studies showing that adolescent rats that consume high-fat/high-sugar diets exhibit delayed spontaneous extinction and impaired extinction retention of fear-related behaviors (Reichelt et al., 2015; Baker et al., 2016; Vega-Torres et al., 2018). Our findings extend beyond those reported to date by showing fear extinction deficits independent of effects associated with obesity and metabolic disturbances.

Evidence supports that impairments in attention and threat discrimination may heighten the risk for anxiety and stress-related psychopathology (López-Aumatell et al., 2009a,b; Block and Liberzon, 2016). This study confirms our previous findings showing that rats that consume obesogenic diets exhibit alterations in ASR plasticity, which may result in an inaccurate assessment of the level of threat (Kalyan-Masih et al., 2016; Vega-Torres et al., 2018). Notably, we found that the ASR neural substrates impacted by the WD seem to be dependent on the age of onset of diet exposure. The adolescent rats that consumed the WD exhibited increased ASR sensitization to the footshocks. This behavioral proxy may represent maladaptive stress reactivity and anxiety (Shalev et al., 2000; Conti and Printz, 2003).

Interestingly, the adult rats that consumed the WD showed greater long-term habituation of the ASR relative to CD rats. Since sensitization and habituation of the ASR require independent neural substrates (Leaton, 1976; Koch, 1999; Pilz and Leaton, 1999), our data indicate that short exposure to WD influences different targets in adolescent and adult rats. Taken together, the ASR behavioral outcomes reported here support the notion that perturbations in attention, threat discrimination, and startle habituation may heighten vulnerability for anxiety and stress-related disorders in individuals that consume obesogenic diets.

Short Exposure to an Obesogenic Diet Attenuates Basomedial Amygdala Activation Following Cued Fear Conditioning

Several studies indicate that the mPFC and the basolateral complex of the amygdala (BLA) are critical to the acquisition and expression of conditioned fear (Sotres-Bayon et al., 2004; Davis, 2006; Quirk et al., 2006). The highly conserved neurocircuitry connecting the mPFC and the amygdala plays a critical role in anxiety and in the extinction of fear memories (Milad and Quirk, 2002; Phelps et al., 2004; Adhikari et al., 2015; Janak and Tye, 2015) and is abnormal in PTSD patients (Gilboa et al., 2004; Koenigs and Grafman, 2009). The PFC and amygdala undergo striking structural changes during adolescence (Jalbrzikowski et al., 2017), providing a biological basis that may underlie their unique vulnerability to the disruptive effects of obesity and the consumption of diets rich in saturated fats and sugars. Paralleling clinical data in humans (Riederer et al., 2016; Geha et al., 2017), we showed that DIO rats exhibit significant and partly irreversible microstructural alterations in mPFC regions and amygdalar nuclei associated with fear learning and fear extinction (Vega-Torres et al., 2018). The impact of obesogenic diets on PFC and BLA neuroplasticity is supported by studies showing reduced dendritic spine density in the PFC (Dingess et al., 2017) and dendritic length in the basal arbors of the BLA (Janthakhin et al., 2017) in rats that consume obesogenic diets rich in fats. Together, these structural alterations may lead to anxiety (Kim and Whalen, 2009), impairments in fear processing (Poulos et al., 2009), and aberrant feeding behaviors (Land et al., 2014).

Age Increases Anxiety and Emotional Reactivity in Lewis Rats

There is some evidence in support of the notion that limbic structures involved in higher processing of emotional cues become deficiently activated with age despite showing a higher basal level of activation (Meyza et al., 2011). The histological results presented in this study provide support to this notion by indicating different c-Fos signatures in adolescent rats, with greater activation in mPFC and amygdalar regions, relative to adult rats. This suggests that the typical age-related increases in anxiety in adult LEW rats may be related to a blunted neuroendocrine response, combined with insufficient top–down regulation of limbic regions fear and anxiety (Adhikari et al., 2015).

It is becoming increasingly clear that obesogenic diets dysregulate critical mediators of the HPA axis in rats (Auvinen et al., 2012; Abildgaard et al., 2014; Boitard et al., 2015; McNeilly et al., 2015; Kalyan-Masih et al., 2016;

Sobesky et al., 2016; Vega-Torres et al., 2018; Khazen et al., 2019). The present findings seem difficult to reconcile with these reports, as they indicate that short exposure to a WD was not sufficient to increase the circulating levels of corticosterone and related neuroendocrine biomarkers in the prefrontal cortex. However, differences in the methodology (fear conditioning paradigm) and diet composition (fat content and source; purified ingredient-matched control diet) may explain this discrepancy. Nonetheless, our findings support an age-related enhancement of the HPA axis tone in LEW rats (Meyza et al., 2011).

Age and Diet Modulate the Expression of the Corticotropin-Releasing Hormone Receptor 1

The corticotropin-releasing hormone (CRH) system has received considerable attention as a promising therapeutic target for anxiety and stress-related disorders (Bale and Vale, 2004; Binder and Nemeroff, 2010). CRH is an essential component of the behavioral and endocrine responses to stress, signaling through the stimulation of the G-protein-coupled CRH receptor 1 (CRHR1). The CRHR1 is abundantly expressed in fear-modulating corticolimbic circuits, including the mPFC (Steckler and Holsboer, 1999). Studies in humans and rodents indicate that CRH-CRHR1 hypersignaling represents a candidate mechanism for PTSD risk (Bremner et al., 1997; Rajbhandari et al., 2015; Toth et al., 2016; Jovanovic et al., 2020). CRHR1 hypersignaling alters brain structural integrity (Chen et al., 2004; Kolber et al., 2010; Toth et al., 2014) and has long-lasting consequences in stress susceptibility (Uribe-Mariño et al., 2016), mainly when it is triggered early in life (Toth et al., 2016).

Although the role of CRH in obesity is very complex, studies demonstrate that the CRH system has anorectic and thermogenic roles (Kuperman et al., 2016). CRH-CRHR1 signaling has emerged as a potential neuromodulator of food intake energy expenditure (Arase et al., 1988). Interestingly, CRHR1 is coexpressed with various metabolic receptors in corticolimbic structures (Koorneef et al., 2018), supporting an interplay between metabolism and fear. Our findings indicate that CRHR1 upregulation represents an early molecular adaptation to the obesogenic diet. Furthermore, our results provide support for further study of this signaling system as a candidate mechanism for anxiety and PTSD risk in obesity. We have demonstrated that exposure to an obesogenic diet during the critical maturational period of adolescence may permanently modify corticolimbic circuits and the response to further stressful stimuli. CRHR1 may play an integral role in the mechanisms of these long-lasting structural changes (Yang et al., 2015). Together, this study provides compelling evidence of the close relationship between energy homeostasis and the function of corticolimbic pathways modulating fear.

Extrahypothalamic CRH signaling modulates the sensitization of mesolimbic dopamine circuits to stress (Burke and Miczek, 2014). Alterations in reward-related brain areas play a major role in stress responsivity, with important implications for PTSD (Corral-Frias et al., 2013; Abraham et al., 2014; Holly and Miczek, 2016). Dopamine and its D1 and D2 receptors play critical roles in fear learning and extinction in both humans (Haaker et al., 2013, 2015) and rodents (Fadok et al., 2009a; Hitora-Imamura et al., 2015; Pignatelli et al., 2017; Zbukvic et al., 2017; Ng et al., 2018). In addition to changes in genes associated with dopamine uptake and metabolism, reduced D1 and D2 receptor expression levels have been documented in obesity (Wang et al., 2001; Huang et al., 2005; Davis et al., 2009; Alsiö et al., 2010; Johnson and Kenny, 2010; Tomasi et al., 2015; Carlin et al., 2016). Please refer to Reichelt (2016) for an influential review on this topic (Reichelt, 2016). Consistent with these studies, our findings demonstrate that 1 week in the obesogenic diet was sufficient to reduce dopamine receptor mRNA levels in the mPFC. Interestingly, this effect was only observed in adult rats, possibly reflecting the dynamic changes in the expression of these receptors across mPFC maturation (Burke and Miczek, 2014). Given the critical role of dopamine signaling in fear extinction, our findings suggest that diet-induced neuroadaptations in this system could have implications for stress-related psychopathology in obese individuals.

Limitations and Future Studies

This study had some limitations to be addressed in future research. The results should be interpreted with caution, as results of this rat model do not necessarily directly translate to the human condition. Whereas our results are consistent with deficits in fear extinction, it is unclear from our data whether the WD effects are related to impairments in fear extinction memory acquisition, consolidation, expression, reconsolidation, and/or retrieval. Continued translational work will inform the basis of these learning and memory deficits. We analyzed mPFC markers only based on our prior studies revealing long-lasting effects of a short-term exposure to an obesogenic diet in this region and its critical role in fear extinction learning, retention, and expression (Vega-Torres et al., 2018). Future studies investigating the molecular landscape of the amygdala and other mesocorticolimbic structures would better characterize the impact of obesogenic diets in brain centers implicated with emotional regulation. Several lines of evidence demonstrate that high-fat diets and fear conditioning alter the HPA axis and dopamine function markers in the brain. Therefore, it is likely that a combination of these factors contributed to the observed differences in mRNA levels. Future studies are required to clarify the relative contribution of diet and fear conditioning to gene expression. Lastly, replication in additional rats, different rat strains, sex, and conditioning paradigms is warranted.

Summary

This study shows that the consumption of obesogenic diets during adolescence heightens behavioral vulnerabilities associated with risk for anxiety and stress-related disorders. Given that fear extinction promotes resilience against PTSD and fear extinction principles are the foundation of psychological treatments for anxiety and stress-related disorders, understanding how obesity and obesogenic diets affect the acquisition and expression of fear extinction memories is of tremendous clinical relevance.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Loma Linda University IACUC.

AUTHOR CONTRIBUTIONS

JF and JV-T planned the experiments, tested the data statistically, and wrote the manuscript. JV-T conducted the behavioral and molecular experiments, including ASR, FPS, and EPM. MA and RR-O performed the histological experiments and quantified c-Fos staining. AR-R contributed to the RT-qPCR studies, discussion, and the manuscript. PO-A conducted and analyzed the ELISA data. All authors contributed to the article and approved the submitted version.

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REVIEW
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Environment and Gene Association With Obesity and Their Impact on Neurodegenerative and Neurodevelopmental Diseases

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Obesity is a multifactorial disease in which environmental conditions and several genes play an important role in the development of this disease. Obesity is associated with neurodegenerative diseases (Alzheimer, Parkinson, and Huntington diseases) and with neurodevelopmental diseases (autism disorder, schizophrenia, and fragile X syndrome). Some of the environmental conditions that lead to obesity are physical activity, alcohol consumption, socioeconomic status, parent feeding behavior, and diet. Interestingly, some of these environmental conditions are shared with neurodegenerative and neurodevelopmental diseases. Obesity impairs neurodevelopment abilities as memory and fine-motor skills. Moreover, maternal obesity affects the cognitive function and mental health of the offspring. The common biological mechanisms involved in obesity and neurodegenerative/neurodevelopmental diseases are insulin resistance, pro-inflammatory cytokines, and oxidative damage, among others, leading to impaired brain development or cell death. Obesogenic environmental conditions are not the only factors that influence neurodegenerative and neurodevelopmental diseases. In fact, several genes implicated in the leptin–melanocortin pathway (*LEP*, *LEPR*, *POMC*, *BDNF*, *MC4R*, *PCSK1*, *SIM1*, *BDNF*, *TrkB*, etc.) are associated with obesity and neurodegenerative and neurodevelopmental diseases. Moreover, in the last decades, the discovery of new genes associated with obesity (*FTO, NRXN3, NPC1, NEGR1, MTCH2, GNPDA2*, among others) and with neurodegenerative or neurodevelopmental diseases (*APOE, CD38, SIRT1, TNFα, PAI-1, TREM2, SYT4, FMR1, TET3,* among others) had opened new pathways to comprehend the common mechanisms involved in these diseases. In conclusion, the obesogenic environmental conditions, the genes, and the interaction gene–environment would lead to a better understanding of the etiology of these diseases.

Keywords: obesity, neurodegenerative diseases, neurodevelopmental diseases, environment, genes

INTRODUCTION

Obesity is an excess of fat body mass that may decrement health. Obesity is caused by an energy imbalance due to an excess of food intake and less physical activity. The World Health Organization (WHO) has reported that worldwide obesity prevalence has tripled since 1975. In 2016, the prevalence of overweight and obesity was over 1.9 billion adults, 39% overweight, and 13% obese (World Health Organization [WHO], 2020). Body mass index (BMI), given by dividing the weight by height (kg/m^2), is used for classifying people as obese (BMI above 30 kg/m^2).

The obesity epidemic has dramatically increased in the last decades, and it goes in parallel with the change in our environment by unhealthy diet (sugar-sweetened beverages, fried foods, etc.), physical inactivity, sedentary lifestyle, and poor sleeping (Hruby et al., 2016).

In addition, genetics is also implicated in the development of obesity, explaining over 40% of the heritability of this disease. Several studies have demonstrated that genes in the melanocortin system control the energy balance [reviewed in Xu et al. (2011) and Hill and Faulkner (2017)]. Mutations in these genes, such as melanocortin 4 receptor (MC4R), have been associated with monogenic obesity (Farooqi et al., 2000). However, studies about common or polygenic obesity have revealed over 900 genetic variants associated with obesity by the approach of genome-wide association studies (GWAS) (Locke et al., 2015; Yengo et al., 2018).

Moreover, obesity is a risk factor to develop several neurological consequences (O'Brien et al., 2017), such as dementia and Alzheimer disease [AD, reviewed in Arnoldussen et al. (2014) and Anjum et al. (2018)]. Obesity is a comorbidity for psychiatric disorders that may influence behavior, cognition, and mood, suggesting that common biological pathways in the central nervous system (CNS) are implicated in obesity and psychiatric disorders (Proulx and Seeley, 2005).

In this journal's issue, a compendium of papers focused on obesogenic environmental conditions that affect neurodevelopment and neurodegeneration was collected. In this review, we will address aspects, such as obesogenic environment leading to develop obesity. In addition, the principal genes that have been associated with obesity and that are implicated in neurodegenerative diseases (NDgDs) or neurodevelopmental diseases (NDvDs) as well as the new genes associated with NDgD or NDvD are described.

OBESOGENIC ENVIRONMENT

To prove that an obesogenic environment could play an important role in developing obesity, dogs exposed to an obesogenic environment (owned by obese people) presented a higher prevalence of obesity compared to dogs with lean owners (Mason, 1970). Then, obesogenic environmental conditions have been part of the increment on this disease.

Two important components, the environment (nurture) and the genes (nature), are risk factors to develop common obesity. These two components are frequently studied independently; however, the interaction between gene and environment can increase the susceptibility to develop the disease. The genes are part of nature and have been defined as the nucleotides that form our DNA (deoxyribonucleic acid). DNA differs in some base pairs among individuals, giving genetic variation and individual differences in a trait (Wood, 2019). However, environment is part of nurture; that is, a non-genetic thing that could modify a trait (Wood, 2019).

The United Kingdom Biobank (UK Biobank) has collected data of close to half a million United Kingdom citizens of middle to old age about lifestyle factors and genetics (Sudlow et al., 2015). More than 130 lifestyle factors, such as socioeconomic status, general health, mental health, sleep, physical activity, alcohol consumption, smoking, diet, the genetic risk score (GRS), among others, were analyzed to study the interaction between gene and environment leading to obesity. Gene–environment interaction is defined as the response of an individual to environmental stimuli based on his genotype. In other words, the genes confer susceptibility, but the environment could influence and modify the genotype (Reddon et al., 2016).

Analysis of the UK Biobank database showed that the most significant environmental factors that could influence the development of obesity, in the presence of genetic risk variants, were physical activity, alcohol consumption, and socioeconomic status (Sudlow et al., 2015; Rask-Andersen et al., 2017). Moreover, parent feeding behavior and diet had also been implicated in the development of obesity (Dalle Molle et al., 2017).

Physical Activity

When it comes to obesogenic environment, we need to think about the main arms of the energy balance, which are energy expenditure and energy intake. With respect to lower energy expenditure, a sedentary lifestyle, or physical inactivity, due to prolonged watching TV hours, interacts with the genetic predisposition causing the development of obesity (Qi et al., 2012b). A study analyzing 20,000 men demonstrated that having a physically active lifestyle reduces 40% of the genetic predisposition for obesity by analyzing 12 variants associated with obesity (Li et al., 2010). A meta-analysis of more than 110,000 individuals supported the previous results, indicating that the physical activity counterbalances the genetic predisposition to develop obesity (Ahmad et al., 2013).

Alcohol Consumption

Alcohol consumption was another gene–environment associated with BMI (Rask-Andersen et al., 2017). In fact, alcohol consumption might reduce the effect of the obesity genetic variants by reducing the BMI. This is consistent with data showing that alcoholic patients present lower physical activity (Liangpunsakul et al., 2010) and a lower BMI (Addolorato, 2000) as a consequence of an increase in lipolysis and disorders of lipid metabolism (Steiner and Lang, 2017). In fact, it has been demonstrated that chronic alcohol consumption could provoke lipodystrophy in rats, triggered by a disturbance on lipogenesis (Zhang et al., 2015). A higher lipolysis and fatty acid release will provoke the transportation of the fatty acids to the liver, leading

to their accumulation and provoking hepatic steatosis over time (Kema et al., 2015).

Socioeconomic Status

Several studies have analyzed the relationship between socioeconomic status and obesity. It has been reported that obesity prevalence increases with higher deprivation levels (National Obesity Observatory, 2012) due to a worse diet (Darmon and Drewnowski, 2008) and less physical activity (Giles-Corti, 2002). Interestingly, in rich countries, the obesity prevalence was higher among poor people and did not change among the wealthiest. However, in poor countries, obesity and overweight is higher in the upper class (Templin et al., 2019). In developing countries, such as Mexico, a new middle class (poor people who became wealthier) has the most risk to become obese (Levasseur, 2015).

To address how education could influence BMI, a study with siblings sharing the same environment was performed. This study demonstrated that higher education levels correlate with a lower BMI (Kim, 2016). Educational level could affect the selection and purchase of healthy food due to the knowledge of healthy food (Bhurosy and Jeewon, 2014). Another study performed in children demonstrated that severe obesity increased with a lower household head education and lower urbanization level (Ogden et al., 2018).

Moreover, many "environmental layers" could affect obesity predisposition, such as intrauterine environment, mother–child interaction, food/community environment, parent feeding behavior, among others, representing the biological or social influence for any person to develop the disease (Dalle Molle et al., 2017). Of these layers, we will briefly describe parent feeding behavior and food (diet) as part of the energy intake, an important arm of the energy balance.

Parent Feeding Behavior

One of the environmental layers influencing child appetite is parent feeding behavior. For example, giving food as a reward of good behavior is associated with a higher intake of unhealthy foods and beverages (Dalle Molle et al., 2017).

A poor eating self-regulation in children has been associated with higher body weight (Hughes and Frazier-Wood, 2016). Picky eating is common in preschool children, which leads to parental anxiety and family conflicts (Kumar et al., 2018). Authoritative parenting has a positive correlation with non-picky eating in their toddler, suggesting that this parent feeding behavior could overcome the feeding difficulties (Podlesak et al., 2017). Lower weight has been predicted with mothers who promote positive child body image; however, feeding practices of pushing to eat have been associated with weight gain (Damiano et al., 2016). Then, nurture plays an important role in child eating behaviors and gene–environment interaction to shape child appetitive traits (Wood, 2019).

Diet

Diet is one of the main environmental factors that influence obesity development. An accelerated lifestyle has changed dietary habits, inciting people to look for ready-to-go food, instead of preparing their food at home. In fact, a United States National Nutritional Survey (from 1965 to 2008) reported that home-consumed diet has decreased 23% and most of the Americans have their calorie intake from processed foods purchased in restaurants or grocery stores (Smith et al., 2013). A study in the United Kingdom demonstrated that consumption of out-of-home (restaurants, cafes, and takeaways) meals has a higher energy intake (75–104 kcal per day) compared to those of people who rarely consume them (Goffe et al., 2017). On the other hand, frequent cooking at home has been associated with better healthy eating index, regardless low or high income (Wolfson et al., 2020).

Epidemiological studies have shown that in the period of 2005–2010, 14% of American young adults obtained their caloric needs from sugar-sweetened beverages (Ervin and Ogden, 2013). In Mexico, a country with one of the world's highest obesity prevalence, 17.5% of children and 19% of the adults obtained their daily caloric intake from sweetened beverages (Stern et al., 2014).

A systematic review of 32 studies reported a positive association between sugar-sweetened beverage consumption and the obesity risk (Bucher Della Torre et al., 2016). Moreover, a meta-analysis showed that consumption of soft drinks has increased in the last decades, and 75% of beverages and food contained added sugar. Then, drinking sugar-sweetened beverages increases the risk of obesity, diabetes, and metabolic syndrome (Bray and Popkin, 2014). In addition, obesity-genetic predisposition is associated with the positive correlation between soft drink and fried food consumption and adiposity traits (Qi et al., 2014; Olsen et al., 2016). A review suggested that eating fried foods at least four times per week gives a higher risk on developing obesity and other chronic diseases, such as type 2 diabetes (T2D) and hypertension, leading to coronary artery disease (Gadiraju et al., 2015).

Another study showed that eating more fruits and vegetables and avoiding red and processed meats could improve cardiometabolic profiles (Schwedhelm et al., 2019).

For this reason, WHO has published dietary recommendations, suggesting a higher intake of fruits, vegetables, legumes, nuts, and whole grains, limiting to less than 10% of free sugars and 30% from fats of the total intake (World Health Organization [WHO]., 2018). Even though these recommendations have been published, it has been shown that there are several barriers to eat healthy food, such as household income and cooking and eating behaviors (Wolfson et al., 2019).

A recent study demonstrated that diets in different populations are "unhealthy, unsustainable, and unequitable" (Fanzo and Davis, 2019). As abovementioned, people are eating "unhealthy," with high intake of sugar and fats. The diet is a link between humans and the environment. The production of plants and animals for human consumption is changing the environment, provoking biodiversity loss and becoming "unsustainable." Finally, socioeconomic status marginalizes some people to access healthy food, the diet becoming "inequitable."

Therefore, interventions to control obesity have to consider several environmental factors that had been associated with obesity. For example, in Latin America, some of the intervention

programs have implemented elevated taxes for sugar-based foods, marketing control, and consumer education to select healthier food (Popkin and Reardon, 2018). The global network, International Network for Food and Obesity Monitoring and Action Support (INFORMAS), implemented in Mexico nine different actions to reduce obesity: provision and promotion of healthy food, increase the offer of healthy food, a comprehensive package, combat obesity using the money obtained from the taxes over the sugar-sweetened beverages, among others (Nieto et al., 2019).

As addressed above, environment has many different edges, so it cannot be simply studied. For example, using the new technologies such as sequencing, it could take decades, trying to sequence the whole environment. Therefore, it has been an easier option to sequence the human genome than the environment (Bogardus and Swinburn, 2017). Sequencing the human genome will help to understand how genetic mutations could provoke a disease (monogenetic) or how different genetic variants give susceptibility to develop the disease (polygenetic).

GENETICS OF OBESITY

When we talk about genetics, we have to define heritability as "the proportion of observed variation in a particular trait that can be attributed to inherited genetic factors" (Merriam-Webster Dictionary, 2020). A systematic review of obesity heritability, based on the BMI of twins studies (140,525 twins) and family studies (42,968 family members), revealed that BMI heritability ranged from 47 to 90%. In addition, they demonstrated that the genetic contribution to BMI was higher during childhood than adulthood (Elks et al., 2012).

Obesity is classified in three different ways: monogenetic, polygenetic, or syndromic obesity. In this review, we will only focus on explaining monogenetic and polygenetic obesity.

Monogenic Obesity

Monogenetic obesity is caused by mutations (not common changes in the DNA) of one gene, typically causing severe early-onset obesity. This type of obesity is rare, and approximately 7.3% of the severe early-onset obesity is affected in one gene (Kleinendorst et al., 2018). Some of the genes associated with monogenetic severe obesity are: leptin (*LEP*), leptin receptor (*LEPR*), proopiomelanocortin (*POMC*), *MC4R*, preproconvertase 1 (*PCSK1*), single minded 1 (*SIM1*), brain-derived neurotrophic factor (*BDNF*), and tyrosine kinase receptor tropomycin-related kinase B (*TrkB*) (Ramachandrappa and Farooqi, 2011).

Most of the proteins involved in the monogenic obesity are involved in the leptin–melanocortin signaling pathway. This pathway is important in controlling the energy balance in the hypothalamus by coordinating the energy intake and the energy expenditure (Morton et al., 2014; van der Klaauw and Farooqi, 2015). Leptin stimulates neurons expressing POMC, producing melanocortin peptides that will bind the MC4R controlling the energy balance. Mutations in *POMC* and *MC4R* genes lead to severe obesity due to hyperphagia (Krude et al., 1998; Farooqi et al., 2003). Mutations in *MC4R* are the most common in monogenetic obesity, present in more than 5% of childhood obesity (Farooqi et al., 2000; Vaisse et al., 2000). Mutations on *PCSK1* lead to a misprocessing of melanocortin peptides, then leading to obesity and abnormalities of glucose homeostasis and adrenal function (O'Rahilly et al., 1995; Jackson et al., 1997). The transcription factor SIM1 causes severe obesity due to hyperphagia and reduces the paraventricular nucleus (PVN) of the hypothalamus (Michaud, 2001; Ramachandrappa et al., 2013). *BDNF* is also expressed in the hypothalamus, and it is downstream MC4R signaling promoting anorectic signal and locomotor activity (Kernie, 2000; Lebrun et al., 2006). Mutations in *BDNF* and its receptor, *TrkB*, are associated with obesity and hyperphagia either in humans or mice (Xu et al., 2003; Yeo et al., 2004).

Details of the function of these genes will be presented in the section *Monogenetic Obesity Genes*.

Polygenetic Obesity

Common obesity is characterized to be associated with several variants or polymorphism of different genes, polygenetic. These polymorphisms or single nucleotide variants (SNVs) are changes of one nucleotide of the DNA sequence and are common variants in the population.

Fifteen years ago, technology had a great impact on genetics by creating new molecular biology methods able to analyze several variants at the same time. In addition, the formation of big consortia, such as the UK Biobank or Genetic Investigation of ANthropometric Traits (GIANT) consortium, helped increase the sample size for epidemiological or genetic studies.

The GWAS were created based on analyzing thousands or millions of variants (covering more than 75% of the human genome) at the same time in thousands of individuals (case-control cohorts). The GWAS are hypothesis-free studies, meaning that we ignore the biological effects of the variants analyzed.

In 2007, F*TO* was the first gene associated with obesity discovered by the GWAS approach (Frayling, 2007). Then in 2009, the first three obesity GWAS were published, reporting 16 *loci* associated with obesity (Meyre et al., 2009; Thorleifsson et al., 2009; Willer et al., 2009). Since then, several meta-analyses had identified different variants associated with obesity in different populations (Locke et al., 2015; Yengo et al., 2018) or with obesity-related traits, such as body fat distribution (Pulit et al., 2019) in more than 300,000 or 700,000 individuals. Now, 941 near-independent variants have been associated with BMI, and 346 loci were associated with body fat distribution (Yengo et al., 2018; Pulit et al., 2019).

Interestingly, many of the new genes associated with obesity were found to be involved in neurogenesis, in the development of the CNS, in pathways such as appetite and food intake regulation (Locke et al., 2015; Yengo et al., 2018). In addition, a human compendium of 578 genes associated with body weight and food intake and expressed in the brain was published by

collecting data from several GWAS and some candidate genes (Ignatieva et al., 2016).

Some of the genes associated with obesity discovered by GWAS approach are *MC4R, POMC, FTO, NRXN3, NPC1, NEGR1, GNPDA2, MTCH2, ETV5*, among others. Some of them are related to synaptic function and neurotransmitter signaling (*NEGR1*, *NRXN3*, *CADM2*, *GRID1*, *ELAVL4*, and *SCG3*) and energy homeostasis (*POMC*, *MC4R*, *BDNF*, *ETV5*, *HNF4G*, and *TLR4*) (Locke et al., 2015).

The GWAS opened a new gate to understand the biological pathways involved in obesity. This hypothesis-free approach reveals new variants that are associated with obesity, without knowing their target gene and the effect that could have over it. Then, the challenge now is to go from genomics to physiology, describing the function of the new genes associated with obesity (Gutierrez-Aguilar et al., 2012, 2014).

Polygenic variants have a modest effect. Then, GRS was created to represent "the number of risk variants across all the genome," meaning that the higher the GRS, the higher susceptibility to develop obesity (Loos and Janssens, 2017). Several efforts have been performed to obtain an algorithm that could predict the risk for developing obesity (Loos and Janssens, 2017; Khera et al., 2019). Finally, Khera et al. (2019) published a genome-wide polygenic score that integrates all available common variants for obesity into a quantitative measure of inherited susceptibility. This score is able to predict the risk of developing obesity, as well as differences in weight during childhood (Khera et al., 2019).

In the last 15 years of the genomic era, hundreds of variants have been associated with obesity. Ultimately, obesity genetic risk prediction could be used to know the individual's genetic susceptibility to develop the disease. Therefore, as a principle of precision medicine, strategies and treatments could be personalized for each individual in the future, known as pharmacogenomics. However, biology is now the missing piece for solving the obesity puzzle. Many years may take to be able to understand the effect of each of the variants associated with obesity and to understand their function and their influence in the biology of obesity.

More details of the function of these genes will be presented in section *Polygenetic Obesity Genes*.

GENE–ENVIRONMENT INTERACTION LEADING TO OBESITY

Many studies have reported that the predisposition to develop obesity is by the influence of either the environment or gene variants. In this section, we will discuss individual variants or the GRS (adding the effect of several gene variants associated with obesity) and their association with some obesity traits, as well as some environmental factors.

Individual variants are studied to simplify the understanding of the interaction between gene–environment. An example of this is the interaction of two well-known obesity variants (*FTO* rs9939609 and *MC4R* rs17782313) with lifestyle measured. The study demonstrated that changes in lifestyle by implementing physical activity and adherence to a Mediterranean diet can modulate the obesity risk conferred by *FTO* and *MC4R* variants (Corella et al., 2012). In addition, gene variants could have an effect on weight loss depending on the diet. *FTO* variant is associated with change in appetite or abdominal fat distribution when exposed to high or low protein diet (Zhang X. et al., 2012; Huang et al., 2014).

The BMI heritability in twin and family studies is 47–90% (Elks et al., 2012). However, an obesity GWAS meta-analysis, analyzing 941 gene variants, explained that those variants represented only [∼6% of the variance of BMI (Yengo et al., 2018)], suggesting that all those variants associated with obesity have modest effects. Then, where is the rest of the heritability? The answer to this question has been called the "missing heritability" that could partly be explained by the influence of the environment over the genes. Several reviews have summarized the state of the art of the interaction between genes and environment in obesity, eating behavior, and diet, among others (Dalle Molle et al., 2017; Heianza and Qi, 2017; Heianza and Qi, 2019; Li and Qi, 2019).

Genetic risk score takes into account several genetic variants that could explain a trait. For example, some of the lifestyle factors that may modify the obesity GRS are sugar-sweetened beverages, where over 30 obesity gene variants were analyzed, and demonstrated interaction between genes and environment (Qi et al., 2012a; Brunkwall et al., 2016). As mentioned above, the UK Biobank data analysis demonstrated the relationship between genetic risk and environment (Sudlow et al., 2015; Rask-Andersen et al., 2017; Tyrrell et al., 2017; Nagpal et al., 2018). For example, the analysis of the GRS of 94 genetic variants were associated with physical activity, sedentary lifestyle, and socioeconomic status (Rask-Andersen et al., 2017).

Therefore, our genes confer susceptibility to develop obesity; however, we can modify the environment to reduce the risk. Then, improving adherence to healthy dietary patterns had a benefit, even in the presence of an obesity genetic risk (Wang et al., 2018c).

Then, gene–environment interactions are explaining part of the missing heritability; however, more research is needed to better understand how the genes give susceptibility to develop a disease in the presence of an environment that enhances the trait. Interestingly, some of the environmental factors associated with obesity are also risk factors to develop NDgD or NDvD, as described below.

NEURODEGENERATIVE AND NEURODEVELOPMENTAL DISEASES

Neurodegenerative diseases and NDvDs are multifactorial disorders where environment and genetics play an important role for their development (Cardoso et al., 2019; Dunn et al., 2019).

Neuronal loss leads to neurodegeneration due to accumulation of abnormal proteins in the brain. Dementia

could be described as the impairment of cognitive function, and the most common NDgDs are AD (60–70% of the cases) and Parkinson's disease (PD).

Alzheimer disease is characterized by an abnormal accumulation and deposition of β-amyloid peptide on the amyloid plaque (Haass and Selkoe, 2007). In addition, TAU protein is hyperphosphorylated, producing paired helical filaments integrated in neurofibrillary tangles (Zenaro et al., 2017). On the other hand, PD is characterized by postural instability, resting tremor, stiffness, bradykinesia, caused by a progressive loss of dopaminergic neurons, and aggregates of α-synuclein (Poewe et al., 2017). Another NDgD is Huntington's disease (HD) characterized by progressive brain disorder leading to movement, loss of cognitive conditions, emotional problems, and psychiatric symptoms (Snowden, 2017).

It has been demonstrated that obesity is a risk factor for mild cognitive impairment, independent of age (Elias et al., 2005; Hassing et al., 2010). Moreover, a meta-analysis reported the association between obesity and neurological disorders, demonstrating that obesity doubles the risk for AD (Anstey et al., 2011), and diabetes also confers risk (Profenno et al., 2010). Metabolic syndrome (obesity, T2D, hypertension, hypercholesterolemia, and hypertriglyceridemia) constitutes a risk factor for developing PD (Nam et al., 2018).

On the other hand, NDvDs are a group of conditions that affect the development of the nervous system, leading to learning disability, affecting self-control, emotions, and memory, as well as impairment in personal, occupational, and social functioning (Maher et al., 2017). Obesity has also been associated with neurodevelopmental disorders, such as autism (Criado et al., 2018) and schizophrenia (SCZ) (An et al., 2018).

Several authors have already reviewed the implications of obesity, and its metabolic dysfunctions could contribute to neurological consequences (O'Brien et al., 2017), as well as the impact on NDgD (Mazon et al., 2017). Briefly, overweight and obesity provoke metabolic changes that damage the CNS by altering synaptic plasticity and leading to neural death by either cell necrosis or apoptosis (Mazon et al., 2017). More details will be explained in Section "*Common Biological Mechanisms Between Obesity and Neurodegenerative and Neurodevelopmental Diseases*." It is evident that obesity provokes metabolic and physiological changes that lead to the development of NDgD or NDvD.

ENVIRONMENTAL FACTORS INFLUENCING NEURODEGENERATIVE OR NEURODEVELOPMENTAL DISEASES

Some of the environmental factors that have been implicated in a cognitive decline, dementia, or NDgD are physical activity, diet, and stress (lack of sleep) (Zhao et al., 2018; Gubert et al., 2020). In particular, for PD, lifestyle factors such as heavy alcohol consumption and cigarette were associated with the risk of developing the disease (Paul et al., 2019). Moreover, other illicit substances, heavy metals (iron, copper, manganese, lead, and mercury), pesticides, are other environmental factors that could provoke PD (Ball et al., 2019).

It has been described that obesity could lead to poorer neurodevelopment abilities, such as attention, inhibitory control, working memory, problem-solving, and fine motor skills (Mina et al., 2017). These abilities are impaired in NDvDs such as autism spectrum disorders (ASD), attention-deficit hyperactivity disorder (ADHD), and diseases associated with obesity (Manu et al., 2015; Criado et al., 2018; Hanć and Cortese, 2018).

A lower intelligence quotient score in childhood is associated with weight gain and obesity in later adulthood (Chandola et al., 2006; Yu et al., 2010). Moreover, midlife obesity is associated with lower cognitive ability, memory, verbal ability, and spatial abilities in late life (Dahl et al., 2010; Hassing et al., 2010). During early-life years, exposure to different factors such as education level, food deficiency, learning ability, family-related factors could increase the risk to develop cognitive impairment and dementia in advance age (Wang X. J. et al., 2019).

Maternal obesity, an environmental factor for the offspring, affects the cognitive function and mental health of the offspring. Maternal obesity could influence memory, learning, and some NDvDs, such as ADHD and ASD, as well as NDgD (Contu and Hawkes, 2017; Edlow, 2017). In addition, offspring of obese mothers present behavioral abnormalities, decreased sociability, anxiety, and feeding disorders (Edlow, 2017). In fact, maternal obesity increases the risk for intellectual disability in 1.3–3.6-fold, ADHD in 1.6–2.8-fold, and 2-fold in the difficulty of regulating emotions (Edlow, 2017).

Another NDvD is SCZ; its risk factors to develop it include obesity, poor diet, physical activity, genetic vulnerability, stress, environmental toxins, among others (Debnath et al., 2015).

Thus, obesity and NDgD/NDvD share the same environmental risk factors (physical activity, diet, socioeconomic status, stress, among others), suggesting that all these factors could modulate common biological mechanisms, regulating the CNS. In the next section, we address the common biological mechanism shared by obesity and NDgD/NDvD.

Neurodegenerative disease or NDvD development is influenced by exposure to environmental factors and by the genetic background of each person. Some of the genes associated with NDgD or NDvD are described in Section "Genes Associated With Neurodegenerative and Neurodevelopmental Diseases."

COMMON BIOLOGICAL MECHANISMS BETWEEN OBESITY AND NEURODEGENERATIVE AND NEURODEVELOPMENTAL DISEASES

Several authors have reviewed the plausible common biological mechanisms between obesity and neurodegeneration/neurodevelopment, which are briefly explained below (Mazon et al., 2017; O'Brien et al., 2017; Pugazhenthi et al., 2017).

Obesity is a consequence of an excessive energy intake, provoking a hypertrophy of the adipose tissue. Consequently,

dysfunction and inflammation of the adipose tissue trigger impaired insulin signaling, altered secretion of adipokines and cytokines, compromised triglyceride storage, and liberation of free fatty acids. The high levels of free fatty acids contribute to insulin resistance (IR) (Schneeberger et al., 2014), which has been linked to neurocognitive dysfunction (Stoeckel et al., 2016). IR and chronic hyperglycemia induce oxidative stress and inflammatory responses, provoking neuronal death and impairing cognitive processes (Treviño et al., 2015). Pro-inflammatory adipokines, including interleukin (IL)-1, IL-6, IL-1β, Tumor Necrosis Factor-Alpha (TNFα), plasminogen activator inhibitors (PAI-1), C-reactive protein, and leptin, have been proposed to be part of the neuroinflammation triggering neurodegeneration [reviewed in Arnoldussen et al. (2014)].

Leptin, a hormone secreted by the adipose tissue, reaches the hypothalamus (to control energy balance) and the hippocampus, regions involved in processes such as synaptic plasticity, memory, and cognition (Irving and Harvey, 2014). Besides the pro-inflammatory responses, other common biological features between obesity and neurodegeneration are oxidative damage, energy metabolism failures, and mitochondrial and neurotransmission systems dysfunction, ultimately leading to cell death [reviewed in Mazon et al. (2017)].

As mentioned above, maternal obesity exposes the offspring to inflammatory cytokines, which has been associated with low birth weight and premature birth, but interestingly is also associated with neural development, leading to disorders like SCZ, ADHD, and ASD (Buka et al., 2001; Donev and Thome, 2010; Blackmore et al., 2011; Angelidou et al., 2012). In fact, inflammatory mediators cross the blood–placenta barrier influencing fetal development. Then, maternal obesity produces a raise on inflammatory markers in the hippocampus of the offspring (Sullivan et al., 2015).

In addition, inflammation regulates serotonin function. Interferon alpha-treated rats showed decreased serotonergic axons in the amygdala and the ventral media prefrontal cortex (Ishikawa et al., 2007). Moreover, maternal high-fat diet consumption diminished the serotonin synthesis, leading to anxiety behaviors in the offspring (Sullivan et al., 2010).

Another reason how obesity affects the brain development is the fact that glucose can cross the blood–placenta barrier. However, the maternal insulin does not cross it, leading to insulin production by the baby. This hormone acts as a growth factor, then hyperinsulinemia in the prenatal period might alter brain development (Stachowiak et al., 2013). On the other hand, in ASD children, higher leptin levels were detected compared to healthy children (Ashwood et al., 2008).

GENES ASSOCIATED WITH OBESITY AND NEURODEGENERATIVE AND NEURODEVELOPMENTAL DISEASES

As we previously mentioned, many genes are involved in the susceptibility to develop obesity and NDgD/NDvD. However, in the next section, we will describe some of the most important genes associated with these diseases, their function, and the plausible mechanism overlapping among these complex diseases.

Genes Associated With Obesity and Its Impact on Neurodegenerative or Neurodevelopmental Diseases

Some of the genes that code for the leptin–melanocortin pathway proteins are associated with monogenetic or polygenic obesity.

In this section, we will briefly describe the genes involved in this pathway and that have been associated with monogenic obesity, as well as their effect in developing NDgD and NDvD (**Table 1**). These genes are *LEP*, *LEPR*, *POMC*, *CAR*T, *NPY*, *MC4R*, *PCSK1*, *SIM1*, *BDNF*, and *TrKB*.

Monogenetic Obesity Genes

Leptin and leptin receptor (LEP and LEPR)

Leptin is a hormone expressed and secreted by adipose tissue (Masuzaki et al., 1995). It is a cytokine/adipokine essential for the regulation of energy balance through feeding behavior and energy expenditure (Zhang et al., 1994). Leptin is an anorexigenic hormone, stimulating the expression of anorexigenic neuropeptides (POMC and α-MSH) and inhibiting the expression of orexigenic neuropeptides (NPY and AGRP) (Jéquier, 2006). The desire to eat is reduced by leptin signals by binding to the leptin receptor in the arcuate nucleus of the hypothalamus and stimulating thermogenesis and satiety.

Leptin receptor β (LEPRβ) is expressed in the neocortex, hypothalamus, medulla, and cerebellum (Burguera et al., 2000). LEPRβ activates the Janus kinase 2 and signal transducer and activator of transcription 3 (JAK-2/STAT3) pathway (Baumann et al., 1996; Bjørbaek and Kahn, 2004; Arnoldussen et al., 2014). This signaling pathway is associated with multiple physiological and pathological regulation processes. It can induce the expression of high-mobility group box 1 (*HMGB1*), which promotes the release of cytokines as Tumor Necrosis Factor-Alpha (TNFα), inducing the inflammatory reaction (Liu et al., 2007).

Many mutations in the *LEP* and *LEPR* genes have a major influence on metabolism, leading to obesity (Ramachandrappa and Farooqi, 2011; Wasim et al., 2016). Both *LEPR*- and *LEP*-deficient individuals exhibit rapid weight gain in the first few months of life, with endocrine abnormalities and severe hyperphagia (Saeed et al., 2014).

Leptin plasma levels are highly correlated to IR, adipocyte number, and fat mass (Friedman and Halaas, 1998). However, in spite of high leptin levels during obesity, a failure in the essential leptin mechanisms (reduction in feeding behavior and increased energy expenditure) is present (Frederich et al., 1995) due to leptin resistance (Morris and Rui, 2009).

In the last few years, many studies have shown the role of leptin and leptin receptor in neurological and NDgD. *In vitro* studies showed that inhibition of the JAK/STAT3 pathway protects against α-synuclein (α-SYN), induced neuroinflammation and dopaminergic neurodegeneration (Qin et al., 2016). α-SYN is a presynaptic protein that plays a central role in the pathophysiology of PD through neuroinflammatory

response (Roodveldt et al., 2008). Genetic missense mutations in α-*SYN* gene have been associated with familial forms of PD (Polymeropoulos, 1997; Singleton, 2003).

Moreover, leptin can reduce TAU phosphorylation triggering neuroprotective effects. A leptin-resistant mouse model (*Lepr db/db*) developed obesity and diabetic phenotypes showing high levels of TAU protein phosphorylation. TAU aggregation and hyperphosphorylation trigger cytotoxicity and development of NDgD. This phosphorylation increases the risk for developing dementia (Platt et al., 2016).

Recently, a study demonstrated that high-fat diet rat offspring showed cognitive damage and lower *Insr*, *Lepr* expression levels in hippocampus, persisting up to postnatal day 150. Thus, maternal exposure to high-fat diet during pregnancy and lactation can modulate cognition and behavior on adult offspring through *Lepr* (Cordner et al., 2019).

Proopiomelanocortin (POMC)

Proopiomelanocortin is a prohormone that suffers posttrans lational proteolysis, generating the active hormones α-, β-, and γ-, melanocyte-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH), which all have a wide range of physiological actions (Cone, 2005; Toda et al., 2017). POMC is a key component of the melanocortin–leptin system, which regulates food intake and energy balance (Mountjoy, 2015). Then, mutations in *POMC* gene have been associated with morbid obesity (Ramachandrappa and Farooqi, 2011; Nordang et al., 2017).

In the brain, *POMC* is expressed in the arcuate nucleus (ARC) of the hypothalamus, the pituitary gland, and the brain stem (Toda et al., 2017). POMC neurons in the ARC integrate peripheral signals such as leptin, insulin, and glucose, which regulate energy balance by inducing satiety and higher energy expenditure. Satiety is mediated by the action of POMC peptides (α and β-MSH) on MC4R in the PVN of the hypothalamus (Andermann and Lowell, 2017; Toda et al., 2017; Candler et al., 2019).

The relationship of POMC with NDgD has been demonstrated with a specific AD animal model. This AD animal model (3xTg-AD) is a transgenic mouse expressing three dementia-relates genes: presenilin-1 (PS_{1m146V}), amyloid precursor protein ($APPS_{we}$), and microtubule-associated protein tau (tau_{P301L}). This model exhibited plaque and tangle pathology, synaptic dysfunction, and showed amyloid (β and TAU pathology

TABLE 1 | Genes associated with obesity and NDgD or NDvD.

GENE	NDgD	NDvD	REFERENCES
Monogenic			
LEP	PD		Polymeropoulos, 1997; Singleton, 2003
LEPR	PD	Cognitive damage	Platt et al., 2016; Cordner et al., 2019
POMC	AD, HD		van der Burg et al., 2008; Do et al., 2018
CART	HD, PD		Lin et al., 2018; Cheong et al., 2019
NPY	AD, PD, HD		Ahmed et al., 2019; Li et al., 2019
MC4R	AD		Giuliani et al., 2011; Do et al., 2018
PCSK1	AD	PWS	Oddo et al., 2003; Hokama et al., 2014; Castillo et al., 2017; Ramos-Molina et al., 2018
SIM1	PD	SCZ	Osterberg et al., 2011; Purcell et al., 2014
BDNF	AD	Depressive disorder	[Reviewed in Balietti et al. (2018) and Zaw and Taneepanichskul (2019)]
TrkB	PD		[Reviewed in Jin (2020)]
Polygenic			
FTO	AD		Vagelatos and Eslick, 2013; Li et al., 2018
NRXN3	AD	ASD	Vaags et al., 2012; Zheng et al., 2018; Hishimoto et al., 2019
NPC1	AD	SCZ	Rouillard et al., 2016; Kawazoe et al., 2018
NEGR1	AD	SCZ, ASD	Karis et al., 2018; Ni et al., 2018; Szczurkowska et al., 2018; Raghavan et al., 2019
MTCH2	AD	SCZ	Purcell et al., 2014; Karch et al., 2016
GNPDA2	PD		Lachén-Montes et al., 2019
APOE	AD		Lambert et al., 2013; Liu et al., 2013; Shi et al., 2017; Wray et al., 2018
CD38	PD		Saad et al., 2011; Chang et al., 2018
SIRT1	AD, PD	SCZ	Kishi et al., 2011; Zhang A. et al., 2012; Rana et al., 2019
TNFα	AD, PD	SCZ, ASD	Baj and Seth, 2018; Bodnar et al., 2018
PAI1	PD		Pan et al., 2018
TREM2	AD		Yeh et al., 2017
SYT4	AD		Zhang et al., 2009
FMR1		FXS	Tassone et al., 2000, 2007; Li et al., 2020
TET3	AD		Hokama et al., 2014; Santos-Cortez et al., 2018

AD, Alzheimer disease; PD, Parkinson disease; HD, Huntington's disease; SCZ, schizophrenia; ASD, autism spectrum disorders; FXS, fragile X syndrome; PWS, Prader–Will syndrome.

(Oddo et al., 2003; Sterniczuk et al., 2010). Hypothalamic gene expression showed higher mRNA expression of genes related to inflammation and apoptosis, as well as lower levels of POMC and NPY-expressing neurons. However, voluntary exercise training reduced apoptosis and increased POMC and NPY-expressing neuronal populations (Do et al., 2018). Early exercise intervention can normalize hypothalamic inflammation, neurodegeneration, and the glucose metabolism in 3xTg-AD model, suggesting that exercise can reduce the progression of dementia and AD (Do et al., 2018).

On the other hand, an HD mouse model (R6/2), which has early dysregulations in the corticostriatal pathway (Cepeda et al., 2007) and hyperactive striatal neurons (Walker et al., 2008), exhibits a reduction of the feeding-related neuropeptides (*POMC, NPY*, and *CART*) (van der Burg et al., 2008). In addition, high-fat diet induced a reduction of synaptic inputs in hypothalamic nuclei [including lateral hypothalamus (LH) and ARC] and apoptosis of NPY/AGRP and POMC neurons. This dysfunction is associated with hypothalamic neurodegeneration (Tabrizi et al., 2011; Sousa-Ferreira et al., 2014). Expression of feeding-related neuropeptides in hypothalamic progenitor cells showed that POMC, CART, and NPY could be involved in the development of HD (Sousa-Ferreira et al., 2011).

Besides POMC, another anorexic neuropeptide is the cocaine and amphetamine regulated transcript (CART). CART is a neuropeptide expressed in the brain that is involved in reducing food intake and regulating energy homeostasis (Douglass et al., 1995; Banke et al., 2013). CART was associated with the development of HD (Gabery et al., 2010). Increased *CART* levels in the cerebrospinal fluid were associated with an increased number of CART immunopositivity neurons in the hypothalamus of HD patients (Cheong et al., 2019).

In patients with PD and ischemic stroke, levels of dopamine (DA) are reduced (Calne and Sandler, 1970; Bhakta et al., 2014). Administration of exogenous DA in *ex vivo* neurons induced *CART* expression and showed protection against brain damage by reducing inflammation activation (Lin et al., 2018). All of these confirm that CART could be involved in the development of NDgD as HD and PD.

On the other hand, NPY is an orexigenic neuropeptide associated with obesity, which is related to appetite regulation and development obesity (Wu et al., 2019). The NPY system is expressed in the peripheral nervous system and in the CNS (hippocampus, basal ganglia, and brain stem (Allen et al., 1986). Some monogenetic studies have reported the association of NPY with neurodevelopment, AD, PD, and HD (Ahmed et al., 2019; Li et al., 2019).

Melanocortin 4 receptor (MC4R)

Melanocortin is produced from the cleavage of the POMC precursor. This protein will then bind to one of its receptors. There are five known MCRs designated as MC1R through MC5R (Gantz et al., 1993, 1994; Roselli-Rehfuss et al., 1993; Yang et al., 2000). *MC4R* is predominantly expressed in the CNS including the hypothalamus, thalamus, hippocampus, brain stem, and cortex, although it is also detected in peripheral tissues. In addition, it could be expressed by neurons, microglia, and astrocytes (Chen et al., 2018). In particular, MC4R is activated by the POMC-derivate neuropeptides (α- and β-MSH) and blocked by agouti-related protein (AgRP) expressed in AgRP/NPY neurons in the ARC. The function of these neurons is modulated by signals from adipose tissue or the gut, such as leptin, ghrelin, and NPY (Clément et al., 2018).

The MC4R signaling pathway is necessary to control the energy balance, thermogenesis, and peripheral glucose metabolism, which involves G protein-mediated activation of adenylate cyclase and augmented cAMP production (Vollbach et al., 2017). As mentioned above, mutations in the *MC4R* gene have been associated with early-onset obesity and severe hyperphagia, causing about 5% of severe obesity in children and adults (Farooqi et al., 2000; Vaisse et al., 2000; Ramachandrappa and Farooqi, 2011).

However, in the brain, MC4R is involved in anorexigenic, anti-inflammatory, and antiapoptotic effects (Caruso et al., 2013). In addition, AD transgenic mouse model 3xTg-AD showed lower levels of *MC4R* and *AgRP* mRNA compared to the control (Do et al., 2018), confirming the important role of MC4R in the development of AD.

Another study showed that MC4R activation can inhibit the overexpression of inflammatory cytokines (*IL-6*, *IL-1*, *IL-1*β, and *TNF*α) in cerebral ischemia and AD (Giuliani et al., 2011; Spaccapelo et al., 2013).

Preproconvertase-1 (PCSK1)

PCSK1 is the gene that encodes the proprotein convertase 1/3 (PC1/3), which is a principal processing enzyme of precursor proteins in the secretory pathway. It is expressed in the brain, neuroendocrine system, and enteroendocrine cells (Creemers, 2008; Choquet et al., 2011). PC1/3 is synthesized as proPC1/3, which is inactive and is quickly converted into PC1/3 by autocatalytic excision of the NH2-terminal propeptide in the endoplasmic reticulum (ER). For fully PC1/3 activation, a second internal rupture of the propeptide is required in the post-ER compartment (Muller and Lindberg, 1999). An example of PC1/3 substrate is POMC, which is expressed in different neural cell populations of the ARC. In addition, PC1/3 acts in concert with PC2 to process POMC and obtain different neuropeptides as α-MSH [reviewed in Stijnen et al. (2016)].

Deficiency of *PSCK1* was associated with recessive monogenic obesity (Ramachandrappa and Farooqi, 2011). Mutations in *PSCK1* were associated with early-onset obesity, hyperphagia, sensitive hypoglycemia, and endocrine disorders (Jackson et al., 1997; Farooqi et al., 2007). However, *PCSK1*-null mice are not obese but showed growth retardation and multiple neuroendocrine abnormalities (Zhu et al., 2002; Choquet et al., 2011; Creemers et al., 2012). Moreover, PCSK1 deficiency has been associated with a major neuroendocrine disease, the Prader–Willi Syndrome (PWS). This disease is a complex genetic disorder characterized by hypogonadism, obesity, hyperphagia, growth impairment, and cognitive impairments (Ramos-Molina et al., 2018).

On the other hand, *PCSK1* expression in the hypothalamus is high in POMC and AgrP/NPY neurons, both leptin-responsive neuronal populations with ARC. PC1/3 activity is essential for

pre-AGRP and POMC processing in the ARC (Ramos-Molina et al., 2016). The expression profiles in postmortem human brains showed downregulation in *PCSK1* and other 11 metabolic genes (Hokama et al., 2014). In the cortex of 3xTg-AD mouse model, *Pcsk1* expression was downregulated, which could correlate with cognitive impairment (Oddo et al., 2003; Hokama et al., 2014; Castillo et al., 2017).

Single-minded 1 (SIM1)

SIM1 is a member of the basic helix-loop-helix Per-Arnt-Sim (β-HLH-PAS) family of transcription factors. SIM1 is critical for the formation of the PVN in the hypothalamus in mice (Michaud et al., 1998). Homozygous *Sim1*-knockout mice ($Sim1^{-/-}$) lack PVN and die perinatally. However, heterozygous $Sim1^{+/-}$ mice are viable, presenting an early-onset obesity, hyperphagia, and increased linear growth, similar to *Mc4r*-mutant mice (Michaud, 2001). A few mutations in *SIM1* gene have been found in obese individuals (Ramachandrappa and Farooqi, 2011), affecting the SIM1 transcriptional activity (Zegers et al., 2014).

SIM1 gene, like other metabolic genes, participates in the development of NDgD and NDvD. Defects in the serotonergic systems are associated with depression, obsessive–compulsive disorder, and SCZ. In addition, degeneration of mesencephalic dopaminergic (mDA) neurons is associated with PD. $Sim1^{-/-}$ newborn mice were used to evaluate *Sim1* impact in mDA neuron differentiation and rostral 5-hydroxytryptamin (5-HT) neurons. They found a reduction in the number of dorsal raphe nucleus (DRN) 5-HT neurons, suggesting that *Sim1* may modulate serotonin release *via* regulator of G protein signaling 4 (RGS4) (Osterberg et al., 2011). The role of RGS4 in not well understood, but it can modulate 5-HT 1A-mediated neurotransmitter release *in vitro* and *in vivo* (Beyer et al., 2004; Ghavami et al., 2004). All of these suggest that *SIM1* could have an important role in the development of NDgD as PD (Osterberg et al., 2011) and NDvD as SCZ (Purcell et al., 2014).

Brain-derived neurotrophic factor (BDNF)

BDNF is a neurotrophic factor that plays a fundamental role in the development and plasticity of the CNS. BDNF binds to the tropomyosin-related kinase receptor (TrkB) (Di Carlo et al., 2019). BDNF is a key factor in brain signaling and synaptic plasticity (Hofer et al., 1990; Kowiański et al., 2018). BDNF/TrkB neurotrophic signaling regulates the migration, development, differentiation, and survival of fetal neurons (Chaldakov et al., 2007), and is a major participant in the regulation of food intake (Rosas-Vargas et al., 2011). Mutations in *BDNF* gene have been associated with monogenetic obesity (Ramachandrappa and Farooqi, 2011).

Recently, numerous studies have shown the important role of BDNF in the development of NDgD and neurodevelopment. BDNF was associated with HD and AD (Couly et al., 2018; Smith-Dijak et al., 2019). Deficient BDNF/TrkB activity triggers neurodegeneration in AD *via* the activation of JAK2/STAT3 pathway and increasing inflammatory cytokines in human AD brains (Wang Z. H. et al., 2019). BDNF levels and its signaling have been modulated in the etiopathogenesis of AD, which suggested that BDNF levels could be a biomarker for AD [reviewed in Balietti et al. (2018)]. Moreover, a recent study showed that the P42 peptide treatment alleviates HD deficits in motor performance by changing BDNF level and activity (Couly et al., 2018).

As previously reported, the exposition to heavy metal can cause cognitive impairment and depressive disorders through BDNF. In early pregnancy, higher arsenic levels in blood were associated with lower levels of BDNF. The heavy metal exposure could trigger maternal depressive disorder and newborn neurodevelopment by lower levels of BDNF (Zaw and Taneepanichskul, 2019).

Tropomyosin-related kinase B (TrkB)

TrkB or neurotrophic receptor tyrosine kinase 2 (NTRK2) is a receptor for neurotrophin (NT) 4, BDNF, and NT-3 (Klein et al., 1990; Squinto et al., 1991). Some neurological diseases, obesity, and eating disorders have been associated with dysregulation of TrkB (Desmet and Peeper, 2006; O'Rahilly and Farooqi, 2006; Luberg et al., 2010). Mutations in the gene encoding TrkB, *NTRK2*, have been associated with monogenetic severe obesity with developmental delay (Ramachandrappa and Farooqi, 2011). These mutations modify TrkB ability to stimulate neurite outgrowth in response to BDNF. Thus, reduced hypothalamic neurogenesis could play a role in obesity and severe hyperphagia (Gray et al., 2007). The dorsomedial hypothalamus (DMH) has a role in the regulation of energy expenditure. A recent study revealed that the activation of *TrkB*-expressing DMH neurons suppresses appetite and maintain physiological satiety. It indicates that BDNF can modulate in part on the DMH to control bodyweight, suggesting that activation of DMH by the TrkB neurons could be a powerful way to treat obesity (Liao et al., 2019).

Recently, studies showed that TrkB can be involved in the development of NDgD. TrkB is widely distributed in different regions of the human brain, specifically in the dopaminergic neurons of the substantia nigra. In PD patients, the *TrkB* expression in the substantia nigra is significantly lower [reviewed in Jin (2020)], suggesting a role of this gene in the development of NDgD.

Polygenetic Obesity Genes

Obesity is a multifactorial and polygenic disease in which variants of different genes are associated with this disease and with the development of NDgD and NDvD (Arnoldussen et al., 2014; Lee and Mattson, 2014). As mentioned above, over 900 genetic variants have been associated with BMI and 346 *loci* were associated with body fat distribution (Yengo et al., 2018; Pulit et al., 2019). In this review, we will briefly described a few of the genes associated with obesity that have been discovered by a GWAS approach and that had been replicated in different populations. The genes reviewed here are *FTO, NRXN3, NPC1, NEGR1, MTCH2*, and *GNPDA2*, which were chosen for their association with obesity and their influence on NDgD or NDvD.

Fat mass and obesity-associated gene (FTO)

FTO gene is one of the most studied genes due to its association with obesity (Locke et al., 2015). A common variant of the *FTO* gene was identified through a T2D GWAS in 2007, and it also

showed a strong association with obesity (Frayling et al., 2007; Scuteri et al., 2007). *FTO* association with obesity is the most replicated in different populations worldwide (Dina et al., 2007; Andreasen et al., 2008; Villalobos-Comparán et al., 2008).

FTO protein function was first described as an N^6-methyladenosine (m^6A) demethylase dependent of iron and 2-oxoglutarate (Gerken et al., 2007; Jia et al., 2011). Then, *FTO*-deficient mouse model was studied to understand its physiological function. These mice display postnatal growth retardation and reduced food intake, with a reduction of adipose tissue (Fischer et al., 2009; Gao et al., 2010). On the contrary, the overexpression of *FTO* showed a reduction of adipose tissue (Church et al., 2010). However, it took 8 years to understand that *FTO* intronic variant associated with obesity does not regulate *FTO* expression. In fact, this variant disrupts the binding site of the ARID5B repressor, which regulates *IRX3* and *IRX5* expression, genes involved in adipogenesis, thermogenesis, and lipid accumulation (Claussnitzer et al., 2015). This is an example that the discovery of new genetic variants could unveil an unexpected impact on physiology.

Over the last few years, *FTO* was associated with NDgD, especially AD. Obesity and T2D in human and mice can activate *FTO* in the brain tissues by defective insulin signaling (Li et al., 2018). It is known that obesity and T2D are commonly associated with the development of AD (Vagelatos and Eslick, 2013). A recent study demonstrated that in the 3xTg-AD mouse model, *Fto* neuronal conditional silencing reduced the cognitive deficits, suggesting its implication on the insulin signaling defect present in AD (Li et al., 2018).

Neurexin 3 (NRXN3)

NRXN3 is a type of neurexins, which are neuron-specific cell surface proteins. Their structure suggests a role in the cell adhesion and cell recognition (Ushkaryov et al., 1992).

NRXN3 gene has been associated with waist circumference as an obesity trait (Heard-Costa et al., 2009). Moreover, this gene was also associated with increased BMI and visceral fat and decreased sleep duration (Prats-Puig et al., 2013).

NRXN3 is involved in synaptic function in the cognitive decline associated with aging and AD. Moreover, mutations in *NRXN3* have been identified in AD patients (Zheng et al., 2018; Hishimoto et al., 2019), and rare deletions have been associated with ASD (Vaags et al., 2012).

Niemann–pick type C1 (NPC1)

NPC1 is a protein that regulates the transport of cholesterol and fatty acids from late endosomes/lysosomes to cellular structures as mitochondria and plasma membrane for maintaining lipid homeostasis [reviewed in King and Sharom (2012)]. In humans, GWAS showed common *NPC1* variants associated with obesity (Meyre et al., 2009).

A rare autosomal recessive mutation in human *NPC1* causes a disorder in lipid storage leading to progressive and lethal neurodegeneration and lung and liver failure [reviewed in Lamri et al. (2018)]. NPC disease is a lysosomal storage disorder, present in childhood with accumulation of visceral lipid and progressive neurodegenration, with characteristic dysphagia, cerebellar ataxia, and dementia resulting in a lower life expectancy (Newton et al., 2018). *NPC1* gene has been associated with NDv and NDgD as AD (Rouillard et al., 2016) and SCZ (Kawazoe et al., 2018).

Neuronal growth regulator 1 (NEGR1)

NEGR1 is expressed in the rat brain. It has been proposed that NEGR1 could modulate the intracellular cholesterol trafficking, suggesting its implication in human obesity (Kim et al., 2017). *NEGR1* was associated with obesity (Thorleifsson et al., 2009; Willer et al., 2009). Moreover, *Negr1*-deficient mice showed increased adiposity (Joo et al., 2019). The genetic variability in *NEGR1* could be associated with psychological traits of patients with eating disorders, like bulimia (Gamero-Villarroel et al., 2015).

Moreover, a GWAS reported a *NEGR1* variant associated with major depression (Wray et al., 2018). Recent studies on humans and animals support the idea that NEGR1 is involved in psychiatric disorders such as SCZ (Karis et al., 2018), ASD (Szczurkowska et al., 2018), and AD (Ni et al., 2018; Raghavan et al., 2019).

Negr1 deficiency in animals leads to impaired cortical development and impaired behavior, a conduct similarly observed in ASD patients (Szczurkowska et al., 2018). On the other hand, SCZ patients showed higher levels of *Negr1* in the dorsolateral prefrontal cortex (Karis et al., 2018). Therefore, these data demonstrate the implication of *NEGR1* in NDvD.

Mitochondrial carrier homolog 2 (MTCH2)

MTCH2 is a relative novel protein located in the inner membrane of mitochondria. It is expressed in white adipose tissue. MTCH2 has an important regulatory role in the differentiation and biology of the adipocyte (Bernhard et al., 2013). *MTCH2* variants have been associated with increased BMI, obesity, and diabetes (Willer et al., 2009; Heid et al., 2010). A study on *Mtch2*-knockout mouse model reported that the animals die at an embryonic 7.5 day, suggesting that *Mtch2* could play a specific role in embryonic development (Ruggiero et al., 2017).

It is relevant mentioning that the lower expression of *MTCH2* was associated with late onset AD status in a GWAS (Karch et al., 2016). A case control study in Swedish population showed several variants associated with SCZ by exome-sequencing (Purcell et al., 2014). These data suggest the possible role that *MTCH2* could have in the development of NDgD and NDvD.

Glucosamine 6 phosphate isomerase 2 (GNPDA2)

GNPDA2 gene encodes the enzyme glucosamine-6-phosphate deaminase (GlcN6P). This enzyme catalyzes the reversible reaction of D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium (Arreola et al., 2003). This enzyme has a hydrolase activity and is involved in metabolic pathways, glucose and nucleotide metabolism. *GNPDA2* is highly expressed by the brain (cortex and hypothalamus) (Willer et al., 2009).

GNPDA2 gene variant was associated with obesity in populations with European ancestry (Willer et al., 2009), and this association was replicated in other populations (Renström et al., 2009; Locke et al., 2015). In a diet-induced obesity model, high-fat diet led to a lower *GNPDA2* hypothalamic expression compared to rats fed with chow diet (Gutierrez-Aguilar et al., 2012).

GNPDA2 was overexpressed in PD and AD cases and showed lower serum levels in PD patients. Interestingly, in PD patients, GNPDA2 showed an inverse correlation with α-synulein protein levels in the cerebrospinal fluid (Lachén-Montes et al., 2019), suggesting its implication in developing PD.

Genes Associated With Neurodegenerative and Neurodevelopmental Diseases

Genetic studies have shown genes implicated in developing NDgD and NDvD (Saad et al., 2011; Arnoldussen et al., 2014; Henriksen et al., 2017; Wu and Pan, 2018).

The heritability for AD is between 60 and 80% (Wingo, 2012). AD genetics has been reviewed elsewhere (Reitz, 2015; Goldman and Van Deerlin, 2018; Jung et al., 2018). However, only 1% of all AD is autosomal dominant genes, such as presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*), and amyloid precursor protein (*APP*) (Hinz and Geschwind, 2017).

With the genomic era, several GWAS and meta-analysis have been performed, identifying up to 29 risk *loci* associated with AD, and *APOE* is one of them (Lambert et al., 2013; Jansen et al., 2019).

Interestingly, some genes associated with obesity were also associated with AD by different genetic approaches: candidate gene approach *FTO* (Li et al., 2018), by differentially expressed genes *NRXN3* (Zheng et al., 2018; Hishimoto et al., 2019), *NPC1* (Rouillard et al., 2016), *NEGR1* (Wray et al., 2018), or by GWAS approach: *LEPR*, *BDNF*, *TNF*α, and *MTCH2* (Gao et al., 2015; Karch et al., 2016; Lemche, 2018).

In respect to PD, this disease only affects 2% of the population over 60 years old. Family-based genetic studies had identified 23 genes associated with PD, having diverse functions as deficiency of synaptic transmission, vesicular recycling, lysosomal dysfunction, mitophagy. Some of those genes are: *synuclein-Alpha* (*SNCA*), inherited in an autosomal dominant; *PARKIN*, autosomal recessive juvenile parkinsonism; PTEN-induced kinase (*PINK1*), recessive early onset, among others (Karimi-Moghadam et al., 2018).

Meta-analysis from PD GWAS report over 30 variants close to genes like *CTSB*, *TMEM175*, *LRRK2*, among others (Nalls et al., 2014; Chang et al., 2018). Some of the common genes that have been associated with obesity and PD are: *GNPDA2* (Lachén-Montes et al., 2019) *CD38*, *TNF*α, and *PAI-1* (Pan et al., 2018) that will be discussed below.

Huntington's disease is another NDgD characterized by progressive neurodegeneration with severe neuronal loss (90%) that occurs in the lateral hypothalamus (Sprengelmeyer et al., 2006), as well as neuronal degeneration and hypothalamic dysfunction (van Duijn et al., 2014). In this disease, the gene *HTT*, encoding for the huntingtin protein, has a CAG trinucleotide expansion mutation, causing behavioral abnormalities, dementia, and progressive movement disorder (Ross et al., 2014). A GWAS reported a few gene variants associated with HD that are close to the *MLH1, FAN1, MTRM10, RRM2B*, and *UBR5* genes. Some of these genes are implicated in DNA mismatch repair, structure-specific DNA handling, mitochondrial energetics, and oxidative stress (Lee et al., 2015). However, genes associated with obesity have been also described to be associated with HD, such as *POMC*, *NPY*, *CART*, and *BDNF* (Sousa-Ferreira et al., 2011).

Neurodevelopmental diseases are a group of early-onset neurological disorders, which also have a genetic background. These disorders include ASD that often present intellectual disability (ID), motor abnormalities, and epilepsy. Fragile X syndrome (FXS) is part of the syndromic ASD classification (Tărlungeanu and Novarino, 2018).

Fragile X syndrome is an inherited disease linked to the X chromosome and is one cause of intellectual disability. This syndrome is associated with a triplet repeats of cytosine-guanine-guanine (CGG) in the *FMR1* gene (O'Donnell and Warren, 2002; Visootsak et al., 2014). Usually, FXS patients have social anxiety, severe communication deficits, and stereotyped behavior (Hall et al., 2009). Some cases with *FMR1* premutation showed lack of satiation, severe hyperphagia, and severe obesity (Martínez-Cerdeño et al., 2017).

Schizophrenia is another NDvD that affects 1% of the worldwide population. It is a chronic and severe mental disorder, characterized by cognitive impairment. More than 1,000 candidate genes have been proposed to be associated with SCZ. However, their functional implication in developing SCZ remains unclear. Hu et al. (2013) analyzed the new genetic variants associated with SCZ and identified pathways that might explain the biological function causing this disease. They propose that pathways involved in fatty acid degradation, glycan degradation, PPAR signaling, among others, could be involved in the development of this disease. However, the genes that have been associated with SCZ are *NRXN3,* and *NEGR1, NPC1, and TNF*α (Hu et al., 2013; Park et al., 2019).

Frequently, the associations between genes and diseases are studied separately. Interestingly, a recent genetic study analyzed the association of millions of genetic variants with BMI and major psychiatric disorders (SCZ, bipolar disorder, and major depression), instead of analyzing separately each disease. This study identified 111 genetic loci overlapping between BMI and the psychiatric disorders. Some of these variants are associated with genes expressed in the brain with plausible functions in CNS development, intracellular processes, and GABAergic and glutamatergic signaling (Bahrami et al., 2020); however, their functions have to be confirmed.

In the next section, we will briefly describe genes associated with NDgD as AD, PD, and HD that were discovered by a GWAS approach and meta-analysis (Qi et al., 2012b; Moss et al., 2017; Chang et al., 2018; Jansen et al., 2019) and that had previously shown strong association with monogenic or polygenic obesity (Tong, 2011; Hatziri et al., 2018; Wang et al., 2018a,b; Liu et al., 2019). Some of the genes mentioned below are *APOE* and *TREM2* associated with AD; *CD38* variants with AD and PD; and *SYT4* with PD (Chang et al., 2018; Jansen et al., 2019). In addition, genetic studies found the association of SIRT1 with PD and AD (Zhang A. et al., 2012; Rana et al., 2019) and with SCZ (Kishi et al., 2011). A similar strategy to select the genes associated with NDvD as SCZ and ASD. However, there are just a few genes highly associated with the development of NDvD and obesity. Some of them were discovered by exome-sequencing studies as *MTCH2*

and *SIM1*, which were associated with the development of SCZ (Purcell et al., 2014). GWAS showed that *FMR1* was associated with ASD and FXS (Tassone et al., 2000; Tassone et al., 2007) and *TET3* gene variants with the development of NDvD (Santos-Cortez et al., 2018). However, little is known about the function of these genes and their implications in NDgD and NDvD.

Apolipoprotein E (APOE)

Apolipoprotein E is a glycoprotein that is produced predominantly by astrocytes in the brain and peripherally in the liver (Huang and Mahley, 2014). There are three *APOE* alleles: E2, E3, and E4. Peripherally, APOE2 and APOE3 bind to high-density lipoproteins (HDLs), responsible for trafficking lipids from the periphery cells to the liver for elimination. APOE4 have greater affinity for very low-density lipoprotein, and it is less efficient at homeostatic maintenance (Huang and Mahley, 2014). However, *APOE4* was associated with higher fasting glucose and insulin levels, as well as an increased metabolic syndrome risk with younger age onset (Torres-Perez et al., 2016). In obese men, *APOE4* carriers have elevated levels of plasma cholesterol, triglycerides, glucose, and insulin and presents IR (Elosua et al., 2003; Jones and Rebeck, 2019).

The most common *APOE3* allele has been associated with an average risk to develop AD. However, *APOE4* homozygotes increase the risk 15 times to the development of AD. In addition, *APOE4* is also associated with increased risk for CVD and metabolic syndrome (El-Lebedy et al., 2016).

Interestingly, *APOE* is one of the metabolic genes associated with the development of NDgD. *APOE4* has shown a strongest association with the development of late-onset AD (Lambert et al., 2013; Jansen et al., 2019). APOE affects TAU pathogenesis, neuroinflammation, and TAU-mediated neurodegeneration (Yoshiyama et al., 2007). *APOE4* carrier has increased Aβ accumulation and decreased clearance in AD brains (Liu et al., 2013; Shi et al., 2017).

Cluster of Differentiation 38 (CD38)

CD38 is a type II transmembrane glycoprotein (Jackson and Bell, 1990). It is a lymphocyte-specific antigen, has ectoenzymatic activity, and functions as a receptor and adhesion molecule (States et al., 1992). *CD38* is highly expressed on plasma cells, red blood cells, platelets (Deaglio et al., 2001), and adipose tissue and in obese people (Nair et al., 2005; Mutch et al., 2009). CD38 is involved in different biological processes including cell proliferation, hormone secretion, muscle contraction, egg fertilization, and immune response. It is also involved in the catabolism of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP) (Howard et al., 1993).

A *CD38*-deficient mouse model has higher metabolic rate and showed protection against diet-induced obesity through increasing NAD-dependent activation of sirtuin (SIRT) proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which is involved in the regulation of mitochondrial biogenesis and energy homeostasis (Barbosa et al., 2007). On the other hand, a recent study showed that CD38 participates in adipogenesis and lipogenesis of adipose tissues through regulating Sirt1-mediated signaling pathway (Wang et al., 2018b).

In addition to its role in obesity, *CD38* is also expressed in neurons, microglial cells, and astrocytes [reviewed in Guerreiro et al. (2020)]. *CD38* is highly expressed in the mouse brain during development (postnatal days 14 and 28). The *CD38*-knockout mice showed that the brain displayed a 10-fold NAD^+ level more than wild type, suggesting that CD38 is one regulator of intracellular NAD^+ levels in the brain.

Genomic studies demonstrated that *CD38* was associated with PD (Saad et al., 2011) and was confirmed by a meta-analysis (Chang et al., 2018). It suggests that *CD38* could play an important role in the development of NDgD as PD.

Sirtuin1 (SIRT1)

SIRT1 is a deacetylase NAD^+-dependent that is expressed in the heart, adipose tissue, muscle, kidney, liver, and brain (basal ganglia, prefrontal cortex, and hippocampus, which are areas associated with NDgD) (Zakhary et al., 2010). SIRT1 is involved in important processes such as cell cycle regulation, energy metabolism modulation, mitochondrial biogenesis, glucose/cholesterol metabolism, etc. (Herranz and Serrano, 2010; Nogueiras et al., 2012). SIRT1 has been associated with obesity (Zillikens et al., 2009) and is involved in food intake regulation, life span, diabetes, and CVD (Pfluger et al., 2008; Nogueiras et al., 2012).

SIRT1 expression and activity were increased by resveratrol treatment, protecting neuronal cells (Seo et al., 2012; Herskovits and Guarente, 2014) and accelerating brain aging (Duan, 2013). SIRT1 has shown an important role in the regulation of other anti-aging genes as *KL* (*Kloto*), *SHC1 or p66Shc* (*transforming protein SCH1*), and *FOXO1a/FOXO 3a* (Forkhead box) by p53 transcription factor deacetylation (Seo et al., 2012; Herskovits and Guarente, 2014). Low expression of SIRT1 in adipose tissue was shown in obese subjects (Stefanowicz et al., 2018).

Moreover, low levels of *SIRT1* are present in NDgD as AD and PD (Lutz et al., 2014; Singh et al., 2017). In addition, genetic studies found the association of *SIRT1* with PD and AD (Zhang A. et al., 2012; Rana et al., 2019) and with SCZ (Kishi et al., 2011).

Tumor Necrosis Factor-Alpha (TNFα)

Tumor necrosis factor-alpha is a potent pleiotropic pro-inflammatory cytokine (Hajeer and Hutchinson, 2000, 2001). TNFα is produced by many cell types such as neutrophils, fibroblasts, keratinocytes, macrophages, natural killer cells, T and B cells, and tumor cells (Anderson et al., 2004). TNFα plays an essential role in chronic inflammation associated with different pathologies, such as obesity, T2D, AD, and PD (Wei et al., 2011).

Tumor necrosis factor-alpha has been associated with NDgD and NDvD in many reports (Arnoldussen et al., 2014). The neuroinflammation in the NDgD, such as AD, PD, amyotrophic lateral sclerosis, and multiple sclerosis is modulated by cytokines like TNFα (Baj and Seth, 2018). Higher levels of cytokines as TNFα, IL-10, TNFβ, and CRP were associated with NDvD, as SCZ and ASD (Bodnar et al., 2018).

Plasminogen Activator Inhibitor (PAI-1)

Plasminogen activator inhibitor is a single-chain glycoprotein belonging to the serine protease inhibitor (serpin) superfamily. There are four PAIs: PAIs-1 to 3 and protease nexins (Feinbloom and Bauer, 2005). PAI-1 inhibits the plasminogen activator and is produced by platelet, hepatocytes, adipocyte, vascular smooth muscle cell, fibroblast, and macrophages (Placencio and DeClerck, 2015). *PAI-1* mRNA expression in visceral and subcutaneous adipose tissue was correlated with BMI and severe obesity (Alessi et al., 2000). Plasma PAI-1 activity and antigen were positively associated with BMI in hypertense men (Skurk et al., 2004). The expression of *PAI-1* can be regulated by lipid/glucose metabolites, environmental factors, inflammation, age, BMI, and lifestyle. Also, chemical messengers like TNFα, hormones, inflammatory cytokines, growth factors, and endotoxins can modulate its expression (Oishi, 2009).

Recently, a new study showed the association of higher levels of PAI-1 with PD (Pan et al., 2018). *PAI-1* polymorphisms can change focal and brain stem neurological signs in patients with traumatic brain injury (Pan et al., 2018). These results suggested that *PAI-1* can participate in the development of NDgD (Arnoldussen et al., 2014).

Trigger Receptor Expressed on Myeloid Cells 2 (TREM2)

TREM2 is expressed on myeloid cells (macrophages, dendritic cells, and microglia) (Bouchon et al., 2001; Daws et al., 2001; Wang et al., 2015; Ulland et al., 2017) and in adipose tissue (Park et al., 2015). TREM2 regulates the behaviors of different cell biologicals: survival, proliferation, differentiation, phagocytosis, and inflammatory response (Zhong et al., 2015; Kober and Brett, 2017). It also acts as a lipid sensing receptor to recognize and bind lipids (Wang et al., 2015).

TREM2 gene expression was upregulated in adipose tissue in obesity animal models (Fujimoto et al., 2011; Grant et al., 2011; Park et al., 2015). *Trem2*$^{-/-}$ mice fed with a high-fat diet showed lower mass but higher hypertrophy in adipocytes and increased adipocyte death. In addition, these mice had deficient inflammatory response of adipose tissue macrophages and severe hepatic steatosis. They showed that the function of TREM2 is a feedback mechanism to control obesity-induced IR by regulating adipose tissue remodeling pathways (Liu et al., 2019).

Furthermore, *TREM2* genetic variants have been associated with a higher risk to develop AD. The amyloid plaque compaction depends on TREM2 mechanism, forming a protective barrier that attenuates toxicity nearby neurons [reviewed in Yeh et al. (2017)].

Synaptotagmin-4 (SYT4)

SYT4 is insensitive to Ca^{2+}. *SYT4* is expressed in brain and neuroendocrine system and has been suggested to have a neuroendocrine role (Tong, 2011; Zhang et al., 2011). The upregulation of *SYT4* inhibits the release of oxytocin, which is a characteristic in obese phenotype (Tong, 2011; Zhang et al., 2011). The negative regulation of *SYT4* could potentially repair or reduce the degree of diabetes neuropathies (Rahimi et al., 2015); however, the physiological function of SYT4 remains unknown.

A specific mouse model showed that *SYT4* upregulation, within dystrophic neurons, could reflect impaired protein degradation that happens in AD (Zhang et al., 2009), suggesting its implication in this disease.

Fragile X Mental Retardation 1 (FMR1)

FMR1 gene encodes the Fragile X mental retardation protein (FMRP). FMR1 is found in most adult and fetal tissues, especially in brain and testes (Berry-Kravis et al., 2002; Berry-Kravis et al., 2011). It has been reported that FXS patients have obesity (Martínez-Cerdeño et al., 2017).

Trinucleotide (CGG) repeat in *FMR1* promotor region is associated with FXS, and a permutation is associated with fragile X-associated tremor/ataxia syndrome (FXSAS). Many individuals with permutation on the *FMR1* gene have high levels of mRNA, but normal FMRP is synthesized (Tassone et al., 2000, 2007). *Fxr1*$^{-/-}$-knockout mice die at 24 h of birth, and heterozygous mice exhibit abnormal limb musculature and learning and circadian rhythm deficits (Berry-Kravis et al., 2011; Francis et al., 2014). Analysis of graph diffusion and multitask clustering of FMR1 Clip-seq and transcriptional targets showed pathways regulated by FMR1 in human neural development (Li et al., 2020).

As we previously mentioned, the function or pathway of many of the genes discovered by GWAS is unknown. Such is the case of *FMR1* with its implication in developing obesity or NDvD.

Ten Eleven Translocate 3 (TET3)

TET3 is a protein which belongs to the TET family, which catalyzes hydroxylation of 5-metylcytosine (5mC) to 5-hidroxymetylcytosine (5hmC) (Ito et al., 2010; Pastor et al., 2013). This is a key step in active DNA demethylation, which needs α-ketoglutarate (αKG) as a cofactor (Tahiliani et al., 2009). However, in maternal obesity, placental *TET3* methylation is increased, without totally understanding its role in obesity (Mitsuya et al., 2017).

TET3 is the most highly expressed enzyme in the brain and is an essential enzyme in neuronal differentiation and *in vivo* early neocortical development (Lv et al., 2014; Li et al., 2015). In fact,*Tet3*-knockout mouse model showed defects in brain morphology, behavior, and motor development (Santos-Cortez et al., 2018).

Interestingly, some genomic studies have shown the association of *TET3* gene variants with the development of NDvD (Santos-Cortez et al., 2018). Moreover, *TET3* expression levels are downregulated in postmortem brains of AD individuals (Hokama et al., 2014). Therefore, *TET3* is one of the new genes discovered to be associated with both NDgD/NDvD, and its mechanism leading to those diseases would have to be unveiled.

DISCUSSION

In this review, we addressed the obesogenic environmental conditions that influence the development of NDgD or NDvD. Interestingly, some of the environmental factors that lead to the development of obesity, NDgD, or

NDvD are common. Some of the environmental factors are physical activity, alcohol consumption, socioeconomic status, parent feeding behavior, and diet. All of these factors influence the development of obesity and impair abilities as memory, fine motor skills, and cognitive function.

Obesity is a comorbidity of NDgD and NDvD due to the common biological mechanisms that affect brain cell death or brain development. The adipose tissue hypertrophy provokes inflammation, impaired insulin signaling. The secretion of adipokines and pro-inflammatory cytokines is altered, as well as the lipid metabolism. High levels of free fatty acids contribute to IR and hyperglycemia and lead to impaired cognitive processes. Moreover, oxidative damage, energy metabolism failures, mitochondrial and neurotransmission systems dysfunction could lead to cell death and NDgD. On the other hand, maternal obesity produces high levels of inflammatory mediators that can cross the blood–placenta barrier, influencing fetal development. This fetal exposure could end in neurodevelopmental disorders like SCZ, ADHD, and ASD.

Genetics plays an important role in developing obesity, NDgD, and NDvD. We described the genes associated with obesity, either on monogenic or polygenic manner. For the monogenetic obesity, several of the genes are involved in the leptin–melanocortin pathway, mainly regulated in the hypothalamus. This pathway controls the energy homeostasis, given by the food intake and the energy expenditure. Surprisingly, all these genes were also associated with NDgD or NDvD by sharing regulation on specific brain regions (hypothalamus, hippocampus, thalamus, cortex, etc.) or by leading to synaptic dysfunction and amyloid β and TAU pathology.

Moreover, we described the new genes associated with obesity, NDgD, or NDvD, as discovered by a GWAS approach. This approach has helped to discover new genes without a previous established hypothesis, thus without knowledge limitations. These genomic studies had discovered gene variants with unknown functions that we might have never imagined to be implicated in a particular disease.

In this review, we mentioned just a few genes associated with obesity, NDgD, or NDvD, but a long list of genes are associated. However, the challenge now is to identify the function of all these genes and their plausible implication in these diseases, as well as the gene–environment interaction.

In fact, gene–environment interactions have explained part of the missing heritability, but more research is needed to better understand how the genes confer susceptibility to develop a disease in the presence of an environment that enhances the trait. Therefore, our genes confer susceptibility to develop obesity, NDgD, and NDvD; however, we can modify the environment to reduce the risk.

The prevalence of obesity has risen dramatically in the last decades. As obesity is a trigger for the development of many complex diseases, as NDgD and NDvD, it is plausible that in the next decades, the prevalence of these diseases will increase. Even though we cannot fully understand the etiology of obesity, many of the risk factors for the development of obesity are already known. Then, we can modify the environmental factors to prevent obesity and avoid the risk of developing NDgD and NDvD.

CONCLUSION

Obesity and NDgD/NDvD are diseases that share environmental and genetic factors, which lead to the development of all these diseases. Understanding the environment, the genes, and the gene–environment interaction involved in obesity and NDgD/NDvD, we will comprehend the etiology of these diseases. Then, by modifying our environmental factors and knowing the genetic susceptibility, we might be able to avoid the development of these diseases.

AUTHOR CONTRIBUTIONS

MF-D analyzed, discussed, and wrote the genetic section. YD-L searched for the bibliography and analyzed the literature. RG-A conceived, analyzed, discussed, wrote, and edited all the manuscript. All authors revised and approved the last version of this manuscript.

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The Appetite Suppressant D-norpseudoephedrine (Cathine) Acts *via* D1/D2-Like Dopamine Receptors in the Nucleus Accumbens Shell

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D-norpseudoephedrine (NPE), also known as cathine, is found naturally in the shrub *Catha edulis* "Khat." NPE has been widely used as an appetite suppressant for the treatment of obesity. Although it is known that NPE acts on α1-adrenergic receptors, there is little information about the role of dopamine receptors on NPE's induced anorectic and weight loss effects. Equally untouched is the question of how NPE modulates neuronal activity in the nucleus accumbens shell (NAcSh), a brain reward center, and a pharmacological target for many appetite suppressants. To do this, in rats, we characterized the pharmacological effects induced by NPE on weight loss, food intake, and locomotion. We also determined the involvement of dopamine D1- and D2-like receptors using systemic and intra-NAcSh antagonists, and finally, we recorded single-unit activity in the NAcSh in freely moving rats. We found that NPE decreased 24-h food intake, induced weight loss, and as side effects increased locomotor activity and wakefulness. Also, intraperitoneal and intra-NAcSh administration of D1 and D2 dopamine antagonists partially reversed NPE's induced weight loss and food intake suppression. Furthermore, the D1 antagonist, SCH-23390, eliminated NPE-induced locomotion, whereas the D2 antagonist, raclopride, only delayed its onset. We also found that NPE evoked a net activation imbalance in NAcSh that propelled the population activity trajectories into a dynamic pharmacological brain state, which correlated with the onset of NPE-induced wakefulness. Together, our data demonstrate that NPE modulates NAcSh spiking activity and that both dopamine D1 and D2 receptors are necessary for NPE's induced food intake suppression and weight loss.

Keywords: anorexigenic drugs, food intake, locomotor activity, weight loss, nucleus accumbens, obesity

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INTRODUCTION

Obesity is currently a pandemic affecting more than 650 million people worldwide. Although obesity is primarily treated with exercise and diet, appetite suppressants can aid in weight loss (Wing and Hill, 2001; Joo and Lee, 2014; Brett, 2019). Amphetamine was the first appetite suppressant widely used in humans, but in the late 1960s, it was restricted because of its highly

addictive properties (Harris et al., 1947; Stowe and Miller, 1957; Sharp et al., 1962; Stark and Totty, 1967; Drevets et al., 2001). Subsequent appetite suppressants were mainly amphetamine congeners but with less intense properties (Zelger and Carlini, 1980; Kalix and Khan, 1984; Balint et al., 2009; Khan et al., 2012). These drugs exert their pharmacological effects by stimulating the release of norepinephrine, serotonin, and dopamine (DA) via uptake inhibition (Baumann et al., 2000; Drevets et al., 2001; Rothman et al., 2001; Broening et al., 2005). The most commonly prescribed appetite suppressants since 1959, are phentermine and diethylpropion (Bray, 2000; Kushner, 2018). However, there is very little information about their mechanism of action, especially on their central effects. To fill this gap, recently, we found that mild stimulants, including diethylpropion, phentermine, and bupropion, suppressed food intake, induced weight loss, and modulated neural activity in the nucleus accumbens shell (NAcSh) (Kalyanasundar et al., 2015; Perez et al., 2019), a brain region with robust dopaminergic innervation involved in feeding, sleep, and locomotor behavior (Kelley et al., 2005; Palmiter, 2007; Tellez et al., 2012). In contrast to the idea that they mainly act via norepinephrine and serotonin neurotransmitters, we found that D1- and D2-like DA receptor antagonists greatly attenuated their anorectic and weight loss effects (Kalyanasundar et al., 2015). However, there is a scarcity of information about D-norpseudoephedrine (NPE), an appetite suppressant introduced in the 1970s, used for weight reduction. Thus, here we have extended our studies to NPE.

The leaves of "Khat" contain several active compounds. Still, the most potent is cathinone [S-(−)-cathinone], followed by two diastereoisomers: cathine [1S,2S-(+)-norpseudoephedrine, abbreviated as NPE] and norephedrine [1R,2S-(−)-norephedrine] (Kalix and Khan, 1984; Balint et al., 2009). Cathinone and its less potent metabolite NPE are referred to as natural amphetamines (Kalix, 1992). However, and despite that, they are structurally related to amphetamine; they exhibit fewer addictive properties (specially NPE), according to the World Health Organization (Eddy et al., 1965; Zelger and Carlini, 1980; Eisenberg et al., 1987; Kalix et al., 1990; Toennes et al., 2003). Eisenberg et al. (1987) showed that rats treated with NPE exhibited increased locomotor activity and reduced food intake at doses of 10–50 mg/kg. Moreover, NPE has diminished potency relative to cathinone but shown to have a longer duration of action (Peterson et al., 1980; Zelger and Carlini, 1980; Pehek et al., 1990). Nevertheless, over the past decades, NPE has received very little experimental attention; only a few clinical studies have examined NPE as an appetite suppressant for the short-term treatment of obesity (Greenway, 1992; Richert, 2011; Lemieux et al., 2015; Onakpoya et al., 2016). Recently, the long-term (24 weeks) efficacy and safety for NPE were studied in humans, with promising results (Hauner et al., 2017). Nonetheless, and despite its widespread use, their evoked behavioral and neuronal effects remain poorly understood.

This study aimed to increase our knowledge about the mechanism of action of NPE and its effects evoked in the brain. We found that blockage of DA receptors partially reversed NPE-induced pharmacological effects. Our results confirm and further extend the idea that most, if not all, appetite suppressants of the phenethylamine class exert their anorectic effects via NAcSh's D1- and D2-like receptors (Kalyanasundar et al., 2015).

MATERIALS AND METHODS

Animals

A total of 62 male Sprague–Dawley rats ∼250–350 g were used for all experiments. Future studies should evaluate the effects of NPE in female rats. Animals were housed individually and had *ad libitum* access to food and water except during multichannel recordings or when locomotion was measured in the open field (see below). Room temperature was maintained at 21 ± 1°C, with 12/12 h light–dark cycle (0600–1,800 h). All procedures were approved by the CINVESTAV institutional animal care and use committee.

Drugs and Chemicals

The NPE hydrochloride was kindly provided by Productos Medix (Mexico). R(+)–SCH-23390 hydrochloride (SCH) and S(−)-raclopride (+)-tartrate salt (RAC) were obtained from Sigma–Aldrich (Mexico). These compounds were dissolved in physiological saline (Sal) (0.9% NaCl) and administered intraperitoneally (i.p.) in a volume of 1 ml/kg or 2.5 μg/0.5 μL per hemisphere in the intra-NAcSh infusion (see below).

Dose–Response Effects of NPE on Weight Loss and Food Intake

To determine whether NPE influences the rats body weight and food intake, these variables were measured approximately at the same time once daily 20 min before the experiment's start. Rats were injected with Sal ($n = 3$), 10 mg/kg NPE, 20 mg/kg NPE, 40 mg/kg NPE, or 80 mg/kg NPE, namely, NPE10, NPE20, NPE40, and NPE80, respectively ($n = 4$/group). Animals were individually housed and received 100 g allotment of standard rat chow (Purina Mexico) per day. Behavioral experiments were carried out between 1,600 and 1,800 h (i.e., 2 h prior to the commencement of the rat's active phase). The daily changes in body weight and food intake were expressed in grams relative to the baseline (BL, i.e., the values recorded 20 min before the experiment's start, in the first injection day).

Locomotor Activity in the Open Field

All locomotor effects were measured using Ethovision XT10 (Noldus Information Technology, the Netherlands) (Perez et al., 2019). Our setup recorded up to six open field arenas simultaneously (40 L × 40 W × 30 H cm). The arenas were placed together in two rows (3 × 2) and a CCD camera (IDS camera, Germany) with a uEye Cockpit software recorded with a top view and 15-fps resolution. After 3 days of habituation to the open field, animals were injected with their corresponding treatment once daily and then were placed in an open field for 90 or 120 min. The videos were analyzed using the center body mass, tracking the position of the animal as "x" and "y" coordinates to compute the total distance traveled (cm). Two videos were lost and thus not included into the analysis. They corresponded to

days 2 and 4 for the same animals; one rat for NPE10, NPE20, and NPE40 groups.

Effects of Systemic Administration of DA D1- and D2-Like Receptor Antagonists on NPE-Induced Pharmacological Effects

To determine the contribution of D1- and D2-like receptors on NPE-induced behavioral effects, we injected SCH or RAC antagonists i.p. over 7 consecutive days. The antagonist concentrations were selected based on previous studies showing that doses 1.5 mg/kg (SCH) and 0.5 mg/kg (RAC) antagonized the locomotor effects induced by amphetamine, methamphetamine, and diethylpropion (Broening et al., 2005; Wright et al., 2013; Kalyanasundar et al., 2015). Based on our dose–response experiment, the NPE's effective dose (ED_{50}) that leads to weight loss was 20 mg/kg. Thus, we used this dose for all subsequent experiments. Animals were divided into six groups: Sal + Sal ($n = 4$), SCH + Sal ($n = 3$), RAC + Sal ($n = 3$), Sal + NPE ($n = 4$), SCH + NPE ($n = 4$), and RAC + NPE ($n = 4$). The first name indicates the initial i.p. injection of the DA antagonist, and the following name represents the second i.p. injection received. Body weight and food intake (both in grams) were measured daily 20 min before each experiment's start. Rats were habituated to the open field for 2 days (data not shown). During the treatment, the rats were daily introduced in the open field during 0–45 min to record the basal locomotor activity (with no injection), and then at 45 min, they received the first i.p. injection with either Sal or two antagonists (either SCH or RAC). Then at 60 min, they received the second i.p. injection (either Sal or NPE), and their locomotor activity was monitored for 1 additional hour.

Intra-NAcSh Infusions of DA Antagonists

Cannula Implantation

To evaluate the role of the DA receptors located in the NAcSh on the NPE-induced changes in weight loss, feeding, and locomotion, we performed intra-NAcSh infusions of DA antagonists. Animals were anesthetized using a cocktail of ketamine (100 mg/kg) and xylazine (8 mg/kg). For the bilateral intra-NAcSh infusions, two holes were drilled at the following coordinates: AP +1.4 mm; L ± 1 mm from bregma. Stainless-steel guide cannulas (0.63 diameter × 11 mm in length) aimed at the NAcSh were bilaterally inserted 5.5 mm DV relative to bregma. Two screws served as anchors in the skull bone, and the whole assembly was cemented with dental acrylic. A stylus was inserted into the cannula to prevent clogging, and it was removed before each daily microinjection. For all animals, enrofloxacin (10 mg/kg, i.p.) and Baytril (5%, i.p.) were administered for 3 days after surgery, and they were allowed to recover for 7 days.

The microinjections were performed via a 30-gauge stainless-steel injector, protruding 1.0 mm from the guide cannula's tip, connected via a Teflon tube to a 10-μL glass microsyringe (Hamilton 80366) attached to a microinfusion pump (KD scientific- KDS200 series). A total volume of 0.5 μL (0.33 μL/min) per hemisphere of RAC or SCH was infused once daily for 7 days. The injector was left into the cannula for 1 additional minute to allow drug diffusion (Gutiérrez et al., 2003).

Following the same procedures used in systemic experiments, 18 naive animals were assigned to six groups, each of n = 3: Sal + Sal, SCH + Sal, RAC + Sal, Sal + NPE, SCH + NPE, and RAC + NPE. Body weight and chow food intake were measured daily just before placement in the open field. Then, rats were introduced in the open field during 0–45 min (with no injection). At 45 min, animals were briefly removed and microinjected with either Sal, SCH, or RAC (2.5 μg/0.5 μL) into the NAcSh, and then they were returned to the open field. Next, an i.p. injection of either Sal or NPE (20 mg/kg) was given at 60 min. The rat's locomotor activity was recorded for 1 additional hour.

Electrophysiology

Surgery

Animals were anesthetized using a cocktail of ketamine (100 mg/kg) and xylazine (8 mg/kg), and implanted with an i.p. catheter following the protocol described by Perez et al. (2019). After the catheter's surgery, we inserted an electrode array in the NAcSh following previously described methods (Tellez et al., 2012). Briefly, a movable 4 × 4 microwire array (tungsten wires of 35 μm in diameter) was unilaterally implanted in the right NAcSh at the following coordinates: AP = 1.4, L = ± 1, and DV = 7.5 mm from bregma. One stainless-steel screw was soldered to a silver wire (203 μm) and implanted on the cerebellum's surface served as ground. Finally, the electrode array was anchored to the skull using dental acrylic and two more screws. To maintain the patency, catheters were flushed daily with Sal (0.9%). Seven days after surgery, rats were habituated for 2 days to the operant box. A polyethylene tube, 30 cm in length, was connected to the catheter attached to a syringe outside the box and was manually operated to infuse the drug (either Sal or NPE) non-invasively during the neuronal recordings.

Single-Unit Recordings in the NAcSh of Freely Moving Rats

Recordings (19 sessions from three rats) were performed using a multichannel acquisition processor (Plexon, Dallas, TX). During recordings, rats were placed in an operant box enclosed in a sound-attenuating cubicle equipped with a webcam. Each session lasted for 3 h and consisted of three epochs: BL (0–1 h), Sal (1–2 h), and treatment (NPE: 2–3 h; 20 mg/kg). Voltage signals were sampled at 40 kHz and digitalized at 12-bit resolution. The action potentials were band-passed filter (154 Hz to 8.8 kHz). Only single neurons with action potentials with a signal-to-noise ratio larger than 3:1 were analyzed. Action potentials were identified online using a voltage–time threshold window and the three principal components contour template algorithm of neuron's wave shape (Gutierrez et al., 2010). Action potentials were then sorted using offline sorter software (Plexon Dallas, TX). No food or water was available during recordings to avoid unintended modulations induced by chewing or licking and those induced by satiety. Thus, the intention was primarily to record the pharmacological effects induced by NPE. The NAcSh's local field potentials (LFPs) were amplified 1,000×, filtered 0.7–300 Hz, and digitized at 1 kHz using a digital acquisition card (National

Instruments, Austin, TX). They were used to compute the brain state hypnograms (see below).

Statistical Analyses for Behavior

All data are presented as mean ± SEM. Statistical differences both within- and between-subjects' factors were assessed by two-way repeated-measures analysis of variance (RM ANOVA), followed by Tukey *post hoc* analysis using GraphPad Prism 7. Complete statistical analyses on body weight, food intake, and locomotor activity can be found in **Supplementary Table 1**.

Calculation of NPE's ED_{50} for Weight Loss

We calculated the effective dose 50 (ED_{50}) on body weight loss using the equation $f = \min + ((\max–\min)/[1 + (ED_{50}/X)\hat{}\text{Hill slope}])$, where f is the expected response to a given dose (X), min and max are the lowest and highest weight loss, and the ED_{50} is the dose at which 50% of the subjects are expected to show the desired response using the program developed for Gadagkar and Call (2015).

Neurons That Increase or Decrease Firing Rates After Administration of NPE

All analyses were performed using MATLAB toolboxes and homemade custom scripts. After the injection of NPE, a significant change in firing rate was identified using a Kruskal–Wallis test, considering a significance level $\alpha < 0.05$. Neurons exhibiting an increased firing rate after treatment relative to BL epoch were classified as "increased," whereas neurons with reduced activity were named "decreased." We used a χ^2-test to assess differences in the percentage of neurons modulated.

Principal Component Analysis for Population Activity Trajectories

Preprocessing

All neuronal responses were concatenated in a single n × m matrix D, where rows were $\boldsymbol{n}$ = neurons, and $\boldsymbol{m}$ = columns were firing rates binned at 1-s size. The neuronal's responses were smoothed with a Gaussian kernel ($\sigma = 100$ ms) as follows:

$$f(x|ts, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} * e^{\frac{-(x-ts)^2}{2\sigma^2}}$$

where $\boldsymbol{ts}$ were timestamps for every spike per neuron, $\boldsymbol{x}$ was a vector containing values to construct each Gaussian kernel (e.g., ts - 3σ: 10 ms: ts + 3σ), and σ was kernel standard deviation. Then, all neural responses were downsampled in bins of 30-s size. The neural responses were z scored as follows:

$$fR_{\text{z-score}} = \frac{fR - \mu}{\sigma}$$

where fR was a vector containing the firing rate of a given neuron, μ is the mean firing rate, and σ is the standard deviation of the firing rate of a given neuron. The standard deviation and mean firing rate were calculated from the BL (time 0–1 h).

Principal Component Analysis

To understand the neural dynamics of the population NAcSh's responses, we used principal component analysis (PCA) to calculate the linear combinations of the population activity, capturing the most variance in the averaged population responses. The population activity was projected onto two axes, corresponding to the first two principal components to describe both temporal dynamics and the relationships across the different task's epochs (BL, Sal, and NPE) present in the population activity trajectories. This analysis was computed in MATLAB using the following line code:

```
[∼, scr, ∼] = pca(D′, 'Rows', 'complete');
plot(scr(:,2), scr(:,1), '-o', 'linewidth', 2);
axis tight; grid on; xlabel('PC 2'); ylabel('PC 1');
```

where $\boldsymbol{D}'$ was the transposed matrix of z scored neural responses, and $\boldsymbol{scr}$ were the principal components, which correspond to the first two principal components explaining the most variance.

Hypnograms: The LFP's Brain State Map

For hypnograms, behavioral states were assigned using information obtained from the LFP as outlined in Gervasoni et al. (2004) and Tellez et al. (2012). In brief, after the elimination of segments with amplitude saturation, a sliding window Fourier transform was applied to each LFP signal to calculate two spectral amplitude ratios (0.5–20/0.5–55 Hz and 0.5–4.5/0.5–9 Hz for ratios 1 and 2, respectively). PCA was then applied to these ratios obtained from all LFP channels, and the PCAs were used as the overall ratios measure. These measures obtained for each second of data were further smoothed with a Hanning window (20 s length). Finally, the two PCAs of the spectral ratios were plotted against each other to construct a two-dimensional (2-D) state space where the density of points reflects the relative abundance of the different brain states. Rapid eye movement (REM) sleep was not included in this analysis because animals spent very little time in REM state (data not shown). The final 2-D brain state maps were selected and validated after visual inspection of animals' behavior in the video within three behavioral states: slow-wave sleep (SWS), quiet wake (qW), and active wake (aW) (Tellez et al., 2012).

Histology

At the end of the experiments, rats were injected with pentobarbital sodium (150 mg/kg i.p.) and perfused with PBS, followed by 4% paraformaldehyde. Brains were placed in a 10% sucrose (vol/vol) solution for 24 h, with sequential increases in sucrose concentration until reaching 30% in 72 h. The brain slices (50 μm) were stained with cresyl violet to verify the cannula locations and recording sites (**Supplementary Figure 1**).

RESULTS

Behavioral Effects

NPE-Induced Weight Loss

To determine the efficacy of NPE, we measured the body weight of rats over 7 consecutive days. **Figure 1A**, left panel, shows the change in body weight relative to initial BL weight after administering either control Sal or one of the following doses of NPE; 10, 20, 40, and 80 mg/kg. We found that Sal-treated rats gained body weight over the 7-day treatment period. Statistical

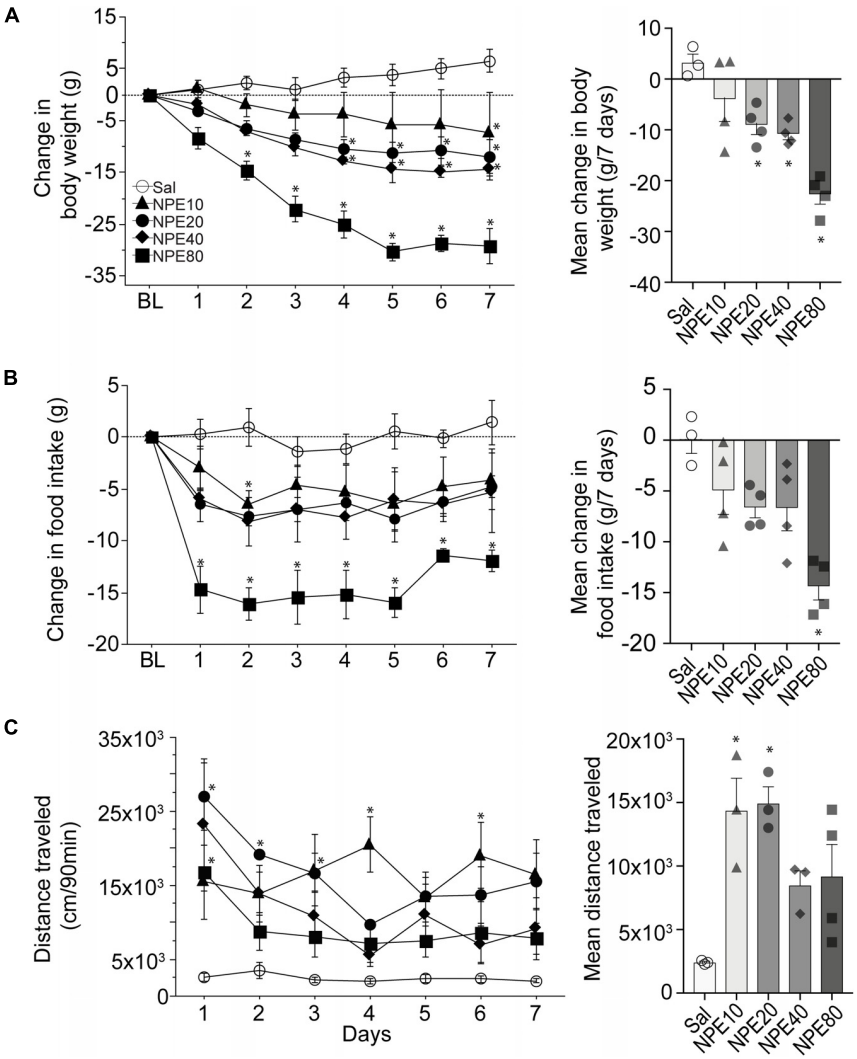

FIGURE 1 | D-Norpseudoephedrine (NPE) leads to weight loss, suppresses food intake, and NPE increases locomotor activity. **(A)** *Left panel:* Change in body weight (g) relative to baseline day (BL) over 7 consecutive injections of saline (Sal) or NPE at 10-, 20-, 40-, and 80-mg/kg doses (NPE10, NPE20, NPE40, and NPE80, respectively). The horizontal dotted line represents no change in body weight relative to BL. *Right panel*: Average body weight loss over 7 consecutive days of treatment. **(B)** *Left panel*: Change in chow intake (g) over 7 days. *Right panel*: Average change in chow intake. **(C)** *Left panel*: Locomotor activity induced by different doses of NPE. *Right panel*: Mean distance traveled follows inverted-U shape response. Bars represent the mean ± SEM. $^*p < 0.05$ significantly different from saline-treated rats.

analysis demonstrated a significant main effect of doses on body weight [RM ANOVA; $F_{(4,14)} = 13.1$, $p = 0.0001$; time (days): $F_{(6,\ 84)} = 21.32$, $p < 0.0001$, and doses × time interaction: $F_{(24,\ 84)} = 4.3$, $p < 0.0001$]. In contrast, NPE20, NPE40, and NPE80 treatments resulted in a significantly greater weight loss than the control Sal group across days (all $p_s < 0.05$). We also plotted the average change in body weight in **Figure 1A**, *right panel*. A *post hoc* analysis unveiled that NPE20 and NPE40 induced a similar weight loss ($p > 0.9$), whereas NPE20, NPE40, and NPE80 induced more weight loss than the Sal group ($^*p < 0.05$).

Administration of NPE Suppressed Food Intake

To study the NPE-induced anorectic effects, we measured the chow food intake. **Figure 1B**, left panel, shows the daily change in food intake (in g) relative to BL (**Figure 1B**, left panel). As expected, the administration of Sal did not change food intake, because values remained around zero. In contrast, NPE10, NPE20, and NPE40 inhibited food intake with a similar magnitude (−4.9 ± 0.8, −6.6 ± 0.4, and −6.6 ± 0.8 g, respectively), whereas the highest NPE dose 80 mg/kg achieved the maximum reduction −14.3 ± 0.7 g (**Figure 1B**, right panel [RM ANOVA; main effect of doses: $F_{(4,14)} = 7.9$, $p = 0.0015$; time (days): $F_{(6,\ 84)} = 3.3$, $p = 0.005$, and no significant doses × time interaction: $F_{(24,\ 84)} = 0.7$, $p = 0.8$]. A Tukey *post hoc* analysis unveiled that NPE at doses of 80 mg/kg was significantly different to Sal-treated rats ($p < 0.05$); whereas the lowest doses exhibited a non-significant trend suppressing food intake.

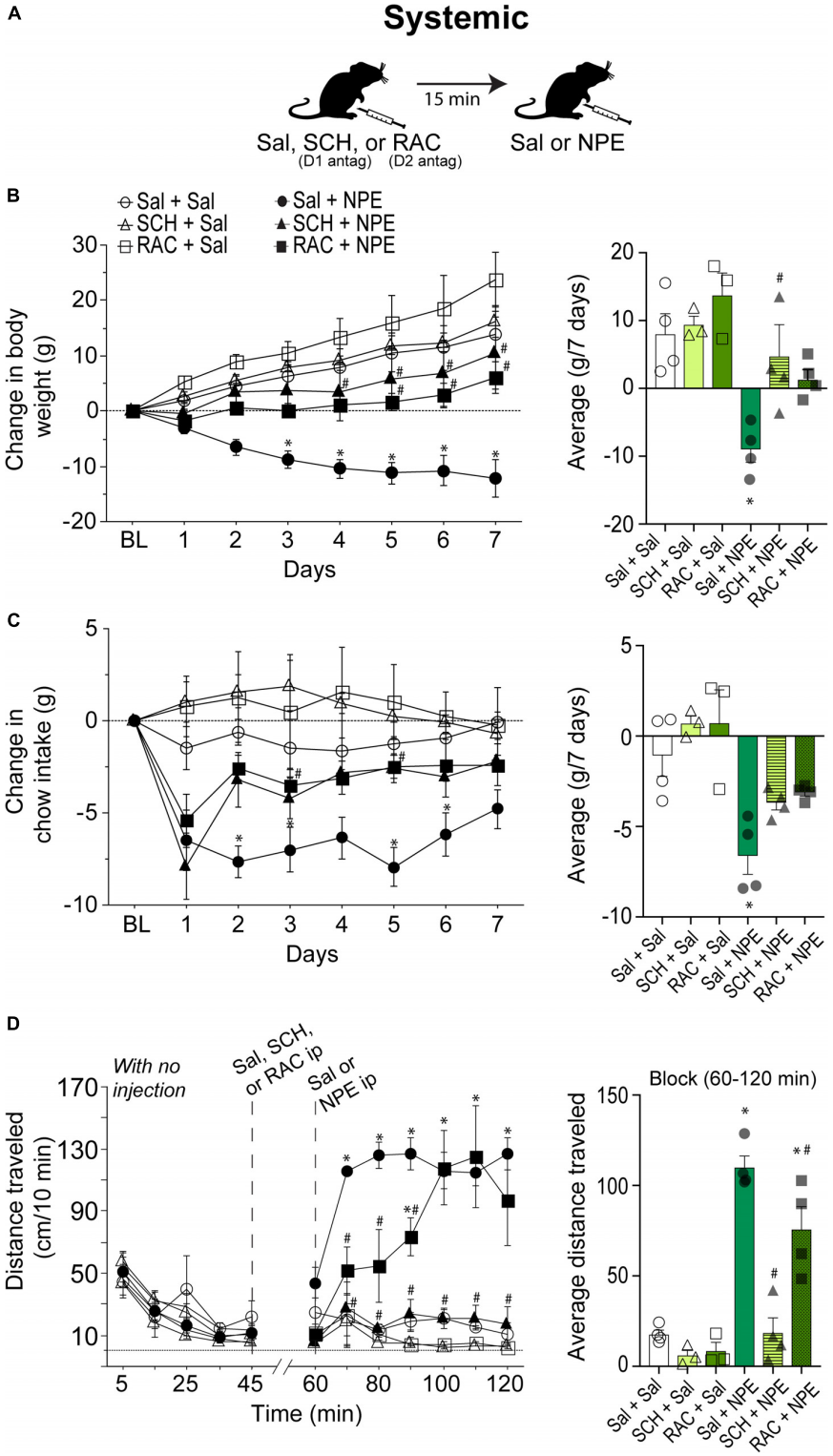

FIGURE 2 | Intraperitoneal injection of either D1 (SCH23390- SCH) or D2-like receptor [raclopride (RAC)] antagonists partially reversed NPE -induced behavioral effects. **(A)** Scheme of i.p. injections. **(B)** *Left panel:* Change in body weight over 7 days relative to BL (first day of injection). NPE was given in a fixed dose of 20 mg/kg. *Right panel*: The average change in body weight. **(C)** *Left panel* shows the change in chow intake (g). *Right panel*: The average change in food intake. **(D)** *Left panel*: Distance traveled (cm/10 min) for a period with no injection (interval 0–45 min), and the evoked distance after NPE or Sal (>60 min). The first i.p. injection (Sal, SCH, or RAC) was given at 45 min, which is 15 min before the second injection at 60 min (Sal or NPE, note: the break-in *x*-axis at 45–60). *Right panel:* Average distance traveled. $^{*}p < 0.05$ significantly different than in Sal + Sal. $^{\#}p < 0.05$ compared to Sal + NPE group.

NPE Increased Locomotor Activity

Previous studies reported that acute administration of appetite suppressants in rats also stimulates locomotor activity (Reimer et al., 1995; Rothman and Baumann, 2006; Kalyanasundar et al., 2015; Perez et al., 2019). We then asked whether NPE modulates this behavior (**Figure 1C**, left panel). As expected, Sal administration (control) did not change locomotor activity. In contrast, we observed that locomotor activity was significantly higher after injection of NPE compared to the control group [RM ANOVA; main effect of doses: $F_{(4,11)} = 6.4$, $p = 0.006$; time (days): $F_{(6,\ 66)} = 4.3$, $p = 0.0009$, and doses × time interaction: $F_{(24,\ 66)} = 1.9$, $p = 0.01$]. Note that rats treated with NPE10 and NPE20 exhibited greater locomotor activity than the Sal-treated rats ($p < 0.05$), whereas the highest doses NPE40 and NPE80 showed a reduced activity compared to the lowest doses (**Figure 1C**, right panel). NPE's increased locomotor activity tends to follow a dose-dependent curve of inverted-U shape, suggesting that normal locomotion was compromised in large doses, as shown in Thiel and Dressler (1994). In rats, our dose–response curve for the weight loss over 7 days of treatment with NPE yielded an effective dose (ED_{50}) of 20 mg/kg. Therefore, we used the ED_{50} dose for all subsequent experiments.

NPE-Induced Weight Loss, Food Intake Suppression, and Locomotion Were Attenuated by Systemic Injection of DA D1 and D2 Receptor Antagonists

We then went to determine whether the NPE's induced pharmacological effects depend on DA D1 and D2 receptors. Thus, either D1 (SCH; SCH23390 1.5 mg/kg) or D2 receptor antagonist RAC (0.5 mg/kg) were administered systemically 15 min before the i.p. injection of NPE or Sal. **Figure 2A** depicts the drug administration protocol. We found a significant treatment effect [$F_{(5,16)} = 7.2$, $p = 0.001$], days [$F_{(6,\ 96)} = 24$, $p < 0.0001$], and a significant interaction between factors [$F_{(30,96)} = 4.9$, $p < 0.0001$]. **Figure 2B**, left panel, shows the change in body weight over 7 consecutive days. The control group continued to gain body weight over time (Sal + Sal: 8 ± 1.3 g, **Figure 1B**, right panel). Likewise, the DA D1 antagonists alone (i.e., SCH + Sal) did not affect the weight gain compared to the control group (Sal + Sal: 8 ± 1.3 g vs. SCH + Sal: 9.4 ± 1 g; $p = 0.99$, n.s.). Although RAC + Sal exhibited a slighter increase in body weight gain (13.7 ± 1.7 g) than rats treated with Sal, the difference did not achieve statistical significance ($p = 0.76$, n.s.). In contrast, Sal + NPE leads to a robust and significant weight loss (−9.0 ± 0.9 g, $p = 0.0063$). Most importantly, blockade of DA D1 receptors attenuated NPE-induced weight loss, because SCH + NPE (4.7 ± 1.7 g; black triangles) was significantly different than Sal + NPE group ($p = 0.03$). Though the RAC + NPE group (1.5 ± 0.7 g; black squares) did not reach statistical significance ($p = 0.143$ n.s. for the full effect) (**Figure 2B**, right panel), the day-by-day analysis uncovered that RAC + NPE group attenuated NPE-induced weight loss until days 5–7 (see # in **Figure 2B**, left panel, black squares). Our data demonstrate that D1-like (and in less degree D2-like) receptors are critical players for NPE-induced weight loss.

We measured the 24 h food intake. **Figure 2C**, left panel, shows the change in food intake over 7 days of treatment. RM ANOVA found a treatment main effect: $F_{(5,\ 16)} = 9.0$, $p = 0.003$; no time effect (days): $F_{(6,\ 96)} = 1.5$, $p = 0.1$, and no significant treatment × time interaction: $F_{(30,\ 96)} = 1.2$ $p = 0.1$. The Sal + Sal, SCH + Sal, and RAC + Sal were not significantly different among the three control groups. In contrast, Sal + NPE group exhibited a significant reduction in chow intake compared to Sal + Sal (see * right panel). Note that on the first day DA antagonists did not eliminate the initial food intake suppression induced by Sal + NPE. However, after the second and subsequent days, blockade of DA receptors partially reversed Sal + NPE-induced food intake suppression. Accordingly, SCH + NPE and RAC + NPE were not statistically different from Sal + Sal group ($p = 0.35$ and $p = 0.6$). These results demonstrated that systemic administration of DA antagonists partially attenuated NPE-induced anorectic effects.

We also measured NPE's induced locomotion effects. **Figure 2D**, left panel, shows the daily locomotor activity. During the 0–45 min period (with no injection), all groups exhibited a similar distance traveled (<70 cm/10 min: all p_s = n.s.). Likewise, Sal + Sal group showed no increase in locomotor activity between BL and after Sal injection (0–45 min: 28.5 ± 5.5 cm/10 min; 60–120 min: 17.7 ± 2.4 cm/10 min). In contrast, from 60 to 120 min, the comparison among the six groups showed a significant effect of treatment on locomotion [$F_{(5,\ 16)} = 32.7$, $p < 0.0001$] and time [$F_{(6,\ 96)} = 5.2$, $p = 0.0001$] and significant interaction between factors [$F_{(30,96)} = 3.3$, $p < 0.0001$]. After systemic administration of DA antagonists alone, rats showed no increase in locomotion; in fact, they showed a rather decreased in locomotor activity compared to Sal + Sal group (SCH + Sal: 6.1 ± 2.4 cm/10 min; RAC + Sal: 8.5 ± 3 cm/10 min, and Sal + Sal: 17 ± 2.4 cm/10 min, all p = n.s.). In contrast, Sal + NPE–treated rats exhibited increased locomotor activity in comparison to Sal + Sal. The D1 receptor antagonist SCH completely reversed the locomotion induced by NPE (SCH + NPE vs. Sal + NPE; $p < 0.0001$; **Figure 2D** *right panel*). The D2 antagonist RAC mainly delayed the NPE-induced locomotion ($p = 0.04$; RAC + NPE). Thus, these data suggest that the NPE-induced locomotion depends more on DA D1 than D2 receptors, suggesting an important role of D1 receptors on NPE-induced locomotor effects.

Blockade of D1- and D2-Like Receptors Directly in the NAcSh Attenuated the NPE-Induced Behavioral Effects

To evaluate the involvement of DA receptors located in the NAcSh for the NPE-mediated behavioral effects, we microinjected either D1 or D2 antagonists directly into the NAcSh, while rats received an i.p. injection of Sal or NPE (**Figure 3A**). **Figure 3B**, left panel, shows the change in body weight over 7 days. Statistical analysis found a significant effect of treatment [RM ANOVA; $F_{(5,\ 12)} = 26.4$, $p < 0.0001$; time (days): $F_{(6,\ 72)} = 122.7$, $p < 0.0001$; and interaction: $F_{(30,\ 72)} = 8.5$ $p < 0.0001$]. DA antagonists alone in the NAcSh did not affect body weight gain compared to the control group (**Figure 3B**, right panel: Sal + Sal: 8 ± 1.g; SCH + Sal: 9.4 ± 1 g; RAC + Sal: 13.7 ± 1.7 g). In contrast, the weight loss induced by NPE was significantly different than Sal + Sal (Sal + NPE; -5.8 ± 0.5 g;

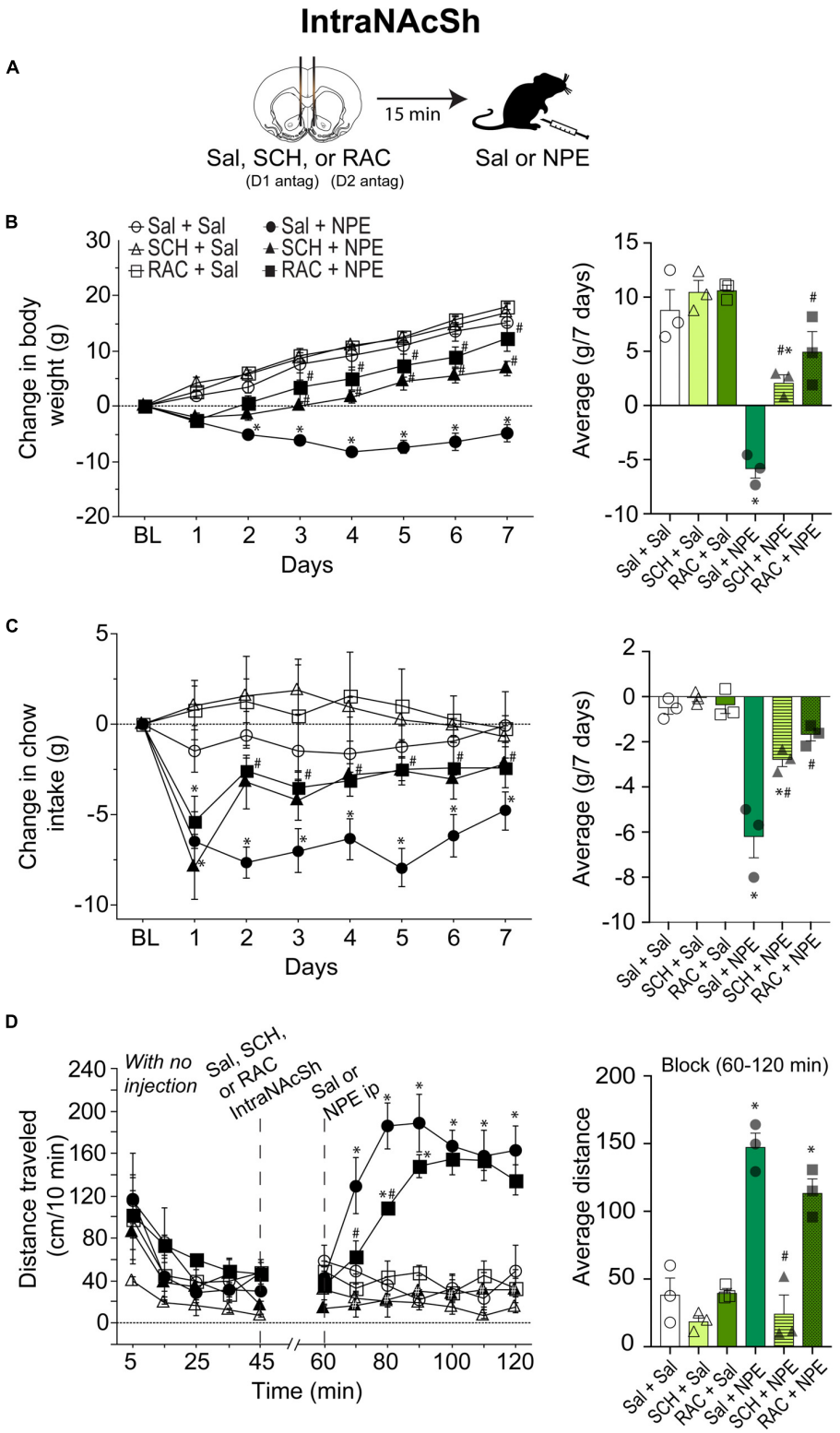

FIGURE 3 | Role of intra-NAcSh dopamine D1- or D2-like receptors on NPE's induced behavioral effects. **(A)** The protocol of drug administration: intra-NAcSh injection either Sal, or RAC, or SCH (at 45 min), followed by i.p. injection of either Sal or NPE (at 60 min, see panel **D**). **(B)** *Left panel:* Change in body weight during 7 consecutive days of treatment. *Right panel* averaged over 7 days. **(C)** *Left panel:* Change in chow intake (g) relative to BL. *Right panel*: The average change in chow intake. **(D)** *Left panel:* The distance traveled (cm/10 min) on a period with no injection (interval 0-45 min) and after injections (60–120 min). *Right panel*: Average distance. $^{*}p < 0.05$ significantly different than in Sal + Sal. $^{\#}p < 0.05$ compared to Sal + NPE group.

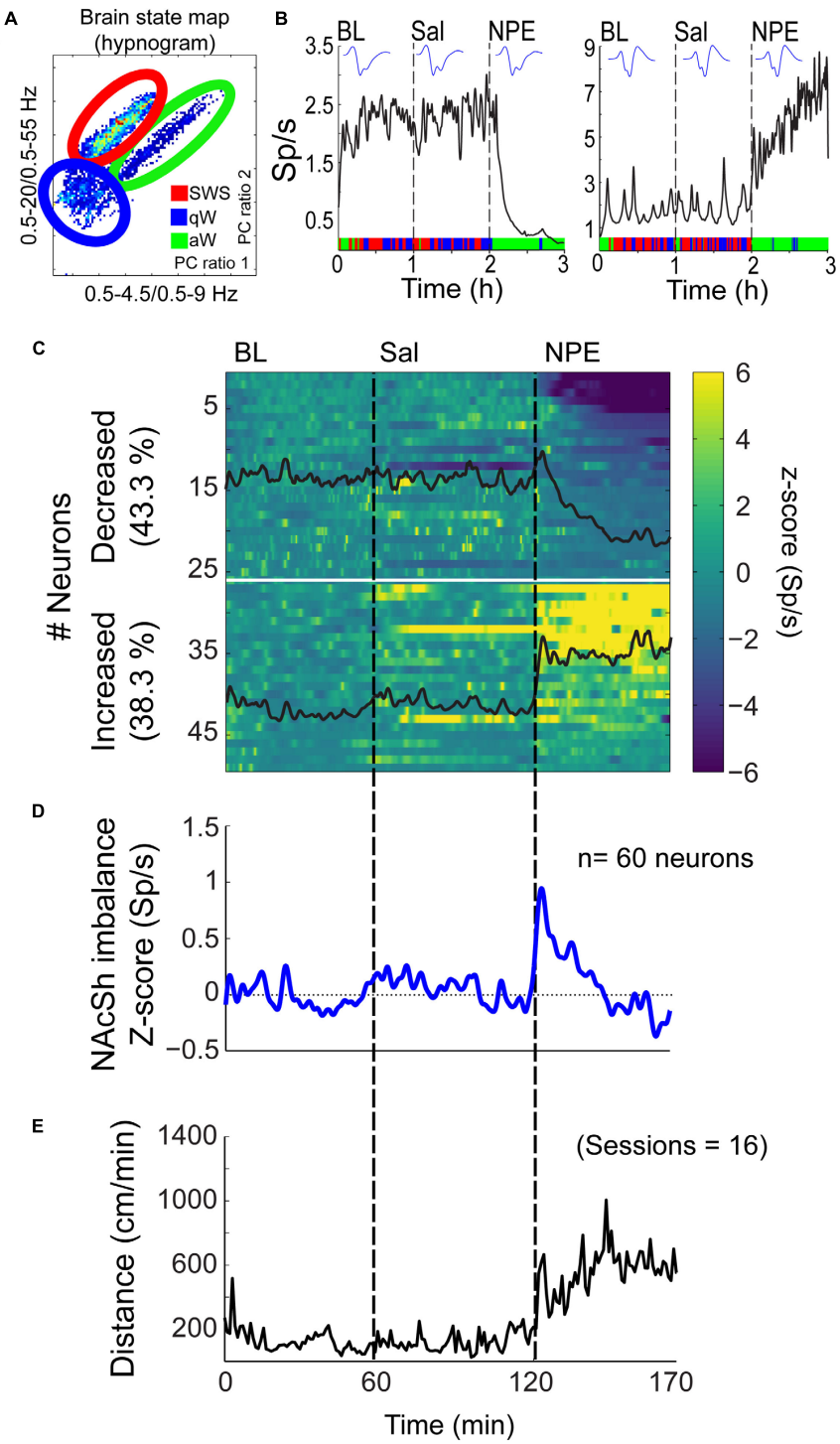

FIGURE 4 | NPE modulates NAcSh spiking activity. **(A)** A brain state map (hypnogram) was computed from the local field potentials (LFPs) from the NAcSh, illustrating the three major behavioral states: slow-wave sleep (SWS), quiet awake (qW), and active awake (aW). Each dot represents the principal component (PC) ratio between two power intervals for each second of NAcSh's LFP activity. Each dot falling into the red ellipsoid corresponded to periods when the animal was in SWS, and in blue and green, ellipsoids represent qW and aW brain states, respectively. **(B)** *Left panel*: An example of one neuron exhibiting decreased firing rates (spikes/s, Sp/s) after NPE injection. The action potential waveform did not change across the session. The color bar on the *x*-axis represents the SWS, aW, and qW states. *Right panel:* A typical neuron with increasing firing rates after NPE injection. **(C)** A color-coded peristimulus time histogram (PSTH) of NAcSh neuronal responses of 49/60 neurons recorded during BL, Sal, and NPE epochs (vertical black lines). The horizontal white line split neurons with decreased from increased neural responses. **(D)** The average population PSTH activity of all 60 NAcSh neurons recorded. **(E)** Average locomotor activity (cm/min) obtained across 16 recording sessions.

$p < 0.0001$). The weight loss induced by NPE (Sal + NPE) was significantly attenuated by intra-NAcSh infusion of either SCH (SCH+ NPE: weight gain; 2.1 ± 0.8 g; $p = 0.0063$) or RAC (RAC + NPE: 4.9 ± 1.2 g; $p = 0.0005$). Our data demonstrate that D1- and D2-like receptors in the NAcSh are involved in the NPE-induced weight loss.

We then evaluate their effects on food intake (**Figure 3C**). Direct infusion of DA antagonists into the NAcSh significantly attenuated the NPE-induced food intake suppression (**Figure 3C**, left panel) {effect of treatment [$F_{(5,12)} = 27.1$, $p < 0.0001$], and days [$F_{(6,\ 72)} = 22.1$, $p < 0.0001$], and significant interaction [$F_{(30,72)} = 3.9$, $p < 0.0001$]}. Briefly, food intake in the three control groups (Sal + Sal, SCH + Sal, and RAC + Sal) showed that they consumed the same amount of chow (i.e., values remained at the same level than BL, p = n.s.; **Figure 3C**, right panel). In contrast, the administration of NPE inhibited food intake compared to Sal + Sal (Sal + NPE; $p < 0.0001$), and its anorectic effects were attenuated by the infusion of DA antagonists. That is, rats treated with D1 or D2 antagonists (SCH + NPE or RAC + NPE groups) consumed more chow food than rats treated with Sal + NPE (all $p_s < 0.01$). These data show that NPE's anorectic effects were markedly reduced by D1 and D2 receptor antagonism in the NAcSh.

The analysis of the locomotor activity further uncovered the major involvement of D1 DA receptors. **Figure 3D**, left panel, shows the distance traveled for each group. During 0 to 45 min, there were no significant differences between the control groups, except that the SCH + Sal group showed lower locomotor activity during the period with no injection (open triangle perhaps as a result of a carryover effect). In the interval 60 to 120 min, RM-ANOVA demonstrated a main effect of treatment [$F_{(5,\ 12)} = 30.3$ $p < 0.0001$], time [$F_{(6,\ 72)} = 8.7$, $p < 0.0001$] and interaction [$F_{(30,\ 72)} = 6.5$ $p < 0.0001$]. During this period, the antagonists administered alone, SCH + Sal (18.8 ± 2.6 cm/10 min), and RAC + Sal (39.9 ± 3.8 cm/10 min) failed to induce locomotor activity compared to Sal + Sal (38.5 ± 5.2 cm/10 min, all p_s = n.s. **Figure 3D**, right panel). In contrast, Sal + NPE group exhibited a robust locomotion (147.7 ± 12.4 cm/10 min) compared to Sal + Sal ($p < 0.0001$). We found that SCH + NPE (24.5 ± 4.9 cm/10 min) completely attenuated the locomotion induced by Sal + NPE (**Figure 3D**, left panel, $p < 0.0001$), whereas the RAC + NPE group only delayed the onset but did not reverse the locomotion induced by Sal + NPE (113.8 ± 10.6 cm/10 min; **Figure 3D**, right panel; $p = 0.20$). Again, these results demonstrated that DA D1-like receptors in the NAcSh are the major contributors for NPE-induced locomotion.

Electrophysiology Characterization of NPE Induced Modulation of Neuronal Activity in the NAcSh

Given that the NAcSh receives strong dopaminergic input from dopaminergic neurons in the ventral tegmental area (Powell and Leman, 1976; Cauda et al., 2011), we demonstrated that the pharmacological effects of NPE were attenuated by both systemic and intra-NAcSh infusion of DA antagonists. Furthermore, to understand how NPE modulates NAcSh neural activity, we recorded single-unit activity after the injection of NPE. **Figure 4A** displays a brain state map obtained from NAcSh LFP's oscillations. Each dot represents 1 s of LFP signal corresponding to a brain state mapping to a particular rat's behavior. It clearly shows that animals exhibited three different behavioral states: (1) SWS, dots falling inside the red ellipsoid; (2) qW (blue); and (3) aW (green). **Table 1** shows the time spent in each behavioral state as a function of epochs: BL period (BL, 0–1 h), Sal (1–2 h), and 20 mg/kg of NPE (2–3 h). Interestingly, after the injection of NPE, the predominant brain state changed from SWS to aW; animals spent most of the time on this active brain state (**Figure 4B**, see bottom for hypnograms of two different experiments). This is also evident in **Table 1** in NPE's epoch; animals rarely exhibited SWS (<1 min), because they stayed most of the time in the aW state reflecting insomnia [54.9 min; $F_{(2,54)} = 113.7$, $p < 0.0001$]. At the single neuronal level, NPE evoked a tonic, and long-lasting, modulation in spiking activity. **Figure 4B** shows two representative modulatory responses induced by NPE and their corresponding brain state map (SWS, aW, and qW). The neuronal activity is depicted as a function of the following epochs: BL, Sal, and NPE, which are illustrated along with their waveform across the three epochs (see blue insets). **Figure 4B**, left panel, shows a representative neuron that reduced their firing rates after NPE administration relative to both BL and Sal period (BL: 2.17 ± 0.005 Sp/s; Sal: 2.28 ± 0.004 Sp/s; NPE: 0.44 ± 0.008, Sp/s). In contrast, the example shown in **Figure 4B**, right panel, comes from a neuron that increased activity after NPE. Specifically, the firing rate during the BL and Sal period was lower and similar (BL: 1.64 ± 0.008 Sp/s; Sal: 1.79 ± 0.007 Sp/s), but after NPE, it gradually increased (2–3 h: 5.78 ± 0.02 Sp/s).

Subsequently, we explored how NPE modulates NAcSh's population activity. A total of 60 neurons were recorded from three rats in the NAcSh, while Sal and NPE were injected. **Figure 4C** displays the normalized NAcSh neuronal activity (only neurons significantly modulated by NPE are shown, $n = 49/60$) using a population color-coded PSTH (peristimulus time histogram), where yellow colors indicate responses above BL activity, and dark blue colors represent decreased responses. After NPE, 43.3% (26/60) of neurons were classified as decreased, while 38.3% (23/60) were increased [$\chi^2_{(1)} = 0.64$, $p = 0.42$, n.s.], and the other 18.3% (11/60) were non-modulated (**Table 2** shows the average firing rate as a function of epochs). Overall NPE

TABLE 1 | Time spent in each behavioral state across the epoch.

Behavioral states	Time (min)		
	BL	Sal	NPE
SWS	37.9 ± 0.9	34.9 ± 1.5	0.7 ± 0.2*
qW	7.9 ± 0.6	9.9 ± 0.8	3.1 ± 1.5
aW	13.4 ± 1.0	14.3 ± 1.8	54.9 ± 1.7*

*Values are mean ± SEM, n = 19, *p < 0.05 significantly different from baseline (BL) and saline (Sal) epochs. SWS, slow wave sleep; qW, quiet wake; aW, active awake.*

TABLE 2 | Firing rate in each epoch.

Type of response	Firing rate (Sp/s) per 60-min epoch		
	BL	Sal	NPE
Decreased (26/60)	2.6 ± 0.004	2.4 ± 0.004	1.1 ± 0.009*
Increased (23/60)	2.4 ± 0.004	2.8 ± 0.004	4.2 ± 0.004*

*Values are mean ± SEM. *p < 0.0001 comparisons to BL and Sal epoch.*

modulated 81.6% of NAcSh neurons. The average population activity of all recorded neurons exhibited an initial bias toward activation (**Figure 4D**). That is, the NAcSh population activity rapidly exhibited an activation imbalance reaching a maximum peak in about 1–2 min after NPE that returned to BL levels around 32 min (*z* scores crossed 0 again), despite that these rats exhibited the highest locomotion level at this time (**Figure 4E**) and some individual neurons still exhibited a gradual ramp-up activity for at least 1 h or more (e.g., **Figure 4B**, right panel). Thus, to gain a better understanding of the population dynamics induced by NPE, we performed a PCA to describe their population trajectories (**Figures 5A,B**). We found that the first PC1 explains the most variance and captures the increasing neuronal responses, whereas the second PC2 reflects the decreasing responses. By plotting the scores of the PC1 against PC2, in 30 s bin size increments, we can now observe that, during BL, most black circles fell within the same PCA subspace for nearly 1 h (see BL overlapping black circles in the scatter plot). During the Sal epoch activity, trajectories slightly moved, however, this change seem not to be related to Sal since it occurred minutes before Sal injection and because it remained stable in the new PCA subspace for nearly 1 additional hour (see Sal labeled overlaid in green circles). More interestingly, 1 min after NPE injection, NAcSh's population activity entered in a non-physiological pharmacological brain state. Further at 32 min (orange circle and big arrow), it jumped again into a different and dynamic PCA trajectory, at the same time the average population activity had appeared to be returned to BL levels (see **Figure 4D**, i.e., excitation and inhibition seem to be canceling each other). Nevertheless, NPE-induced modulation at the single-unit level was sustained for at least an hour (**Figure 4C**), while the animal was awake and moving (**Figure 4E**). Thus, from the PCA analysis, it is evident that NPE induces a pharmacological brain state that correlated with wakefulness (**Figure 5**).

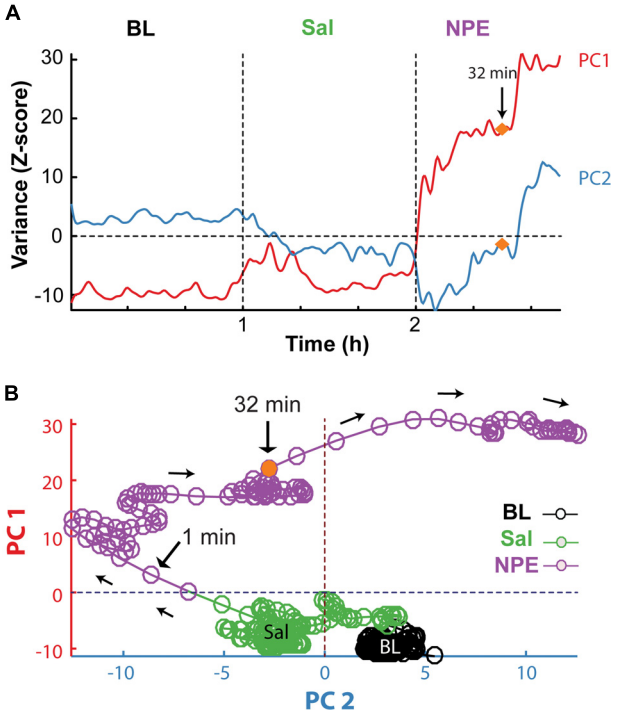

FIGURE 5 | PCA population trajectory analysis unveil that NPE induced a dynamic pharmacological brain state. **(A)** The two first principal components as a function of time computed from the normalized NAcSh population PSTH activity. The first PC1 contains the contribution of neurons exhibiting increasing responses (PC1), whereas the second PC2 reflects the activity of decreasing responses after the onset of NPE. **(B)** Population trajectories projected into two-dimensional spaces, along the PC1 (increasing responses) vs. PC2 (decreasing responses). Each circle represents the population activity (in a 30 s bin size), and the color represents different epochs; BL (black circles), Sal (green circles), and NPE (purple circles). Note that neuronal activity in BL epoch remained around an attractor state. Likewise, activity slightly changed in the Sal epoch reflecting a non-stationary activity of the NAcSh. However, they again stayed in a new attractor state. In contrast, 1 min after administration of NPE, the population trajectories entered a different position in the PCA space. In both panels, the orange circle indicates 32 min after the onset of the NPE administration, where population trajectories changed once more.

DISCUSSION

Obesity has reached epidemic proportions worldwide. The current recommendations for the treatment of obesity and overweight include physical activity and reduced caloric intake. When behavioral intervention is not sufficient, pharmacotherapy is recommended (Derosa and Maffioli, 2012; Kushner, 2018; Brett, 2019). In the present study, we found that rats treated with NPE decreased food intake showed greater weight loss and more locomotion than Sal-treated rats. Most importantly, antagonism of both DA D1- and D2-like receptors, either systemic or intra-NAcSh, partially reversed NPE-induced behavioral effects. Electrophysiological recordings further uncovered that NPE evoked a strong modulation on NAcSh's single-unit and population activity that correlated with the onset of the active awake brain state, indicative of insomnia. Our data, in rats, give further support of NPE as a robust appetite suppressant.

NPE as a Robust Appetite Suppressant

NPE was originally used in the 1970s for the short-term treatment of obesity (Zelger and Carlini, 1980; Greenway, 1992; Richert, 2011). However, there is very little information about their behavioral and neuronal responses elicited in the NAcSh. We found that NPE has a significant weight-reducing effect for the doses tested, where intermediate doses (20 and

40 mg/kg) induced the same weight loss, but at 80 mg/kg NPE was more effective than the other doses (**Figure 1**). The reason for this phenomenon is not clear, but the same results were found in humans (Hauner et al., 2017). Moreover, we observed that NPE induced less tolerance over 7 days on food intake (Nencini et al., 1996) than other appetite suppressants such as diethylpropion and phentermine (**Figure 1B**; see also Kalyanasundar et al., 2015). Our results confirm previous studies showing that NPE decreased the food intake and could lead to weight loss in both rodents and humans (Zelger and Carlini, 1980; Eisenberg et al., 1987; Kalix, 1992; Hauner et al., 2017). Furthermore, our results also agree with the findings of Schechter (1990a), who found that rats trained to discriminate against the interoceptive cues produced by cathinone or amphetamine "generalized" to NPE. Likewise, acute tolerance, i.e., tolerance after a single dose, occurs when NPE is tested 24 h after cathinone or amphetamine administration (Schechter, 1990b). The "generalization" effect depends on DA release because CGS10746B, an inhibitor of presynaptic DA release, blocked this effect. Altogether, these results raised the possibility of dopaminergic signaling nature of the NPE's cue and/or its production of tolerance (Pehek et al., 1990; Schechter, 1990a). Our findings confirm that DA D1/D2 receptors mediate NPE induced food suppression, which is in line with the idea that DA plays a major role in regulating food intake and caloric energy balance (Fernandes et al., 2020). Moreover, a state of DA dysregulation has been observed in obese rats (Geiger et al., 2009; Alsiö et al., 2010). Thus, it is tempting to propose these appetite suppressants may help to restore the lower dopaminergic tone observed in obese rats (Axel et al., 2010; Hansen et al., 2013). Taking together, the pharmacological and behavioral effects induced by NPE reflect the importance of DA signaling on feeding behavior.

D1- and D2-Like DA Receptors Are Responsible for NPE's Induced Locomotor Activity

Locomotion is a motor behavior often observed spontaneously in rodents and known to be induced by drugs such as DA agonists (Beninger, 1983; Liu et al., 1998; Schwienbacher et al., 2002). Here we demonstrated that NPE increased locomotor activity at 10 and 20 mg/kg doses, but at the high doses (40 and 80 mg/kg) reduced locomotor activity (**Figure 1C**). This inverted-U shape is a hallmark effect of amphetamine congeners on locomotion (Kalix, 1996; Perez et al., 2019). Our results are consistent with other studies demonstrating that drugs that enhance DA transmission increase locomotor activity or produce stereotypy depending on the dose (Daberkow et al., 2013). Likewise, amphetamine congeners increase the release of DA and stimulate locomotor activity at lower doses. However, at higher doses, locomotion is suppressed and replaced by stereotypy behavior in the form of head weavings (Segal and Mandell, 1974; Kalyanasundar et al., 2015; Perez et al., 2019). Behavioral studies, using systemic injections of drugs (amphetamine and cocaine), suggest that both DA D1- and D2-like receptors play an important role in locomotor activity (Dreher and Jackson, 1989; Baldo et al., 2002; Lecca et al., 2004; Knab et al., 2012; Moratalla et al., 2017). Moreover, the role of the NAc in motivated movement is well known (Meyer et al., 1993; O'Neill and Shaw, 1999; Baldo et al., 2002). For example, in rats, the administration of dopaminergic agonists promotes multiple behaviors, including locomotion, grooming, rearing, and stereotypy. Likewise, the infusion of DA or its agonists into the NAc enhances locomotor activity (Hoffman and Beninger, 1985; Dreher and Jackson, 1989; Meyer et al., 1993; O'Neill and Shaw, 1999; Baldo et al., 2002). Here we found that NPE increases locomotor activity, and the blockage of D1- and D2-like receptors, either systemic or into NAcSh, had a significant effect in NPE-induced locomotor activity (**Figures 2D**, **3D**). Similar results were found by O'Neill and Shaw (1999) demonstrating that a systemic administration of D1 antagonist SCH 23390 reduced the locomotion induced by amphetamine, cocaine, and SKF82958 (a D1 agonist). In contrast, the D2 antagonist RAC only attenuated amphetamine hyperactivity. Moreover, Baldo et al. (2002) found that ambulatory effects are blocked by injecting DA D1 and D2 antagonists into NAcSh, with a more prominent effect of DA D1 receptors than D2 on locomotor activity. Likewise, our data demonstrate that NPE induces locomotor activity via activation of both D1 and D2 receptors, but DA D1 receptors are necessary for the NPE-induced locomotion.

The food intake inhibition and increased locomotion induced by systemic administration of NPE were comparable to that induced by other amphetamine congeners, which are known to increase brain DA levels in the striatum (including the NAcSh), resulting in decreased food intake by promoting arousal, locomotion, and stereotypy (Kelley et al., 2005). Thus, it has been proposed that DA can be a neurotransmitter that mediates most pharmacological effects induced by appetite suppressants. Recently, it has been suggested that DA is also involved in the control of body weight, feeding, wakefulness, locomotion, and stereotypy (Seiden et al., 1993; Costa, 2007; Nicola, 2010; Tellez et al., 2012). Our results also suggest these appetite suppressants inhibited food intake, perhaps by promoting locomotion, a behavior that could compete with feeding (Kalyanasundar et al., 2015). To dissect the role of DA receptors, we blocked them, either systemically or intra NAcSh, and both yielded comparable results. Despite the limitations of restricting the diffusion of drugs at the NAcSh, our study points out DA receptors as important contributors to the NPE-induced locomotion and food intake suppression. Of course, our data did not preclude the participation of other brain regions in NPE's effects. In this regard, the dorsal striatum would be an interesting target to explore its participation in the stereotypy induced by these appetite suppressants (Girasole et al., 2018; Engeln et al., 2020).

NPE Evokes Neuronal Responses in the NAcSh

In the present study, we found that NPE modulated nearly 81.6% of NAcSh neurons recorded, either reducing (43.3%) or increasing (38.3%) their firing rates, with no differences in the

percentage of inactive/active neurons (**Figure 4C**, p = n.s.). Despite the similar proportion of neurons modulated with either a positive or negative sign, the NAcSh population activity exhibited a net firing rate imbalance toward activation after NPE injection (that lasted < 30 min). Likewise, previous studies have shown that amphetamine, a DA releaser (Daberkow et al., 2013), activates some NAc neurons (Sombers et al., 2009). However, we also observed neurons with reduced responses after NPE. The inactive responses could be important to maintain the net inactivation/activation balance because, after 32 min, the population activity returned to BL activity levels; this is despite individual neurons continued responding over the 1 h of recordings. Accordingly, PCA trajectory analysis uncovered that NAcSh population activity, in fact, went into a dynamic pharmacological brain state (**Figure 5**). Given that the majority of cell types composing the NAcSh are GABAergic medium spiny neurons (either MSN-D1 or MSN-D2), it is most likely that NPE modulates them. We speculate that NPE activates MSN-D1 neurons because activation of these neurons is responsible for most, if not all, motor side effects induced by increasing DA levels in the striatum (Girasole et al., 2018; Engeln et al., 2020). However, and because our extracellular recordings could not distinguish among cell types, we do not know the identity of the cells that were either excited or inhibited or whether NPE affected NAcSh's interneurons (Nicola and Malenka, 1997). Future studies should uncover what cell type(s) NPE is directly targeting.

Comparison of NPE Versus Other Appetite Suppressants

Although NPE shares some similarities with other appetite suppressants, we also found major differences between them. A similarity was that all of them lead to weight loss, decreased food intake, and stimulated locomotor activity. Thus, NPE induces anorectic effects in the same manner as other phenethylamine derivatives such as diethylpropion, phentermine, bupropion, and cathinone; this is perhaps not surprising because chemically, these substances are all structurally related to amphetamine (Khan et al., 2012). Studies in humans and in rodents revealed that amphetamine congeners produce weight loss and decreased food intake at different levels with the following strength (amphetamine > cathinone > diethylpropion ≥ phentermine > NPE > bupropion) (Zelger and Carlini, 1980; Chen et al., 2001; Cercato et al., 2009; Hendricks et al., 2009; Kalyanasundar et al., 2015, 2016; Hauner et al., 2017; Lucchetta et al., 2017; Perez et al., 2019). In summary, what they all have in common is that their pharmacological effects on weight loss and food intake require DA signaling (Balcioglu and Wurtman, 1998; Baumann et al., 2000; Chen et al., 2001; Kalyanasundar et al., 2015; Lemieux et al., 2015). Another similarity among phentermine, diethylpropion, bupropion, and NPE is that they all promote an active awake state (reflecting insomnia) and also stimulate locomotion and produce stereotypy (Eisenberg et al., 1987; Kalyanasundar et al., 2015, 2016; Perez et al., 2019). They also modulate population NAcSh's activity (Kalyanasundar et al., 2015; Perez et al., 2019). Although they modulate NAcSh activity, they do not seem to do it in the same manner (or magnitude). For example, a major difference is that diethylpropion, > phentermine, and > bupropion evoked a net inhibitory imbalance, respectively (Kalyanasundar et al., 2015). In contrast, here we found that NPE elicits a unique net NAcSh's activation imbalance and a rapid return to population BL activity levels, not seen in the other appetite suppressants. The reason for these differences is not clear, but it can be hypothesized that it reflects the different degrees with which each appetite suppressant releases DA (Baumann et al., 2000; Rothman et al., 2001; Santamaría and Arias, 2010), as well as their effects in other neurotransmitters (e.g., norepinephrine, serotonin, and acetylcholine). Thus, the different neurochemical profiles of each appetite suppressant should determine its final modulatory pattern observed in the NAcSh population activity. Nevertheless, all these appetite suppressants share a common DA signaling in the NAcSh as an important component of most, if not all, amphetamine congeners to exert their anorectic and weight loss effects.

In summary, our results, in rats, provide evidence supporting a dopaminergic mechanism of action underlying the suppression of feeding and locomotion induced by NPE, which depends on its potency to release DA that in turn stimulates D1- and D2-like DA receptors in the NAcSh.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the CINVESTAV institutional animal care and use committee.

AUTHOR CONTRIBUTIONS

BK and RG designed the research. MM and BK performed the research. CP, BA, and BK analyzed the data and made the figures. CP, BK, and RG wrote the article. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.572328/full#supplementary-material

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High-Fat Diet-Induced Weight Gain, Behavioral Deficits, and Dopamine Changes in Young C57BL/6J Mice

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Chronic exposure to a high-fat diet (HFD) may predispose individuals to neuropathologies and behavioral deficits. The objective of this study was to determine the temporal effects of a HFD on weight gain, behavioral deficits, and dopamine changes in young mice. One-month old C57BL/6J male and female mice were fed either a control diet (containing 10% calories from fat) or a HFD (containing 45% of calories from fat) for 5 months. Physiological measures such as food consumption, body weight, blood glucose, and behaviors such as motor activity, sensorimotor integration, and anxiety-like behaviors were evaluated monthly. Dopamine (DA), dopamine receptor D2 (DRD2), and dopamine transporter (DT) protein expression levels were measured in the midbrain after 5 months of dietary exposure. Results showed that body weight was significantly greater in the HFD-exposed group compared to the control-group at the end of the 4th month, while food consumption was similar in both groups. For behavioral effects, the HFD group exhibited a significant decrease in motor activity in the open field test after 3 months, and rearing frequency after 4 months of dietary exposure. The HFD group also showed deficits in sensorimotor integration after 3 months. Specifically, chronic HFD exposure increased contact time and time to remove the first adhesive tape in the adhesive-tape removal test ($p < 0.05$). Furthermore, the HFD group showed significant deficits in balance/coordination compared to the control group after 4 months of dietary exposure using the beam traverse test, and increased anxiety-like behavior tested by both the open field and light/dark box tests ($p < 0.05$). Neurochemical measurements showed that HFD-exposed mice had significantly higher midbrain DA and DRD2 protein levels compared to the control group after 5 months of dietary exposure ($p < 0.05$). These results indicate that the impact of HFD on the C57BL/6J mouse strain began at the 3rd month of dietary exposure. Behavioral deficits occurred at a similar time point as increased body weight, at about 3–4 months. Overall, this study provides a critical understanding on how HFD-induced changes in weight gain and behavioral deficits in this strain occur over time. The behavioral changes support the idea that changes also occurred in neurochemical pathways such as dopamine dysregulation.

Keywords: high-fat diet, motor coordination, sensorimotor, anxiety-like behavior, C57BL/6J mice, dopamine, dopamine D2 receptor, dopamine transporter

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INTRODUCTION

A high fat diet (HFD) has been shown to increase the risk of neurodegenerative disorders such as Alzheimer's and Parkinson's disease in humans (1, 2). Symptoms of these neurodegenerative diseases include motor and cognitive behavioral deficits resulting from a disruption in the biological functions of neurotransmitters in the brain. Dopamine (DA) is an important neurotransmitter regulating the food eating reward circuit, motor activity, and emotion (3–5). Loss of dopaminergic neurons in the brain, especially in the midbrain including the substantia nigra nuclei, is responsible for reduced motor activity, impaired motor and sensory balance, and abnormal changes to food reward circuitry (6). HFD has been shown to disrupt dopaminergic pathways and generate motor behavioral deficits, but the duration of chronic HFD exposure needed to cause these effects is unclear.

HFD has been shown to impair dopaminergic pathways in various rodent models. For example, HFD-induced insulin resistance slowed down nigrostriatal function and favored loss of DA neurons in mice (7, 8). Mice fed with a HFD show deficits in pre-attentive central information processing (9). Decreased dopamine receptor D2 (DRD2) was observed in HFD and high sugar diet-induced obese rats (10). The C57BL/6J mice fed with a high fat and sugar diet for 16 weeks showed a significant increase of DA release from the striatum, but a much slower uptake of DA compared to controls (11). Tweleve-week-old adult Sprague Dawley rats showed that 2 weeks of HFD consumption disrupted DA networks (9, 12). In comparison to controls, obesity-prone rats exhibited 42% lower striatal DRD2 density and 30% lower total dopamine transporter (DAT) total expression of DAT following 8 weeks on a HFD (13). Collectively, these studies show that chronic intake of a HFD can disrupt dopaminergic function in rodents, but the initial time point when disturbances occur is not clear.

A disruption in dopaminergic function has been positively associated with behavioral deficits (14). For example, injection of DA into the nucleus accumbens restored normal motor activity in rats with reduced motor activity from the injection of 6-hhyroxydopamine causing an 83% DA depletion (15). The loss of dopaminergic neurons and subsequent depletion of DA in the substantia nigra are known to cause the motor deficits observed in Parkinson's disease in both humans and rodent models (16–18). Dopamine D1 and D2 receptors in the hippocampus and amygdala are associated with anxiety-related behaviors. In the case of rodent studies, it has been shown that amphetamine withdrawal results in depression and anxiety-like behavior associated with dysregulation of DA (19, 20). Therefore, behavioral deficits in locomotion and anxiety are potential indicators of altered brain neuro-activity related to DA dysregulation. Other neurochemical pathways related to behavior may also be affected, or it may be HFD-induced obesity itself that is causing the deficits due to increased weight, movement problems, and anxiety.

This study aimed to assess the occurrence of motor behavior deficits over time in a young mice given chronic exposure of HFD. The central hypothesis is that HFD will induce behavioral deficits within 5 months of HFD intake which reflect neurochemical changes in the brain, possibly related to dopaminergic pathways. The choice of a 5-month timeline was based on previous research that showed significant elevations in body weight, glucose, insulin resistance, and adipose tissue weight in C57BL/6J mice after 5 months on a HFD (21). The results from this study will contribute to a greater understanding of when and how HFD contributes to behavioral deficits and changes in brain neurochemistry.

MATERIALS AND METHODS

Animals, Diets and Experimental Design

One-month-old male and female C57BL/6J mice were fed with either a control or a HFD diet ($n = 15$ including 8 male and 7 female for each dietary treatment) for 5 months. Diets were purchased from Research Diets Inc. (New Brunswick, NJ). The energy density for both control (Catalog# D12450H) and HFD (Catalog# D12451) was 4.7 Kcal/g diet. The control diet had 10% calories from the fat, while HFD had 45% calories from the fat. The main source of fat was lard. Mice were housed in individual cages in an animal facility maintained on a 12 h light (7A.M.−7P.M.)/dark cycle and proper temperature control of 24–26°C. Food consumption and body weights were measured weekly. Mice were fasted overnight for 10–12 h and blood glucose was measured from the tail blood using a Relion Prime Blood Glucose Monitoring System (Catalog# 556621084, Walmart, Bentonville, AK). Three categories of behavioral tests for motor activity, sensorimotor integration, and anxiety-like behavior, were measured at the time points shown in **Figure 1**. All behavioral tests were performed between 9 A.M. and 5 P.M. during the day and estrus was not determined in female mice. Biomedical assays for DA, DRD2, and DAT were performed at the end of the dietary exposure. The animal protocol (#18-006) was proved by the Institutional Animal Care and Use Committee at the North Carolina Agriculture and Technical State University.

Behavior Analysis
Motor Activity

Open Field Test

The open field test was conducted in Plexiglas chambers (40.6 × 40.6 cm). Motor Monitor software (Kinder Scientific, CA) was used to record motor and anxiety-like activities for 60 min. The total distance traveled and rearing frequencies were recorded as motor activities. Time spent in the center zone, distance traveled, and entries into the center zone were recorded as anxiety-like behaviors (22).

Stride Length Test

The stride length test was used to identify gait inconsistency. Reduced length of strides indicate basal ganglia dysfunction (23, 24). The stride length test was conducted separately for the front paw and rear paw. The front and rear paws were painted with red or black ink, respectively. The mice were allowed to walk on a white strip of paper (4.5 cm wide, 40 cm long) placed in an alleyway toward a dark goal box. The distance between the same paw prints were measured, and the four longest distances for each paw were averaged (24).

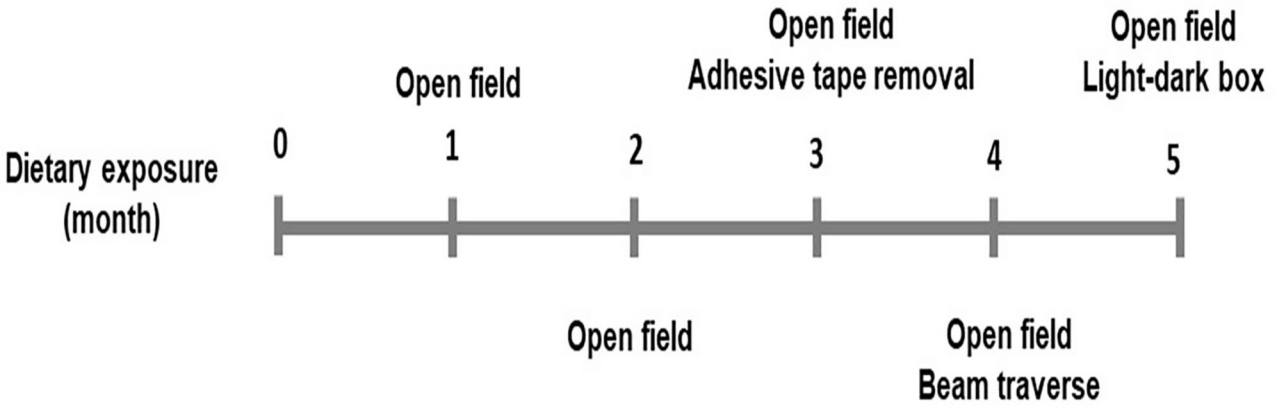

FIGURE 1 | Time points of behavior tests performed.

Sensorimotor Integration

Adhesive Removal Test

The adhesive removal test signifies accurate paw sensitivity and dexterity (25). Two different colored adhesive tapes (5 mm^2) were placed on the left and right front paws of the mouse. The time taken for the mouse to realize the adhesive tapes were on the paws was recorded as the contact time. The time it took the mouse to remove the adhesive tapes from both paws was recorded as total removal time, which included the contact time. If the mouse did not remove the adhesive tape by 120 s, the trial was terminated and 120 s was recorded as the removal time. There were 20% of HFD mice ($n = 3$) unable to remove the tape, but all control mice removed the tape within 120 s.

Beam Traverse Test

The beam traverse test analyzes fine motor coordination and balance in mice (26). The beam contained two sections, with the wider section 12 mm in width and the narrow section 6 mm in width. Each section was 1 m long. The beam was suspended 50 cm above the floor and secured to the tabletop. A black goal box was placed at the narrow end of the beam as the finishing point. The home cage nesting materials were placed in the dark goal box to attract the mouse to walk toward the finish point. A lamp was used to shine light above the starting point and served as an aversive stimulus (26). Mice were placed under the light at the wider end of the beam and trained over three trials to walk across the beam toward the narrow portion of the beam and end at the dark goal box. The fourth trial served as the test trial and was video-recorded for offline analysis. The time taken by the mouse to cross the beam was analyzed for each training trial. For the test trial, the time to cross and number of slips for the wide and narrow portions of the test were analyzed separately.

Anxiety-Like Behavior

Light-Dark Test

The light-dark box tests the levels of exploratory and anxiety-like behaviors expressed by a mouse (27). The light-dark test was conducted in the same apparatus used for the open field test, but with a black plexiglass divider that separated the apparatus into light and dark sections of equal size. The dark box included a door separator and a lamp was used to brightly illuminate the light side. Each mouse was placed in the dark side for 30 s to allow the mouse to acclimate to the dark environment. The separator wall was removed and the mouse was allowed to freely explore both compartments for 10 min. From this test, three parameters were analyzed: time spent by mice in both the light and dark sides, distance traveled by the mice on both sides, and the number of crossovers/entries into the light side. The data were extracted using the Motor Monitor software (Kinder Scientific, CA).

Brain Dopamine and Related Markers

At the end of the 5th month of dietary exposure, mice were euthanized with CO_2 followed by decapitation. The brains were removed, and the midbrain region was dissected under a dissection microscope and immediately frozen on dry ice. The midbrain region was processed for ELISA assays to detect DA (Catalog# MBS269234, MyBioSource, San Diego, CA), DRD2 (Catalog# MBS9301506, MyBioSource, San Diego, CA), and DAT (Catalog# MBS2703507, MyBioSource, San Diego, CA) levels following the manufacturer's protocols (28, 29).

Statistical Analysis

Data were analyzed using two-way ANOVA for Exposure (Control diet, HFD) and Month as a repeated measure, followed by appropriate *post-hoc* tests (Sidak's and Tukey's multiple comparison tests). In cases where behavior and biochemical data were analyzed at one time point, data were analyzed using an independent samples unpaired *t*-test. The level of significance was set at $\alpha = 0.05$.

RESULTS

Food Intake, Body Weight, and Blood Glucose

As shown in **Figure 2A**, HFD-exposed mice began to weigh significantly more than control-diet mice at the 4^{th} and 5^{th}

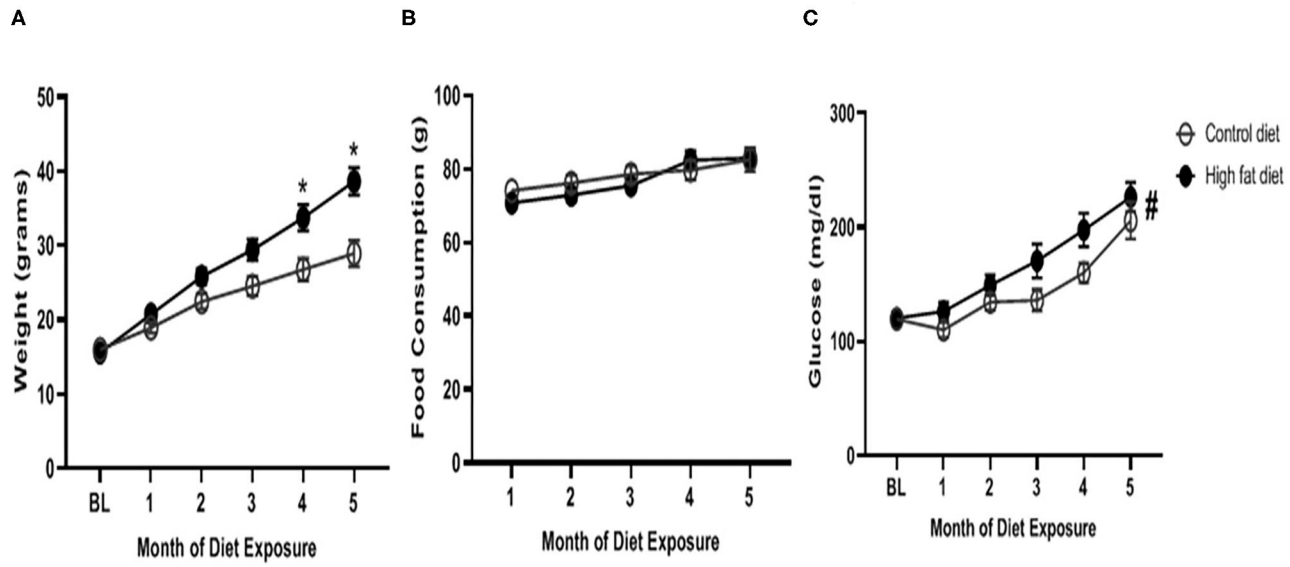

FIGURE 2 | Physiological measures of **(A)** weight, **(B)** food consumption, and **(C)** blood glucose levels across months of exposure to control diet (open circles) and high fat diet (closed circles). Data are presented as mean ± SEM. Control diet-exposed $n = 15$, High fat diet-exposed $n = 15$. * indicates a significant difference between control diet and high fat diet at that month of exposure. # indicates a main effect of diet.

months of dietary exposure as supported by an exposure by month interaction [$F_{(5, 140)} = 17.21$, $p < 0.0001$], and main effects of diet [$F_{(1, 28)} = 7.93$, $p < 0.001$] and month [$F_{(5, 140)} = 199.20$, $p < 0.0001$]. There was a trend for HFD-exposed mice to weigh more at the 3rd month of dietary exposure ($p = 0.08$). As shown in **Figure 2B**, HFD-exposed mice consumed similar amounts of diet as control-diet exposed mice at each month, however, diet consumption did increase in both groups across months as supported by an exposure by month interaction [$F_{(4, 112)} = 2.77, p < 0.05$], and main effect of month [$F_{(2.718, 76.09)} = 27.97, p < 0.0001$]. As shown in **Figure 2C**, blood glucose levels were generally higher in HFD-exposed mice compared to control diet-exposed mice as supported by a main effect of diet [$F_{(5, 140)} = 31.98, p < 0.0001$]. Additionally, blood glucose levels increased in both groups over time as supported by a main effect of month [$F_{(1, 28)} = 5.62, p < 0.03$]. The diet by month interaction failed to reach statistical significance.

Behavior Analysis

The HFD group showed significant changes in motor activity, sensorimotor integration, and anxiety-like behavior as described below.

Motor Activity

Overall, HFD-exposed mice showed decreased motor behavior across months compared to control-diet exposed mice. As shown in **Figure 3A**, HFD-exposed mice showed a significant decrease in distance traveled beginning after the 3rd month of exposure as supported by a diet by month interaction [$F_{(3, 84)} = 10.55$, $p < 0.001$], and main effects of diet [$F_{(1, 28)} = 7.30$, $p < 0.02$] and month [$F_{(3, 84)} = 109.10$, $p < 0.0001$]. Rearing behavior decreased more in HFD-exposed mice compared to control-diet exposed mice (**Figure 3B**) as supported by a main effect of diet [$F_{(1, 28)} = 13.06$, $p < 0.002$] and month [$F_{(3, 84)} = 73.62$, $p < 0.0001$]. The diet by month interaction failed to reach statistical significance. There were no changes in stride length for the front paw across months in either dietary group (**Figure 3C**) but, in general, HFD exposure decreased stride length for the rear paw (**Figure 3D**) as supported by a main effect of diet [$F_{(1, 28)} = 4.89$, $p < 0.05$].

Sensorimotor Integration

A time course for sensorimotor integration was performed but only the significantly different time point was described below. After 3 months of dietary exposure, HFD-exposed mice showed some deficits in sensorimotor integration. Specifically, chronic HFD exposure increased contact time [**Figure 4A**; $t(28) = 2.35$, $p < 0.03$] and time to remove the first adhesive tape [**Figure 4B**; $t(28) = 2.65$, $p < 0.02$]. There was no difference in total time, which included the contact time, to remove both adhesive tapes between control and HFD groups (**Figure 4C**). Twenty percent of the HFD mice reached to maximum testing time of 120 s, and this ceiling effect could contribute to no difference in total time between control and HFD groups. After 3 months, the HFD group tended to show deficits in contact time and total time to remove the first adhesive tape at 4 and 5 months, but the differences between control and HFD groups were not significant ($p > 0.05$).

Using the beam traverse test following 4 months of diet exposure, HFD-exposed mice showed deficits in balance/coordination compared to control diet-exposed mice. As shown in **Figure 5**, HFD-exposed mice showed an increase in time to cross both the wide [**Figure 5A**; $t(28) = 2.07$, $p < 0.53$] and narrow [**Figure 5B**; $t(28) = 5.23, p < 0.0001$] sides

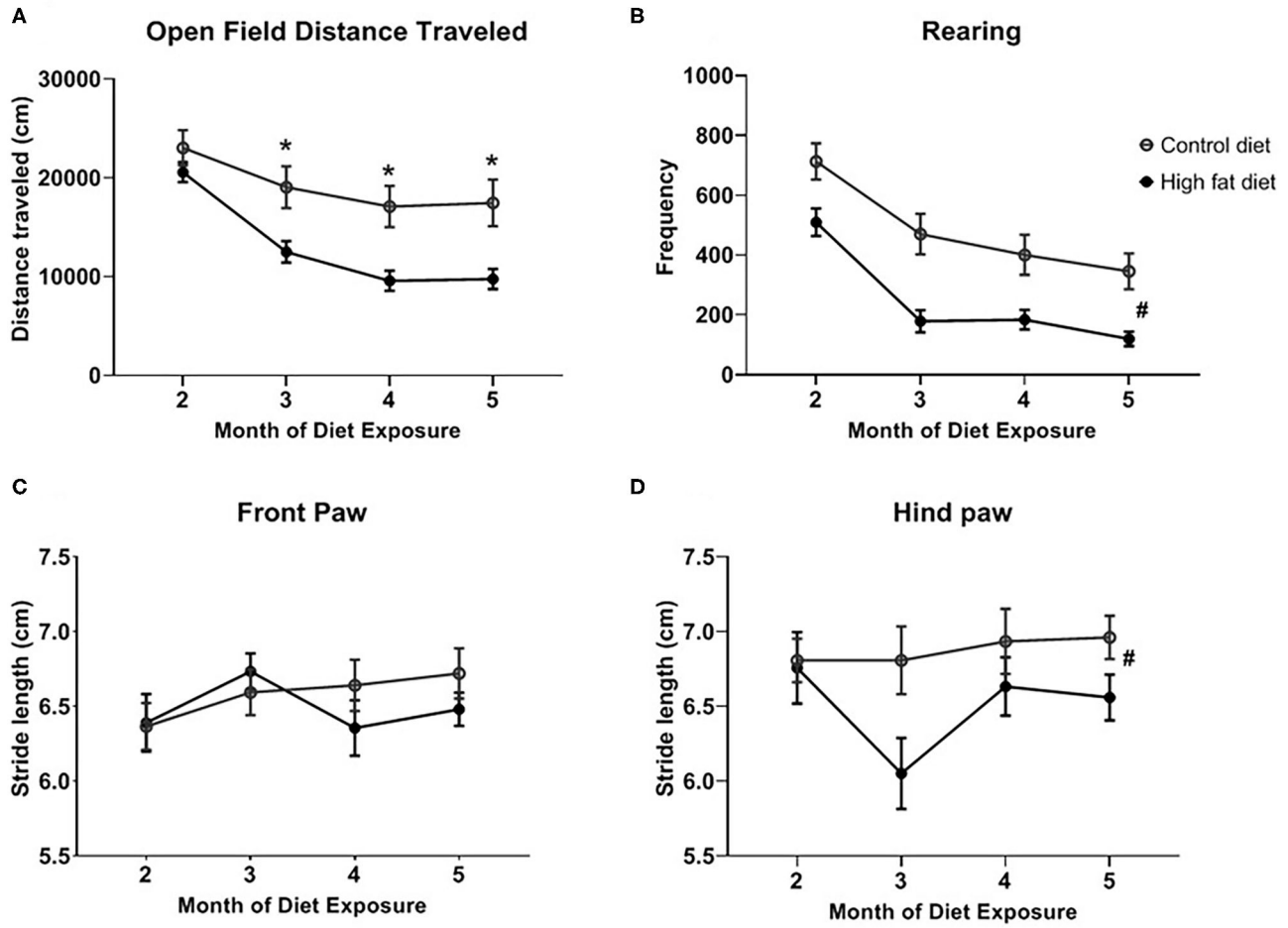

FIGURE 3 | Measures of motor behavior for **(A)** open field distance traveled, **(B)** rearing in the open field test, and stride length for the **(C)** front paw and **(D)** rear paw across months of exposure to control diet (open circles) and high fat diet (closed circles). Data are presented as mean ± SEM. Control diet-exposed $n = 15$, High fat diet-exposed $n = 15$. * indicates a significant difference between control diet and high fat diet at that month of exposure. # indicates a main effect of Diet.

of the beam. Additionally, HFD-exposed mice had a greater frequency of slips on the wide [**Figure 5C**; $t(28) = 2.85$, $p < 0.01$] and narrow [**Figure 5D**; $t(28) = 4.23$, $p < 0.0003$] portions of the beam. At the 5th month, the difference between two treatment groups was not significant ($p > 0.05$).

Anxiety-Like Behavior

Using the center zone in the open field test and the light/dark test, HFD-exposed mice showed significantly increased anxiety-like behavior compared to control diet-exposed mice after 5 months of dietary exposure. As shown in **Figure 6A**, HFD-exposed mice showed decreased time in the center zone compared to control diet-exposed mice across months as supported by a main effect of diet [$F_{(1, 28)} = 13.37$, $p < 0.003$] and a main effect of month [$F_{(3, 84)} = 6.57$, $p < 0.002$] at the end of the 5th month of dietary treatment. In the light/dark test, HFD-exposed mice spent less time on the light side [**Figure 6B**; $t(26) = 4.11$, $p < 0.0005$] and fewer entries into the light side (**Figure 6C**; $t(26) = 5.79$, $p < 0.0001$) compared to control diet-exposed mice. There was no significant difference in anxiety-like behavior during months 1–4 of the dietary exposure.

Brain Dopamine and Related Markers

Following 5 months of dietary exposure, HFD-exposed mice showed increased DA levels [**Figure 7A**; $t(18) = 3.09$, $p < 0.01$] and DRD2 receptor levels [**Figure 7B**; $t(18) = 2.12$, $p < 0.05$]. However, there were no differences in DAT levels between the groups after 5 months of dietary exposure (**Figure 7C**, $p > 0.05$).

DISCUSSION

This study aimed to investigate the early changes in behavior and brain dopaminergic function in young mice fed a HFD. Our results showed that initial signs of weight gain and behavioral deficits started at the 3rd month of HFD intake. At the end of the 5th month, the mice fed a HFD showed increased DA and DRD2 levels in the midbrain, but there was no change in DAT levels. This study contributes to the field of

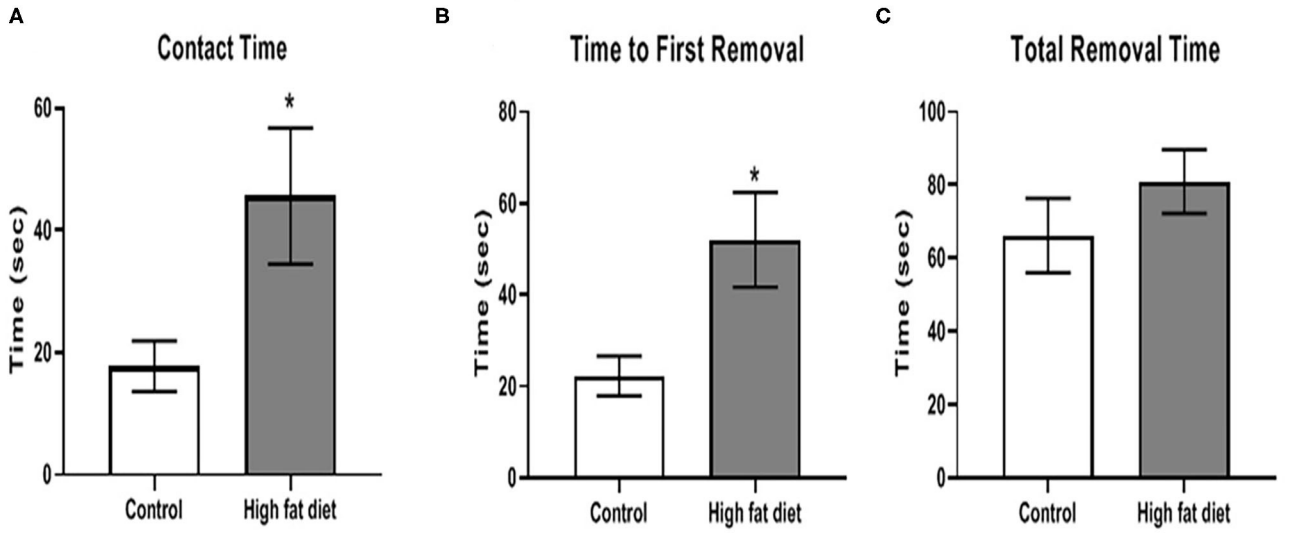

FIGURE 4 | Measures of sensorimotor integration using the adhesive removal test for **(A)** contact time, **(B)** time to removal of first adhesive tape, **(C)** total time to remove both adhesive tapes after 3 months of exposure to control diet (clear bars) and high fat diet (gray bars). Data are presented as mean ± SEM. Control diet-exposed $n = 15$, High fat diet-exposed $n = 15$. * indicates a significant difference between control diet and high fat diet.

nutrition and neuroscience because the identification of early signs of behavioral deficits may reflect initial dysregulation of dopaminergic or other neurochemical pathways, and this information is critical in understanding the progression of brain disorders induced by HFD.

The physiological data collected in this study are consistent with our previous research (21). Previously, we found that 1-month old C57BL/6J mice fed a HFD gained significantly more body weight after 4 months than mice fed the control diet, and also found no difference in food consumption between HFD and control groups and control-diet exposed groups. The increase in body weight on the HFD group is consistent with other reports. A study using young adult C57BL/6J mice (2-month-old) showed significant increases of body weight after 2 months on a HFD (30). Another study found that HFD caused young C57BL/6J mice to weigh 12.4% more than controls after 6 weeks even though the former consumed less food per day (31). Finally, a study using male Wistar rats aged 14–16 weeks also found significant differences in body weight after 4 weeks of HFD exposure (32). These results indicate that rodent models from different age groups chronically fed HFD display greater body weight compared to animals fed control diets. The rate of weight gain on any diet is always a function of age. For example, adult animals might gain weight more quickly due to their slower metabolic rate compared to younger animals (33).

Identifying the initial time point for body weight gain and motor behavioral deficits is fundamental knowledge concerning the impact of HFD in C57BL/6J mice. It may help to reveal underlying conditions of neurophysiology and behavior that are dependent on caloric intake. For example, calorically restricted C57BL/6J mice showed improved cardio-metabolic rates, hippocampus RNA expression, nutrient sensing pathways, age-dependent cognitive function, and dendritic spine density compared to mice fed a control diet (34). In contrast, HFD fed mice showed weight gain, impaired glucose tolerance (IGT), deficits in hippocampal-dependent memory/learning and mood states, and depression-like behavior (31). Results such as these indicate that caloric intake and the time course of weight gain play a crucial role in the etiology of proper brain function (or disorder).

Three categories of behavior were tested in our study as indicators of initial signs of dopamine-related behavioral activities: motor activity, sensorimotor integration, and anxiety-like behavior. Two tests were used in each category to confirm the results, and we found similar times of occurrence for behavioral deficits in each pair of tests. In the open-field test, the HFD-exposed mice showed a decrease in total distance traveled starting at the 3rd month of HFD, and decreased rearing frequency that served as a measure of exploratory behavior. Similarly, in another study, when 8-week-old adult male C57BL/6J mice have been placed on HFD diet for 3 months, they also showed significant decreases in these open-field tasks (30). In our study, both the open-field and the stride length tests support that HFD caused the initial signs of motor deficits beginning at the 3rd month of HFD exposure. These findings are important because deficits in motor activity are one of the important symptoms in many neurodegenerative diseases (35). As we mentioned previously, body weight also increased significantly in HFD mice at the 3rd month of dietary exposure. The motor behavioral deficits and increases in body weight occurred at the same time indicating that these changes may be linked.

Individuals with neurodegenerative disorders often show deficits in sensorimotor integration. Two tests were used to test sensorimotor integration in this study: the adhesive tape

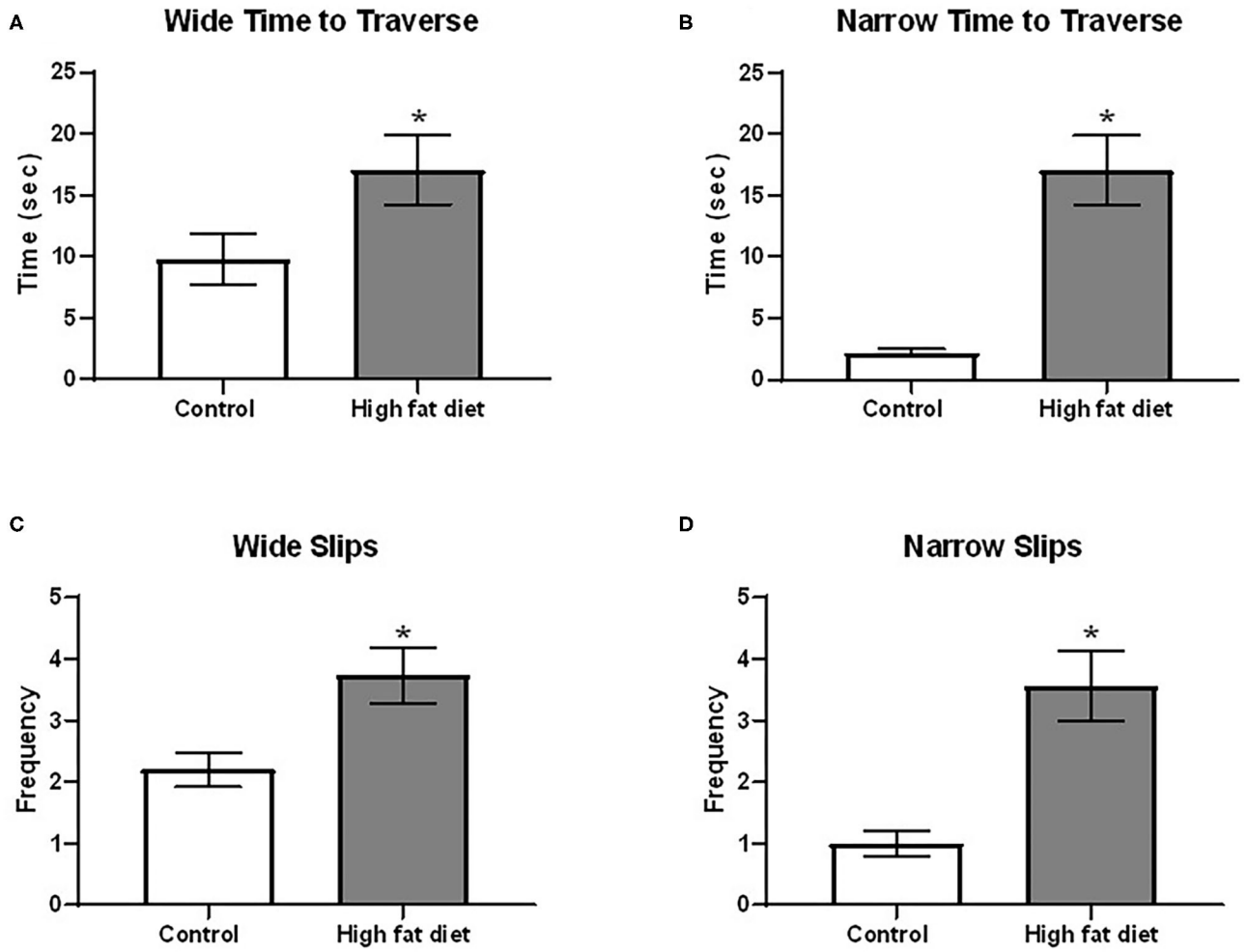

FIGURE 5 | Measures of balance/motor coordination using the beam traverse test following 4 months of exposure to control diet (clear bars) and high fat diet (gray bars). **(A)** time to traverse the wide portion of the beam; **(B)** time to traverse the narrow portion of the beam. **(C)** frequency of slips on the wide portion of the beam; **(D)** frequency of slips on the narrow portion of the beam. Data are presented as mean ± SEM. Control diet-exposed $n = 15$, High-fat diet-exposed $n = 15$. * indicates a significant difference between control diet and high fat diet.

removal and beam traverse tests. After 3 months, HFD-exposed mice showed increased time to remove the first adhesive tape from their paws. Other studies have also shown that obesity deteriorates sensorimotor integration. Following the 4th month of dietary exposure in our study, HFD-exposed mice showed deficits in balance/motor coordination in the beam traverse test. The HFD-exposed mice took a longer time to cross the beam and had a higher number of slips than the control group. Mice with destroyed dopaminergic neurons in the substantia nigra by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine spent more time crossing the beam compared to control animals (36). These results were also observed in rats. Male Sprague-Dawley rats fed a high fat and sucrose diet performed worse in removing the adhesive tape compared to the control group (37). In male Sprague-Dawley rats fed with a HFD for 3 months, the rats showed significant impairments in the beam-traverse test (38). Overall, these results showed that sensorimotor integration and balance can be disrupted after 3 and 4 months, respectively, of HFD exposure.

Mood states are also impacted by HFD. HFD has been shown to cause an increase in anxiety that is associated with increased body weight and decreased motor activity in mice (39). In adult C57BL/6J mice fed a HFD for 3 months, increased anxiety-like behavior was observed as decreased time spent in the center zone in the open field test (30). Chronic exposure to HFD has also been shown to result in depression-like behavior (31). Similarly, our study showed an increased anxiety-like phenotype demonstrated by decreased time spent in the center zone of the open field test, and less time spent on the light side in the light/dark box test. Although the light-dark box test is commonly used to test anxiety-like behavior, both the open field and light-dark box tests still rely on locomotor exploratory behavior (27). Therefore, an

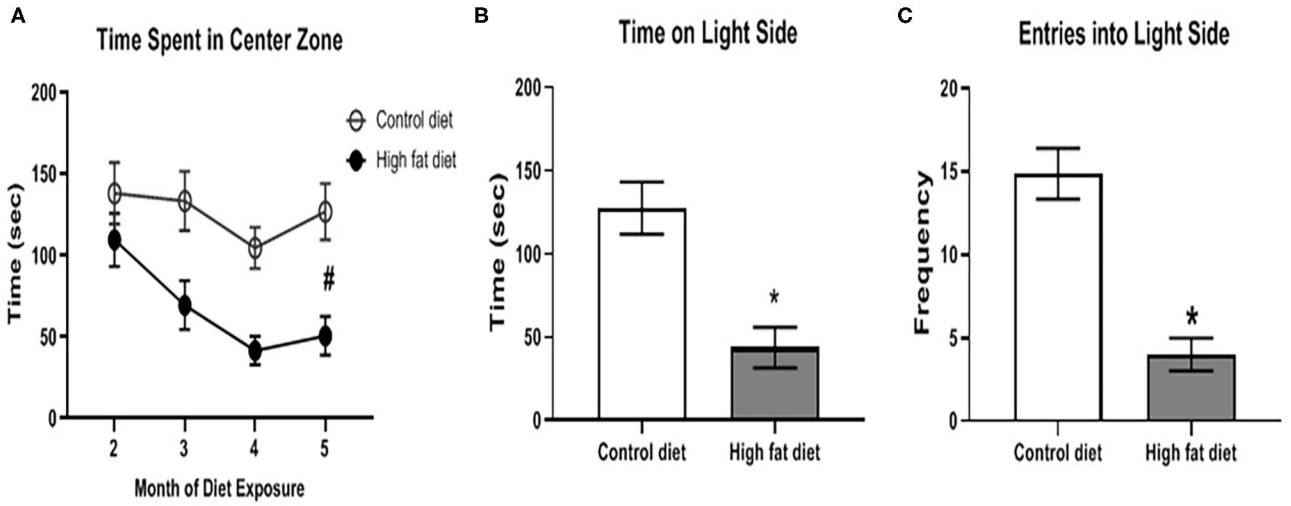

FIGURE 6 | Measures of anxiety-like behavior using the open field test and light/dark test. **(A)** amount of time spent in the center zone of the open field test across months of diet exposure. **(B,C)** amount of time spent on, and number of entries into, respectively, the light side of the two-chambered light/dark test following 5 months of diet exposure. Control diet (open circles/clear bars) and high fat diet (open circles/gray bars). Data are presented as mean ± SEM. Control diet-exposed $n = 15$, High fat diet-exposed $n = 15$. * indicates a significant difference between control diet and high fat diet; # indicates a main effect of diet.

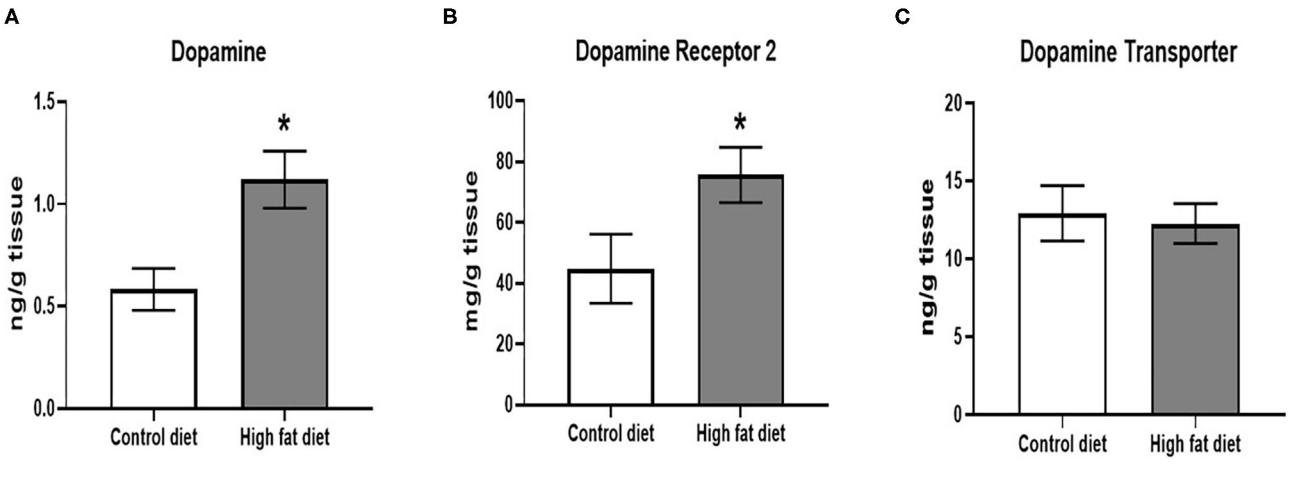

FIGURE 7 | Measures of dopamine and dopamine receptor D2 using ELISA assays. **(A)** amount of dopamine protein per gram of midbrain tissue. **(B)** amount of dopamine receptor D2 protein per gram of midbrain tissue. **(C)** amount of dopamine transporter protein per gram of midbrain tissue. Control diet (clear bars) and high fat diet (gray bars). Data are presented as mean ± SEM. Control diet-exposed $n = 10$, High-fat diet-exposed $n = 10$. * indicates a significant difference between control diet and high fat diet.

alternative explanation is that the increased anxiety-like behavior, such as less time spent in the light side of the box, was due to decreased locomotor activity induced by chronic exposure to HFD. In the future, other tests of anxiety-like behavior that do not rely on locomotor behavior (e.g., light-enhanced startle response) will be used as better indicators of anxiety.

In summary, our data showed that initial behavioral deficits and increased body weight are observed after 3 months of HFD exposure. Mice given the HFD diet exhibited significant decreases in motor activity and increases in anxiety-like behavior. However, sensorimotor integration and balance did not differ between control and HFD groups until the 4th month of exposure.

We aimed to link the behavioral deficits induced by HFD with a disturbance in midbrain dopaminergic pathways in this study. However, since we only had measurements of dopaminergic molecules at the end of the dietary exposure, we can only suggest that the decreased motor activity, decreased sensorimotor integration, and increased anxiety-like behavior were associated with dopaminergic changes. To verify an association, more time

points will be needed for a kinetic study of the expression of dopaminergic molecules in the future. We did find increased DA and DRD2 protein levels in the midbrain at the end of 5th month of HFD exposure, but the expression of DAT did not change compared to controls. A study in adult C57BL/6J mice given a HFD diet for 3 months showed significantly higher levels of DRD2 in the striatum (40). Increased striatal DA levels were also found in adult C57BL/6J mice after 4-months of a high fat and sugar diet (11). These studies supported our findings, but our model is a developmental model starting with younger, normal weight, 1 month old mice. Increased DA expression may have occurred to compensate for the HFD-induced weight gain and potentially rewarding effects of the HFD (6). However, unchanged DAT level along with increased DA and DRD2 levels in our study could imply that the dopaminergic pathway may have begun to show signs of dysfunction.

In conclusion, our study determined that young adult mice fed HFD developed signs of behavioral deficits, especially in motor activity and anxiety-like behavior. This occurs at the 3rd month of HFD exposure in association with increased weight gain. Our findings demonstrate the initial time point of significant body weight and behavioral deficits related to HFD exposure in this strain. Due to the fact that most rodent models are inbred, this initial point might differ by genetic background. However, we predict that our overall finding that early exposure to HFD leads to increased weight and associated deficits in behavior, is robust. In the future, longer exposure to HFD, more extensive biomolecular assays across associated physiological pathways and systems, increased numbers of time points and larger sample sizes, as well as testing different strains of mice will aid in a more robust detection and mechanistic modeling of behavioral deficits. We expect this would further elucidate the potential involvement of DA, DRD2, and DAT in these behavioral deficits.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Use and Care Committee (IACUC), North Carolina Agricultural and Technical State University.

AUTHOR CONTRIBUTIONS

JH and AM-D designed the experiments, analyzed the data, and wrote and revised the manuscripts. PN who was a graduate student carried out the behavioral experiments, and analyzed the behavioral and physiological data. AO who was a graduate student carried out the biomedical experiments and analyzed the biomedical data. FF and DB were undergraduate students who performed the behavioral experiments and animal husbandry. JG provided general project guidance and support and assistance with the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Western Diet Consumption During Development: Setting the Stage for Neurocognitive Dysfunction

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The dietary pattern in industrialized countries has changed substantially over the past century due to technological advances in agriculture, food processing, storage, marketing, and distribution practices. The availability of highly palatable, calorically dense foods that are shelf-stable has facilitated a food environment where overconsumption of foods that have a high percentage of calories derived from fat (particularly saturated fat) and sugar is extremely common in modern Westernized societies. In addition to being a predictor of obesity and metabolic dysfunction, consumption of a Western diet (WD) is related to poorer cognitive performance across the lifespan. In particular, WD consumption during critical early life stages of development has negative consequences on various cognitive abilities later in adulthood. This review highlights rodent model research identifying dietary, metabolic, and neurobiological mechanisms linking consumption of a WD during early life periods of development (gestation, lactation, juvenile and adolescence) with behavioral impairments in multiple cognitive domains, including anxiety-like behavior, learning and memory function, reward-motivated behavior, and social behavior. The literature supports a model in which early life WD consumption leads to long-lasting neurocognitive impairments that are largely dissociable from WD effects on obesity and metabolic dysfunction.

Keywords: obesity, high fat diet, adolescent, anxiety, cognition, memory

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INTRODUCTION

Children in the United States are exposed to a dietary environment where there is an overabundance of highly palatable foods that are easily affordable and readily accessible. Observations from earlier National Health and Nutrition Examination Surveys (2003–2004, 2005–2006) report that the highest sources of energy for 2- to 18-year-olds were grain desserts, pizza, and soda, which are low in beneficial nutrients, but high in solid fats and/or added sugars (Reedy and Krebs-Smith, 2010). More recent data indicates that consumption of saturated fat and sugar in children continues to exceed the recommended limit of fewer than 10% of total calories for anyone 2-years-old or older, as boys and girls (age 1–18) obtain a range of about 11–12% of their total calories on average from saturated fat and a range of about 11–17% of their total calories on average from added sugar (health.gov, 2015). This type of dietary environment, along

with a shift towards larger food portions, has undoubtedly contributed to the alarming increased prevalence of childhood obesity, which is now approximately 18% in children aged 2–19 years (CDC, 2019). In addition, the majority of children with obesity remain obese, both as adolescents and as adults (Simmonds et al., 2016). Emerging evidence reveals that both childhood and adult obesity are associated with impaired performance in various cognitive tasks (Morris et al., 2015; Wang et al., 2016; Dye et al., 2017). However, given that obesity is strongly associated with consumption of a Western Diet (WD; specified in more detail below), a standing question arises as to whether the WD *per se* may impart neurocognitive dysfunction independent of obesity and/or its associated metabolic impairments. Indeed, evidence from both humans and preclinical rodent models described herein indicates that habitual consumption of a WD during early life developmental periods can lead to long-lasting neurocognitive dysfunction even independent of obesity and severe metabolic dysfunction (Francis and Stevenson, 2013; Noble and Kanoski, 2016). Thus, in order to better inform policies relating to dietary recommendations, it is imperative to understand the dietary and neurobiological mechanisms linking perinatal and childhood WD consumption with impaired cognitive abilities throughout the lifespan.

To study the link between WD patterns during early life periods and neurocognitive development, rodent models are often used to target discrete periods of development during which dietary components can be administered with rigorous control and with objective quantification of the amount of calories consumed. The perinatal period in rodents, lasting from gestation to weaning (weaning at ∼postnatal day [PN] 21–24), is a time during which maternal exposures can have lasting effects on cognitive processes. Indeed, the perinatal developmental stage is a critical period for neuronal programming of regions involved in learning and memory, such as the medial prefrontal cortex (mPFC) and the hippocampus (HPC) (Reynolds et al., 2017; Sharp and Lawlor, 2019). Although the exact timing varies slightly by strain, in rats PN 22–27 is considered the approximate juvenile stage of development, PN 28–42 equivalent to the early-mid adolescent period (∼12–17 years in humans), and PN 43–55 comparable to the late adolescence/emerging adulthood period in humans (∼18–25 years) (Spear, 2016). The juvenile and adolescent phases of development are critical periods through which complex cognitive abilities such as working memory, sociability, and inhibitory control develop (Semple et al., 2013).

In laboratory rodents, several different dietary manipulations have been used to model aspects of the WD. A rodent "high fat diet" model typically involves increasing the amount of fat (as a % of total kcal, e.g., 45% or 60% kcal from fat) while reducing the amount of carbohydrates compared to low fat and high carbohydrate standard rodent control diets. However, the carbohydrate content of a common rodent high fat diet is predominantly comprised of simple sugars, vs. the complex polysaccharide-based carbohydrate content of a typical rodent control diet (with the exception of some low-fat control diets that are high in sucrose). Another common rodent WD model is a cafeteria diet, which is a free-choice diet with combination of highly palatable, energy dense foods (e.g., high saturated fat, high sugar) that are commonly consumed by humans. Modeling the obesogenic environment omnipresent in modern Westernized cultures, these diets are provided in the home cages and are therefore easily accessible to the animals. Relative to a control group on a healthy diet, rodents exposed to these WD models may, but do not always, display one or more of the following outcomes associated with metabolic syndrome and obesity: increased caloric intake, body weight gain, increased adiposity, hyperinsulinemia, hyperglycemia, glucose intolerance, and inflammation contributing to hepatosteatosis (Sampey et al., 2011).

While commonly referred to as "diet-induced obesity" (DIO) models in the literature, rodent WD models lead to differential metabolic and body weight outcomes based on species (e.g., rats vs. mice), strain, sex, and/or age and duration of dietary consumption. For example, the same dietary manipulation may lead to obesity and metabolic dysfunction in adult male rats, but not in adolescent female rats. Thus, in this review we will refer to these dietary models as "WDs" and not DIO, and we further define a WD as a rodent model with access to either a diet high in fat (greater than 30% of total kcal from fat), a diet with carbohydrate consumption coming predominantly from monosaccharides (glucose, fructose) or disaccharides (sucrose), or more commonly, a combination of the two (as in the examples described in the previous paragraph).

Herein we review insights from carefully controlled rodent studies that inform on the impact of WD consumed during early life developmental stages on various cognitive domains, including anxiety-like behavior, learning and memory function, reward-motivated behavior, and social behavior (**Supplementary Table 1**). A second overarching goal of the review is to describe potential underlying neurobiological mechanisms linking dietary models with cognitive outcomes, and thus we focus exclusively on preclinical rodent models as these models offer a distinct advantage in this regard. Perinatal exposure will refer to maternal WD consumption during gestation, lactation, or both, with additional focus on prenatal exposure in Section 5. Given that the overwhelming majority of rodent model studies do not distinguish between the juvenile and adolescent stages, we use the term "adolescence" to refer to the developmental period from weaning until early adulthood, which in rodents is approximately postnatal (PN) days 21–60. We also describe neurocognitive results (behavioral, molecular) with regards to whether or not these outcomes were accompanied by obesity, thus leading to a concluding framework in which early life WD effects on cognition are largely dissociable from effects on body weight and metabolism.

ANXIETY-LIKE BEHAVIOR

Behavioral Models and Neural Substrates

While anxiety can be an adaptive emotional response to stressful situations, excessive and/or chronic anxiety can have detrimental health effects in humans and manifest as a clinical anxiety disorder (Fox and Kalin, 2014; Sharp et al., 2015;

Juruena et al., 2020). In humans, a number of lifestyle factors are associated with anxiety, including diet. For instance, consumption of added sugars and saturated fat is associated with higher anxiety levels (Masana et al., 2019; Fatemi et al., 2020), and evidence from carefully controlled rodent experiments suggests a causal relationship between diet and anxiety-like behavior. In rodents, anxiety-like behavior is assessed via measurable behavioral changes, including measures of exploration, hypoactivity, suppressed consumption of novel foods (neophagia), and fear-associated freezing behavior. One common assessment of anxiety-like behavior is the open field (OF) test, where increased thigmotaxis, or time spent by the walls of an enclosed apparatus, is indicative of increased anxiety-like behavior. Other common anxiety-like behavioral tests are the elevated plus maze (EPM) procedure, and the conceptually similar elevated zero maze (EZM). Each test is rooted in the positive drive for rodents to explore novel environments, as well as the drive to avoid exposed areas without walls or enclosures. Spending more time in the enclosed arms while making relatively few and infrequent crossings to the open arms of the EPM or EZM is indicative of anxiety-like behavior. Other common assessments of anxiety-like behavior include the novelty suppressed feeding (NSF) task, which measures a rodent's aversion to eating in a novel environment, the social interaction test, where decreased time spent engaging in social behavior is indicative of anxiety-like behavior, and the light-dark transition task, where a decrease in the willingness to explore the illuminated, unprotected area of the apparatus is suggestive of anxiety-like behavior (Bailey and Crawley, 2009). Finally, measuring corticosterone plasma levels following a stressor (e.g., restraint stress) provides a read-out of hypothalamic-pituitary-adrenal (HPA) axis reactivity, which tends to be heightened in anxiety (Packard et al., 2016).

A dietary influence on anxiety may be indicative of lasting changes to brain structures involved in anxiety-like behavior. Briefly, anxiety-like behavior is mediated by a network of brain regions (Adhikari, 2014; Calhoon and Tye, 2015) that is still incompletely understood, but includes the basolateral amygdala (BLA) (Singewald et al., 2003; Hale et al., 2008), the ventral HPC (Nascimento Häckl and Carobrez, 2007; Lowry and Hale, 2010), and the mPFC (infralimbic and prelimbic areas) (Kim et al., 2011; Jiao et al., 2015; Liu et al., 2020). In particular, BLA inputs to the ventral HPC is associated with anxiolytic behavior (Pi et al., 2020) whereas inputs from the ventral HPC to the lateral hypothalamic area (LHA) has been shown to mediate anxiogenic behavior (Jimenez et al., 2018). Additionally, excitation of the BLA terminals in the central amygdala (CeA) is associated with anxiolytic behavior (Tye et al., 2011) whereas CeA projections to the bed nucleus of the stria terminalis are associated with anxiogenic behavior (Ahrens et al., 2018). Finally, brainstem regions are also involved in the control of anxiety-like behavior, with the locus coeruleus being associated with anxiogenic behavior (Itoi and Sugimoto, 2010; McCall et al., 2017). The following sections describe several rodent studies that investigate both the impact of WD on anxiety-like behavior and putative underlying neurobiological mechanisms. Interestingly, several studies discussed below reveal that WD consumption affects similar brain regions in rodents as previously described to be associated with anxiety in humans (Bas-Hoogendam et al., 2017; Besteher et al., 2017, 2020).

Perinatal WD Exposure

Studies on the effects of perinatal exposure to WD suggest increased anxiety-like behavior in offspring (Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012; Sasaki et al., 2013; Glendining et al., 2018; Guedine et al., 2018; Winther et al., 2018). For example, when rodent dams are fed a high dietary fat composition (60% kcals fat) before mating until weaning of the offspring, the male (Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012; Sasaki et al., 2013; Winther et al., 2018) and female (Peleg-Raibstein et al., 2012; Sasaki et al., 2013; Winther et al., 2018) progeny as adults display increased anxiety-like behavior in the EPM apparatus and in a food neophobia task (Peleg-Raibstein et al., 2012) relative to progeny born to dams that received a low-fat diet. In mice, perinatal exposure to diet with a lower fat content (45% kcals fat) also resulted in greater anxiety-like behavior in the EPM at adulthood, especially in females (Glendining et al., 2018). Similarly, male and female offspring from dams consuming a 60% fructose diet presented an anxiety phenotype in the EZM when tested during early adolescence (PN 26–34) (Bukhari et al., 2018). Interestingly, these findings can occur independent of the potential obesogenic effects of the maternal diet on the offspring, as weight gain was not observed in adulthood in one study that found increased anxiety-like behavior in the OF and EPM tasks in adult male and female offspring after perinatal exposure to a 60% kcals fat diet (Sasaki et al., 2013).

While an obesogenic phenotype is not necessary for the development of anxiety-like behavior, the duration of the maternal diet may be an important factor. For instance, if a cafeteria diet is provided to rat dams strictly during lactation only (PN 1–21), the male offspring do not display anxiety-like behavior in the EPM during adulthood (Guedine et al., 2018). This occurs despite hyperphagia and significant weight gain and greater adiposity in the offspring, thus further supporting the notion that while obesity is not a requirement for effects of perinatal WD on anxiety-like behavior, the diet duration from gestation to lactation is critical. In some cases, maternal consumption of a cafeteria diet only during lactation has an anxiolytic impact (decreasing anxiety) on male and female offspring behavior at weaning (Speight et al., 2017) or 10 weeks of age (Wright et al., 2011). The anxiolytic effects associated with maternal cafeteria diet during lactation only may be based on maternal behavior, as Speight and colleagues (Speight et al., 2017) observed enhanced licking and grooming of pups by WD-fed dams.

In addition to the effect of perinatal WD exposure on anxiety-like behavior, studies suggest that maternal WD consumption throughout gestation and lactation may impact the HPC and amygdala, brain regions that are strongly linked with anxiety. For example, WD-associated anxiety-like behavior is accompanied by increased expression of 5HT-r1a and GABAa alpha2 receptor subunit in the ventral HPC (Bannerman et al., 2004), as well as elevated brain-derived neurotrophic factor (BDNF) expression in the dorsal HPC (Peleg-Raibstein et al., 2012), a region

where BDNF levels correlate with the magnitude of anxiety-like behavior in the EPM task in wildtype mice (Yee et al., 2007). Exploration of the open arm of the EPM apparatus (decreased by WD) also correlates negatively with HPC gene expression for inflammatory markers TNFa and MCP-1 (Winther et al., 2018). Pups perinatally exposed to WD have elevated hippocampal microglial activation at birth, as demonstrated by increased expression of CD11b, a microglial activation marker, and TLR4, an endogenous pattern recognition receptor involved in metabolic-inflammatory signaling (Bilbo and Tsang, 2010). Additionally, pups exposed to a perinatal WD also show increased circulating peripheral cytokine expression (IL-1β in the liver and serum IL-6) during adulthood (Bilbo and Tsang, 2010). In the amygdala, perinatal WD elevates mineralocorticoid and glucocorticoid receptors during adulthood in rats, possibly due to an overall heightened HPA axis response to stress supported by the elevated basal corticosterone levels also seen in the adult rats (Sasaki et al., 2013). Taken together these findings suggest that perinatal exposure to a WD promotes inflammatory processes and alters stress responsivity markers. Indeed, others have found that maternal obesity is associated with increased inflammatory signaling during pregnancy that likely impacts the development and health of the offspring (Segovia et al., 2017), and that the HPA axis during development is vulnerable to maternal nutrition and/or metabolic status (Long et al., 2012; Balsevich et al., 2016). Collectively, these findings suggest that maternal WD consumption impacts the brain in a multitude of ways that may impact anxiety-like behavior, including increasing inflammatory signaling pathways, modification of the serotonergic, GABAergic, and neurotrophin signaling systems, and elevating the HPA axis responsiveness. Further research is necessary to determine the extent to which these neurobiological changes are causally related to the impact of perinatal WD exposure on anxiety-like behavior.

While the majority of the studies described above found anxiogenic effects (increased anxiety) as a consequence of perinatal WD exposure, some have reported mixed results in various anxiety measures (Sasaki et al., 2014; Zieba et al., 2019). For example, perinatal exposure to a 60% fat diet reduced anxiety-like behavior in the light-dark box while having anxiogenic effects in the EPM and OF tasks (Sasaki et al., 2014). Similarly, although anxiety measures in the OF and NSF tests were unchanged by maternal consumption of a WD, a trend for increased open arm time in the EPM was observed at adulthood (Zieba et al., 2019). Such findings raise the question as whether certain behavioral assays are more sensitive to the anxiogenic impact of perinatal WD consumption.

In contrast to the anxiogenic effects associated with perinatal WD exposure described above, maternal exposure to a WD may be anxiolytic (decreasing anxiety) for the offspring in the presence of perinatal stress. For example, rat offspring that underwent maternal separation, which normally induces anxiety, did not display increased anxiety-like behavior in the OF test if the dams consumed a WD (40% kcals fat) from gestation to postpartum day 21 (Rincel et al., 2016). These results were accompanied by a WD-associated prevention of maternal separation-associated changes in the expression of several genes in the PFC that are linked with abnormal anxiety-like behavior in adulthood, including BDNF and 5HT-r1a (Rincel et al., 2016). Moreover, the maternal separation-induced upregulation of Rest4 in the PFC, whose expression is associated with anxiety in adulthood (Uchida et al., 2010), was reversed with perinatal WD (Rincel et al., 2016). These data suggest a potential relationship between perinatal WD and stress on anxiety-like behavior later in life.

Adolescent WD Consumption

Similar to perinatal exposure, adolescent consumption of WD can lead to increased anxiety-like behavior. For example, adolescent male rats that consumed a cafeteria diet consisting of 45% fat, a 15% weight by volume (w/v) sucrose solution, and standard chow during adolescence displayed increased anxiety-like behavior in the EPM during adulthood (Ferreira et al., 2018). Similarly, consumption of a 45% fat diet combined with a 10% w/v sucrose solution for 8 weeks promoted anxiety-like behavior in adulthood (Gancheva et al., 2017). In male mice, 7 weeks of exposure to a 60% fat diet enhanced anxiety-like behavior in both the EPM and the OFT (Yang et al., 2020). Male, but not female, adolescent rats given free access to chocolate cookies (high in both fat and sugar) presented an elevated anxiety phenotype in the EPM as well as greater plasma corticosterone levels following restraint stress (Kim et al., 2018). Similarly, enhanced HPA axis reactivity was also observed in male rats fed a lard-based high fat diet (60% kcals fat) from adolescence through adulthood, although no diet effects were reported in the OF task (Abildgaard et al., 2014). In addition, ad lib access to a 5% w/v sucrose solution in adolescent male rats from PN 30–46 was sufficient to induce an anxiety phenotype in the NSF task months later when tested during adulthood (PN 204) (Gueye et al., 2018), and male rats that consumed a 10% w/v sucrose solution from PN 25–50 also displayed anxiety-like behavior in the OF test in adulthood (PN 75) (Kruse et al., 2019). However, the aforementioned study also showed that the long-term effect of increased anxiety-like behavior was not seen in adult male rats that received the 10% w/v sucrose drink from PN 75–100, highlighting early life as a critical time period during which Western dietary patterns influence anxiety-like behavior (Kruse et al., 2019). Together, these studies support that, similar to perinatal WD consumption, exposure to WD factors during early adolescence generally promotes anxiety-like behavior in adulthood.

Similar to effects associated with perinatal WD, anxiety-like phenotypes are observed independent of weight gain and obesity outcomes caused by adolescent WD consumption. For example, rats that consumed a cafeteria diet consisting of 45% fat, a 15% w/v sucrose solution, and standard chow during adolescence had significantly increased caloric intake and body weight (Ferreira et al., 2018), but consumption of a marginally high fat diet (21.1% from fat) from 1 to 5 months old in male mice did not result in differences in body weight relative to controls (Vinuesa et al., 2016). However, both studies found that these rodents developed anxiety-like behavior in adulthood after consuming the WD. In addition, the increased anxiety-like behavior discussed above in adolescent male rats consuming a lard-based high fat diet (45% kcals fat) with a 10% w/v sucrose solution for 8 weeks was associated with features of the metabolic syndrome such as reduced insulin sensitivity,

hypercholesterolemia, hypertriglyceridemia and greater visceral adiposity (Gancheva et al., 2017) despite no differences in body weight, thus implying a potential role for metabolic impairments rather than increased body mass *per se*.

While weight gain may be less relevant to the development of anxiety-like behavior associated with adolescent WD consumption, WD may be contributing to anxiety-like behavior by affecting neurobiological processes in the HPC, the nucleus accumbens (ACB) and the mPFC. For example, Ferreira and colleagues (Ferreira et al., 2018) found that anxiety-like behavior induced by adolescent WD consumption is associated with reduced neurogenesis in the subgranular region of the dentate gyrus. In accordance, cell proliferation was diminished in the dentate gyrus of adult rats exposed to a sucrose solution during adolescence (Gueye et al., 2018). Kim and colleagues (Kim et al., 2018) observed anxiogenic phenotype associated with chocolate cookies consumption that coincided with increased BDNF expression in the ACB, a feature reminiscent of rodent stress-induced depression models (Eisch et al., 2003). Male rats fed a 60% fat diet for 7 weeks displayed greater senescence-related gene expression in the mPFC, especially in astrocytes and microglia (Yang et al., 2020). Furthermore, Kruse and colleagues (Kruse et al., 2019) found that anxiety-like behavior seen in male rats that consumed a 10% w/v sucrose solution during adolescence may be explained, in part, by increased mPFC, but not ventral HPC, expression of calretinin, an important developmental calcium-binding protein that is increased after stressful situations such as maternal separation (Xu et al., 2011) and whose protein expression is usually reduced in adulthood (Caballero et al., 2014). Importantly, differences in calretinin expression were not observed in males that consumed the high sucrose diet during adulthood, suggesting that excessive sucrose consumption during adolescence impacted normal calretinin development. Despite these findings, more research is needed to identify the physiological relevance of each of these candidate pathways to early life WD-induced effects on anxiety-like behavior.

While many studies show that WD consumption during adolescence generally promotes anxiety-like behavior, it should be noted that there are instances where an elevated anxiety phenotype did not develop after consumption of WD. For example, brief exposure (11 days) to a 41% fat diet initiated at adolescence (PN31) had no impact on anxiety-like behavior in the EPM (Vega-Torres et al., 2020). In male mice, prolonged consumption of a 45% fat diet failed to induce behavioral changes in the OF test and the EPM, when testing occurred after 8 and 10 weeks of diet, respectively (Del Rio et al., 2016). Similarly, intake of a 55% fructose diet initiated at adolescence did not alter anxiety measures in the OF and EPM tests, although basal corticosterone plasma levels were increased after 10 weeks on the diet in male rats (Harrell et al., 2015). One study showed that a cafeteria diet resulted in anxiolytic behavior in adulthood in the OF and EPM test in male and female rats when fed from weaning until early adulthood (3–11 weeks old) (Lalanza et al., 2014). Results revealed that the cafeteria diet increased adiposity and metabolic disturbances, such as hypertriglyceridemia, hyperglycemia and insulin resistance, in both males and females. However, a 1-week removal of the cafeteria diet during adulthood led to increased anxiety in the OF test, suggesting that cafeteria diet withdrawal, but not the cafeteria diet itself, can prompt anxiety-like behavior (Lalanza et al., 2014). However, additional studies are needed given that anxiety-like behavior can still be seen in animals that still consume a WD into adulthood (Vinuesa et al., 2016). Other studies have shown that excessive sugar consumption (11% w/v sucrose or high fructose corn syrup solution) during adolescence has no impact on anxiety-like behavior during adulthood in male rats in the EZM (Hsu et al., 2015; Noble et al., 2019). Whether or not anxiety-like behavior is developed after chronic sugar access may depend on the time of testing following sugar removal. For instance, Kruse et al. (2019) found anxiety-like behavior in male rats following 25 days of 10% w/v sucrose removal, whereas Noble et al. (2019) did not find any differences in anxiety-like behavior almost 4 months after removal of an 11% w/v high fructose corn syrup solution. Interestingly, using a similar experimental design to Noble et al., Hsu et al. saw no differences in anxiety like behavior in rats fed either 11% sucrose solution or 11% HFCS solution when testing occurred with no delay following sugar consumption (Hsu et al., 2015). Altogether, these studies suggest that withdrawal from WD may in part explain the increased anxiety-like behavior seen in rodents, although this effect may depend on the type of WD (sugar, high-fat, or combination of the two) and the anxiety-like phenotype may be alleviated given significant time consuming a healthy control diet.

Similar to effects associated with perinatal WD, consumption of WD during adolescence in rodents may reduce anxiety in circumstances associated with early life stress (ELS), fostered by either maternal separation, restraint, social isolation, or a disrupted nest anywhere from PN 2–28. For instance, in male rats, consumption of a high sucrose WD during social isolation from PN 21–28 resulted in reduced anxiety-like behavior in the OF and EPM tests at PN 28 relative to animals that received standard chow and animals that received stress without the WD (Marcolin et al., 2012). Similarly, social anxiety was attenuated in male rats maintained on a WD (45% kcal from fat) after weaning after having previously undergone a 3-day ELS test from PN 27–29 where on each day they were subjected to either forced swim, elevated platform stress, or restraint adulthood (Ali et al., 2018). In male rats subjected to limited nesting from PN 2–9, free access to a high fat/high sucrose diet (43% kcals fat, 17% kcals protein and 40% kcals from sucrose) initiated at weaning also prevented the expression of an anxiety phenotype at adulthood (Maniam et al., 2016). Similarly, female rats subjected to ELS from PN 2–14 also display reduced anxiety, as assessed by EPM in adulthood, following consumption of a continuous cafeteria-style WD (32% kcals from fat) (Maniam and Morris, 2010). Thus, similar to what occurs with perinatal WD, these findings suggest that the relationship between dietary factors and anxiety in adolescents interacts with the effects of ELS. However, this is not always the case, and may be stressor- or age-dependent. For example, exposure to predator stress during adulthood in male rats fed a WD since weaning exacerbated anxiety behaviors in the EPM and OF tests (Kalyan-Masih et al., 2016).

While the underlying mechanisms for the anxiolytic effects of WD consumption in cases of ELS are incompletely understood, WD consumption, either during or following ELS, resulted in rats consuming more food (Marcolin et al., 2012; Maniam et al., 2016), gaining more weight (Maniam and Morris, 2010; Marcolin et al., 2012; Maniam et al., 2016), having increased adipose tissue (Maniam and Morris, 2010; Ali et al., 2018), higher plasma glucose levels (Marcolin et al., 2012), and elevated plasma leptin and insulin (Maniam and Morris, 2010). Normally ELS will result in an elevated corticosterone response, however, consumption of WD following ELS reduces the corticosterone response in adulthood (Maniam and Morris, 2010; Ali et al., 2018). The reduced corticosterone response may be related to the normalized hypothalamic corticosterone releasing hormone mRNA and reduced hippocampal glucocorticoid receptor gene expression seen in adulthood following consumption of a WD in females (Maniam and Morris, 2010), although hippocampal glucocorticoid receptor protein expression was increased in males (Maniam et al., 2016). Moreover, WD may prevent an imbalance of antioxidant enzymes in the PFC (Marcolin et al., 2012) or lead to increased D1R and D2R mRNA expression in the ACB, suggesting that dopamine signaling may also have a role in protecting against stress-induced anxiety-like behavior (Ali et al., 2018). Importantly, these studies suggest that ELS in combination with a WD prevents anxiety-like behavior despite leading to a compromised metabolic phenotype, as demonstrated by weight gain, adiposity, and elevated plasma insulin, leptin, and glucose levels. Collectively, these studies reveal that WD consumption may function as a reaction to stress that can relieve anxiety-like behavior associated with ELS.

Summary

The development of anxiogenic or anxiolytic behavior in association with early life WD exposure is likely dependent on whether or not the WD is accompanied by ELS (**Figure 1**). More specifically, evidence suggests that exposure to WD, either perinatally or during adolescence, is associated with increased anxiety-like behavior during adulthood unless the rodents undergo a period of ELS, in which case the diet may reduce anxiety like behavior. Therefore, WD consumption may be a coping mechanism in response to ELS. Notably, while obesity is often not observed following early life WD consumption, in the cases of ELS, obesity and metabolic dysfunction are often present in those consuming WD factors, thus further highlighting that WD influences on anxiety are not directly tied to the presence vs. absence of obesity and associated comorbidities. Further research is needed to determine the precise mechanisms through which early life WD factors, either with or without ELS, impact the brain and responsivity to stress during adulthood.

LEARNING AND MEMORY

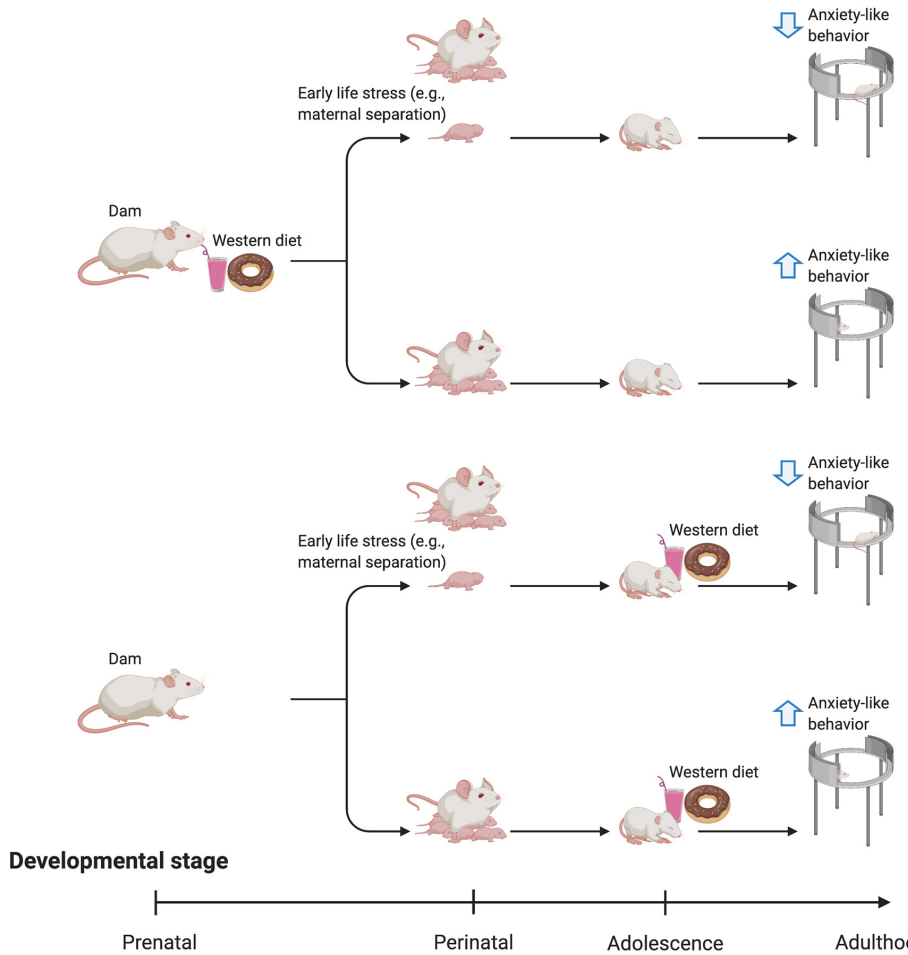
Behavioral Tests

The process of learning and remembering information is perhaps as well-studied as it is persistently mysterious. In rodent models, memory is typically assessed based on an observable behavioral change that is attributable to prior experience, and a variety of behavioral tasks are commonly implemented depending on the type of memory being studied. Common tests of recognition memory that involve passive reinforcement (not inherently appetitive/rewarding, or aversive/punishing) are the Novel Object Recognition (NOR), Novel Place Recognition (NPR), or Novel Object in Context (NOIC). The latter two of these tasks, which involve contextual-based associations, relies on the function of the HPC and interactions with the perirhinal and prefrontal cortices, whereas object recognition memory relies on the function of the perirhinal cortex, and is independent of the HPC when conducted without a temporal or contextual component (Barker and Warburton, 2011).

Spatial memory, or memory based on the location of visuospatial cues in the environment, is commonly assessed via the Y-Maze Spontaneous Alternation task, Morris Water Maze (MWM), the radial arm maze, and the Barnes Maze. These tasks require allocentric navigation, are hippocampal-dependent, and involve either escape from aversive reinforcement (MWM, Barnes Maze), appetitive reinforcement (e.g., food; radial arm maze), or passive reinforcement (Y-Maze Spontaneous Alternation task) (Broadbent et al., 2004; Ofen, 2012; Quillfeldt, 2016). Other memory tests discussed in this section include fear conditioning and avoidance learning (typically based on brief foot shock or predator odor) and Pavlovian stimulus-reinforcement learning. These latter learning and memory tasks are mediated by complex network of forebrain structures that include the amygdala, striatum, insula, HPC, and mPFC among other regions (Kong et al., 2014; Quillfeldt, 2016). WD consumption has been shown to impact the neural substrates associated with the behavioral tasks described above, and early life stages of development are particularly sensitive to these effects in both human and rodent models (Noble and Kanoski, 2016). Moreover, the HPC is a canary in the coal mine in the sense that, at least in some cases, WD factors have a negative influence on hippocampal-dependent memory processes following very short periods of consumption, prior to the effects of the diet on body weight, metabolic function, and integrity in other brain regions (Kanoski and Davidson, 2011). In this section, we discuss the neural substrates and potential mechanisms through which early life WD factors impart learning and memory dysfunction while highlighting some critical gaps in current knowledge.

Perinatal WD Consumption

Exposure to western dietary factors through maternal nutrition has long-term consequences for learning and memory function, even when the animals are maintained on standard, low-fat chow after weaning (Tozuka et al., 2010; Peleg-Raibstein et al., 2012; Kuang et al., 2014; Page et al., 2014; Lépinay et al., 2015; Bengoetxea et al., 2017; Janthakhin et al., 2017; Vuong et al., 2017; Rincel et al., 2018; Cordner et al., 2019). For example, conditioned odor aversion in which an animal avoids an odor that was previously paired with an aversive memory, is impaired in adult male rats that received perinatal exposure to a WD containing 45% kcal from fat (Janthakhin et al., 2017). Moreover, the ability to extinguish an avoidance response to a previously paired odor is impaired in rats that receive the same perinatal WD



Anxiety-like behavior

- Early life Western diet consumption increases anxiety-like behavior.*
- However, early life Western diet consumption can also alleviate the effects of early life stress on anxiety-like behavior.*
- These outcomes can occur independent of obesity and metabolic impairments
- Underlying neurobiological mechanisms involve altered signaling pathways in the hippocampus, amygdala, nucleus accumbens, and medial prefrontal cortex.

*Effects may vary based on behavioral test, sex as a variable, rodent species, strain, diet composition, and diet duration

FIGURE 1 | Summary of the effects of early life Western diet consumption on anxiety-like behavior.

exposure for the same duration (Rincel et al., 2018). Similarly, memory in the NOR task is also impaired in both adolescent male and female rats (Moreton et al., 2019) and adult male rats (Teixeira et al., 2020) when their dams consumed a cafeteria diet throughout lactation. Male offspring from dams fed a 45% kcal fat diet during both gestation and lactation failed to discriminate a novel object in NOR at 14 weeks of age as well (Vuong et al., 2017). Interestingly, male progeny from dams fed a 45% kcal fat diet (GD14 – PN21) demonstrated impairments in the NOR task at PN19–20 but not 1–2 months of age in both sexes (Bengoetxea et al., 2017). Together, these studies suggest that object-based episodic memory and the ability to learn and extinguish conditioned odor aversion is weakened with perinatal WD exposure.

In contrast to conditioned odor aversion and NOR, other types of memory may not be as vulnerable to perinatal WD consumption. Spatial memory retention in the MWM task, for example, is actually enhanced in male rats after perinatal

exposure to a 60% kcals high-saturated-fat or high-trans-fat diet (Bilbo and Tsang, 2010). In support of this, another study has shown that MWM spatial memory retention was only impaired in adulthood when obese male rats were reintroduced to a WD (60% kcals from fat) later in life at 8 weeks old, despite having received perinatal exposure to the same WD during gestation and lactation (White et al., 2009). Furthermore, while spatial learning in the Barnes Maze was impaired in adolescent male offspring of mouse dams that received a 57% kcals fat WD from 6 weeks prior to mating up to lactation day 16, there were no differences in spatial memory/retention (Tozuka et al., 2010). In addition, perinatal WD feeding (45% kcals fat) had no effect on MWM spatial memory retention in male and female offspring tested at an older age (20 months), but did appear to rescue the memory deficits in the MWM task induced by unpredictable prenatal stress (a combination of restraint, swimming, wet bedding, noise, food restriction, and lights on overnight) (Bengoetxea et al., 2017). Pavlovian fear conditioning in adult male and female offspring was also not affected by a 60% kcals fat WD during the perinatal period (Peleg-Raibstein et al., 2012). Similarly, maternal consumption of a 43% kcals fat diet had no impact on both contextual and cued fear conditioning in 9.5 months old male offspring (Zieba et al., 2019). While the aforementioned studies suggest that perinatal exposure to diets that are 57–60% kcal fat do not have an effect on spatial memory, one study found that a 60% kcal HFD, given to dams during pregnancy and lactation, resulted in impaired cognitive performance in the NOR task and the Barnes Maze in adult male rats even after being provided a standard diet at PN 21 (Cordner et al., 2019). Overall, these studies provide mixed evidence that perinatal exposure to a WD impairs hippocampal-dependent spatial memory in offspring, highlighting a need for further investigation to identify critical mediating variables.

In addition to the type of learning and memory being evaluated, one major difference between the aforementioned studies that found effects of perinatal WD on various types of memory vs. those that did not is the ratio of fat and sugar content of the WDs. Those that found no effect of perinatal WD exposure on hippocampal-dependent memory utilized diets that were extremely high in fat (around 60% kcal from fat), whereas those that found impaired odor-based avoidance memory utilized a more moderate WD with regard to fat calorie % (45% kcals fat, respectively). In order to have higher fat content in rodent diets, normally this is achieved by reducing the carbohydrate content, which in a WD is typically predominantly sugar. For instance, a 45% kcals fat WD would have higher sugar content (∼17% kcals sucrose) than a 60% kcals fat WD containing ∼7% kcal sucrose. In fact, studies investigating the effects of perinatal WD on spatial learning and memory that used a more moderate WD with regards to fat content (39% kcal from fat) showed that male rats had impaired spatial memory retention in the MWM task, but this was only the case when the animals were maintained on the WD into adulthood as opposed to animals that had their WD replaced with standard chow after lactation (Lépinay et al., 2015). In another study where perinatal exposure to a 45% kcals fat WD was initiated 1 month before mating and continued throughout gestation and lactation, male rat offspring had impaired spatial memory retention in the MWM as adults regardless of whether they were weaned onto standard chow or maintained on the WD (Page et al., 2014). Interestingly, a perinatal diet high in sucrose (20% w/v sucrose solution), but not fat, given to the rat dams from gestational day 1–21 was not sufficient to promote spatial memory impairment in the MWM in either adolescent or adult male offspring (Kuang et al., 2014). Thus, the fat/sugar ratio may have a significant role in determining if learning and memory is impacted, with perinatal exposure to a 45% kcals fat, 17.5% kcals sucrose during the entire perinatal period being particularly detrimental to learning and memory in adulthood.

The ratio of sugar and fat in the diet is also a critical variable for WD effects on metabolism and neuronal outcomes. For instance, odor memory impairment induced by perinatal exposure to a 45% kcals fat WD is associated with dendritic atrophy in the BLA and the CA1 region of the HPC during adulthood (Janthakhin et al., 2017) as well as a reduction in dendritic spines and dendritic length in the mPFC at weaning (Rincel et al., 2018) in male rats. While the ratio of sugar to fat was not specified, mPFC dopamine metabolism was reduced and serotonin metabolism in the mPFC was increased in male and female rats that were exposed to a cafeteria diet perinatally during lactation (Moreton et al., 2019). These results are independent of the obesogenic effects of the diet, as the time on the diet was insufficient to promote obesity in the dams or the offspring who were being tested in a few of aforementioned studies (Janthakhin et al., 2017; Rincel et al., 2018). However, the offspring of rat dams on a 45% kcals fat WD showed reduced hippocampal protein expression of an array of genes that are associated with synaptic plasticity and spatial memory, including BDNF, activity-regulated cytoskeletal-associated protein (Arc), nerve growth factor, synaptophysin, and the NR2B subunit of the glutamate NMDA receptor in adulthood (Page et al., 2014). Cordner et al. found that a 60% kcal perinatal HFD resulted in rats having increased body weights throughout their lifetime as well as having decreased expression of the leptin and insulin receptor in the dentate gyrus and CA3 region of the dorsal hippocampus of offspring at PN 21, which persisted at PN 150, long after the HFD was removed (Cordner et al., 2019). Together, these studies highlight neurobiological mechanisms through which perinatal WD consumption may impact the BLA, mPFC, and HPC to contribute to impaired learning and memory, outcomes that may or may not coincide with poor metabolic outcomes.

In studies that found that learning and memory processes were not impaired following exposure to a perinatal WD, in some cases there were still long-term effects on metabolism and on the brain. For example, despite showing better spatial memory retention, male rat offspring of obese dams on a 60% kcals fat WD exhibited increased neuroinflammation and microglial activation in the HPC (Bilbo and Tsang, 2010). Lack of impairments in fear conditioning in male progeny from dams fed a 43% kcals fat diet was also associated with increased body weight at adulthood (Zieba et al., 2019). In the ventral HPC, male mice subjected to a perinatal 60% kcals fat WD had increased mRNA expression of 5-HT1AR and GABAa alpha2 receptor, suggesting differences in the GABAergic and serotonergic systems despite there not being a group effect

on Pavlovian fear conditioning (Peleg-Raibstein et al., 2012). While a perinatal high sucrose diet (20% w/v solution) did not impact spatial memory, exposure to the diet led to increased plasma levels of glucose in the dams, increased body weight in the offspring, and increased apoptosis and activated caspase-3 in the HPC (Kuang et al., 2014). In addition, although maternal consumption of a 60% kcals fat diet did not alter progeny's cognitive performance in the NOR and Y-maze tasks, these animals still presented greater anxiety-like behaviors and hippocampal inflammation (Winther et al., 2018). Thus, in some cases where perinatal WD exposure is not associated with learning and memory impairments, perinatal exposure to a high fat or high sucrose WD nevertheless leads to metabolic impairments and neurobiological alterations in the HPC, the consequences of which may influence learning and memory at time points later in life that were not investigated in these studies. Regardless, these studies support a framework in which the effects of perinatal WD exposure on learning and memory are largely dissociable from the effects of WD on obesity and associated metabolic dysfunction.

In some cases, the detrimental aspects of the perinatal WD exposure on brain and behavioral outcomes are reversed during adulthood after dietary intervention. For example, the oxidative stress, lipid peroxidation, and decreased BDNF protein levels in the HPC observed following perinatal 57% kcals fat WD exposure were normalized in adulthood when animals were weaned on healthy chow (Tozuka et al., 2010), corresponding to improvements in Barnes Maze memory performance. Together, data highlighted in this section suggest that WD exposure during the perinatal period may negatively impact learning and memory function and HPC neurobiological signaling pathways during adulthood, particularly when the diet contains high percentages of fat and sugar, and even in the absence of obesity. Furthermore, the mixed evidence on learning and memory outcomes after perinatal HFD exposure may depend on the impact maternal HFD exposure has on programming the neural correlates underlying learning and memory and whether or not the maternal programming persists in adulthood. The conditions required for reversal of these long-term disturbances requires further investigation.

Adolescent WD Consumption

Evidence from multiple studies suggests that adolescent consumption of a WD containing a high % kcal from fat impairs hippocampal-dependent learning and memory in rodents, and that these effects even occur following acute exposure. For example, short-term feeding (1 week, from PN 21–28) of a WD containing 60% kcals from fat in male mice impaired spatial memory in the Y-maze alteration task and object recognition memory impairment in the NOR task during adolescence (Kaczmarczyk et al., 2013). Similarly, impaired object location memory and impaired hippocampal long-term potentiation was reported in adolescent male rats with a similar dietary exposure (Khazen et al., 2019). Finally, impaired extinction of cued fear conditioning is observed in male rats after only 1 week of exposure (PN31–38) to a 41% kcals saturated fat diet in male rats (Vega-Torres et al., 2020). Together, these reports suggest that short-term exposure to high-fat WDs post-weaning impairs spatial and episodic memory during adolescence.

While the aforementioned short-term WD exposure studies suggest that the diet significantly impacted memory, these impairments were likely independent of metabolic effects, as the short duration on the diet was insufficient to promote weight gain (Kaczmarczyk et al., 2013; Khazen et al., 2019; Vega-Torres et al., 2020) or aberrant glucose metabolism (Kaczmarczyk et al., 2013; Vega-Torres et al., 2020). Kaczmarczyk and colleagues reported impaired performance in the NOR task in adolescent mice after both 1 and 3 weeks of exposure to a 60% kcals fat WD, which could be improved by switching animals to a healthy low-fat diet for 1 week. On the other hand, spatial memory deficits in the Y-maze task were present after 1 week, but this effect could no longer be observed after 3 weeks of WD exposure. The 3 weeks of WD timepoint coincides with elevated activity of monoamine oxidase A and B, the enzymes that metabolize dopamine, in the HPC and hypothalamus. In combination with decreased levels of hypothalamic dopamine and increased levels of its metabolic homovanilic acid in the HPC at the 1 week timepoint only, these results suggest spatial memory deficits may be consequent to reduced dopamine signaling after 1 week of WD exposure, whereas dopamine levels are restored after 3 weeks with increased activity of dopamine metabolizing enzymes (Kaczmarczyk et al., 2013). Another possible mechanism for memory impairment following short-term WD feeding involves glucocorticoid receptors. Khazen and colleagues found that intraperitoneal treatment with a glucocorticoid receptor antagonist was able to reverse impaired long. term potentiation and memory deficits, suggesting that glucocorticoid signaling may mediate the effects of WD on hippocampal dysfunction. Similarly, Vega-Torres and colleagues reported dampened neuronal activity in the amygdala following foot shock delivery, as well as increased gene expression for the corticotropin release hormone receptor-1 within the mPFC. In sum, short-term exposure to a WD post-weaning can impair memory independent of the obesogenic effects of the diet, and these memory deficits are associated with changes in dopamine, glucocorticoid signaling, and long-term potentiation in the HPC and mPFC.

Given that short-term feeding of WD impacts memory function, it is not surprising that long-term WD feeding also impairs memory. For example, while binging on a WD (45% kcals from fat) for 2 hrs daily throughout adolescence did not result in spatial memory deficits (Blanco-Gandía et al., 2019), *ad libitum* access (for 1+ month) to this diet after weaning promotes deficits in spatial learning in the MWM task (Boitard et al., 2014), the NOL task (Del Rio et al., 2016), the radial arm maze task (Boitard et al., 2012), and the Hebb Williams Maze (Blanco-Gandía et al., 2019) in adult male rodents. Impairments are also seen in reversal learning in the MWM (Boitard et al., 2014) and enhanced aversive and auditory fear memory (Boitard et al., 2015), as assessed by COA and auditory fear conditioning, respectively. Although 2 h daily access to high fat and high sugar pellets for 28 days during adolescence did not affect odor recognition, rats under this diet regimen failed to demonstrate novelty preference in the NOR task (Reichelt et al., 2020).

Adolescent (PN28–56) consumption of a 63% kcal fat diet in male mice impaired discrimination in the Y-maze, reversal learning in the MWM, and cued fear extinction (Labouesse et al., 2017). Alterations in fear extinction were also reported in male rats fed a 41% kcal fat diet for 82 days (PN28–110) (Vega-Torres et al., 2018). Interestingly, adolescent exposure to a lard-enriched WD for 13 weeks (well into adulthood) showed memory deficits in the radial arm maze and the NOL task, despite animals undergoing a 70% caloric restriction over the last 5 weeks of the diet period (Valladolid-Acebes et al., 2011, 2013). Importantly, in some cases switching from a 45% fat diet to a standard rodent diet for 2 weeks can reverse the spatial memory deficits (Blanco-Gandía et al., 2019). Similarly, chronic consumption of a WD initiated during adolescence and consisting of powdered chow, lard, and dextrose (with 41.7% of the calories were derived from fat) is also associated with episodic memory impairments in adulthood (Marwitz et al., 2015). Switching the rats to a control diet for 5 months after an initial 3 months of exposure to a lard-enriched 45% fat WD initiated at weaning, normalized memory impairments in the MWM and COA task (Boitard et al., 2016). These data suggest that male rats develop impairments in spatial memory, reversal learning, and aversive and auditory fear memory in adulthood in response to long-term high-fat, high-sugar consumption starting during adolescence, and moreover, that these effects may be reversible in some cases with dietary intervention. Importantly, learning and memory deficits in male rodents were not observed when WD consumption for a similar duration was confined to adulthood, despite similar diet-induced elevations in body weight and metabolic disruption (Boitard et al., 2012, 2014, 2015; Valladolid-Acebes et al., 2013; Labouesse et al., 2017). These findings corroborate that adolescence is a developmental period of particular vulnerability for WD effects on learning and memory function.

In addition to memory impairment, long-term feeding of a WD also leads to significant disruptions in metabolism and neurobiological systems associated with memory control. While short-term feeding of a WD during adolescence does not promote weight gain, long-term feeding from adolescence to adulthood typically promotes weight gain in male rodents (Valladolid-Acebes et al., 2011, 2013; Boitard et al., 2012, 2014, 2015; Marwitz et al., 2015; Labouesse et al., 2017; Vega-Torres et al., 2018; Blanco-Gandía et al., 2019). Additionally, prolonged intake (3+ months) of a WD started during adolescence and maintained well into adulthood in male rodents imparts metabolic alterations in adulthood such as increased circulating leptin (Valladolid-Acebes et al., 2011, 2013; Boitard et al., 2012), corticosterone, cholesterol, and insulin (Boitard et al., 2012) as well as hyperglycemia (Valladolid-Acebes et al., 2013; Vinuesa et al., 2016) and insulin resistance (Marwitz et al., 2015; Vinuesa et al., 2016). Memory deficits following adolescent WD exposure are also found in the absence of significant weight gain, such as impaired NOR in male rats with intermittent access (2 hrs daily) to high fat and high sugar pellets (Reichelt et al., 2020). Long-term consumption of a WD, initiated during adolescence, resulted in molecular alterations in the HPC, amygdala and mPFC that accompanied memory impairments at adulthood. For example, reduced neurogenesis (Boitard et al., 2012, 2016; Vinuesa et al., 2016), increased microglial activation (Vinuesa et al., 2016) and diminished gene expression of monoamine oxidase A (Reichelt et al., 2020) can be observed in the HPC of rodents fed a WD since adolescence and displaying spatial memory deficits. Alterations in aversive and auditory fear memory in adult male rats fed a WD since adolescence are attenuated by glucocorticoid receptor antagonism in the amygdala (Boitard et al., 2015). Rodents with impaired extinction and reversal learning, but also spatial memory and NOR, due to WD exposure during adolescence, presented with downregulation of the synaptic modulator reelin and altered long term depression (Labouesse et al., 2017) and reduced BDNF and monoamine oxidase A gene expression (Reichelt et al., 2020) in the mPFC. Altogether, these results demonstrate weight gain and metabolic impairments often accompany but are not conditional for WD initiated during adolescence to induce learning and memory deficits. These data further suggest neurogenesis, microglial activation, glucocorticoid signaling, as well as synaptic transmission and neural plasticity in the HPC, amygdala and mPFC as potential mechanisms.

Given that rodent WDs high in fat are often also high in sugar, it is important to investigate the contribution of dietary sugars to the effects on learning and memory. Common obesity-promoting diet compositions from Research Diets consist of the 45% kcal HFD, which contains 17.5% of kcal from sucrose, the 58% kcal HFD, which contains about 13% of kcal from sucrose, and the 60% kcal HFD, which contains about 7.5% of kcal from sucrose. Interestingly, the effects of sugar alone have been shown to impact learning and memory independent of weight gain when given during the adolescent period (Hsu et al., 2015; Reichelt et al., 2015; Abbott et al., 2016; Alten et al., 2018; Buyukata et al., 2018; Noble et al., 2019). Furthermore, the effects of adolescent dietary sugar on learning and memory function persist into adulthood. For example, male rats given free access to an 11% w/v high fructose corn syrup drink for at least 30 days during adolescence had episodic and spatial memory impairments, assessed by the NOIC, Barnes Maze, and Morris Water Maze tasks (Hsu et al., 2015; Alten et al., 2018; Noble et al., 2019). Furthermore, NOIC memory impairments persisted even when animals were tested after several months without access to sugar solutions (Noble et al., 2019). Notably, adult rats fed sugar solutions for a similar length of time did not show memory deficits (Hsu et al., 2015). Rats consuming a high in sugar, but low in fat diet (26.7% sucrose/lactose, 6.5% fat) starting at weaning impaired episodic and spatial memory in the object recognition and Y-Maze tasks and impaired learning in the contextual fear conditioning task in adulthood (Altermann Torre et al., 2020). Overall, these studies suggest that WDs high in sugar (independent of elevated fat content vs. a control diet) have adverse effects on learning and memory that last into adulthood and are not easily reversible by removal of the diet.

While the studies described above typically involve *ad libitum* access to the experimental diet, some studies have examined the effects of intermittent access to a sugar solution during adolescence on learning and memory function. Results of these studies suggest that intermittent sugar access similarly conferred lasting impairments in learning and memory function later in life

(Reichelt et al., 2015; Abbott et al., 2016; Buyukata et al., 2018; Noble et al., 2019). For example, male rats given intermittent access (2 hrs daily) to a 10% w/v sucrose drink during adolescence were impaired in both the place recognition (Abbott et al., 2016) and object-in-place recognition tasks (Reichelt et al., 2015; Abbott et al., 2016) and were unable to use contextual information to discriminate between the context-appropriate and context-inappropriate levers in a context devaluation task, which requires communication between the mPFC and HPC (Reichelt et al., 2015). Similarly, male rats with intermittent access to a 10% sucrose solution for 28 days (PN28–55) presented impairments in both learning and memory in the MWM task at adulthood (Kendig et al., 2013). Despite conferring impairments in learning and memory, male rats that had free access to an 11% w/v sugar solution have normal body weights throughout the dietary exposure period (Hsu et al., 2015; Noble et al., 2019), with one study finding that consumption of a high fructose corn syrup solution actually led to a decrease in body weight despite the rats showing glucose intolerance and increased adiposity (Alten et al., 2018). Intermittent access to an 10–11% w/v sugar solution also did not promote weight gain during the 30 days of access in either males (Kendig et al., 2013; Abbott et al., 2016; Noble et al., 2019) or females (Abbott et al., 2016), yet one study has found that significant weight gain occurred in male rats after the intermittent access period to a 10% sucrose solution (Reichelt et al., 2015). Collectively, these studies show that intermittent access to a sugar solution during adolescence can impart long-lasting memory deficits, and that these effects can occur independent of body weight gain.

The influence of sex with regards to adolescent WD exposure effects on memory function is poorly understood. Males, but not females, on a chronic 45% kcals fat WD have reduced freezing behavior compared with chow fed controls in a contextual fear conditioning task in adulthood (Hwang et al., 2010). However, given that WD consumption can be anxiolytic, it is difficult to determine whether the reduced freezing behavior was due to improved memory function *per se*, or was a function of reduced anxiety. Moreover, Buyukata et al. found that both male and female rats on an intermittent sucrose access schedule showed impaired NOR memory during adolescence when the objects shared multiple similar features. However, when the objects were arranged with either small or large spatial separations (spontaneous location recognition task), males that consumed sugar performed worse in tasks with small spatial separations, whereas females performed worse in tasks with large spatial separations (Buyukata et al., 2018). In another study, both male and female rats previously on a chronic 58% kcals fat WD during adolescence displayed impaired memory in a spatial object recognition task in adulthood (Underwood and Thompson, 2016). In addition, female mice fed for 12 weeks of a 60% kcals fat WD showed altered reversal but not initial learning in the MWM task (Klein et al., 2016). While these studies suggest that the effects of adolescent WD exposure are sexually dimorphic depending on the task and type of memory being tested, further research is clearly needed in this area.

Estrogen may be a critical factor mediating sex differences in vulnerability to adolescent WD-induced memory impairments. For example, in female rats intermittent dietary sucrose access during adolescence (10% w/v sucrose solution, 2 hrs daily) did not impact NPR performance, however the rats were only able to perform place recognition correctly during the proestrus phase of the estrous cycle, a stage that contains higher levels of circulating estrogens (Abbott et al., 2016). Taken together, similar to males, female rats given intermittent sugar access display impairments in episodic memory. However, the episodic memory deficits may be determined by the stage of the estrous cycle.

Metabolically, female mice exhibit similar deficits to males in response to a 45% kcal from fat WD from adolescence to adulthood, having significant weight gain relative to controls despite comparable caloric intake (Hwang et al., 2010). However, WD-fed males are distinguished from females by having higher glucose levels relative to controls fed a healthy low fat diet (Hwang et al., 2010). Unlike male rats, female rats did not gain significant weight or display glucose intolerance after chronic WD exposure (58% kcal fat), suggesting that female rats may develop a less severe metabolic phenotype under these conditions compared to males (Underwood and Thompson, 2016). However, female mice fed a 60% kcal fat WD for 12 weeks did develop hyperphagia and greater body weight gain relative to animals receiving the control diet, and alterations in reversal learning in the MWM task were prevented by wheel running (Klein et al., 2016). As for a potential mechanism as to how male and female rodents differ in regard to contextual fear conditioning, Hwang et al. (Hwang et al., 2010) found that WD-fed males, but not females, had reduced long term potentiation but were also lacking a normal long term depression response. Together, these studies suggest that chronic exposure to WD starting in adolescence may alter learning and memory processes in female rodents, although the specific types of memory involved may be sex-dependent, and like males, memory function is dissociable from metabolic impairments.

With regard to underlying neurobiological mechanisms for how high sugar diets during adolescence impact memory function, memory deficits induced by free access to the 11% w/v sugar solution in adolescence are associated with increased plasma insulin and pro-inflammatory cytokines such as interleukin 6 and interleukin 1β in the dorsal HPC (Hsu et al., 2015). Moreover, another study found systemic inflammation after adolescent consumption of a 11% w/v sugar solution. Using in-vivo electrophysiology, the authors revealed that concurrent with systemic inflammation, high fructose corn syrup consumption induced hyperexcitability in hippocampal CA3-CA1 synapses (Alten et al., 2018). Furthermore, the effect of a diet high in sugar on plasticity depended on the developmental stage, such that during adolescence, 1 week of consumption reduced synaptophysin, BDNF, protein kinase B (AKT), and phosphorylated AKT in the HPC. However, when access to the simple sugar-enriched WD is maintained into adulthood, there is increased synaptophysin, spinophilin/neurabin-II, and decreased BDNF and neuronal nitric oxide synthase, suggesting that plasticity markers change depending on stage of development (Torre et al., 2020). As opposed to free access to sugar, the memory deficits associated with intermittent access to sugar in adolescent males were accompanied by deficits

in parvalbumin-immunoreactive cell density in the HPC and mPFC in adulthood (Reichelt et al., 2015). Altogether, these findings suggest that the HPC is a region that is particularly sensitive to perturbations by adolescent dietary sugar consumption, with plasticity and inflammatory signaling pathways implicated as putative mechanistic links between diet and memory dysfunction.

Cafeteria diets are often obesogenic when consumed by rodents during adulthood, however, whether or not adolescent consumption of cafeteria diets in rodents promotes cognitive impairment is still controversial. For example, adolescent male rats on a cafeteria diet consisting of a variety of high fat and high sugar palatable food options, standard chow, and a 15% (w/v) sucrose solution weighed more and had greater adiposity compared with rats maintained on chow and water alone. The cafeteria diet-fed rats also exhibited impairments in hippocampal-dependent spatial learning and memory in the MWM, but not object novelty detection or fear acquisition, during adulthood (Ferreira et al., 2018). In contrast, a similar study, using very similar dietary parameters of highly palatable human foods along with standard chow and a 12% w/v sucrose solution, found no effect of adolescent consumption of a cafeteria diet on spatial memory in adulthood using the Barnes Maze (Gomez-Smith et al., 2016). Despite intact spatial learning, the cafeteria diet rats displayed an obesogenic phenotype as indicated by increased body weights, visceral adiposity, hyperinsulinemia, glucose intolerance, and dyslipidemia with elevated serum triglyceride levels and reduced HDL cholesterol, and greater hippocampal neuroinflammation in adulthood. Moreover, replacing the cafeteria diet with a standard rodent diet appeared to reverse all of the metabolic deficits mentioned before as well as the neuroinflammation (Gomez-Smith et al., 2016). These studies reveal that the effects of an adolescent cafeteria diet on memory are variable and highlight that more work is needed to identify critical mediating variables. However, given that in some cases obesogenic effects have been observed in the absence of memory impairments (and vice versa), these findings further highlight a framework in which the effects of early life WD consumption on cognition and metabolism are dissociable.

Summary

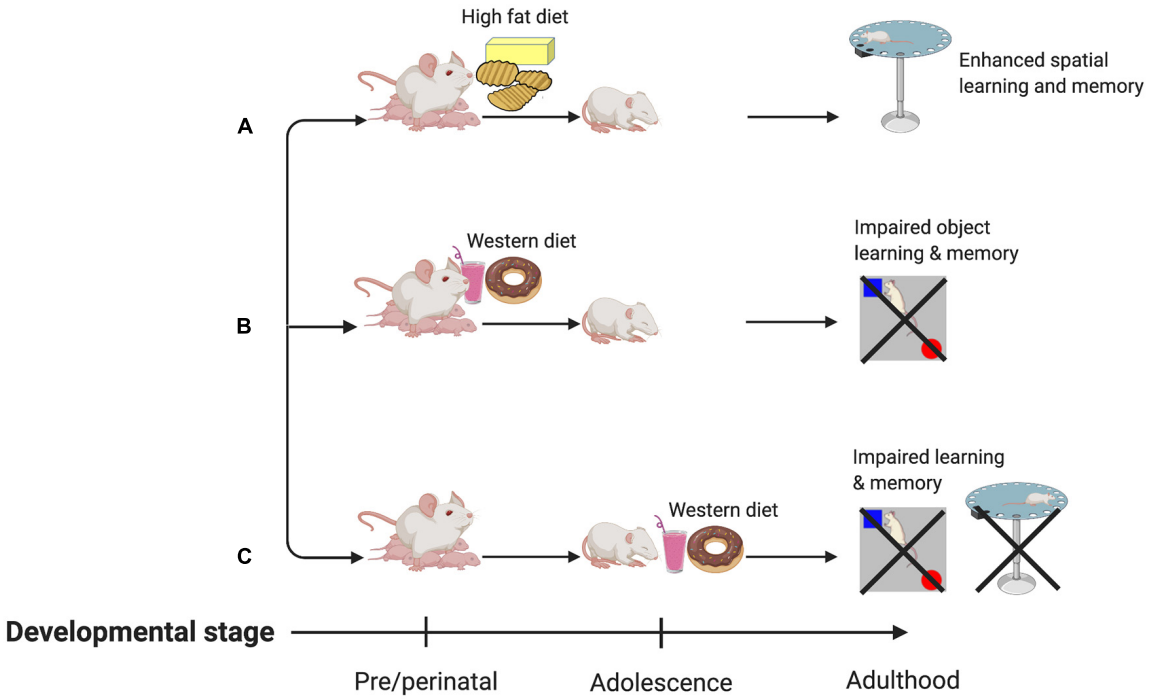
Adolescent exposure to WD factors contributes to memory impairments, even in the absence of weight gain (**Figure 2**). While our focus here is on rodent models, studies have identified a deleterious impact of early life WD consumption on memory function in humans as well (Baym et al., 2014; Haapala et al., 2015; Khan et al., 2015; Cohen et al., 2018). Given that the rodent and human HPC have similar development patterns during the adolescent period, the insights garnered from rodent studies may provide insight into how WD factors might impact human adolescent brain development. The studies discussed in this section further highlight that rodent models have elucidated potential mechanisms for the effects of WD on learning and memory impairments, and these relate to region-specific changes in Ca2+ dysregulation (long term potentiation), glucocorticoid receptors, dopamine metabolism, neuroinflammation, microglial activity, and other factors that affect plasticity and ultimately alter the network dynamics of neural ensembles that support cognition, with the HPC, mPFC, and amygdala being particularly affected. In conclusion, several studies have identified adolescence as a period of high vulnerability for deleterious effects of WD consumption on memory and neural processes associated with memory control. More research is needed to fully understand the extent that sex and sex hormones are critical variables, as well as the effectiveness of various interventions (dietary, etc.) to reverse the long-lasting memory impairment associated with early life WD consumption.

REWARD-MOTIVATED BEHAVIOR

Behavioral Models and Neural Substrates

Consumption of palatable food such as a rodent WD engages hedonic- and reward-associated neural systems, and therefore it is not surprising that early-life WD consumption can have lasting impacts on these systems and associated behaviors (Corsica and Hood, 2011; Tompkins et al., 2017; Lowe et al., 2020). Alterations in reward-associated neural systems affect susceptibility to binge eating (Ames et al., 2014; Bodell et al., 2018), obesity (Matton et al., 2013), and addiction to substances of abuse (De Cock et al., 2016). In rodent studies, many behavioral tasks are commonly utilized to investigate these neural reward systems. Those discussed in this section include: sensitization, defined as an increase in locomotion following repeated administrations of drugs that upregulate dopamine signaling; conditioned place preference (CPP), which measures the strength of an association between a rewarding stimulus (e.g., palatable food consumption) and a context; the operant runway task, which measures the time it takes for the animal to reach the goal area and obtain a food reward (Kuhn et al., 2019); visual discrimination reversal learning and attentional set shifting, which are tests of behavioral flexibility (Heisler et al., 2015); operant responding on a progressive ratio reinforcement schedule, which tests for motivation to work for a reinforcer (Killeen et al., 2009); Pavlovian Conditioned Approach where animals show appetitive responses to cues that predict a food reward (Fitzpatrick and Morrow, 2016); outcome devaluation, which tests, among other things, whether or not the animal is exhibiting habitual behavior (Rossi and Yin, 2012); taste reactivity, which indicates an animal's hedonic evaluation of the taste of a food based on their orofacial reactions; macronutrient preference testing (e.g., carbohydrates vs. fat) (Grill and Norgren, 1978) and monitoring intake during chronic access to palatable food. Some additional behavioral tasks discussed in this section measure impulsive behavior, including: the delay-discounting task, which assesses preference for smaller more immediate over larger more delayed food reinforcers; the 5-Choice Serial Reaction Time Test, which discerns impulsivity (via incorrect trials and premature trials) from inattention (via omitted trials) (Dent and Isles, 2014).

Dopaminergic projections from the ventral tegmental area (VTA) to the ACB are involved in motivated behavior, especially Pavlovian cue-reward associative learning (Saunders et al., 2018). On the other hand, opioid signaling in the ACB, within



Learning and Memory

- Consuming high fat diets during the pre/perinatal period can enhance spatial memory (A), whereas consuming a Western diet during the pre/perinatal period can impair object recognition-based learning and memory (B).*
- Adolescent Western diet consumption impairs spatial and episodic memory (C).*
- These outcomes can occur independent of obesity and metabolic impairments
- Underlying neurobiological mechanisms involve altered signaling pathways in the amygdala, medial prefrontal cortex, and hippocampus, including glucocorticoid signaling and neuroinflammation

*Effects may vary based on behavioral test, sex as a variable, rodent species, strain, diet composition, and diet duration

FIGURE 2 | Summary of the effects of early life Western diet consumption on learning and memory.

the ventral striatum, is identified to play a role in linking sweet tastants to hedonic orofacial 'liking' reactions (Smith and Berridge, 2007; Berridge, 2009). There is also evidence to suggest in addition to the striatum, the amygdala is critical for reinforcement learning (Averbeck and Costa, 2017). In a broader context, regions activated by palatable foods are nodes within a larger neural network that encompass connections to other regions such as the hypothalamus, mPFC and HPC that modulate behavior related to reward learning, food intake, impulse control, and novelty (Kelley et al., 2005). In this section, we discuss potential mechanisms for how early life obesogenic diets influence reward-motivated behavior, in part through changes in the dopamine and/or opioid systems.

Perinatal WD Exposure

Perinatal exposure to a WD can dysregulate reward-motivated behavior, even when offspring are reared on a standard chow diet (Naef et al., 2008, 2011; Ong and Muhlhausler, 2011; Morganstern et al., 2013; Wu et al., 2013; Grissom et al., 2015; Peleg-Raibstein et al., 2016; Roversi et al., 2016; Paradis et al., 2017; Sarker et al., 2019; Gawliński et al., 2020). For example, when dams were exposed to a WD (60% kcals from fat) pre-conception there

were no differences in sensitivity to amphetamine, as indicated by amphetamine-induced locomotor activity, or preference for alcohol in offspring. However, maternal exposure to a WD diet during late gestation (gestational days (GD) 12–21) increased alcohol preference and amphetamine sensitivity in male and female mice offspring when tested in adulthood (Sarker et al., 2019). These effects likely involve epigenetic mechanisms as behavioral effects carried on into the 3rd generation where females, but not males, still showed elevated addictive-like behaviors if the WD was provided from preconception to weaning (Sarker et al., 2018). However, another study using a WD (30% kcals fat) found the opposite, where a longer exposure from GD 13 to PN 21 resulted in the offspring being less sensitized, as seen by reduced locomotor activity, to both an initial low-dose exposure to amphetamine and with repeated exposure to amphetamine in adulthood (Naef et al., 2008). Subsequent work with similar dietary conditions demonstrated that male offspring from WD-fed dams display blunted ACB dopamine release following amphetamine administration (Naef et al., 2011). This was accompanied by altered D2 receptor function and signaling, as well as increased motivation for fat rewards. Together these studies show that perinatal WD exposure during late gestation can lead to lasting alterations in reward-motivated behavior in the offspring in adulthood, and that the % kcals fat in the WD may be a critical factor.

WD consumption by dams through an extended perinatal period that includes several weeks of pre-conception, gestation, and lactation yields long-lasting effects on reward-motivated behavior. For example, male and female rats born to dams that received hydrogenated vegetable fat (3 g/kg), a trans-fat, by oral gavage during the extended perinatal period preferred a morphine-associated context in CPP during late adolescence (PN 41+) to a larger degree than control female offspring (Roversi et al., 2016). Similarly, male and female offspring from dams consuming a 60% kcals fat WD initiated prior to conception demonstrated enhanced CPP acquisition for cocaine, as well as greater cocaine and amphetamine sensitization (Peleg-Raibstein et al., 2016). In addition, these animals also display a stronger preference for fat, sugar, and ethanol relative to control offspring. Also, when dams are exposed to a WD where 60% of calories are derived from fat throughout the extended perinatal period, male rat offspring were impaired in behavioral flexibility for sucrose reinforcement in the visual discrimination reversal learning and attentional set shifting tasks (Wu et al., 2013). Additionally, a 60% kcals fat WD during gestation and lactation resulted in both male and female mouse offspring displaying decreased motivation to earn a liquid food reward in operant responding under a progressive ratio schedule of reinforcement, as well as increased impulsivity in the 5-Choice Serial Reaction Time task as measured by incorrect and impulsive premature responses. These effects were observed despite the offspring being food restricted to 80–95% of their free-feeding body weights (Grissom et al., 2015). Finally, if a cafeteria diet was provided during the extended perinatal period, male and female rat offspring responded to a junk food challenge later in adolescence and adulthood by increasing their caloric consumption relative to controls (Ong and Muhlhausler, 2011, 2014), suggesting that WD exposure during the extended perinatal exposure leads to increased consumption of palatable food by the offspring later in life.

Even when WD exposure is initiated at the beginning of gestation, reward functions can be disturbed in the offspring. For example, when dams are fed a WD from gestational day 1 until end of lactation, male pups show a transient enhanced fat preference at PN25 that normalizes by PN95 (Paradis et al., 2017). Female offspring of dams fed a high sugar diet (44% kcals from sucrose) were more responsive to cue and cocaine-induced reinstatement of lever pressing (Gawliński et al., 2020). Finally, maternal consumption of a high fat/high sucrose diet from gestation day 5 until end of lactation increased operant responding for nicotine infusion in the male offspring (Morganstern et al., 2013). Altogether, these studies demonstrate that perinatal WD exposure starting as early as before pregnancy and terminating around the time of weaning increases drive for rewards (increased preference for a place associated with reward, impaired behavioral flexibility, increased impulsivity, and increased consumption of palatable if made freely available), with the exception of one study that observed reduced motivation to work for liquid food reward and no differences in Pavlovian conditioned approach behavior (Grissom et al., 2015).

Similar to the effects described in the earlier sections on anxiety, learning, and memory, an obesogenic phenotype is not required for there to be differences in reward-motivated behavior associated with perinatal WD consumption. While some studies observed that a perinatal WD increases body weight and adiposity and promotes disturbances in metabolism (e.g., insulin insensitivity) in offspring at weaning (Naef et al., 2008; Ong and Muhlhausler, 2011; Morganstern et al., 2013; Peleg-Raibstein et al., 2016; Paradis et al., 2017; Song et al., 2020) and as adults (Naef et al., 2011; Ong and Muhlhausler, 2011; Morganstern et al., 2013; Peleg-Raibstein et al., 2016; Paradis et al., 2017; Sarker et al., 2019; Song et al., 2020), other studies found no such differences (Wu et al., 2013; Ong and Muhlhausler, 2014; Grissom et al., 2015; Roversi et al., 2016; Gawliński et al., 2020), even when the dams were heavier as a result of the obesogenic diet (Wu et al., 2013; Ong and Muhlhausler, 2014). Again, these differences could be reflective of the type of WD being used, with those higher in fat leading to more severe metabolic consequences, and/or also the different rodent strains being used. Regardless, all of the aforementioned studies found differences in reward-motivated behavior, with the exception of 2 studies that found no effects in reward-motivated behavior when dams were fed a 60% kcals fat WD (Sarker et al., 2019; Song et al., 2020). Despite fat preference being only affected in offspring from WD dams who had access to a running wheel, juveniles from the WD-sedentary group still displayed changes in dopamine and opioid-related gene expression in the mPFC, ACB and VTA, with some of these changes lasting throughout adulthood (Song et al., 2020). Sarker and colleagues (Sarker et al., 2019) observed no impact of perinatal WD on high fat diet, sucrose and alcohol preference as well as locomotor response to amphetamine in offspring when maternal WD access was confined to lactation. While lactation may be too short of an exposure period to induce behavioral deficits, WD exposure still resulted in weight gain, a higher fat mass ratio, a higher lipid profile, and high insulin

insensitivity relative to controls (Sarker et al., 2019), thus further highlighting that the effects of perinatal WD consumption diets on reward-motivated behavior and obesity can be dissociated.

Effects on reward-mediated behavioral outcomes associated with perinatal WD exposure may be driven, in part, by altered striatal dopamine signaling. Studies have shown that the ventral striatum, which contains the ACB, is important for encoding food-related reward value and that dopamine is released in response to food-associated cues (Wyvell and Berridge, 2001; Ostlund et al., 2014; Alonso-Alonso et al., 2015; Kosheleff et al., 2018) whereas the dorsal striatum is related to food craving and consumption (Small et al., 2003; DiFeliceantonio et al., 2012; Contreras-Rodríguez et al., 2017). In the striatal regions of rodents, increased sensitization to amphetamine after gestational WD (gestational day 12 to 21) exposure was accompanied by a hypodopaminergic state where both male and female offspring had reduced dopamine levels in both the ventral and dorsal striatum relative to controls. These outcomes were accompanied by higher levels of dopamine and its metabolites in the VTA, which was suggested to be compensating for the reduced dopamine levels in the striatum (Sarker et al., 2019). In contrast, when reduced sensitization to amphetamine was observed after WD exposure from gestational day 13 to PN 21, male offspring had increased levels of dopamine in both the ventral striatum and VTA (Naef et al., 2008). Together, these findings suggest that WD exposure restricted to the gestational period reduces levels of dopamine in the ventral striatum and increases sensitization, whereas longer-term exposure that includes the gestational period increases levels of dopamine in the ventral striatum and decreases sensitization to amphetamine.

When perinatal WD feeding is extended from 3 weeks prior to conception until end of lactation, hyper-responsiveness to cocaine and amphetamine occurs in a context of reduced basal striatal and VTA dopamine levels, but enhanced ACB deltaFosB expression following cocaine exposure relative to control offspring (Peleg-Raibstein et al., 2016). In addition to highlighting a dissociation between basal content and reactivity, these neurochemical observations were associated with lower content of dopamine metabolites, suggesting molecular adaptations to maintain dopamine signaling. In addition to altered locomotor response to amphetamine, impairments in the visual discrimination reversal learning task after an extended perinatal WD exposure may also be due to reduced dopamine uptake in the dorsal striatum in male offspring, suggesting abnormal dopamine clearance being involved in reversal learning impairment (Wu et al., 2013). Another study found that expression of D1R was lower in the ACB, but higher in the VTA after an extended perinatal cafeteria diet, which may be related to male and female rat susceptibility for increased caloric consumption of junk food in adulthood (Ong and Muhlhausler, 2014). Transient increased fat preference coincides with greater D2R gene expression in the ACB, while normalization of fat preference occurs when VTA and ACB tyrosine hydroxylase expression is downregulated (Paradis et al., 2017). Despite offspring showing enhanced preference for junk food at both time points, ACB gene expression for mu opioid receptor is increased and dopamine transporter is decreased during adolescence, while changes occur in the opposite direction at adulthood (Ong and Muhlhausler, 2011). In addition to facilitating cocaine-induced reinstatement, maternal exposure to a high sucrose diet is associated with higher striatal levels of melanocortin 4 receptor in the adult offspring (Gawliński et al., 2020). Grissom and colleagues found that attention and impulsivity deficits observed after perinatal WD exposure during gestation and lactation were linked with overexpression of DNMT1 in the mPFC, which is involved in DNA methylation, catechol-o-methyltransferase, which is an enzyme that degrades catecholamines like dopamine, δ-opioid receptor, whose activation can lead to increased extracellular dopamine (Burns et al., 2019), and cannabinoid receptor 1, which regulates the expression of D2R (Blume et al., 2013; Grissom et al., 2015). In rat offspring, heightened operant responding for nicotine infusions induced by maternal WD was accompanied by dampened acetylcholinesterase activity in the striatum, VTA and hypothalamus, as well as increased nicotinic acetylcholine receptor binding in the VTA (Morganstern et al., 2013). Finally, oxidative stress in the VTA was seen in male and female rats that showed stronger preference for the morphine-associated side in CPP when they were born to dams that received extended perinatal exposure to trans-fat, even in the animals that received trans-fat exposure without any morphine exposure (Roversi et al., 2016). While not evaluated in the aforementioned study, oxidative stress can lead to the degeneration of dopaminergic neurons and dopamine metabolism (Dias et al., 2013), warranting further research on the association between perinatal WD, oxidative stress, and altered dopaminergic signaling. Altogether, there are several mechanisms that could be involved in the development of reward-motivated deficits associated with perinatal WD exposure, with dopaminergic signaling being a prime suspect.

Adolescent WD Exposure

Similar to perinatal exposure, adolescent consumption of WDs also impacts reward-motivated behavioral outcomes. For example, adolescent access to a WD, where 40–45% of total kcals are derived from fat, alters diet preference for fat and carbohydrates (Teegarden et al., 2009; Steele et al., 2019) and increases impulsivity (Steele et al., 2019). Specifically, after only 1 week of exposure to a WD (44.9% kcals from fat) at 3 weeks old, WD mice consumed significantly more calories from relative to controls when given a macronutrient choice test, which included options for a high fat diet, a high carbohydrate diet, or high protein diet in adulthood for 10 days. However, this preference did not lead to overconsumption of a WD (44.9% kcals fat) when provided with chronic access (15 weeks) in adulthood (Teegarden et al., 2009). Food preference shifts can also occur without increased hedonic orofacial liking responses in the taste reactivity test, as adolescent male rats exposed to a chronic WD (40% of calories from hydrogenated vegetable fat) for 8 weeks after weaning did not display differences in taste reactivity for fat or sugar, yet still preferred fat over sugar in a preference test in adulthood compared to age-matched rats that received a high sugar diet (40% of the calories from a powdered sugar and water mixture) or a healthy low fat diet, both of which preferred sugar over fat (Steele et al., 2019). Importantly, these

animals were habituated to both the sugar and the fat a day before preference testing, so the results were not confounded by the novelty of the foods. In addition to alterations in food preference, both the high fat and high sugar groups were also more impulsive in an impulsive-choice test, where the reward was grain pellets, as indicated by increased delay sensitivity/delay discounting. In addition to having an increased discounting rate for delayed rewards, the high fat diet-fed rats were also more impulsive relative to the high-sugar group and the control group when the magnitude of the reward was manipulated (Steele et al., 2019). On the other hand, 8 weeks of daily access to a cafeteria diet did not impact delay discounting for grain-based pellets, however these animals were more sensitive to the preference for smaller, immediate rewards induced by D2R antagonism (Robertson and Rasmussen, 2017). Another study found that male and female mice exposed to an even higher fat diet (60% kcals fat) for 12 weeks beginning at weaning preferred water over a 4% w/v sucrose solution in adulthood, suggesting possible anhedonia and a hypo-reward response. However this effect dissipated after switching to a healthy low fat diet for 4 weeks (Carlin et al., 2016). Accordingly, exposure to a WD reduced sucrose and saccharin preference during adolescence, a phenotype that was no longer observed at adulthood (Rabasa et al., 2016). These studies indicate that adolescent consumption of a WD increases impulsivity for food and dietary preference in adulthood, with adolescent exposure to a high fat leading to increased preference for a high fat diet in adulthood. Additional studies where chronic access is reintroduced in adulthood to determine whether or not these preferences influence long-term dietary choices would be intriguing.

In addition to increasing impulsivity and altering food choice, consuming a WD during adolescence affects motivation for food rewards (Tantot et al., 2017) and increases drug-seeking behavior (Blanco-Gandía et al., 2017; Naneix et al., 2017). For example, long-term exposure to a WD (45% kcal fat) from weaning and continuing into adulthood in male rats alters appetitive instrumental behavior such that WD-exposed rats show reduced motivation for food rewards (grain or sucrose) when under mild deprivation (90% of their *ad libitum* feeding body weight) in a progressive ratio test, where reinforcement during training was based on a random interval, but not random ratio, schedule (Tantot et al., 2017). Tantot and colleagues also found that training on a random interval schedule led WD-exposed rats to develop habitual food-seeking behavior faster than control rats, based on results from an outcome devaluation task (Tantot et al., 2017). However, both of these effects are reversed by subsequent training on a random ratio schedule. Furthermore, consuming a WD higher in fat (60% kcals fat) during adolescence impaired CPP for a food reward high in fat, despite consuming more of the reward during training, which was taken into consideration since the rats did not finish consuming the food in any given trial (Privitera et al., 2011). Importantly, the authors found that adult rats subjected to a 60% kcals WD in adulthood displayed normal CPP for the high fat reward, highlighting that CPP was only impaired when the high fat WD was consumed during adolescence. In the case of drug rewards, however, others have found that mice maintained on a WD (45% kcals fat) where access was given for 2 h/day thrice weekly since adolescence (PN 29) increased sensitivity to the reinforcing effects of a subthreshold dose of cocaine in the CPP task in adulthood (Blanco-Gandía et al., 2017). However, rats maintained on an *ad libitum* 40% kcals fat WD since adolescence (PN 21) had no impact on CPP acquisition for cocaine in adulthood, although this result may have been confounded by the rats being placed on significant water restriction (20 min access per day) during behavioral training and testing (Clasen et al., 2020). Further, when given the opportunity for intravenous self-administration of cocaine, WD-fed male mice elevated levels of self-administration. Ad libitum WD (45% kcals fat) exposure from weaning to adulthood can also lead to heightened amphetamine sensitization at adulthood in male rats relative to rats fed a control diet (Naneix et al., 2017). Taken together, these results suggest that chronic experience with consuming a WD with at least 45% of energy provided by fat starting at adolescence can lead to impaired goal-directed actions for food rewards and increased sensitivity to psychostimulant drugs in adulthood.

As WDs high in fat often contain a higher kcal percentage of sugar compared to control diets, it is important to consider the specific role of sugar in WD-associated effects on reward-motivated behavior. Indeed, there are studies suggesting that sugar affects reward-motivated behavior independent of elevated fat content in the diet (Frazier et al., 2008; Vendruscolo et al., 2010; Kendig et al., 2013; Naneix et al., 2016, 2018; Gueye et al., 2018; Steele et al., 2019). For instance, male rats on a 5% w/v sucrose solution during adolescence (PN 30–46) showed reduced motivation to obtain the low-calorie sweetener saccharin (sweet reinforcer) and maltodextrin (non-sweet carbohydrate reinforcer), but not cocaine in adulthood, as assessed by performance on fixed- and progressive-ratio schedules of reinforcement (Vendruscolo et al., 2010). These findings are supported by another study reporting diminished operant responding for saccharin under a progressive ratio schedule of reinforcement, as well as blunted sucrose preference in adults who had access to a 5% sucrose solution for 16 days during adolescence (Gueye et al., 2018). Adolescent exposure to 5% sucrose solution in male rats also resulted, at adulthood, in reduced intake of either saccharin or sucrose compared to water during two-bottle choice tests, as well as a decrease in positive 'liking' orofacial reactions to sucrose or saccharin relative to controls (Naneix et al., 2016). While there was no effect of brief adolescent access to a 5% sucrose solution, adults rats previously exposed to the sucrose during adolescence showed blunted sensitivity to D1R and D2R pharmacological manipulations on operant responding for 0.13% w/v saccharin solution at adulthood (Naneix et al., 2018). Furthermore, chronic access to *ad libitum* sucrose pellets in male and female mice starting at weaning for 4–7 weeks before being maintained on standard chow reduced motivation for sucrose under a progressive ratio schedule, but there were no differences in sucrose preference over water, nor differences in the amount of food consumed with a 3-week high-sugar, high-fat dietary challenge relative to controls (Frazier et al., 2008). This contrasts with findings in male rats, where a chronic high-sugar diet (∼40% kcals consumed from liquid sugar solution, ∼60% kcals consumed

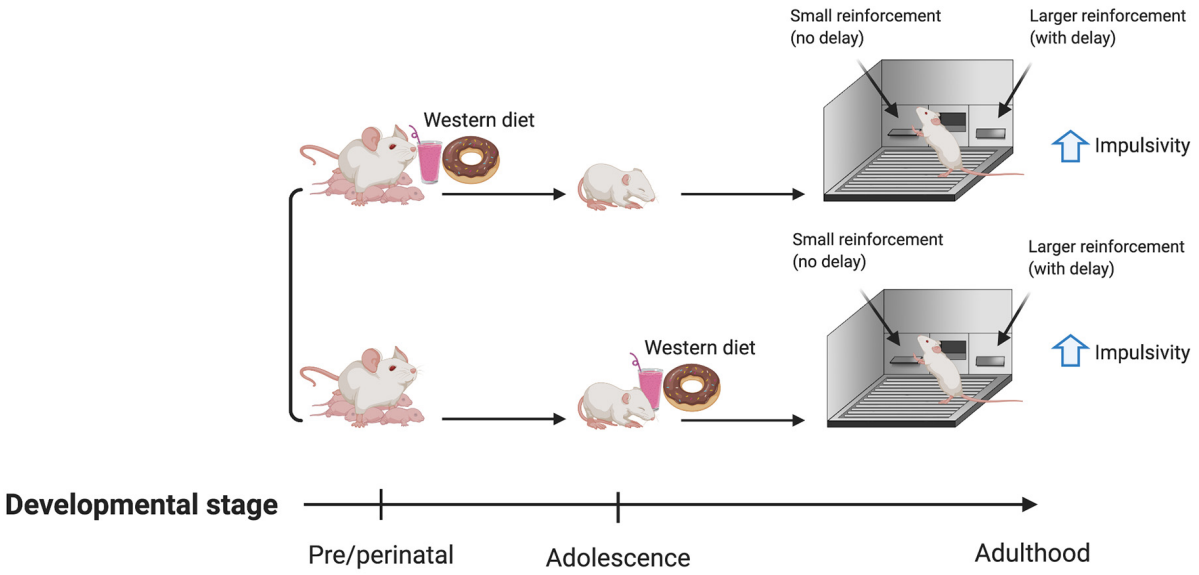
from standard chow for 8 weeks starting at PN 21), promoted a choice consumption bias for sugar over fat without differences in taste reactivity for sugar or fat (Steele et al., 2019). On the other hand, intermittent access to a 10% w/v sucrose or 0.1% w/v saccharin solution during adolescence failed to alter operant responding and delay discounting for food rewards, however, unlike the previous studies, reward pellets were sugar-enriched grain-based rather than pure sucrose (Kendig et al., 2013). The aforementioned studies show that adolescent WDs high in sugar reduce motivation for sweet taste in adulthood and lead to differences in preference for sweet taste, with some rodents also displaying decreased positive orofacial reactions to sweet taste. However, this may be dependent on the food choices available given that when sugar was provided during adolescence as a sweet taste preference was altered relative to control animals fed a healthy diet when sugar was offered as a beverage but not as a solid food.

While there are clear impacts of WD consumption during adolescence on reward-motivated behavior in rodents, obesity (or even increased body weight) is not required for these effects to occur. For instance, rodents displaying altered reward-motivated behavior following adolescent access to sugar either with a 5% w/v sucrose solution, sucrose pellets, or with a 40% kcals sugar diet had similar body weights relative to controls (Frazier et al., 2008; Vendruscolo et al., 2010; Naneix et al., 2016, 2018; Gueye et al., 2018; Steele et al., 2019). These outcomes are mirrored by comparable total energy intake, as the rats in these studies compensate for sugar calories by reducing chow intake (Vendruscolo et al., 2010; Naneix et al., 2016, 2018). However, after a high fat, high sugar WD challenge in adulthood, rats previously exposed to *ad libitum* sucrose pellets during adolescence gained more weight relative to controls (Frazier et al., 2008). Animals on a WD during adolescence or from adolescence to adulthood who showed reward impairments, either gained substantial weight relative to controls ((Privitera et al., 2011; Carlin et al., 2016; Rabasa et al., 2016; Naneix et al., 2017; Robertson and Rasmussen, 2017; Tantot et al., 2017) or did not (Teegarden et al., 2009; Blanco-Gandía et al., 2017), with less exposure (i.e., thrice weekly binge model or a 1 week exposure) likely contributing to normal weight gain in the latter studies. In addition to weight gain and reward deficits, rats fed a WD (45% kcals fat) through adolescence and early adulthood showed increased levels of leptin, insulin, and cholesterol, but not triglycerides (Naneix et al., 2017; Tantot et al., 2017) with one study finding increased adiposity (Carlin et al., 2016). Even though intermittent WD access (2 hrs daily, thrice weekly) during adolescence and early adulthood did not increase body weight, alterations in cocaine CPP in these male mice was still accompanied by greater plasma ghrelin levels (Blanco-Gandía et al., 2017). Altogether, these studies show that an obesogenic phenotype is not required for adolescent onset high sugar or high fat WD exposure to have an effect on reward-mediated behavior in rodents, yet alterations in reward processes may drive WD consumption and weight gain and metabolic alterations later in life.

Similar to perinatal WD exposure, adolescent WD consumption influences reward-motivated behaviors, in part, via alterations mesolimbic dopamine, cannabinoid and opioid signaling. For example, WD-exposed rodents that showed increased locomotor sensitization to amphetamine displayed a concomitant sensitized mesolimbic dopamine response as reflected by increased bursting activity of dopaminergic neurons in the VTA and increased ACB c-FOS expression (indicative of increased neural activation) in response to amphetamine administration. The WD-fed rats also showed increased dopamine release and tyrosine hydroxylase and D2R expression in the ACB (Naneix et al., 2017). On the other hand, similar exposure to sucrose during adolescence reduced D1R and D2R protein expression in the ACB under basal conditions (Naneix et al., 2018). Similarly, male mice that binged on a 45% kcals WD for 2 h a day from adolescence to adulthood were more sensitive to the reinforcing effects of another dopamine-enhancing drug, cocaine, as assessed by CPP and drug self-administration (Blanco-Gandía et al., 2017). These mice had reduced cannabinoid receptor 1 and mu opioid receptor in the ACB, but greater ghrelin receptor expression in the VTA. Mice that received 1 week exposure to a WD (approximately 45% kcal fat) at 3 weeks of age displayed a preference for fat over sugar in a macronutrient choice test, which was related to changes in dopamine-associated signaling pathways in the ventral striatum, including increased ΔFosB, cyclin-dependent kinase 5 (CDK5), and phospho-DARPP-32 Thr-75 (Teegarden et al., 2009). In another study, adolescent access to a 5% w/v sucrose solution led to sucrose anhedonia during adulthood, accompanied by reduced neurogenesis in the HPC, both of which were reversed by chronic antidepressant treatment (Gueye et al., 2018). In rats exposed to a high fat and high sucrose WD during early adolescence, blunted preference for sucrose and saccharin to coincide with reduced catechol-o-methyltransferase, an enzyme that can degrade dopamine, gene expression in the mPFC, a molecular feature which was absent when preference was restored at adulthood (Rabasa et al., 2016). Finally, female mice that had an anhedonic response to sucrose after having been exposed to a 60% kcals WD during adolescence exhibited increased dopamine transporter and reduced tyrosine hydroxylase expression in the VTA, reduced D1R and D2R expression in the ACB, and reduced D1R and D2R in the mPFC, all of which suggest reduced dopamine availability in these regions. However, a chronic 60% kcals fat WD during adolescence led to sucrose anhedonia and increased dopamine protein levels in the mPFC, which was later reversed after switching to a chow diet for 4 weeks (Carlin et al., 2016). Overall, it is clear that WD influences reward-mediated behavior in part by inducing long-lasting changes in dopamine signaling in the ACB, VTA, and PFC.

Summary

Both perinatal and adolescent WD exposure in rodents has lasting effects on reward-motivated behavior in adulthood (**Figure 3**). Perinatal WD exposure has been shown to increase preference for drugs of abuse (Roversi et al., 2016; Sarker et al., 2019) and increase (Sarker et al., 2019) or decrease (Naef et al., 2008) sensitivity to drugs of abuse later in adulthood depending on the duration of perinatal WD exposure. Perinatal WD exposure is also associated with increased impulsivity



Reward-motivated behavior

- Early life Western diet consumption increases impulsive responding for palatable food.*
- These outcomes can occur independent of obesity and metabolic impairments
- Underlying neurobiological mechanisms involved altered signaling pathways in the striatum, ventral tegmental area, and medial prefrontal cortex, including dopamine and opioid signaling

*Effects may vary based on behavioral test, sex as a variable, rodent species, strain, diet composition, and diet duration

FIGURE 3 | Summary of the effects of early life Western diet consumption on reward-motivated behavior.

(Grissom et al., 2015) and a decrease in behavioral flexibility (Wu et al., 2013). Furthermore, perinatal WD exposure in rats can lead to increased consumption of a WD later in life (Ong and Muhlhausler, 2014). Similar outcomes are associated with adolescent WD exposure in rodents. For example, sensitivity to the reinforcing effects of drugs of abuse is increased after WD exposure during adolescence and early adulthood (Blanco-Gandía et al., 2017) as well as locomotor sensitization to amphetamine (Naneix et al., 2017). WD consumption in adolescent rodents can alter macronutrient preference (e.g., carbohydrates vs. fat) depending on the type of macronutrient predominantly consumed during adolescence (Teegarden et al., 2009; Steele et al., 2019). However, increased preference does not necessarily lead to overconsumption as was seen in mice offered chronic WD access in adulthood (Teegarden et al., 2009). Findings from some studies also suggest that WD exposure from adolescence to adulthood can lead to anhedonia (Carlin et al., 2016; Naneix et al., 2016; Rabasa et al., 2016; Gueye et al., 2018). Additionally, adolescent WD consumption may reduce motivation for food rewards (Frazier et al., 2008; Vendruscolo et al., 2010; Privitera et al., 2011; Tantot et al., 2017; Gueye et al., 2018) and promote habitual as opposed to goal-directed behavior (Tantot et al., 2017). Finally, even in the absence of behavioral phenotype under basal conditions, adolescent consumption of a WD or sucrose solution during adolescence is linked to altered sensitivity to the effects of DA pharmacological agents on operant responding for food (Robertson and Rasmussen, 2017; Naneix et al., 2018) but not locomotor activity (Rabasa et al., 2016).

While perinatal and adolescent WD exposure can impact reward-motivated behavior, an obesogenic phenotype, as indicated by increased body weight and a perturbed metabolism, is not required for these effects to occur in rodents. Neural connections involving dopamine, opioid, and other neuropeptide systems between the VTA, mPFC, ACB, and various structures embedded within the brain reward circuitry regulate motivated behavior (Antonopoulos et al., 2002). Depending on the behavior observed, perinatal and adolescent WD exposure has been shown to either increase or decrease dopamine

neurotransmission across regions of the reward circuitry by regulating dopamine uptake and metabolism, as well as the expression of dopaminergic, but also opioid and cannabinoid receptors. In summary, the altered reward-motivated behavioral effects seen with adolescent onset WD exposure is associated with various changes in DA transmission in reward circuitry, and can occur independent of WD effects on obesity and metabolic derangement.

SOCIAL BEHAVIOR

Behavioral Tests and Neural Substrates

In both humans and rodents, it is well known that dietary choices are influenced by social factors (Wouters et al., 2010; Salvy et al., 2012; Hsu et al., 2018; Pompili and Laghi, 2019). One mechanism by which social behavior can impact feeding behavior is by affecting reward systems. For example, in the case of "peer pressure," being surrounded by peers has been shown to reduce inhibitory control by increasing reward sensitivity (Thiel et al., 2009; Logue et al., 2014). In addition, social isolation during adolescence can either increase or decrease sucrose drinking in rats during adulthood, suggesting that early life social isolation can change the incentive value of sucrose rewards (Van den Berg et al., 1999; Hong et al., 2012). However, while social behavior impacts dietary patterns and reward, the question as to whether dietary patterns impact social behavior remains largely underexplored. Given that the reward centers of the brain, such as the ACB, VTA, dorsal raphe nucleus, the lateral habenula, and mPFC are known to be activated by social reward (Chen and Hong, 2018) as well as food rewards (Kelley et al., 2005), it is reasonable to suspect that diet can impact social behavior and that this may be mediated by differences in oxytocin signaling, which modulates the neurons in the VTA and NAc to promote social reward (Dölen et al., 2013; Hung et al., 2017). In this section, we review the currently known effects of an obesogenic diet on social behavior, focusing on studies that examine rodent play behavior (three-chamber social interaction and social reciprocal interaction test), social memory (social-novelty preference test), and social avoidance (social defeat paradigm). We also discuss how diet may impact social behavior through the reward centers of the brain that are known to mediate the preference or reinforcement of social interactions.

Prenatal/Perinatal WD Exposure

Prenatal and perinatal exposure to a WD has been shown to reduce social play behavior in rodents. For example, mouse dams maintained on a 60% kcal from fat WD diet had male and female offspring that displayed decreased sociability in the three-chamber social interaction test during young adulthood (Kang et al., 2014). Similarly, male rats born to dams that received a cafeteria diet of standard chow, milk chocolate, peanuts, and sweet biscuits during lactation spent less time socializing and had fewer social interactions in adulthood (Teixeira et al., 2020). Maternal consumption of a 60% kcal fat WD diet, initiated 8 weeks prior to conception, also reduced sociability and impaired social memory, deficits that were rescued by housing WD offspring with those from dams consuming a regular diet (Buffington et al., 2016). On the other hand, perinatal exposure to a 43% fat kcal WD diet had no impact on social behaviors during adulthood (Zieba et al., 2019), suggesting the % kcal from fat may be a key variable. Interestingly, WD exposure on the paternal side (pre-conception) also influences the social behavior of the offspring, as there were fewer play attack behaviors at PN 24–29 in both sexes and more defensive play behaviors in male rats if the rats were sired by males on a WD (60% kcals fat) for 60 days before mating (Korgan et al., 2018). In contrast, male and female rats that received a high-fat, high-sugar WD (60% kcals fat from solid diet, plus access to a 20% w/v sucrose solution) *in utero* up until PN 40 had increased play behavior (number of play attacks initiated) during adolescence (PN 37) compared to rats that received a control diet for the same duration (Hehar et al., 2016). One major difference between these studies is that the exposure to the diet was maintained in this study through the time of testing. Lastly, maternal WD feeding prevented the negative effect of early-life stress on social interactions in offspring (Rincel et al., 2016). Taken together, these studies suggest that prenatal/perinatal exposure to WDs may lead to reduced social behavior, though perhaps only when exposure to the diets are removed prior to testing, when diet fat content is high (∼60% kcal), and in the absence of early-life stress.

In each of the aforementioned studies, maternal/paternal obesity is a confounding factor, and thus it is difficult to determine whether observed effects are a result of the diet *per se*. For example, in the studies that found reduced social interactions as a result of a pre/perinatal WD, body weights (Kang et al., 2014; Korgan et al., 2018; Teixeira et al., 2020) and fat pads (Korgan et al., 2018; Teixeira et al., 2020) were increased in the offspring relative to controls. However, body weights and fat pads were also increased in the males that were provided the paternal WD and sired the offspring (Korgan et al., 2018), and increased weight was also seen in the dams provided with a maternal WD (Kang et al., 2014). Additionally, early life milk overnutrition starting at PN 2, induced by a reduction in litter size, results in overweight offspring and the males have a lower frequency of social play behavior (pouncing and pinning) without any differences in social behaviors unrelated to play (sniffing and grooming) (Carvalho et al., 2016). Such findings support the notion that weight gain alone may be sufficient to impact social behavior in male, but not female, rats. However, Buffington and colleagues (Buffington et al., 2016) observed profound social deficits in the absence of weight or adiposity differences in male offspring, even though WD fed dams had greater body weight. Taken together, these studies suggest that pre/perinatal exposure to WDs negatively impacts social behavior, and these effects are likely dependent on the duration of dietary exposure (whether the offspring are still on the diet at the time of testing) and the dietary fat content. Further studies are needed to determine the degree to which the effects of WD on social behavior are dependent on the development of obesity in either the parent or offspring.

The neural mechanisms by which pre/perinatal exposure to a WD impacts social behavior are thus far poorly understood. Among the reward system-related brain structures, the developing mPFC is a candidate mechanistic link as it is

known to be involved in understanding social cues in rodent play behavior (Bicks et al., 2015). The HPC, which is known to be involved in social recognition (Uekita and Okanoya, 2011), may also be a neurobiological link between perinatal WD and altered social behavior. Supporting these notions, after a perinatal cafeteria diet, which reduced social interactions in adult offspring, levels of glutathione transferases, which are involved in cellular detoxification and oxidative stress, were greater in the mPFC (Teixeira et al., 2020). In the same study, levels of oxidative stress markers such as malondialdehyde (MDA) and superoxide dismutase (SOD) were higher in the HPC. Other potential mechanisms for reduced social behavior include increased whole brain neuroinflammation induced by IL-1β, TNFα and microglial activation, which was observed following *ad libitum* WD exposure 6 weeks prior to mating, during gestation, and during lactation (Kang et al., 2014). Importantly, both the reduced social behavior and elevated inflammatory marker expression across the brain normalized by switching to a healthy chow diet during lactation (Kang et al., 2014). Interestingly, social deficits in offspring of dams fed a WD were accompanied by dampened VTA LTP in DA neurons upon presentation of a novel mouse, as well as reduced oxytocin cell count in the hypothalamus and intestinal dysbiosis (Buffington et al., 2016). All of these impairments were restored by treatment with *Lactobacillus reuteri*, highlighting a role for gut flora on VTA function and associated social behaviors. Overall, while further research is required, these data suggest that early life WD exposure may impact neural processing in the mPFC, HPC, and VTA, and that a role for gut microbiota composition is an area for future investigation.

Adolescent WD Exposure

Similar to perinatal exposure, consuming an obesogenic diet during adolescence impairs social memory and influences social interaction. More specifically, in adolescent male rats, either brief exposure to a WD (60% kcals fat) for 7–8 days starting at PN 27–28 or prolonged intermittent access to a WD (20% kcals fat, 39.6% kcals sucrose) diet for 2 h daily from PN 28–56 promoted deficits in social recognition memory, measured by the time spent exploring a novel rat vs. a familiar rat (Reichelt et al., 2019, 2020; Yaseen et al., 2019). Moreover, rats on an intermittent WD diet (2 hrs daily from PN 28–56) showed a reduction in the total duration of social contact when they were tested in a social interaction test with a novel rat (Reichelt et al., 2020). These findings were specific to conditions where the animal was allowed to interact with the rat prior to the WD feeding period on the day of the test. When tested after WD access, social investigation (sniffing, licking, grooming) was increased in rats fed WD. These data support that social interactions can be mediated by adolescent WD exposure, but it is important to note that only some social variables are affected, as the frequencies of social play behavior (pinning, pouncing) and of aggressive-like behavior (biting, boxing, overt physical harm) were not affected by the dietary exposure (Reichelt et al., 2020). Additional work revealed that intermittent access to a WD during adolescence impaired both social interaction and social memory, but had no impact on social odor preference as all rats preferred bedding soiled by a conspecific (Reichelt et al., 2020). Adolescent consumption of a 45% fat diet, but not 38% fat and 20% fructose diet, also reduced social interaction during adulthood (Gancheva et al., 2017), suggesting a predominant role for dietary lipids. Consistent with this framework, free access to a 5% sucrose solution during adolescence had no effect on social behaviors during adulthood (Gueye et al., 2018), while consuming a 60% fat diet for 7 weeks reduced social interactions in both the 3-chambers test and the social reciprocal interactions test, in addition to reducing ultrasonic vocalization upon female encounter (Yang et al., 2020). Conversely, male mice on a WD (45% kcal from fat) or cafeteria diet from PN 28–49 before switching to standard chow did not display differences in social behavior in the three-chamber social interaction test in adulthood at PN 73+ relative to controls (Fülling et al., 2020), suggesting that it is possible for deficits in social behavior to be reversed by a dietary intervention. Thus, additional studies to determine the duration of exposure to WDs necessary to impact social behavior, as well as whether or not the effects are reversible with dietary intervention are warranted.

Although adolescent WDs lead to impaired social memory and differences in social interactions, WDs can also protect against social defeat stress. For example, male rats that received 4-h daily access to a 45% kcals fat WD daily for 9 weeks after weaning were more resilient to social defeat stress in adulthood (PN 63), as measured by resident-intruder interactions (spending less time in submissive postures and having a longer latency to submit to the resident) (MacKay et al., 2017). Resiliency to social defeat stress was also demonstrated by another study that found that consuming a 45% kcals fat WD *ad libitum* from weaning onwards in male mice did not result in social avoidance behavior in adulthood when tested using a social interaction test after social defeat stress (Finger et al., 2011). Together, these studies show a protective effect of adolescent WD on social defeat stress such that a WD can be anxiolytic under stressful circumstances, a notion supported by the fact that the rodents in both of the aforementioned studies displayed reduced anxiety with WD exposure after social defeat stress as measured by open field (MacKay et al., 2017) and the light-dark box test (Finger et al., 2011).

While some of the animals that showed deficits in social recognition memory after having consumed a WD during adolescence did not show differences in body weight (Reichelt et al., 2019, 2020; Yaseen et al., 2019), fasting glucose, or cholesterol (Yaseen et al., 2019), those that were impaired in social memory did have higher energy intake and fat mass (Gancheva et al., 2017; Reichelt et al., 2019, 2020; Yang et al., 2020) and increased systemic levels of leptin (Yaseen et al., 2019). However, the rodents resilient to social defeat stress after WD exposure either showed increased weight gain with increased plasma leptin and insulin levels (Finger et al., 2011) or no differences in body weights, body fat, glucose tolerance, caloric intake, or basal corticosterone levels compared to controls (MacKay et al., 2017). In cases where no differences in social interactions were found, mice fed a 45% kcals fat WD but not cafeteria diet had increased body weight and energy intake relative to controls

(Fülling et al., 2020), suggesting that obesogenic phenotypes after adolescent WD exposure likely do not influence social behavior.

Similar to the consequences of perinatal WDs on social behavior, adolescent WDs may affect social behavior by impacting the PFC. For instance, social memory deficits were associated with impaired long-term potentiation in the mPFC and reduced protein levels of oxytocin in mPFC tissue. These deficits were reversed by administration of oxytocin systemically or the selective oxytocin receptor agonist, [Thr4,Gly7]-oxytocin in the mPFC (Yaseen et al., 2019), which supports the idea that intact oxytocin signaling is necessary for rodents to recognize a familiar conspecific animal (Ferguson et al., 2000). Furthermore, the effects on social behavior and memory were associated with reduced expression of monoamine oxydase and COMT genes in the mPFC (Reichelt et al., 2020), suggesting that monoamine activity possibly related to dopamine was altered by WDs. In another study, the same group reported similar molecular outcomes in the mPFC, as well as reduced hippocampal monoamine oxydase and changes in gut microbiota (Reichelt et al., 2020). Impairments in social memory were further determined to be associated with reduced parvalbumin interneurons in the mPFC and increased co-expression of perineuronal nets on parvalbumin interneurons, both of which are involved in neuroplasticity (Reichelt et al., 2019). Male mice showing alterations in social behaviors also displayed blunted neuronal activity in the mPFC upon presentation of a social stimulus, and mPFC expression of senescence-related genes was upregulated, especially in astrocytes and microglia (Yang et al., 2020). Finally, given increased serum levels of both triglycerides and reactive species, it has been hypothesized that altered

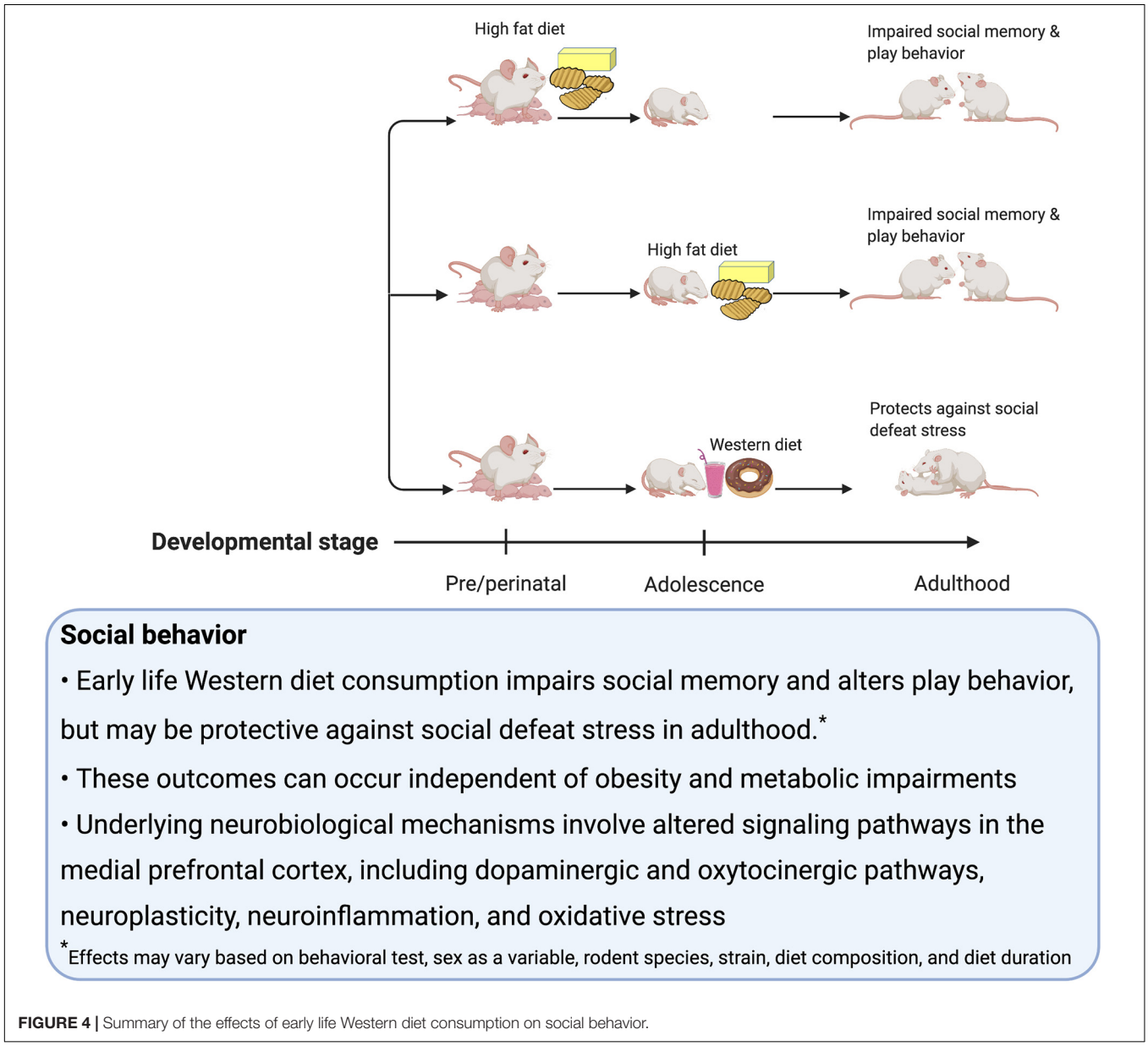


FIGURE 4 | Summary of the effects of early life Western diet consumption on social behavior.

lipid peroxidation mediates the social impairments associated to adolescent WD feeding (Gancheva et al., 2017). Overall, these findings suggest that adolescent WD exposure may impact social behavior through oxytocin signaling, monoamine enzymes, and changes in mPFC neuroplasticity.

Summary

The studies reviewed here suggest that consumption of a WD during early life periods of development impair social-based memory and alters play behavior (e.g., reduces play attacks), but also that these same diet exposures may be protective against social defeat stress (**Figure 4**). These effects are likely independent of an obesogenic phenotype and may include a mechanism involving mPFC dopaminergic and oxytocinergic pathways as well as changes in neuroplasticity, neuroinflammation, and oxidative stress. Given that the majority of these studies in this section only observed male rodents, further research investigating how early life WD consumption impacts social behavior in females is necessary in the future.

CONCLUSION

In this review, we present behavioral and neurobiological evidence from preclinical rodent models highlighting that consumption of (or perinatal exposure to) a WD during critical periods of development can lead to neurocognitive dysfunction later in adulthood. In particular, we highlight the detrimental effects of WDs high in fat (particularly saturated), sugar, or a combination of the two on the following cognitive domains: anxiety-like behavior, learning and memory function, reward-motivated behavior, and social behavior (**Supplementary Table 1**).

During the perinatal period, exposure to WDs results in impairments in each of these cognitive domains in rodents when tested during adolescence and/or adulthood. For example, increased anxiety-like behavior is associated with prenatal/perinatal WD exposure unless the rodents were also subjected to early life stress, where prenatal/perinatal WDs ameliorates anxiety-like behavior. Additionally, WD exposure during the perinatal period impairs episodic memory and conditioned odor aversion learning and extinction but has no effect on contextual or cued fear conditioning. Furthermore, effects on reward-motivated behavior are also common after prenatal/perinatal WD exposure, including addictive-like behaviors to drugs of abuse, increased impulsivity, reduced behavioral flexibility, and reduced motivation to work for food reward despite increased consumption when the rewarding food is made freely available. Finally, prenatal/perinatal WD exposure reduces social memory and prosocial behavior in rodents. Importantly, these effects are often seen despite the progeny being maintained on a standard, low-fat chow after weaning. Further research is necessary to evaluate whether behavioral effects are specific to a certain stage within the prenatal/perinatal period, as some studies found certain effects were specific to late gestation or lactation.

Long-term access to WDs during adolescence increases anxiety-like behavior in adulthood, although this effect may not be long-lasting given that anxiety-like behavior is not seen in rodents that had the WD removed for an extended period of time. Similar to what occurs after prenatal/perinatal WD exposure, adolescent WD exposure is protective against the development of anxiety-like behavior when consumed during periods of early life stress. Both acute and chronic consumption of WDs has deleterious effects on learning and memory processes, including impaired spatial and episodic memory, cued fear learning and extinction, aversive memory, and reversal learning. With regards to reward-motivated behavior, WD exposure during adolescence shifts dietary preferences in adulthood towards a diet similar to what the animal was exposed to during adolescence. Additionally, adolescent WD exposure increases impulsive responding for food rewards, reduces motivation for food rewards, and increases drug-seeking behavior. WD consumption during adolescence also impairs social memory and affects social interaction behavior in adulthood, yet reduces stress induced by social defeat. Altogether, the literature indicates that adolescent exposure to WDs promotes enduring cognitive impairments but may also mitigate stress responses. More research is necessary to determine whether similar effects are observed in female rodents, and whether sex hormones are a critical variable.

In many instances, behavioral deficits associated with early life WD exposure occurred independent of obesity-associated outcomes such as increased body weights, adiposity measures, hyperphagia, or negative metabolic outcomes. Further, in many cases the neural substrates associated with each cognitive domain are impacted by WD exposure during development, such as increased oxidative stress, inflammation and microglial activation, as well as altered neurotransmission, neurotrophic signaling, synaptic plasticity, and reduced neurogenesis. What remains to be understood is whether or not these cognitive deficits can be reversed given some type of intervention (e.g., dietary, exercise, microbiome) and whether or not these cognitive consequences have transgenerational effects.

Evidence reviewed herein establishes that WD exposure in rodents during the perinatal and/or adolescent period promotes long-term deficits in anxiety-like behavior, learning and memory, reward-mediated behavior. While this review focused on rodent studies, similar changes in behavior in humans have been reported, where healthier diets are positively associated with better cognitive outcomes (Baym et al., 2014; Zahedi et al., 2014; Tandon et al., 2016; Borge et al., 2017; Cohen et al., 2018). The rodent literature reviewed here adds important insight into the mechanisms underlying poor cognitive outcomes following early life WD consumption and may help guide translational research and dietary recommendations in humans.

AUTHOR CONTRIBUTIONS

All authors contributed to the idea for the manuscript. LT and LD-S wrote and edited the manuscript. SK and EN edited

the manuscript, provided vital input to shape the manuscript, and contributed to the writing. All authors approved the final manuscript. The figures were created using BioRender.com by LT, and reviewed by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.632312/full#supplementary-material

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Effects of Long-Term Endogenous Corticosteroid Exposure on Brain Volume and Glial Cells in the AdKO Mouse

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Chronic exposure to high circulating levels of glucocorticoids has detrimental effects on health, including metabolic abnormalities, as exemplified in Cushing's syndrome (CS). Magnetic resonance imaging (MRI) studies have found volumetric changes in gray and white matter of the brain in CS patients during the course of active disease, but also in remission. In order to explore this further, we performed MRI-based brain volumetric analyses in the AdKO mouse model for CS, which presents its key traits. AdKO mice had reduced relative volumes in several brain regions, including the corpus callosum and cortical areas. The medial amygdala, bed nucleus of the stria terminalis, and hypothalamus were increased in relative volume. Furthermore, we found a lower immunoreactivity of myelin basic protein (MBP, an oligodendrocyte marker) in several brain regions but a paradoxically increased MBP signal in the male cingulate cortex. We also observed a decrease in the expression of glial fibrillary acidic protein (GFAP, a marker for reactive astrocytes) and ionized calcium-binding adapter molecule 1 (IBA1, a marker for activated microglia) in the cingulate regions of the anterior corpus callosum and the hippocampus. We conclude that long-term hypercorticosteronemia induced brain region-specific changes that might include aberrant myelination and a degree of white matter damage, as both repair (GFAP) and immune (IBA1) responses are decreased. These findings suggest a cause for the changes observed in the brains of human patients and serve as a background for further exploration of their subcellular and molecular mechanisms.

Keywords: glucocorticoid, glia, myelin basic protein, glial fibrillary acidic protein, ionized calcium binding adaptor molecule 1

INTRODUCTION

Glucocorticoid hormones (GCs) are mediators of the response to stress, a state following real or perceived threat to homeostasis (Smith and Vale, 2006). GCs act throughout the body *via* the widely expressed glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Pujols et al., 2002). GC binding to its receptors results in a wide array of genomic and non-genomic signaling changes at the cellular level (Kadmiel and Cidlowski, 2013). A major aspect of GR-mediated effects concerns its metabolic effects on carbohydrate, lipid, and protein metabolism (from their action on liver, adipose tissue, and muscle), which in the long term predispose for obesity and metabolic syndrome. Chronic exposure to high levels of GCs increases visceral adipose tissue (Debono et al., 2013) and upregulates the expression of lipogenic pathway genes (Hochberg et al., 2015). Accordingly, central obesity and dyslipidemia are very common signs in Cushing's syndrome (CS) patients, a disorder caused by prolonged exposure to excess GCs (Angeli et al., 1997; Garrapa et al., 2001; Sharma et al., 2015). By screening populations of patients with simple obesity, prevalence rates of CS might be as high as 9% (Tiryakioglu et al., 2010).

GCs also affect brain function. The effects on peripheral energy metabolism and immunity alone may already do this (Koorneef et al., 2018), but MR and GR are also widely (but differentially) expressed in neurons and other cell populations in the brain. Cortisol affects a wide range of brain processes, including food intake (mirroring peripheral effects on metabolism), cognition, emotion, and autonomic responses. The effects may involve biochemical and structural changes (de Kloet et al., 2005). The study of GC actions in the brain has served to illustrate two closely related concepts: (1) The effects of GC can be adaptive or deleterious, and (2) cellular responses to GCs are different depending on the duration of exposure (acute vs chronic) (McEwen et al., 2015). These two concepts are also illustrated clinically by changes in the brain of CS patients.

Cushing's syndrome patients in active disease have been reported to present with smaller hippocampal volumes (Starkman et al., 1992), larger ventricular diameters, and cerebral atrophy (Simmons et al., 2000; Bourdeau et al., 2002). Strikingly, patients in long-term remission present smaller volumes in total gray matter (Resmini et al., 2012), particularly in the anterior cingulate cortex (Andela et al., 2013), as well as decreased cortical thickness (Crespo et al., 2014; Bauduin et al., 2020). Changes are not limited to gray matter though, as these patients also present significant reductions in white matter integrity (van der Werff et al., 2014).

Recently, we developed mouse models of endogenous CS: the AdKO mice, which carry an inactivating deletion of the gene encoding the regulatory subunit 1 alpha of the PKA, specifically targeted to the adrenal cortex. These mice present adrenal hyperplasia, chronic hypercorticosteronemia, lack of negative feedback after exogenous GCs, and even fat accumulation in the back of the neck ("buffalo hump"), which is one of the most visible signs in CS patients (Sahut-Barnola et al., 2010; Drelon et al., 2016; Dumontet et al., 2018). Here, we used brain magnetic resonance imaging (MRI) in order to find whether the changes observed in human patients were mirrored in our mice. We also performed immunohistochemical staining of glial cells in an attempt to explore the origins of such changes.

MATERIALS AND METHODS

Mice

All animal work was conducted according to French and European directives for use and care of animals for research purposes and received approval from the French Ministry of Higher Education, Research and Innovation (APAFIS#21153-2019061912044646 v3). The *Sf1-Cre* (Cre expression in all steroidogenic cells of the adrenal cortex from its inception) (Bingham et al., 2006) and *Prkar1afl/fl* (floxed allele of *Prakar1a* that allows Cre-mediated inactivation of R1α subunit and subsequent constitutive activation of PKA catalytic activity; Kirschner et al., 2005) were used as breeders. Mice were all maintained and bred on a mixed background. Throughout the manuscript, $AdKO_{2.0}$ refers to *Sf1-Cre*::*Prkar1afl/fl*, and WT refers to littermate control animals. ACTH-independent CS features of mice bearing R1α inactivation in the adrenal cortex using various Cre-expressing lines were previously described (Sahut-Barnola et al., 2010; Drelon et al., 2016; Dumontet et al., 2018). $AdKO_{2.0}$ mice were used here because of their more severe CS phenotype compared to the original AdKO line that used the *Akr1b7* Cre driver (Sahut-Barnola et al., 2010). Mice from both sexes were analyzed at 8–10 weeks of age ($n = 6$ for males, $n = 6$ for females). Femur length was measured for a subset of the mice ($n = 3$ for male, $n = 4$ for females).

Magnetic Resonance Imaging

Mice were anesthetized with 2% isoflurane and perfused for 1 min with 1 × phosphate-buffered saline (PBS) and for 4 min with 4% paraformaldehyde (PFA). The skull was freed from the skin and fat tissue and stored in 4% PFA overnight and then transferred to 4% PFA + 1:40 v/v gadoteric acid 0.5 mmol/ml (Dotarem, Guerbet, France) at 4°C for 3 weeks. Then they were transferred to a solution of 1 × PBS, 1:40 v/v Dotarem, and 0.01% sodium azide, and after 2 days, an MRI scan was acquired. MRI acquisitions were performed on a 9.4-T Bruker magnet (Bruker, Germany). The magnet was equipped with a 15-mm microimaging radiofrequency (RF) coil and maximum gradients up to 1.5 T m^{-1} along the three axes. The fixed brain was introduced inside a flat-bottom 15-mm NMR tube (Hilgenberg, Germany), and Fomblin® (Sigma-Aldrich, France), a perfluorinated polyether, was added to limit the magnetic susceptibility at the interfaces without generating any NMR signal. 2D fast low-angle shot (FLASH) acquisition pilot scans were performed in three planes: axial, sagittal, and coronal. A whole-brain image was acquired based on a 3D-FLASH protocol. The echo and repetition times were set to 5.3 and 15.0 ms, respectively. The flip angle was 30° with a bandwidth of 3 kHz. The field of view (FOV), covering the whole brain, was 16 mm × 14 mm × 15 mm with a matrix of 320 × 280 × 300 points, leading to an isotropic resolution of 50 μm. Eight averages were performed, leading to an experimental time of

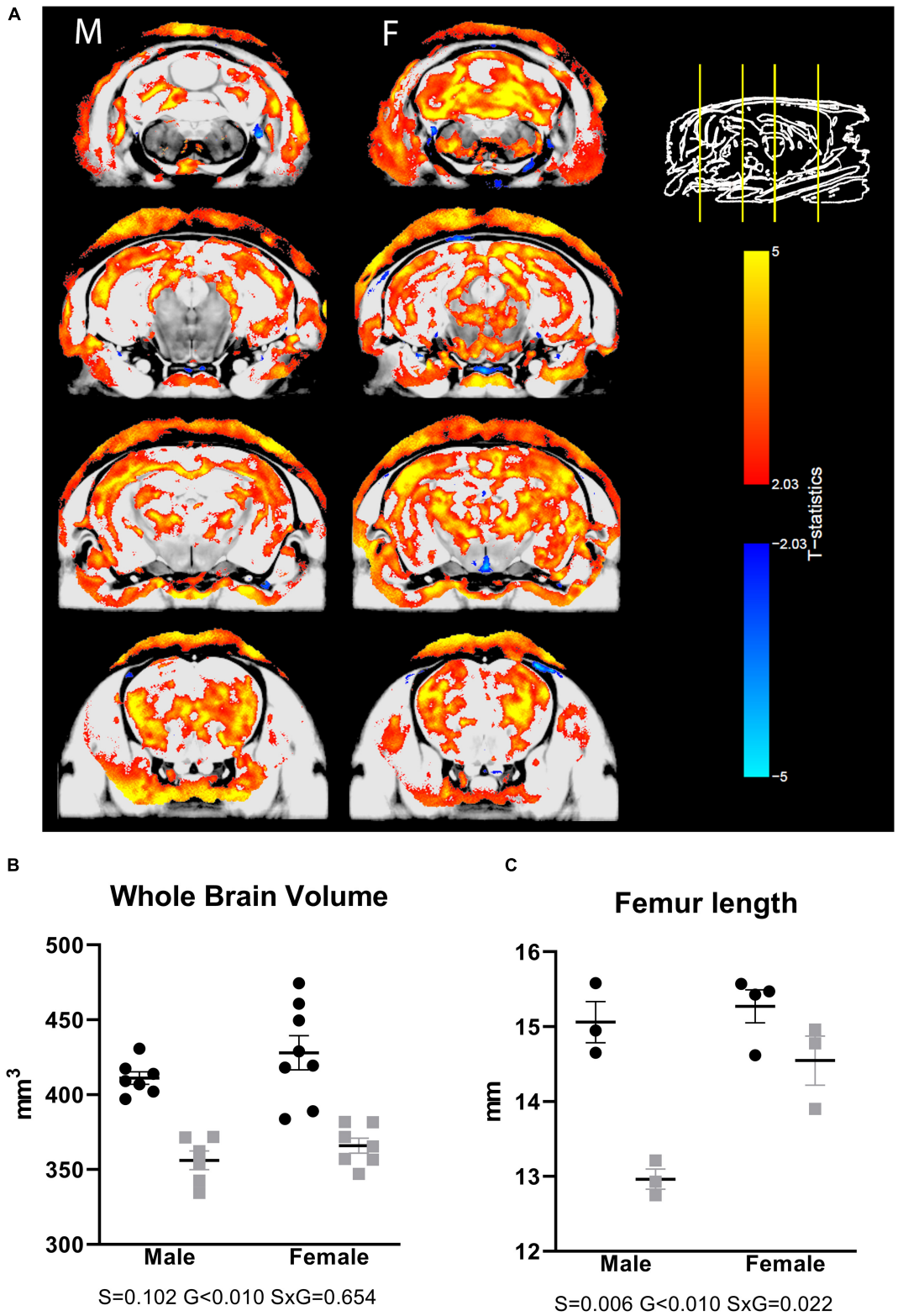

FIGURE 1 | Differences in brain volumes in $AdKO_{2.0}$ mice. **(A)** Coronal MRI slices showing significant differences (FDR 1%) in absolute volume between $AdKO_{2.0}$ mice and age-matched controls. M: male, F: female. The images show coronal MRI slices with the level of significance superimposed. Positive *t*-statistics are shown in red and indicate absolute volume decreases in $AdKO_{2.0}$ mice compared to controls. Negative values are shown in blue and indicate an increase of absolute volume compared to controls. **(B)** Absolute whole-brain volumes. **(C)** Femur lengths for a subgroup of mice. Black dots: wild type. Gray squares: $AdKO_{2.0}$.

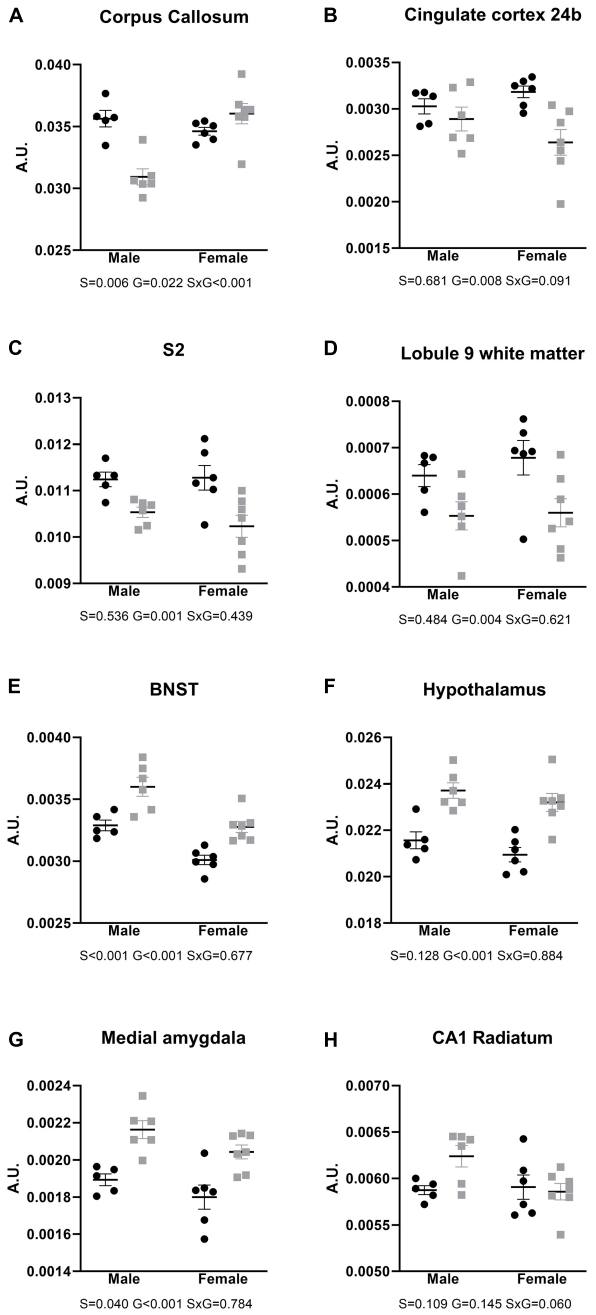

FIGURE 2 | Relative volumes of brain areas and regions, compared with two-way ANOVA. **(A–D)** Areas with smaller relative brain volumes in the $AdKO_{2.0}$ mice. **(E–G)** Areas with bigger relative brain volumes in the $AdKO_{2.0}$ mice. **(H)** CA1 s. radiatum as representative for the hippocampus. S2: secondary somatosensory cortex; BNST: bed nucleus of the stria terminalis; CA1: cornu ammonis 1. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$. AU, arbitrary units.

around 2 h and 50 min. One MRI data set (WT male) was excluded from further MRI analysis due to the presence of imaging artifacts.

The MRI scans were linearly (six parameters followed by 12 parameters) and subsequently nonlinearly registered using a combination of mni_autoreg tools (Collins et al., 1994) and advanced normalization tools (ANTs; Avants et al., 2008, 2011), resulting in unbiased alignment of all scans. A population atlas was created by resampling all MRI scans with the appropriate transform and by averaging the scans. Regional measurements were calculated by registering a preexisting classified MRI atlas on to the population atlas, which parcellated the brain into a volumetric measurement of 182 different brain regions (Dorr et al., 2008; Richards et al., 2011; Ullmann et al., 2013; Steadman et al., 2014). Subsequently, the deformation of the individual brains to the final atlas space was calculated and analyzed (Nieman et al., 2006) using the Jacobian determinant for each voxel between the genotypes. All statistical analyses were performed in R. We ran a linear model separately for each sex including genotype as contrast. Multiple comparisons were controlled for by using the false discovery rate (FDR) at a stringent setting of 1% (Genovese et al., 2002). FDR limits the expected number of false positives in a set of results to a certain predetermined percentage. For example, if a result is considered significant at a 1% FDR, this means that no more than 1% of the results would be expected to be a false positive. The entire pipeline was made available by the Mouse Imaging Centre in Toronto ([1]Lerch et al., 2017).

Immunohistochemistry

Brains were frozen and cut in a cryostat, and 12-μm sections were collected directly on glass slides, at coordinates Bregma 1.10 (anterior brain) and -1.82 (hippocampus), according to the Paxinos and Franklin Mouse Brain Atlas (Paxinos and Franklin, 2001). Glass slides were washed in PBS + Tween 20 0.3% (PBST), 3 min × 5 min. After washing, they were boiled in citrate buffer (Sigma) for 20 min and cooled in ice to room temperature. Slides were then washed again in PBST, 3 min × 5 min, and then incubated in PBST + 2% goat serum (Sigma) (PBSTG) at room temperature for 90 min. Sections were then incubated with antibodies for MBP (MAB386, Merck Millipore; 1:1,000), GFAP (PA1239, Boster Bio, 1:200), or Iba1 (019-19741, Wako Chemical, 1:1,000) diluted in PBSTG, overnight at 4°C. The sections were washed in PBST, 3 min × 5 min, and incubated in H_2O_2 3% for 30 min. After another wash in PBST, slides were incubated with Impress™ HRP anti-rat (MBP; Vector Labs) or EnVision+ system HRP anti-rabbit (GFAP and Iba1; Dako) for 45 min. After this, they were washed once again in PBST for 3 min × 5 min and colored with NovaRED™ solution (Vector Labs). After 7 min, reaction was stopped by adding excess deionized water. Slides were air-dried and mounted with coverslips using Histomount® (National Diagnostics). Due to long-term immersion in fixative supplemented with contrast medium, an elevated level of background was obtained. Digital images of selected fields were acquired in an Olympus microscope equipped with an Olympus digital camera connected to a Dell OptiPlex PC and were displayed using cellSens Standard 1.14 software (Olympus). A 10× objective was used. Digital images

[1]https://github.com/Mouse-Imaging-Centre

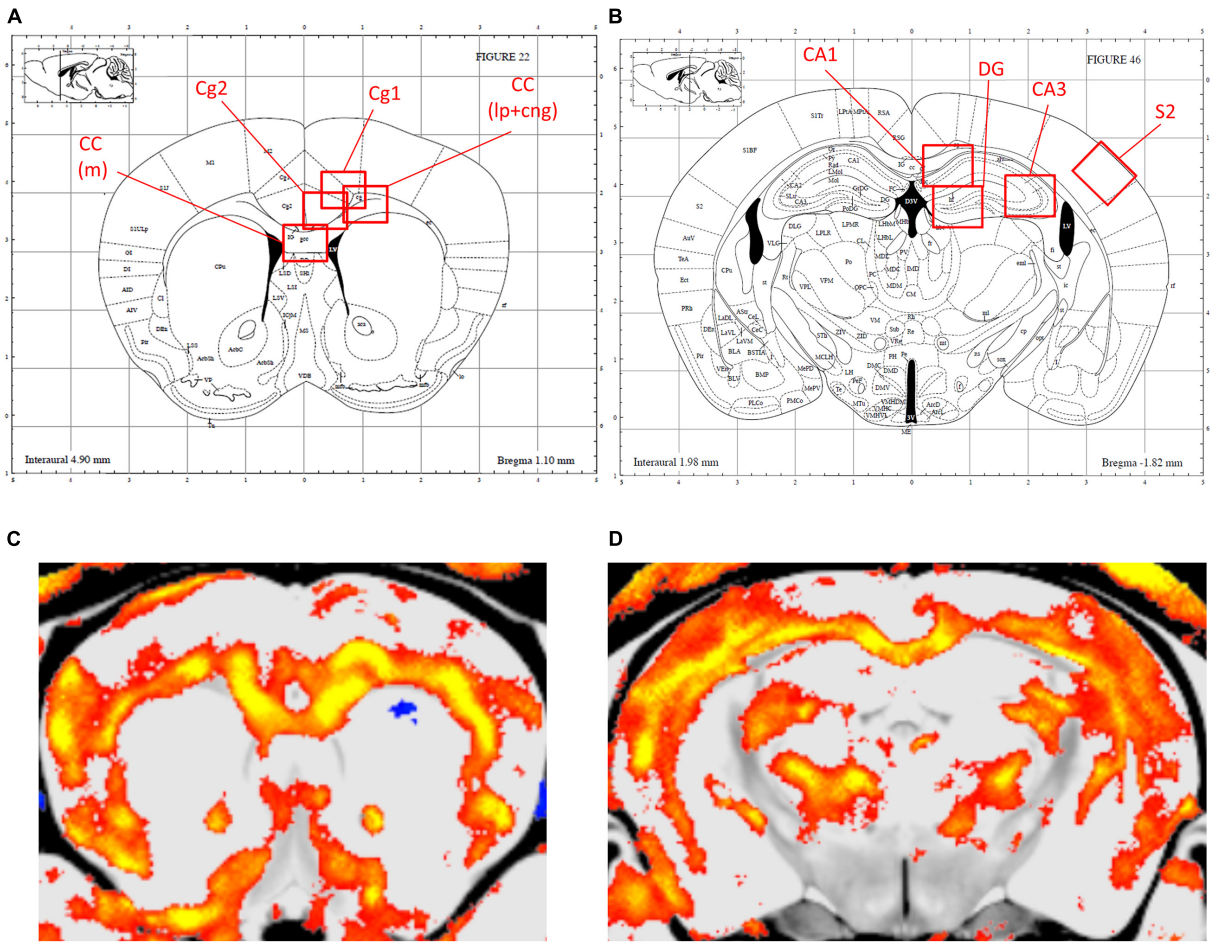

FIGURE 3 | Localization of the areas that were analyzed for immunohistochemical staining. **(A,B)** Indications of the regions of interest (ROIs) in the corresponding levels of the Paxinos atlas for the anterior brain (Bregma 0.98–0.86 mm), and dorsal hippocampus (Bregma -1.82 to -1.94) (Reprinted with permission by Elsevier Ltd.). **(C,D)** orresponding MR images, with smaller volumes indicated in color. Cg1, cingulate cortex 1; Cg2, cingulate cortex 2; CC (lp), corpus callosum lateroproximal; CC (m), corpus callosum medial; S2, secondary somatosensory cortex; CA1, cornu ammonis 1; CA3, cornu ammonis 3.

were then analyzed with ImageJ. In order to bypass the high amount of background in our images, we devised a method of signal quantification. Briefly, images were transformed from RGB to 8 bit, with a resolution of 2,560 × 1,920 pixels, and mean, median, mode, and standard deviation of the gray values in the entire picture were obtained. With these values, the signal threshold for each picture was determined by subtracting a fixed amount of standard deviations from the mode of the entire picture, which represents the background staining. The number of standard deviations was kept constant within the entire series of pictures for each antibody (2 for MBP, 2.5 for GFAP, and 3 for Iba1). The amount of positive signal marked by the threshold was expressed as the percentage area of total ROI and compared with two-way ANOVA with sex and genotype as factors. In GFAP and Iba1, we estimated the total number of cells, by counting the number of objects (cell bodies) above 250 pixels ($\sim$20 μm^2). The threshold for a significant effect was set at $p < 0.05$. Each animal from each subgroup contributed with only one section per brain area for this analysis.

RESULTS

Magnetic Resonance Imaging

To assess whether a CS phenotype results in neuroanatomical changes, we scanned *ex vivo* brain samples using high-resolution MRI. We observed volumetric differences that were widespread over the brain, including both gray and white matter areas (**Figure 1A**), with prominent differences, particularly in the corpus callosum and large parts of the cortex. Looking at absolute volumes, we found a significantly reduced brain size in males, with a trend toward significance in females (**Figure 1B**). For a subgroup of animals, the femur length was measured, showing a significantly smaller value in the transgenic animals (**Figure 1C**), indicating that the large difference in brain volume between WT and transgenic mice may be due to generalized growth retardation and not brain specific. Therefore, we also normalized the segmented structures for the whole-brain volume. Relative volume differences were then apparent for a number of brain regions. **Supplementary Table 1** provides an overview

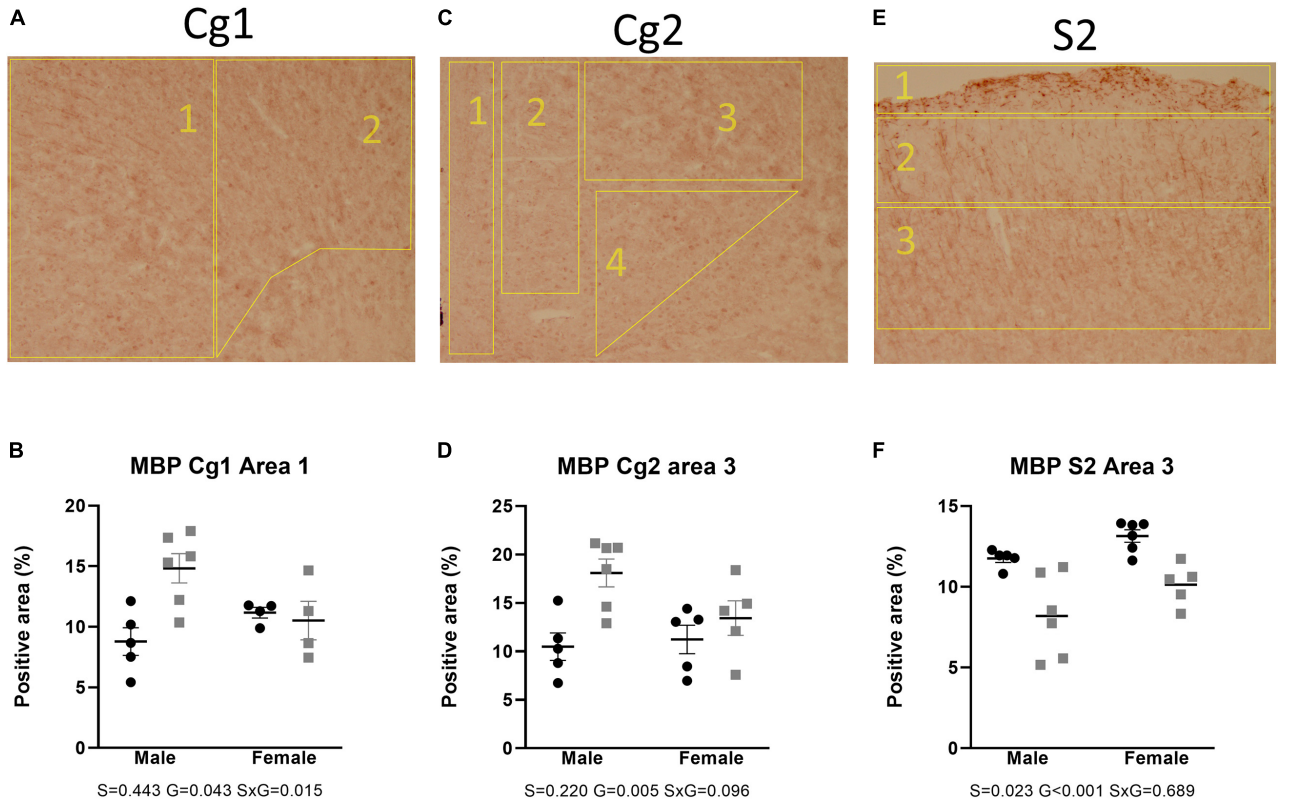

FIGURE 4 | Staining and quantification of MBP at the anterior level. Numbered polygons in stained sections indicate neuroanatomical parcellations. **(A–D)** In the cingulate cortex (Cg), $AdKO_{2.0}$ mice had a male-specific *increase* in MBP-positive area compared to WT mice. **(E,F)** In the somatosensory cortex (S2), there was a reduction in MBP-immunopositive area in $AdKO_{2.0}$ mice of both sexes. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$.

of all brain structures with differential relative volumes and uncorrected significance level. A list of all segmented structures with absolute and relative volumes and uncorrected *p*-values is given in **Supplementary Table 2**.

After FDR correction for multiple comparisons, the corpus callosum showed a robust significant difference in males, with a significantly smaller relative volume in $AdKO_{2.0}$ males than in WT males (**Figure 2A**). Lower volumes were also observed in frontal cortical areas, independent of sex, as well as cerebellar white matter (**Figures 2B–D**). In contrast, the bed nucleus of the stria terminalis, hypothalamus, and medial amygdala were found to be larger in $AdKO_{2.0}$ mice brain (**Figures 2E–G**). Finally, no hippocampal structures differed in relative volumes between genotypes; although the CA1 stratum radiatum appeared larger in male $AdKO_{2.0}$ mice compared to WT male counterparts in the pairwise comparisons available in the MRI pipeline. However, subsequent two-way ANOVA showed no significant effects for factors sex and genotype or their interaction (**Figure 2H**).

Immunohistochemistry

Figures 2, **3** show the relative volumetric differences between the brain structures that were chosen for immunohistochemistry, selected on prior data in active disease (hippocampus) (Starkman et al., 1992, 1999; Burkhardt et al., 2015), long-lasting effects in CD patients (Cg) (Andela et al., 2013), and reduced volumes in the present MRI analysis (S2). As mentioned before, we obtained a high level of background in our preparations due to long-term immersion in fixative supplemented with contrast medium, which we corrected for by using our thresholding method. MBP as a marker for oligodendrocyte function showed a main effect for genotype in cingulate cortex areas (Cg1 $p = 0.043$, Cg2 $p = 0.005$), indicating increased immunoreactivity in the $AdKO_{2.0}$ mice. For the Cg1 area, this effect was male specific (interaction effect $p = 0.015$). This interaction between sex and genotype was present at the trend level of the Cg2 area ($p < 0.1$) (**Figure 4**). At the hippocampal level, however, there was decreased MBP immunoreactivity in the CA1 and CA3 fields (CA1: s. oriens $p = 0.005$, s. pyramidalis $p = 0.033$, s. radiatum $p = 0.014$; CA3: s. lucidum $p = 0.048$, s. radiatum $p = 0.012$) (**Figure 5**). At this frontocaudal level, a decrease of MBP signal in $AdKO_{2.0}$ mice was also observed in the S2 cortex (main effect $p < 0.001$) (**Figure 5**). MBP signal showed a main effect of sex in the hippocampal CA3 area (CA1: s. moleculare $p = 0.047$; CA3: s. radiatum $p = 0.003$, s. moleculare $p = 0.001$) and S2 cortical area (area 3 $p = 0.023$), reflecting higher immunoreactivity in female mice. Overall, at the hippocampal level and somatosensory cortex, MBP immunohistochemistry was reduced in $AdKO_{2.0}$ mice, while in the cingulate cortex, there

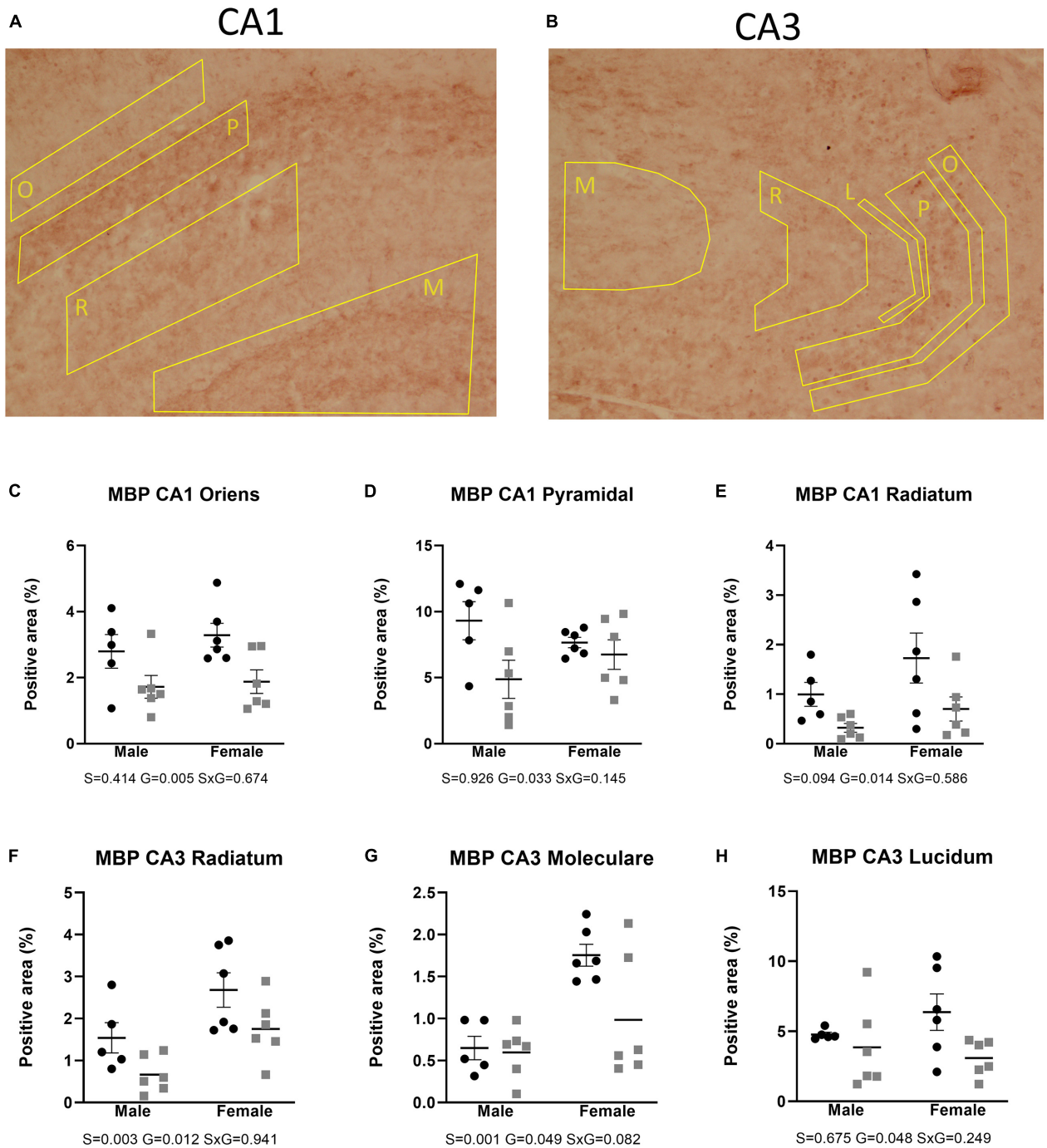

FIGURE 5 | Expression of MBP in the dorsal hippocampus. Coded polygons in stained sections indicate neuroanatomical parcellations. **(A,C–E)** In CA1, $AdKO_{2.0}$ mice showed a decrease in MBP-positive areas in both sexes in oriens (O) and radiatum (R) strata and a male-specific decrease in the pyramidal layer, compared to WT mice. **(B,F)** In CA3, $AdKO_{2.0}$ mice showed a decrease compared to WT mice in expression in both sexes in the s. radiatum. **(G,H)** In the s. moleculare (M) and s. lucidum (L), a female-specific decrease was shown. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$.

was a male-specific increase. All significant effects are listed in **Supplementary Table 3A**.

GFAP immunohistochemistry was used as a reactive astrocytic marker. Two-way ANOVA tests showed a main effect of genotype, reflecting a decrease of immunoreactivity in the $AdKO_{2.0}$ mice throughout the brain regions. This was found in the corpus callosum (p = 0.036) and in the hippocampal CA1 (s. radiatum p = 0.014) and dentate gyrus (DG; granular layer

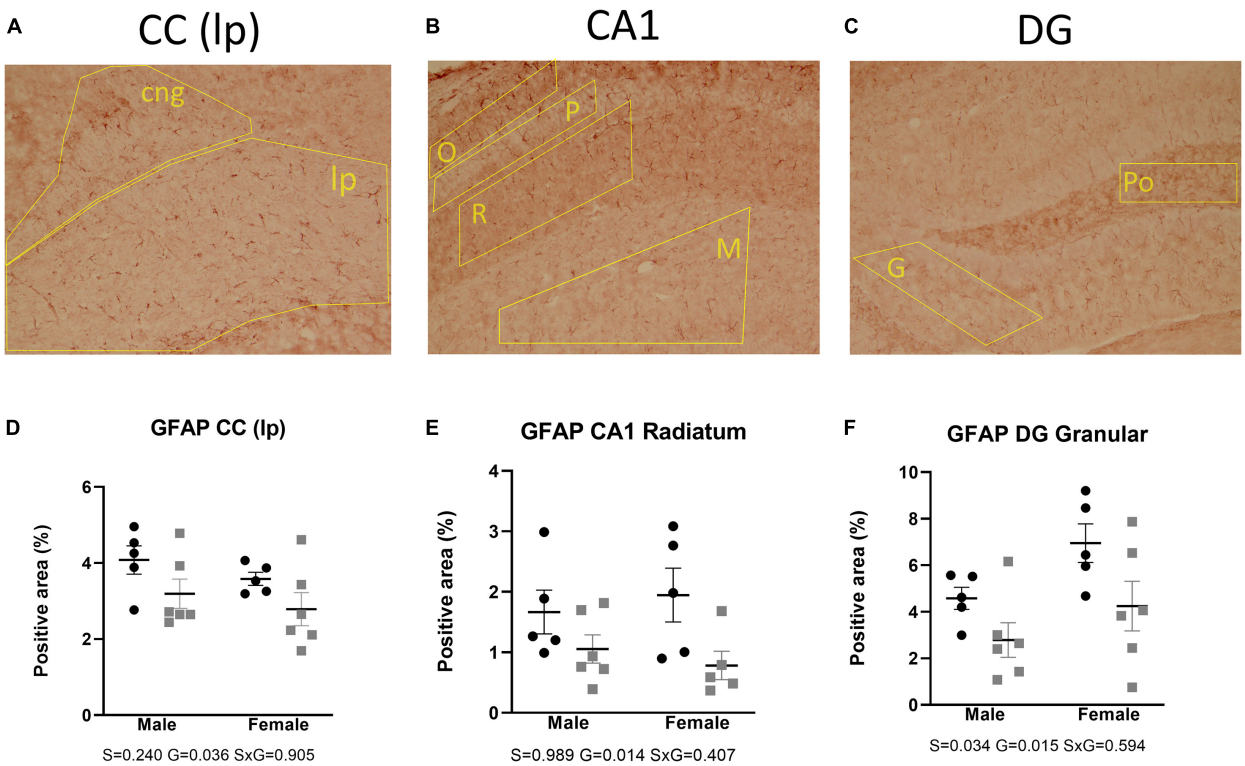

FIGURE 6 | Expression of GFAP in the corpus callosum **(A)**, hippocampal CA1 area **(B)**, and DG **(C)**. Coded polygons in stained sections indicate neuroanatomical parcellations. In the three regions, reduced immunostaining in the $AdKO_{2.0}$ mice of both sexes was observed compared to that in WT mice **(D–F)**. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$.

p = 0.015) (**Figure 6**). Also, a main effect of sex was found in CA1 (s. moleculare $p < 0.001$), CA3 (p = 0.030), and DG (granular layer p = 0.034), which indicates a higher expression in females. The number of GFAP-positive cells was not different between genotypes (**Supplementary Table 4A**). Overall, in both frontocaudal levels, GFAP staining was diminished in $ADKO_{2.0}$ mice. All significant effects are listed in **Supplementary Table 3B**.

Microglial activity was assessed by means of Iba1 immunohistochemistry. Two-way ANOVA showed that Iba1 immunoreactivity in the anterior brain of the $AdKO_{2.0}$ mice was significantly decreased in the corpus callosum (medial p = 0.001, lateroproximal p = 0.003, cingulate p = 0.017) (**Figure 7**). Likewise, at the hippocampal level, decreases were also observed in CA3 (s. moleculare p = 0.033) and DG (polymorph p = 0.040) (**Figure 8**) in $AdKO_{2.0}$ mouse brains. There were no effects of sex or interaction. In the corpus callosum, we observed a reduced total number of Iba1-positive cells (**Supplementary Table 4B**). Overall, in both frontocaudal levels, Iba1 staining was diminished in $ADKO_{2.0}$ mice. All significant effects are listed in **Supplementary Table 3C**.

DISCUSSION

The present study used $ADKO_{2.0}$ mice to study the consequences of long-term GC exposure for the brain. The $AdKO_{2.0}$ mice had decreased relative volumes in a number of anterior cortical brain structures (cingulate cortex and somatosensory cortex) and in a number of white matter structures (corpus callosum, but also cerebellar white matter tracts). In contrast, we observed higher relative volumes in the medial amygdala, BNST, and hypothalamus. Markers for non-neuronal cell types showed that there were bidirectional effects on MBP, that is, a male-specific increase in immunoreactive area in the cingulate cortex and a sex-independent decrease in the somatosensory cortex and the hippocampus. Activated astrocyte and microglial markers GFAP and Iba1 were suppressed at both the anterior and hippocampal levels independent of sex.

Our analysis was based in one section per brain area per animal. This may increase technical variation, which would increase the chance of a false-negative result. For most markers, data provided clear indication of widespread changes, and we may underestimate the number of brain areas in which they might occur.

The volumetric changes found in the $AdKO_{2.0}$ mouse brains were extensive and widespread. In principle, the whole-brain volume reduction reflects previous findings in human studies during active CS. Both pediatric and adult CS patients have a decrease of total brain volume (Bourdeau et al., 2002; Merke et al., 2005). In the analysis of individual brain areas, our results are in line with human studies that found changes in cerebellum and cortex volumes (including insular and cingulate regions) but

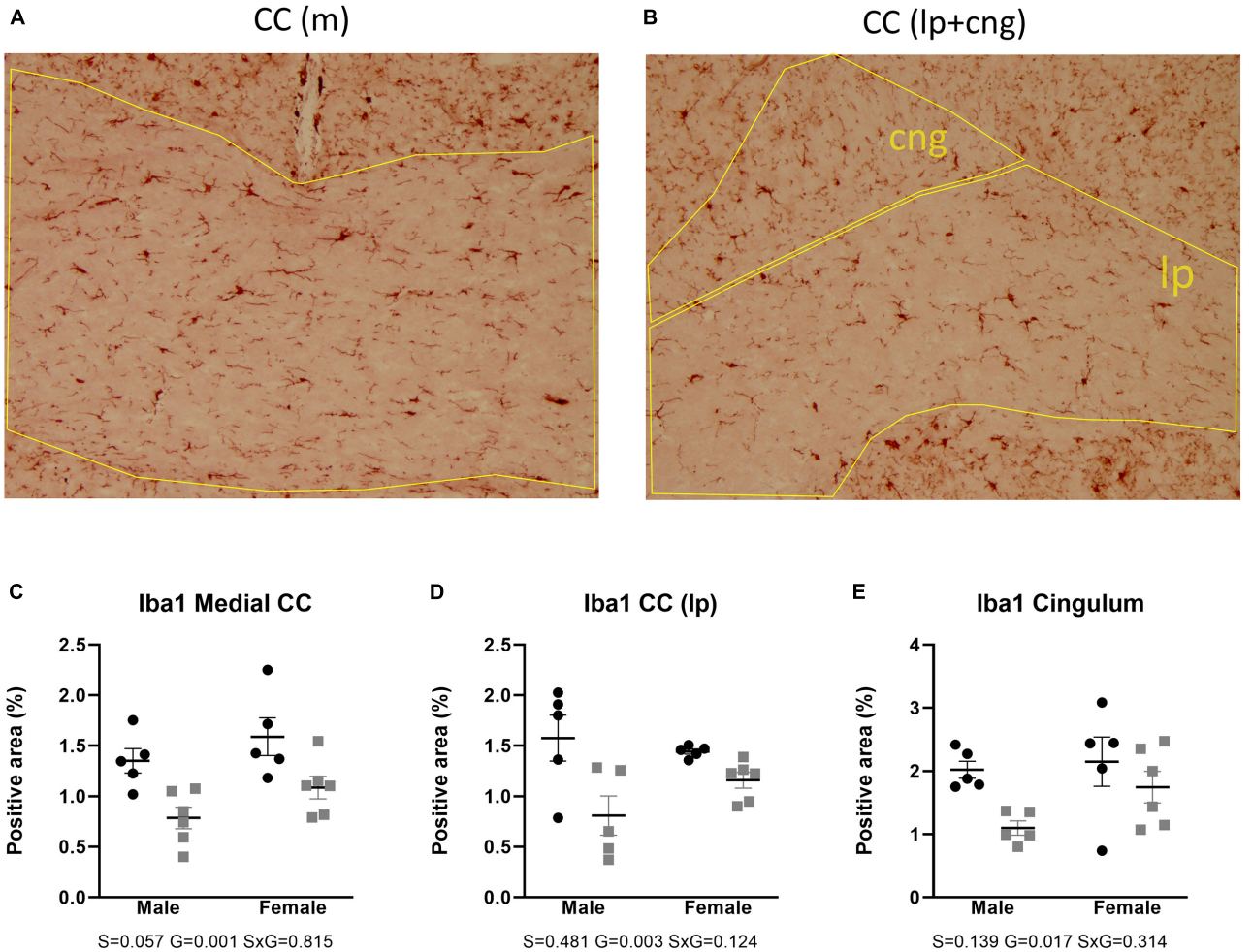

FIGURE 7 | Expression of Iba1 in the corpus callosum (medial and lateroproximal) **(A,B)**. Coded polygons in stained sections indicate neuroanatomical parcellations. In medial and lateroproximal (lp) areas, a decrease in expression in $AdKO_{2.0}$ mice compared to that in WT mice was observed **(C,D)**. In the cingulum (cng), there was a male-specific decrease in the $AdKO_{2.0}$ mice **(E)**. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$.

found no effects when measuring the hippocampus or amygdala (Merke et al., 2005; Santos et al., 2014; Jiang et al., 2017a,b; Hou et al., 2020). However, other studies reported decreases in hippocampal volume (Starkman et al., 1992, 1999; Burkhardt et al., 2015), and this was also observed after chronic exogenous GC exposure (Zhang et al., 2015). In the $AdKO_{2.0}$ brains, volumetric decreases were prominent in white matter areas, such as the corpus callosum and cingulate cingulum. In human studies, patients show smaller absolute volumes of white matter (Jiang et al., 2019; Tirosh et al., 2020), as well as reduced fractional anisotropy and increased mean diffusivity, which indicate white matter deterioration (Pires et al., 2015).

The overall parallels between the data from human imaging studies and ours indicated that the $AdKO_{2.0}$ is an adequate model of CS effects on the brain and opened the opportunity to study the origins of such changes in further detail. It was particularly interesting that the corpus callosum was smaller than in wild-type mice, because this region has been described to still be affected in long-term remission (Andela et al., 2013; van der Werff et al., 2014). We decided therefore to focus our analyses on glial cells in areas that showed reduced relative volumes. We also included the hippocampus, given the extensive literature on its response to GCs.

We observed a decrease in MBP expression in the hippocampus, which may relate to the long-term loss of white matter integrity in remitted CS patients. We did not acquire diffusion tensor imaging (DTI) data in our mice; thus, a direct comparison is not possible. A body of literature suggests inhibiting effects of GCs on oligodendrocyte function and proliferation (Miguel-Hidalgo et al., 2019; hippocampus and pyriform cortex) and white matter (Dunlop et al., 1997; Alonso, 2000; Quinlivan et al., 2000). Work in multiple sclerosis models also supports negative effects of GCs on MBP expression and white matter (Sieve et al., 2004; Chari et al., 2006). The reduced MBP expression seems a logical correlation to the reduced white matter volume in the MRI data of our mice.

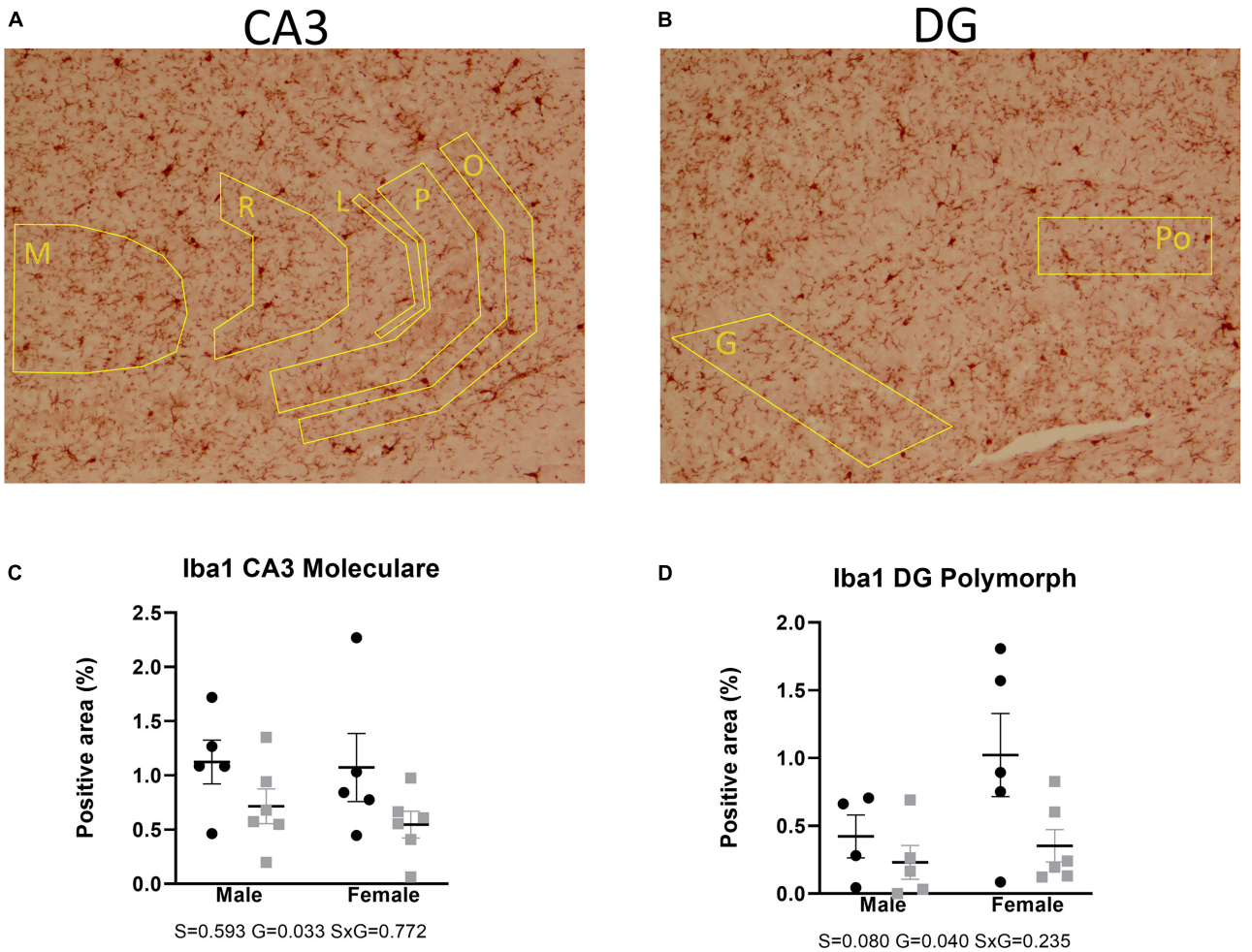

FIGURE 8 | Expression of Iba1 in the hippocampal CA3 **(A)** and DG **(B)**. Coded polygons in stained sections indicate neuroanatomical parcellations. In the stratum moleculare (M) of CA3, $AdKO_{2.0}$ of both sexes showed a decrease in expression, compared to WT mice **(C)**. In the polymorph (Po) layer of DG, there is a female-specific decrease in $AdKO_{2.0}$ mice **(D)**. Data were analyzed by two-way ANOVA, α: 0.05. S, G, and S × G: *p* value of the source of variation (sex, genotype, and interaction, respectively). Black dots: wild type. Gray squares: $AdKO_{2.0}$.

However, the literature also reports protective effects of GCs, mainly in oligodendrocyte cultures (Kumar et al., 1989; Byravan and Campagnoni, 1994; Lee et al., 2008; Xu et al., 2009). A protective role was also observed in *in vivo* settings (Raschke et al., 2008; Sun et al., 2010). The contradictory literature on GC effects on MBP expression may reflect a biphasic effect, with low doses stimulating and high doses being detrimental (Almazan et al., 1986).

MBP immunoreactivity was higher in parts of the prefrontal cortex of $AdKO_{2.0}$ mice, in apparent contrast with the lower volume observed in both active and remitted patients (Andela et al., 2013; Hou et al., 2020), as well as in our mice. The increased MBP immunoreactive surface area in conjunction with lower volume might be due to a different myelin organization, perhaps a more open myelin conformation ("loose myelin"). In fact, it has been observed that myelin lamellae fail to associate and compact after chronic GC treatment (7 days) in rats (Chari, 2014). Overall, our data suggest that in the mouse brain, this cingulate cortex has a particular sensitivity to long-term GC exposure.

The decrease in expression of GFAP is also in line with previous reports on the effects of GCs on astrocytes. It has been shown in other studies that GCs, e.g., corticosterone and dexamethasone, decreased cell viability and proliferation in astrocyte cultures and GFAP expression *in vivo* (Unemura et al., 2012; Guo et al., 2013; Zhang et al., 2015; Freitas et al., 2016). Similarly, chronic stress has been shown to decrease astrocytic cell size, count, and process length in the hippocampus and prelimbic cortex (Czeh et al., 2006; Banasr and Duman, 2008). Chronic stress also decreased expression of GFAP in the hippocampus and brainstem (Ye et al., 2011; Imbe et al., 2013; Lou et al., 2018). However, in some experimental settings, stressors and GCs led to increased astrocyte activation, in a GR-dependent manner (Revsin et al., 2009; Shields et al., 2012; Zia et al., 2015; Huet-Bello et al., 2017). It is well-known that GC effects can be context dependent (Meijer et al., 2019). Our data

are in line with the notion that in the adult brain, in the absence of overt stressors or pathology, GCs suppress astrocyte activity.

Microglial immunoreactivity was clearly lower in the $AdKO_{2.0}$ mice, an effect that is in line with a general immunosuppressive effects of chronically elevated GCs. In agreement with our data, previous works have described that microglia shrink and stop proliferating when corticosterone is added to microglial cultures (Tanaka et al., 1997; van Olst et al., 2018). Cell viability was compromised due to a cytotoxic effect of GCs, and this effect could be blocked by mifepristone (Nakatani et al., 2012; Cerqueira et al., 2013). Chronic corticosterone administration induces retraction of microglial processes in the hippocampus of mice (Park et al., 2011; Freitas et al., 2016), and adrenalectomy greatly increases microglial activation in the hippocampus and hypothalamus after acute stress; this response is ablated by exogenous corticosterone administration in rats (Sugama et al., 2013). In models of neuronal damage, the same trend has been observed for synthetic GCs (methylprednisolone and triamcinolone) (Schroter et al., 2009; Li et al., 2011).

A number of reports, however, described different effects. GCs may potentiate microglial responses *via* a priming mechanism, concomitant with an increase of Iba1 but suppression of cytokine production (Frank et al., 2014). Moreover, chronic restraint stress paradigms increased Iba1 immunoreactivity in various areas of the brain, including the anterior cingulate cortex and hippocampus (Tynan et al., 2010; Hinwood et al., 2012) and stimulated microglial process lengthening and branching out (Hinwood et al., 2013). However, this effect was observed only in large cells, and there might be a timing factor involved, as it has been also observed that induction of microglial proliferation by stress is time limited (Nair and Bonneau, 2006). The conflicting data may be resolved by taking time of exposure into account, since some studies showed stimulatory effect of short-term exposure but inhibitory effects of long-term exposure (Kreisel et al., 2014; Winkler et al., 2017). Our mutant mouse data are compatible with the latter, as they had been exposed to chronic hypercorticosteronemia (8–10 weeks).

The diversity of effects of GCs and stress on brain volume and on cell morphology might be related to age, duration of treatment, sex, stress context, or type of GC molecule, as synthetic GCs may differ from endogenous steroids in some of their effects (Meijer and de Kloet, 2017). Our model closely resembles the conditions of endogenous GC excess in humans in terms of physiological traits and, particularly, the time course of the endocrine imbalance. It remains to be determined to what extent the observed effects depend on direct activation of brain GRs and to what extent the context of, for example, changed metabolism-related factors contributes to the observed effects, as it is known that GR activity is also influenced by its association to (tissue-specific) coregulators and other transcription factors (Spaanderman et al., 2018; Viho et al., 2019). Thus, further studies are necessary to identify specific transcriptional changes that are associated with, and perhaps causal for, the changes in brain morphology that are apparent in these mice.

The present work is, to our knowledge, the first translational study to assess brain volumetric differences together with alterations in glial cell markers. In both aspects, we found significant changes in the selected brain regions. The hippocampus and prefrontal cortex are key regions in the brain for cognitive processing and integration, respectively. In human studies in CS patients both with active disease and in remission, various degrees of cognitive deficit are found. Cognitive performance has been related to myelin integrity, particularly in the hippocampus and prefrontal cortex (Nickel and Gu, 2018). In a similar manner, microglial morphological changes in the prefrontal cortex have been reported in obesity-related cognitive impaired rats (Bocarsly et al., 2015). We consider that our present results provide an opportunity to contribute to the study of participation of glial cells in cognition; thus, in the future, a number of neurobehavioral assays should be tested in the AdKO mice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by C2E2A (Comité d'Ethique pour l'Expérimentation Animale en Auvergne), Commission Nationale de Réflexion Ethique sur l'Expérimentation Animale, and Ministère français de l'Enseignement supérieur, de la Recherche et de l'Innovation. République Française.

AUTHOR CONTRIBUTIONS

JA, LW, and OM designed experiment. IS-B, TD, and NM generated mouse models, managed mouse cohorts and tissues preparation for MRI, and histological analyses. GP and CK conducted MRI measurements. ES and LW analyzed MRI data. JA performed histological procedures and analyzed histological data. JA, LW, AP, AM, and OM prepared manuscript. LW, AM, and OM supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.604103/full#supplementary-material

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MINI REVIEW
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Understanding the Link Between Maternal Overnutrition, Cardio-Metabolic Dysfunction and Cognitive Aging

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Obesity has long been identified as a global epidemic with major health implications such as diabetes and cardiovascular disease. Maternal overnutrition leads to significant health issues in industrial countries and is one of the risk factors for the development of obesity and related disorders in the progeny. The wide accessibility of junk food in recent years is one of the major causes of obesity, as it is low in nutrient content and usually high in salt, sugar, fat, and calories. An excess of nutrients during fetal life not only has immediate effects on the fetus, including increased growth and fat deposition *in utero*, but also has long-term health consequences. Based on human studies, it is difficult to discern between genetic and environmental contributions to the risk of disease in future generations. Consequently, animal models are essential for studying the impact of maternal overnutrition on the developing offspring. Recently, animal models provided some insight into the physiological mechanisms that underlie developmental programming. Most of the studies employed thus far have focused only on obesity and metabolic dysfunctions in the offspring. These studies have advanced our understanding of how maternal overnutrition in the form of high-fat diet exposure can lead to an increased risk of obesity in the offspring, but many questions remain open. How maternal overnutrition may increase the risk of developing brain pathology such as cognitive disabilities in the offspring and increase the risk to develop metabolic disorders later in life? Further, does maternal overnutrition exacerbate cognitive- and cardio-metabolic aging in the offspring?

Keywords: maternal, obesity, overnutrition, cardiovascular disease, offspring, animal models, cognition, aging

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INTRODUCTION

The prevalence of obesity is increasing worldwide and has reached epidemic proportions posing a major problem for healthcare (Ng et al., 2014; Hruby and Hu, 2015). The last World Health Organization (WHO) report classified 1.9 billion adults worldwide as overweight and more than 650 million as obese (WHO, 2020). Obesity is associated with an increased incidence of comorbidities like dyslipidemia, hypertension and type 2 diabetes mellitus (T2DM; Hruby and Hu, 2015). These conditions are associated with higher cardiovascular disease risk and

mortality. A particular concern emerged in the past two decades, namely obesity during pregnancy (Heslehurst et al., 2010; Huda et al., 2010; Gregor et al., 2016; Lindberg et al., 2016; Kominiarek and Peaceman, 2017). Nearly two thirds of women of childbearing age (19–44) are overweight and 36.5% are obese. It is therefore not surprising that this epidemic also affects children of all ages with nearly 38 million children under the age of 5 years classified as overweight or obese (WHO, 2020). It is already known that the obesity epidemic cannot be explained only as a result of an affluent lifestyle, reduced physical activity or a genetic predisposition (Albuquerque et al., 2017; Sheikh et al., 2017). Evidence has suggested that it may originate from environmental factors present already early in development during fetal life. In the early 1980s the Barker hypothesis, also referred as the "developmental origins of adult disease," alluded to an association between fetal undernutrition and low birth weight with the risk of developing adult obesity, including the metabolic syndrome and cardiovascular disease (Barker et al., 1990; Barker, 2007). Programming is a process whereby an insult at a critical time period of development has lifelong significance. According to the Barker hypothesis, variations in the transfer of food from the mother to her baby have profound and long-term implications for the health of the next generation (for review see McMillen et al., 2005). Since the original observations of David Barker, various human and animal studies shifted their investigations to studying the effects of maternal overnutrition and obesity, since diets high in calories and fats combined with limited physical activity and increased sedentary behavior have become more prevalent in developed as well as under-developed countries. Conforming to this hypothesis, studies in the past two decades suggested a link between maternal obesity and/or overnutrition during pregnancy and the increase risk of obesity later in life (Kominiarek and Chauhan, 2016). Epidemiological studies report a positive relationship between weight at birth and adult body mass index (BMI). Moreover, maternal BMI, in particular its increase during pregnancy is positively related to obesity in the offspring as babies, through childhood and into adulthood (Lawlor et al., 2007). More specifically, in a United States cohort, maternal first-trimester obesity led to a two- to threefold increase in the risk of childhood obesity in their progeny; 24% of the children of obese mothers were themselves obese at age 4, compared with 9% of the children of normal-weight mothers (Whitaker, 2004). A maternal hypernutritional state, due to pre-existing T2DM, gestational diabetes or obesity, generates a long-term risk of obesity for the child. Gestational weight gain, irrespective of pre-pregnancy body mass, is positively associated with obesity in 3 years old children (Oken et al., 2007). Maternal obesity increases a child's risk for developing obesity as a consequence of either shared genes, environmental factors, or a combination of both. Parents not only create food environments for their children but also influence their eating behaviors, taste preferences, and food choices (Kral and Rauh, 2010). The *in utero* environment, which profoundly affects the fetal developmental processes, is considered as important as genes or family habits in determining the predisposition to long-term health outcomes. Thus, the *in utero* environment is a key player leading to increased risk of obesity, T2DM and cardiovascular disease in the adult offspring upon exposure to increased nutrient supply before birth (Reynolds et al., 2013; Godfrey et al., 2017). A relation between different insults during pregnancy, including famine, different types of infections, prenatal stress, obstetric complications, smoking, were shown to impact the adult life of the progeny (Boksa, 2004). However, it appears virtually impossible in human studies to establish a cause-effect relationship, to disentangle the direct effects of maternal obesity from the influence of shared genes and postnatal lifestyle on the developing child. Therefore, animal models are necessary to answer these crucial questions and generate results that then may be translated to humans.

This review will summarize findings on the long-term effects of maternal overnutrition and obesity on metabolic states and cognitive function in the offspring from human and rodent studies. It will also try to identify whether one trait can be the consequence of the other.

THE MATERNAL OVERNUTRITION/OBESITY ANIMAL MODEL

Maternal obesity can be induced by exposing female animals to different nutritional diets for example hyperenergetic and highly palatable diets such as high-fat (varying between 45 and 60% calories from fat) (Tozuka et al., 2009, 2010; Peleg-Raibstein et al., 2012, 2016; Kang et al., 2014; Graf et al., 2016; Janthakhin et al., 2017; Robb et al., 2017; Sarker and Peleg-Raibstein, 2018; Sarker et al., 2018, 2019a,b; Wolfrum and Peleg-Raibstein, 2018; Moreton et al., 2019; Zieba et al., 2019), a combination of high-fat high-sugar diet, high-sugar diet or a cafeteria diet which supplement the normal chow diet with a variety of palatable foods (Wright et al., 2014; Ribeiro et al., 2018; Lewis et al., 2019; Moreton et al., 2019). These different diets promote weight gain and depending on the length of exposure induce obesity and other metabolic disorders to the mother. These diets are trying to mimic the human modern food habits and consumption. Each of these types of diets have their advantages and disadvantages. For example, in the high-fat diet (HFD) and high-fat high-sugar diet (HFHS) models, the macronutrients and micronutrients contents can be controlled between the obesogenic diets and the control chow diet. They are commercially available and can be easily utilized. Whereas, the cafeteria diet model has different variations depending on the laboratory and can be tailored depending on the specific research question and adapted accordingly. Female mice exposed to the cafeteria diet gain faster body weight and develop increased body fat composition with changes of other metabolic markers (i.e., insulin levels, triglycerides levels etc.) compared to the HFD model. However, in this diet it is more difficult to control for intake of macronutrients and micronutrients. In addition, by using this diet one needs more careful planning and executing of the diet schedules because otherwise the changes in diet composition throughout the experiment will have too much variations.

An overview of the current literature on animal studies investigating the effects of maternal obesity or overnutrition

utilizes different maternal diets (as mentioned above) as well as different exposure time periods such as exposure only during gestation, only during lactation or prior to mating (conception) and during gestation and lactation (Ghosh et al., 2001; Siemelink et al., 2002; Buckley et al., 2005; Gregersen et al., 2005; Khan et al., 2005; Chen et al., 2008, 2009; Naef et al., 2008, 2011; Dunn and Bale, 2009, 2011; Elahi et al., 2009; Morris and Chen, 2009; Niculescu and Lupu, 2009; Tamashiro et al., 2009; White et al., 2009; Bilbo and Tsang, 2010; Chechi et al., 2010; Gregorio et al., 2010; Vucetic et al., 2010; Ashino et al., 2011; Dunn et al., 2011; Simar et al., 2011; Strakovsky et al., 2011; Wahlig et al., 2011; Zhang et al., 2011; Peleg-Raibstein et al., 2012, 2016; Vogt et al., 2014; Sarker and Peleg-Raibstein, 2018; Sarker et al., 2018, 2019a,b; Wolfrum and Peleg-Raibstein, 2018; Zieba et al., 2019). In addition, also the time periods of exposure prior to mating differ between the studies. Thus, these studies have led to considerably inconsistent metabolic and behavioral outcomes in the offspring.

THE LONG TERM-EFFECTS OF MATERNAL OVERNUTRITION ON COGNITIVE FUNCTION OF THE OFFSPRING

With rates of global obesity constantly increasing, maternal obesity, and excessive gestational body weight leading not only to obesity but also to other long-term health outcomes such as cardiovascular disease and mental disorders including cognitive decline in the progeny. In the past few years, human studies linked maternal obesity with cognitive abnormalities in the offspring (for review, Van Lieshout, 2013; Contu and Hawkes, 2017). More specifically, maternal obesity was linked to reduction in child intelligence quotient (IQ) scores (Neggers et al., 2003; Gage et al., 2013; Bliddal et al., 2014; Pugh et al., 2015), cognitive test scores (Tanda et al., 2013), impaired neuropsychological development (Hinkle et al., 2012; Casas et al., 2013; Huang et al., 2014) and autism spectrum disorder and other intellectual disabilities (Brion et al., 2011; Li et al., 2016; Kong et al., 2020) in the children. Additionally, other studies suggested that an association might exist between maternal obesity and increased symptoms of attention-deficit hyperactivity disorder (ADHD) in children (Rodriguez et al., 2008; Rodriguez, 2010; Buss et al., 2012; Jenabi et al., 2019; Kong et al., 2020; Li et al., 2020; Robinson et al., 2020). Although the association between maternal obesity and offspring's cognitive disabilities was described, the current literature is still scarce, and findings are inconsistent (Brion et al., 2011; Keim and Pruitt, 2012; Van Lieshout, 2013; Bliddal et al., 2014). The underlying mechanisms leading to a higher susceptibility of the progeny to develop cognitive abnormalities later in life is still unknown. The observed cognitive impairments in the adult offspring could also be a result of obesity. Therefore, the understanding how maternal weight and weight gain might contribute to offspring's cognitive development is important, however, knowledge gaps remain. It was shown that excessive food intake and obesity leads to increased risk for cognitive impairments and to various types of neurodegenerative dementia (Stranahan and Mattson, 2008; Sellbom and Gunstad, 2012; Wraw et al., 2018). Therefore, to date, it is still not clear whether the cognitive decline is a trait that precede obesity and/or the metabolic syndrome, or whether the metabolic state itself is leading to cognitive disabilities. Another important question is whether maternal overnutrition exacerbate aging, metabolically and cognitively in subsequent generations.

Maternal nutrition is important for an optimal neurodevelopment of the offspring. In recent years, an increased interest emerged looking at the effects of maternal nutrition and offspring cognitive function. When assessing learning and memory, most of the studies investigated the effects of maternal overnutrition and obesity by examining hippocampal-dependent learning and memory mainly employing the Morris water maze paradigm. Bilbo and Tsang (2010) demonstrated that maternal diet high in either saturated or trans fats induced increased spatial memory performance and a concomitant increased inflammation within the hippocampus compared to control offspring. In contrast, decreased spatial memory performance in the Barnes maze was found in young and adult offspring born to obese mothers (Tozuka et al., 2010). Similar impaired spatial memory performances were observed in offspring exposed to a HFD throughout both the pre- and postnatal period (White et al., 2009; Page et al., 2014; Lépinay et al., 2015). Working memory assessment employing the novel object recognition task also lead to contradictory findings between studies. One study described reduced novel object exploration in young adult offspring exposed to maternal HFD (MHFD) (Graf et al., 2016) while offspring exposed to maternal cafeteria diet during lactation reported increased novel object exploration (Wright et al., 2014). Another study employing exposure led to a wide spectrum of cognitive abnormalities in an age-depend manner in the offspring (Wolfrum and Peleg-Raibstein, 2018). The behavioral abnormalities observed in the adult offspring readily suggests that the perturbations caused by MHFD exposure are diverse and fundamental to normal brain development. It was shown that offspring exposed to MHFD were severely impaired in acquisition of avoidance learning in an aversive learning task, a two-way active avoidance paradigm, as compared to control offspring at adulthood (Wolfrum and Peleg-Raibstein, 2018) while working memory and fear memory remained intact (Peleg-Raibstein et al., 2012; Wolfrum and Peleg-Raibstein, 2018) during adulthood and impaired in aged-adult HFD offspring (Wolfrum and Peleg-Raibstein, 2018). This study had several unique strengths: the longitudinal nature allowed to assess offspring cognition at different developmental ages utilizing different behavioral cognitive testing. The authors could pinpoint when different cognitive dysfunctions such as working memory, spatial memory and associative memory were evident. In summary all these findings can show that cognitive abilities can be influenced by obesity of the offspring. Most of these behavioral tests are dependent of hippocampus function (Vorhees and Williams, 2014). A brain region important in cognitive processing, learning and memory and that was also shown to be sensitive to changes in dietary energy intake (Miller and Spencer, 2014) and in aged-related cognitive ability (Gerstein et al., 2013). In addition,

it was shown that exposure to a high-calorie diet in middle-aged rats led to impaired hippocampus-dependent cognitive functions such as spatial learning and which was accompanied by reduced hippocampal neurogenesis and synaptic plasticity (Stranahan et al., 2008). Since the development of the hippocampus is sensitive to *in utero* nutritional insults, it is not surprising that maternal HFD induced cognitive function impairments which was associated with inhibition of hippocampal neurogenesis and increased apoptosis in the offspring (Tozuka et al., 2009; Kim and Park, 2018).

These discrepancies might be due to methodological differences between the studies such as different gender used, timing of maternal dietary exposure, strain of the animals, different maternal diets employed, different testing protocols and age of the offspring at the time of testing. Another important consideration for the interpretation of the findings is that not all of the maternal diets utilized induce differences in gestational weight between female dams exposed to diet-induced obesity or control diet with no difference in any other metabolic parameters [i.e., fat mass, plasma glucose, insulin, triglycerides, cholesterol, and FFA levels (Peleg-Raibstein et al., 2016)]. While other animal models of maternal obesity lead to an obese state of the mothers (Bilbo and Tsang, 2010; Tozuka et al., 2010; Simar et al., 2011). This makes it difficult to dissociate between the direct effect of maternal obesity and that of overnutrition leading to obesity in the offspring.

Until now, relatively little is known about how maternal overnutrition/obesity can lead to lifelong consequences in cognitive abilities of the offspring. In this respect, this field of research is still in its early stages, and additional studies are required to examine the long-term effects of maternal overnutrition/obesity on memory and learning abilities of the offspring that may lead to advanced cognitive aging and also to increased risk to develop Alzheimer's disease (AD).

NUTRITIONAL PROGRAMMING OF ALZHEIMER'S DISEASE AND RELATED COGNITIVE DECLINE

To this date, investigations on the impact of maternal overnutrition and maternal obesity on offspring cognitive performance and mental health in animal models have focused mainly on anxiety, depression and motivation with some newer studies also examining the effects on learning and memory. Alzheimer's disease, the most common form of dementia, is a chronic progressive neurodegenerative disorder that develops slowly over decades. The observable pathological brain changes and the symptoms often do not follow the same time-course, which makes the diagnosis difficult. One of the clinically observed symptoms is memory loss, with the hippocampus and the cerebral cortex being the brain regions primarily involved. Interestingly, classic clinical studies indicate that human AD is also associated with damage in brain areas related to nutrient-sensing and the motivation to move, especially the hypothalamus which is also linked to the ability to form memories (Ishii, 1966; Saper and German, 1987). The exact etiology of AD is still not fully understood, however, ample evidence points to amyloid-beta peptide (Aβ) as a key player in the pathogenesis of AD and is the earliest lesion in the disease process (Hardy and Higgins, 1992; Ballard et al., 2011). In the past, AD was thought to be a disease that developed in later life, but there is increasing evidence that the disease probably begins many years before clinical symptoms appear. Thus far, very little is known about this "silent" stage of the disease. Exactly when AD begins, and why some people get it and others do not, is still not fully understood. There are many environmental risk factors, such as nutrition, that are thought to have their major effects long before the disease can be diagnosed (Killin et al., 2016). For example, elevated dietary intake of fat has been shown to increase the risk of developing AD and facilitate age-related cognitive impairments (Luchsinger et al., 2002; Hooijmans and Kiliaan, 2008; Gustafson, 2012). Therefore, it is not surprising that obesity and consumption of a Western-style diet, especially in mid-life, are associated with increased risk of AD later in life (Laitinen et al., 2006). The prevalence of AD is greater in countries with a high intake of high-fat/high-calorie diets and lower in those that consume low-fat diets (Grant, 1999; Panza et al., 2004). Recently, evidence suggests that maternal diet may also impact accelerating aging and (McAninch et al., 2020) the subsequent appearance of AD in late life (Borenstein et al., 2006; Lahiri et al., 2008; Miller and O'Callaghan, 2008; Tolppanen et al., 2016). In the previous section we discussed the association from human studies and evidence from preclinical animal models between maternal obesity and unhealthy eating with cognitive impairments and disturbances of cerebral cortex and hippocampus function (Van Lieshout et al., 2011; Van Lieshout, 2013; Cordner and Tamashiro, 2015; Contu and Hawkes, 2017). These leads to neuropsychological impairments as deficits in attention, working memory and executive function. However, to date the underlying mechanisms linking maternal diet to these pathophysiological changes of the brain and behavior are not fully understood. As obesity is a risk factor for AD and excessive intake of fats in adulthood worsen AD in animal models (Tolppanen et al., 2016; Baranowski et al., 2018; Lloret et al., 2019), it is possible that maternal overnutrition affects the development of AD in the offspring. Animal models are fundamental for our understanding the effects of an obesogenic environment during fetal life and the effects depend on multiple and not-exclusive pathways that may lead to long-term neuropathological outcomes. Thus, by employing the overnutrition/obesity animal model with a common genetic background, carefully controlled dietary and activity conditions and a controlled postnatal environment is fundamental for examining how overnutrition prior and during pregnancy increases the development of obesity, cardiometabolic disease and the risk to develop AD in the offspring. Thus far only a handful of preclinical studies investigated the impact of maternal overnutrition/obesity on AD-like cognitive pathology. It was shown that MHFD led to impairment in memory in 2- and 12-month-old triple transgenic mouse model of AD (3xTgAD) offspring compared to control offspring (Martin et al., 2014). The memory impairments were accompanied by a

significant increase in the number of hippocampal tau positive neurons. These findings may imply that MHFD can induce the onset and progression of AD later in life (Martin et al., 2014). In addition, it was demonstrated that the pathological AD marker, clearance of the β-amyloid peptide, was impaired in brains of MHFD offspring (Hawkes et al., 2015). Additionally, offspring born to MHFD Tg2576 mothers (i.e., the Tg2576 mouse model of AD which express the Swedish mutation in the human amyloid precursor protein) developed higher levels of hippocampal β-amyloid pathology compared to control offspring (Nizari et al., 2016).

A LINK BETWEEN CENTRAL LIPIDS AND COGNITIVE FUNCTION?

The brain is the most cholesterol rich organ in the body. The majority of central cholesterol accumulates during embryonic development and in the early postnatal period, while the metabolism of cholesterol in adulthood is characterized by a low turnover and minimal loss of cholesterol (Zhang and Liu, 2015). Brain cholesterol is proposed to be derived by *de novo* synthesis, since only small amounts of plasma cholesterol can transfer through the blood–brain barrier (BBB; Dietschy and Turley, 2001). Cholesterol supply to the central nervous system (CNS) is believed to be mediated by astrocytes and microglia, which predominantly secrete cholesterol and phospholipids together with apolipoprotein E (ApoE). ApoE, a protein involved in fats metabolism in the body, in turn serves as a ligand for these lipoproteins to affect the uptake of lipoproteins via the low-density lipoprotein receptor (LDLR) and the lipoprotein related protein (LRP; Lane-Donovan et al., 2014). Currently it is believed that ApoE-containing lipoproteins redistribute lipids and regulate cholesterol homeostasis within the brain (Mahley, 2016b). As cholesterol is essential for normal brain function including learning and memory, dysfunction in central cholesterol metabolism might lead to deficiencies that will induce structural and functional brain disorders such as AD (Kim et al., 2009a; Mahley, 2016a; Yamazaki et al., 2019). In this context, the expression of ApoE in the brain concomitant with LDLR function has been implicated. For example, one study demonstrated that overexpression of ApoE in the brain had beneficial effects on cognitive function as well as neural circuit function by enhancing the clearance of Aβ (Cramer et al., 2012). Furthermore, LRP1 forebrain knockout mice show alterations in central lipid metabolism in brain regions important for cognitive function paralleled with impairments in memory (Liu et al., 2010). In addition, neuron-specific 2 (LPL) deficient mice display learning and memory deficits (Xian et al., 2009) and overexpression of brain LDL receptors reduces amyloid deposition and may represent a novel treatment for AD (Kim et al., 2009b). Thus, current evidence points to the fact that central cholesterol homeostasis is important for cognitive function. However, the link to peripheral cholesterol metabolism is not yet understood. More specifically, it remains unknown, by which mechanisms lipoproteins are produced in the brain and whether there may be a transport or a link to cholesterol precursors across the BBB thus connecting central and systemic lipid homeostasis?

THE EFFECTS OF MATERNAL HFD EXPOSURE AND THE INCREASED RISK FOR CARDIO-METABOLIC DISEASE IN THE OFFSPRING

In human epidemiological studies maternal BMI was positively correlated with cardiovascular events and premature death in the progeny (Reynolds et al., 2013). Maternal overnutrion/obesity was shown to induce a cardiometabolic phenotype (increased fat disposition, alterations in plasma metabolic markers, increased body weight, hyperinsulinemia, and fasting glucose levels) (Godfrey et al., 2017). In preclinical studies offspring born to over nourished mothers showed increased risk to develop cardiovascular pathology compared to their control counterparts (Samuelsson et al., 2008; Liang et al., 2009; Blackmore et al., 2014; Loche et al., 2018).

In order to try to investigate how maternal overnutrition/obesity influences the cardio-metabolic function of the offspring the body weight is not always a predictor of body composition. Some studies have shown that, despite similar body weights of the offspring of over nourished mothers and control offspring, elevated percentages of body fat can be detected (Buckley et al., 2005; Howie et al., 2009). These findings suggest that elevated body fat percentages may be more likely a result of reduced lean mass than an increase in fat mass *per se*. The pathophysiological processes underlying a cardio-metabolic state (obesity, diabetes, and cardiovascular dysfunction), cognitive decline and structural brain changes are most likely multifactorial. There are some evidence that inflammation (i) may be the cause of decline in cognitive processes in aging (Cornejo and von Bernhardi, 2016), (ii) is a risk factor for acceleration in cognitive decline (Stacey et al., 2015), and (iii) may be a cause for neurodegenerative disorders (Schain and Kreisl, 2017). Inflammation may cause alterations in brain structures such as reduced hippocampal and prefrontal volume (Bruehl et al., 2009), decrease in white matter (Kullmann et al., 2016), and reduction in total brain volume (Jefferson et al., 2007). In addition, inflammation processes were also associated with obesity and the metabolic syndrome (Guillemot-Legris and Muccioli, 2017). Taken together, these findings may suggest that one potential mechanism for explaining the exacerbation of cognitive decline observed in the offspring is alteration in pro-inflammatory markers in plasma and/or brain (Contu and Hawkes, 2017). Expression of brain pro-inflammatory markers such as TNFα, IL1, and IL-6 will in brain structures that predominantly underline memory, learning, and attentional processes, such as the hippocampus, prefrontal cortex, and the hypothalamus in different age stages of the offspring will enable the identifications of how longitudinal changes in inflammation markers might correlate with cognitive acceleration and/or cardiovascular dysfunction. C-reactive protein (CRP) is acting as an indicator for inflammation. In humans increased levels of

circulation CRP was associated with reduced cognitive function and is used as a marker to predict future risk for stroke and ischemic attack (Kuo et al., 2005).

There is still very little known on the role of circulating lipoproteins and cholesterol on neuropathological disorders, such as aging and cognitive decline on the one hand and increased risk to develop cardiovascular disorders on the other hand. Enhancing our understanding on the role and function of lipoproteins and cholesterol in the brain and their transport to the brain, may have an enormous impact in this field. In addition, a potential target for treatment for age-related cognitive and cardio-metabolic disabilities.

CONCLUSIONS AND FUTURE DIRECTIONS

The epidemic of obesity is spreading fast through developed and developing countries. The latest projections from the WHO predict that 1 in 10 is obese (this is an average as in developed countries it is considerably higher). Obesity is a dangerous condition because it creates a permissive environment for many other health-related diseases, such as type 2 diabetes, hypertension and heart disease. Even more worrying are the facts that health problems not only affect the adult population, but also children (Ogden et al., 2014). Childhood obesity is of major concern because it will most likely be translated to higher rates of adult obesity and related comorbidities such as diabetes and cardiovascular disease (Freedman et al., 2007; Li et al., 2008; Pulgarón, 2013). Growing evidence in the past two decades has shown that maternal overnutrition and/or obesity has a long-term impact on offspring health, demonstrating that the *in utero* environment might be a key determinant of long-term health outcomes. However, that far epidemiological human studies cannot distinguish between the effect of overnutrition during pregnancy and postnatal nutrition (such as an obesogenic household). Many human studies investigating the long-term implications of developmental insults on the health of the offspring (such as famine or infection), can point to causality since the insults occur during a specific point in time, while the rest of the pregnancy and childhood is normal. In the case of maternal overnutrition in humans, there is chronic consumption of palatable food during pregnancy that continues into childhood and adolescence, thereby preventing a conclusive association between the metabolic phenotype of the adult offspring exposed to prenatal overnutrition. There are several other limitations to human observational studies due to methodological differences in the publish research that make conclusion difficult or impossible such as socio-economic factors, adjustment for maternal intelligence, inadequate reporting of nutrition evaluations (through self-reported questionnaires), sample selection, low power due to a small sample size, etc. Thus, the establishment of the effect of maternal overnutrition on the offspring can only be studied in isolation in animal models. The health consequences of *in utero* exposure to maternal overnutrition on future generations are thus an area of intense research.

Until now, there are a few intervention studies utilizing the maternal obesity animal model. Those studies employ dietary (for review Zambrano et al., 2010; Nathanielsz et al., 2013; Kang et al., 2014; Liu et al., 2020), physical exercise (Carter et al., 2012; Vega et al., 2015; Fernandez-Twinn et al., 2017)[151; 152; 153], or therapeutic/supplementation interventions during different developmental stages (Vickers et al., 2005; Sen and Simmons, 2010). Some of these studies report potential beneficial effects on metabolic and cognitive outcomes in the offspring (Vickers et al., 2005; Vickers and Sloboda, 2012). Different interventions, a combination of approaches and the time of intervention may act through different mechanisms, which limit the intervention strategies to prevent the detrimental effects of maternal obesity. To date there are only a handful of interventional human studies that try to mitigate the health adversities in the offspring due to maternal obesity (Han et al., 2012; Briley et al., 2014; Chiswick et al., 2015; Poston et al., 2015, 2017; Thangaratinam, 2015; Dodd et al., 2016).

Prevention of obesity in women of childbearing age and the prevention of obesity during childhood are essential to fight the global obesity epidemic. Further, it is of outmost importance to elucidate the specific mechanisms linking *in utero* exposure to an hyperphagic diet to the development of obesity as well as cardiovascular disorders. The latter is the number one cause of global mortality. Outcomes from animal studies have a potential to unravel novel and yet unknown mechanisms involved in the pathophysiology of cardio-metabolic disease and how they are linked to cognitive impairments. A deeper understanding of whether and how maternal overnutrition exerts noxious effects on the health of the offspring may allow the identification of new targets for future interventions against the development of obesity-related cardiovascular disorders and neuropathology.

AUTHOR CONTRIBUTIONS

DP-R conceptualized the project, conducted the systemic review of the literature, and wrote the manuscript.

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Spatial Memory and Gut Microbiota Alterations Are Already Present in Early Adulthood in a Pre-clinical Transgenic Model of Alzheimer's Disease

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The irreversible and progressive neurodegenerative Alzheimer's disease (AD) is characterized by cognitive decline, extracellular β-amyloid peptide accumulation, and tau neurofibrillary tangles in the cortex and hippocampus. The triple-transgenic (3xTg) mouse model of AD presents memory impairment in several behavioral paradigms and histopathological alterations from 6 to 16 months old. Additionally, it seems that dysbiotic gut microbiota is present in both mouse models and patients of AD at the cognitive symptomatic stage. The present study aimed to assess spatial learning, memory retention, and gut microbiota alterations in an early adult stage of the 3xTg-AD mice as well as to explore its sexual dimorphism. We evaluated motor activity, novel-object localization training, and retention test as well as collected fecal samples to characterize relative abundance, alpha- and beta-diversity, and linear discriminant analysis (LDA) effect size (LEfSe) analysis in gut microbiota in both female and male 3xTg-AD mice, and controls [non-transgenic mice (NoTg)], at 3 and 5 months old. We found spatial memory deficits in female and male 3xTg-AD but no alteration neither during training nor in motor activity. Importantly, already at 3 months old, we observed decreased relative abundances of Actinobacteria and TM7 in 3xTg-AD compared to NoTg mice, while the beta diversity of gut microbiota was different in female and male 3xTg-AD mice in comparison to NoTg. Our results suggest that gut microbiota modifications in 3xTg-AD mice anticipate and thus could be causally related to cognitive decline already at the early adult age of AD. We propose that microbiota alterations may be used as an early and non-invasive diagnostic biomarker of AD.

Keywords: 3xTg-AD, dysbiosis, novel-object localization, Actinobacteria, TM7, alpha-diversity, beta-diversity, high-throughput DNA sequencing

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INTRODUCTION

Alzheimer's disease (AD) is an age-related and neurodegenerative disorder characterized by β-amyloid plaques and tau neurofibrillary tangle formation (Serrano-Pozo et al., 2011; Martins et al., 2018) with a progressive decline in cognitive functions (Querfurth and Laferla, 2010; Serrano-Pozo et al., 2011). One model for the study of AD is the triple-transgenic mouse (3xTg-AD), which contains three mutations associated with familial AD (APP Swedish, MAPT P301L, and PSEN1 M146V mutations). In this pre-clinical model, extracellular Aβ-peptide accumulation within the hippocampus appears by 6 months old, and changes in tau occur at 12–15 months old; with hyperphosphorylated tau aggregates detected also in the hippocampus (Oddo et al., 2003). These changes are associated with cognitive impairment in several behavioral paradigms, such as elevated maze, object recognition memory, Morris water maze, and T-maze tasks (Oddo et al., 2003; Chen et al., 2013; Cantanelli et al., 2014; Morin et al., 2016; Davis et al., 2017; Wei et al., 2017; Bello-Medina et al., 2019a). Histopathological and cognitive alterations have been mainly reported in the symptomatic stage of AD, i.e., at 6–9 months old, while learning and memory deficit have not been observed in the pre-symptomatic stage (3–5 months old) (Guzmán-Ramos et al., 2012; Cantanelli et al., 2014). Furthermore, symptoms and pathological manifestations are stronger in female than in male mice in this AD pre-clinical 3xTg-AD model. For instance, in 3xTg-AD mice from 6 to 23 months old, Aβ peptide largely accumulates within the hippocampus and cortex of females compared to males (Hirata-Fukae et al., 2008; Carroll et al., 2010a; Creighton et al., 2019). Likewise, in comparison to males, 3xTg-AD female mice from 6 to 14 months old display worse performance in peak interval procedure, novel-object recognition (NOR) task, spontaneous alternation task, and Morris water maze (Clinton et al., 2007; Carroll et al., 2010a; Creighton et al., 2019; Gür et al., 2019).

Alzheimer's disease etiology is still unknown, with multiple factors proposed as triggers of its development and contributors to its progression, such as oxidative stress (Sonnen et al., 2008; Chen and Zhong, 2014; Cheignon et al., 2018; Pohanka, 2018), lipid (Hamilton et al., 2015; Sah et al., 2017), and glucose metabolism alterations (Chen and Zhong, 2013), synaptic plasticity impairment (Min et al., 2013; Cantanelli et al., 2014; Guillot et al., 2016; Morin et al., 2016), and neuroinflammation (Cuello, 2017). Interestingly, environmental factors involved in the development of age-related disorders as is AD concur with increased intestinal permeability (Tran and Greenwood-Van Meerveld, 2013) and gut microbiota alterations (Syeda et al., 2018). Gut microbiota includes all microorganisms thriving within the intestine, which are functionally related to the host (Sampson and Mazmanian, 2015). To maintain host health, bacteria consortia should be in a dynamic balance between symbiotic, commensal, and pathogenic bacteria (Koboziev et al., 2014). In contrast, dysbiosis occurs when such homeostasis is lost (Degruttola et al., 2016). Gut microbiota contains Gram-positive and -negative bacteria, such as Firmicutes and Bacteroidetes, respectively. In this regard, a relevant component of the outer membrane of Gram-negative bacteria is lipopolysaccharide (Tlaskalová-Hogenová et al., 2004). This endotoxin travels from a leaky intestine, via the bloodstream, to cerebral regions, inducing inflammatory and microglia-mediated innate immune responses that are associated with Aβ oligomers, dimers, and monomers. In this context, microbiota metabolites and/or their components might be part of etiological factors of AD contributing to amyloid neurotoxicity (Colangelo et al., 2002; Dasari et al., 2011; Ferrera et al., 2014; Zhao and Lukiw, 2015) that results in the AD progression or its acceleration.

Currently, the gut microbiota is under characterization in several mouse pre-clinical models of AD to define pathological relationships and their potential causalities. In this context, most data focused on describing dysbiotic gut microbiota within the AD symptomatic stages. For instance, 5xFAD transgenic mice of 6 months old exhibit more abundance of the Proteobacteria and Firmicutes populations than control mice, while the Bacteroidetes population is lower (Lee et al., 2019). APPPS1 transgenic mice of 8 months old displayed significant proportion reductions in Firmicutes, Proteobacteria, and Actinobacteria, with increases in Bacteroidetes and Tenericutes phylum in comparison to wild-type (WT) mice. Regarding bacterial abundance, Rikenellaceae increases, while *Allobaculum* and *Akkermansia* genera decrease as compared to WT mice (Harach et al., 2017). Furthermore, in the 3xTg-AD mice at the symptomatic age of 9 months old, a significant increase in the abundance of Bacteroidetes and Firmicutes but a decrease of Cyanobacteria, Proteobacteria, Tenericutes, and Verrucomicrobia in comparison to WT mice were reported (Syeda et al., 2018).

However, so far, it is unknown if gut microbiota alterations precede cognitive deficits; therefore, we aimed to characterize gut microbiota at an early adulthood age in the 3xTg-AD mice. Furthermore, we analyzed whether sex might elicit dimorphism in the cognitive and gut microbiota parameters studied.

MATERIALS AND METHODS

The experiments reported in this study were carried out following the international (National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Research Council, 2011) and the Mexican Official standard (NOM-036-SSA-2-2002) normative and were approved by the local ethics committee (Comité de Bioética del Instituto de Neurobiología, Universidad Nacional Autónoma de México), approval N° 117.A.

Animals

The study subjects were female (n = 10) and male (n = 10) 3xTg-AD mice harboring APP_{Swe} and Tau_{P30L} transgenes on a mutant $PS1_{M146V}$ knock-in background and female (n = 10) and male (n = 10) non-transgenic mice (NoTg) from the same genetic background B6129SF1/J (both Jackson Laboratory, Bar Harbor, ME, United States). B6129SF1/J hybrid mice are the offspring of the breeding between C57BL/6J females (B6) and 129S1/SvImJ males (129S). All mice were housed in groups of 3–5 per cage with water and food (LabDiet 5001) *ad libitum* and maintained in a room with 12-h dark/12-h artificial light cycles beginning

at 19:00 h. We performed all behavioral procedures between 9:00 and 13:00 h.

Genotyping

We performed genotyping as previously reported (Guzmán-Ramos et al., 2012). Briefly, for DNA extraction, a 5-mm-long caudal tail segment was sectioned, and lysis was made in an alkaline reagent (25 mM NaOH, 0.2 mM disodium EDTA) with heat (95°C, 1 h) and neutralization with a suitable buffer (1 M Tris–HCl at pH 7.5). This DNA sample was used immediately in a polymerase chain reaction (PCR) to test for the presence of the amyloid precursor protein (APP) and tau DNA and mutation in the presenilin 1 (PS1) gene.

Collection of Fecal Samples for Microbiota Analysis

We collected fecal samples from NoTg and 3xTg-AD mice when they were 3 and 5 months old. Fresh fecal pellets were collected by holding the mouse in one hand while the mouse defecates directly in a 1.5 ml polypropylene tube held in the other hand; 7–8 fresh fecal pellets were gathered. Tubes were kept in dry ice until transfer to a −80°C freezer where the samples were maintained until their DNA microbiota profile analysis.

High-Throughput V3-16S rDNA Libraries Sequencing

Fecal DNA was extracted from 100 mg of homogenized feces using Favor prep stool kit (Cat. #FASTI001-1; Favorgen Biotech Corp., Ping-Tung, Taiwan) according to the manufacturer's instructions and stored at −70°C until sequencing (Hernández-Quiroz et al., 2020). We measured DNA concentration using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and DNA quality was evaluated by electrophoresis in 0.5% agarose gel. All PCR reactions were performed in a final volume in 50 μl final volume of 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.8), 2 mM $MgCl_2$, 0.2 μM of each barcoded primer, 0.2 mM each dNTP, 0.025 U/μl of recombinant Taq polymerase (Thermo Scientific EP0402), and 20–50 ng of total nucleic acids. The PCR program was 95°C, 5 min, followed by 30 × [94°C, 30 s; 62°C, 15 s; 72°C 15 s], 72°C, 10 min extension using a GeneAmp PCR System 2700 (Applied Biosystems). For library preparation, each of the 1–45 barcoded amplicons were quantified by gel densitometry and equal mass amounts pooled. The mixture was purified using E-Gel iBase Power System (Invitrogen). The library's size and concentration were checked using the Agilent 2100 Bioanalyzer system and High Sensitivity DNA Kit (Agilent, United States). High-throughput sequencing of the ∼281-bp amplicons was performed using Ion OneTouch™ 2, Ion PGM™ Template OT2 200 Kit v2 DL (Life Technologies, Carlsbad, CA, United States), Ion 318 Chip Kit v2, and Ion Torrent PGM System (García-Mena et al., 2016). The amplicon was not observed for the negative controls and was not sequenced. After sequencing, reads were filtered by the PGM software to exclude low-quality and polyclonal sequences. All reads were analyzed using FastQC software v0.11.9 (Andrews, 2010) and trimmed to 200 nt using Trimmomatic v0.38. The sequencing summary is shown in **Table 1**. Demultiplexed FASTQ files were converted into FASTA files, concatenated into a single file, and then processed with multiple Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 scripts (Caporaso et al., 2010). DNA sequences were classified into operational taxonomic units (OTUs) using closed-based picking parameters with a 97% similarity level against the Greengenes database v13.8.

The corresponding FASTQ sequence files for all samples used in this study were deposited in the NCBI BioProject repository (Accession Number: PRJNA648144). In link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA648144.

Gut Microbiota Relative Abundance and Diversity

We analyzed sequenced data using QIIME (v1.9.0) pipeline (Caporaso et al., 2010) to determine the relative abundance of gut bacterial taxa. Before calculation of alpha diversity, the OTU table was rarefied at 10,000 sequences per sample using a "single_rarefaction.py" QIIME script. Diversity was characterized by alpha diversity, including Shannon, Simpson, Chao1 indexes, and observed species using phyloseq (v1.22.3) and ggplot2 (v3.1.0) packages in R (v3.4.4) (McMurdie and Holmes, 2013). The beta diversity dissimilarity index was calculated by UniFrac distance metric as a percentage of the total variability in different axis of the plot and visualized by principal coordinate analysis as described in Chávez-Carbajal et al. (2019).

Analysis of Significant Enrichment in Gut Microbiota Taxa

We used linear discriminant analysis (LDA) effect size (LEfSe) (v1.0) to disclose significant differences in the relative abundance of bacterial taxa among groups. These LEfSe analyses are represented in the form of bar plots and the parameters set with default $p < 0.05$, LDA scores ≥ 2.0 (Segata et al., 2011).

Novel-Object Localization (NOL) Task Apparatus

The apparatus was an open acrylic box (33 cm × 33 cm × 33 cm) with black walls. The floor of the box was covered with a 1-cm

TABLE 1 | Sequencing summary after trimming[#] (n = 81).

Parameter	Fecal samples, n = 81
Number of reads	5,189,402
Length mean[a]	142 bp (28.01)[b]
Reads mean[a]	42,705.63
Reads min–max[a]	2,950–237,259
Identified OTUs	5,840
Total OTUs count	2,195,820
Samples with <10,000 reads	13

[#]*Reads were analyzed using FastQC software v0.11.9 and trimmed to 200 nt using Trimmomatic v0.38.*
[a]*Length expressed as bases.*
[b]*Standard deviation, for all samples.*
OTUs, operational taxonomic units.

layer of sawdust, the box was cleaned with 7% acetic acid (v/v), and sawdust was replaced between trials. There were four visual cues on the walls of the experimental room, within the visual field of mice. A video camera was positioned above the box, and each trial was video recorded for post-training analysis. The objects used were glass scintillation vials of 2.8 cm × 6.1 cm; these stimuli were denominated familiar localization 1 (Fam 1) and familiar localization 2 (Fam 2), respectively. These objects were attached with Velcro® to the box floor and were cleaned after each trial with 70% alcohol solution (v/v).

Training and Retention Test

Handling of 5-month-old mice took place for 5 min daily on three consecutive days. The NOL task consisted of three sessions: habituation, training, and retention. During the habituation session, the mice could freely explore the open box, without objects, for 5 min. In this session, motor activity parameters such as speed, distance traveled, and resting time were evaluated. Twenty-four hours after habituation, during the training session, the mice were placed in the open box, which contained the two-sample objects (Fam 1 and Fam 2), for 10 min. Retention took place 24 h after the training session; mice were placed in the open box where one of the familiar objects remained in the same localization it was located in training (Fam), and the other familiar object was moved to a novel localization (Nov) (**Figure 1**). The results were expressed both as total exploration time per object.

Immunohistochemistry for β-Amyloid Peptide Detection

After NOL retention, and under anesthesia, we euthanized the mice (n = 4 per group) and transcardially perfused 4% paraformaldehyde in 0.1 M phosphate buffer via the ascending aorta. Brains were removed, postfixed in the same solution overnight. They were cryoprotected with 30% sucrose in phosphate buffer for 6 days. Four frozen sagittal sections of 30 μm from the left hemisphere that contained the *subiculum* and CA1 of the dorsal hippocampus (lateral from 0.72 to 1.80 mm to interhemispheric line; Paxinos and Franklin, 2001) were cut with the aid of a Leica cryostat and were placed on slides. Sections on slides were rinsed in phosphate-buffered saline (PBS) and incubated in 90% formic acid for 7 min and rinsed with water after were incubated in 1% H_2O_2, and then washed with PBS. After blocking with a tyramide signal amplification kit (TSA) blocking buffer (PerkinElmer Life Sciences), the sections were incubated in a monoclonal mouse anti-BAM10 antibody (1:500; Sigma-Aldrich) overnight at 4°C. Subsequently, sections were washed with PBS and incubated for 2 h at room temperature with Alexa Fluor 488-coupled goat anti-mouse antibody (1:500; Life Technologies), then washed with PBS, and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:5,000; Sigma-Aldrich). The stained sections were covered with fluorescence mounting medium (Fluoromount-G, Electron Microscopy Sciences).

The subiculum and CA1 mosaic images used in the analyses were obtained with a 40×/1.25 apochromatic objective lens and the MosaiX module for the Apotome system (Zeiss). Analysis of the β-amyloid accumulation was performed on the images using the ImageJ software, the proceeding was carried out as described previously (González-Franco et al., 2017; Prado-Alcalá et al., 2017). The results were expressed as the area that is occupied for β-amyloid in the subiculum or CA1 of the dorsal hippocampus.

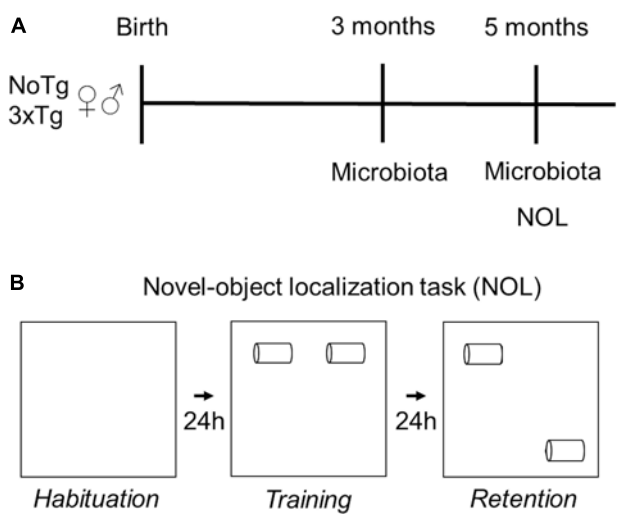

FIGURE 1 | Experimental design. **(A)** Fecal samples of female and male non-transgenic (NoTg) or Alzheimer's disease triple-transgenic (3xTg-AD) mice were collected when they were 3 and 5 months old. **(B)** NOL task was performed in 5-month-old mice. NOL consists of three sessions: habituation, training, and retention test.

Statistical Analysis

We performed the Kolmogorov–Smirnov test to prove the normality parametric assumption. The data analysis of motor activity was made with a two-way ANOVA, where factor 1 was genotype (NoTg or 3xTg-AD) and factor 2 was sex (female or male). The data analysis of NOL performance was made with a three-way ANOVA, where factor 1 was genotype (NoTg or 3xTg-AD), factor 2 was sex (female or male), and within-subject factor 3 was object localization (Fam 1 vs. Fam 2 in training or Fam vs. Nov in the retention test). For the β-amyloid histological analysis, we applied a two-way ANOVA, where factor 1 was genotype (NoTg or 3xTg-AD) and factor 2 was sex (female or male) for each dorsal hippocampus area (*subiculum* or CA1). We used the *post hoc* Bonferroni test when appropriate. The $p < 0.05$ was considered statistically significant.

For gut microbiota analyses, all data were statistically analyzed using t-test or Mann–Whitney U test. Data were expressed in means ± standard deviation, the $p < 0.05$ was considered statistically significant. Sequenced data were analyzed using QIIME pipeline (v1.9.0). OTU picking was made against the Greengenes (v13.8) database. Bioinformatic analyses were made in R environment (v3.4.4); gut bacterial diversity (alpha diversity) was assessed with phyloseq (v1.22.3). Images were plotted using ggplot2 (v3.1.0) and RColorBrewer (v1.1-2) packages.

RESULTS

Motor Activity

In habituation session of NOL, two-way ANOVA showed no differences in speed for genotype [$F_{(1,36)} = 0.77$; $p = 0.38$], sex [$F_{(1,36)} = 2.71$; $p = 0.11$], nor interaction [$F_{(1,36)} = 1.42$; $p = 0.24$] factors (**Figure 2A**). Two-way ANOVA showed no alterations in traveled distance for genotype [$F_{(1,36)} = 2.35$; $p = 0.13$], sex [$F_{(1,36)} = 1.60$; $p = 0.21$], nor interaction [$F_{(1,36)} = 0.66$; $p = 0.42$] factors (**Figure 2B**). The two-way ANOVA showed no significant differences in resting time and showed no statistical differences in genotype [$F_{(1,36)} = 0.08$; $p = 0.77$], sex [$F_{(1,36)} = 0.72$; $p = 0.40$], nor with interaction [$F_{(1,36)} = 0.42$; $p = 0.52$] factors (**Figure 2C**).

Training of NOL

A three-way ANOVA showed a significant effect in total time object exploration during training for genotype [$F_{(1,72)} = 36.63$; $p < 0.0001$], but not for object [$F_{(1,72)} = 0.03$; $p = 0.87$] nor sex [$F_{(1,72)} = 3.00$; $p = 0.08$] factors. The genotype × sex [$F_{(1,72)} = 0.02$; $p = 0.87$], genotype × object [$F_{(1,72)} = 2.18 \times 10^{-4}$; $p = 0.99$], sex × object [$F_{(1,72)} = 0.12$; $p = 0.73$], and genotype × sex × object [$F_{(1,72)} = 0.13$; $p = 0.72$] interactions were not statistically significant (**Figure 3A**). The *post hoc* Bonferroni test showed that exploration time was higher in Fam 1 object in female NoTg than female 3xTg-AD ($p = 0.001$) and Fam 2 object in female NoTg in comparison with female 3xTg-AD ($p = 0.005$) (**Figure 3A**). The same effect was observed in male NoTg vs. 3xTg-AD in Fam 1 ($p = 0.04$) and Fam 2 ($p = 0.01$) objects (**Figure 3A**). The *post hoc* Bonferroni test showed that the exploration times for Fam 1 and Fam 2 were not significantly different in each genotype group; this result reflects a good familiarization process, which is very relevant for the acquisition of the recognition memory.

Retention Test of NOL

In the retention test, a three-way ANOVA showed significant differences in the genotype [$F_{(1,72)} = 15.56$; $p = 0.0002$], object [$F_{(1,72)} = 8.69$; $p = 0.004$], but not in sex [$F_{(1,72)} = 0.24$; $p = 0.63$] factors. The genotype × object [$F_{(1,72)} = 39.26$; $p < 0.0001$] and genotype × sex × object [$F_{(1,72)} = 6.98$; $p = 0.010$] interactions were statistically significant; however, the genotype × sex [$F_{(1,72)} = 2.41$; $p = 0.13$] and sex × object [$F_{(1,72)} = 0.52$; $p = 0.47$] interactions were not significant. The *post hoc* Bonferroni test showed that exploration time NOL was higher than familiar-object place in female ($p < 0.0001$) and male ($p < 0.0001$) NoTg mice (**Figure 3B**). These results indicate a preference for the displaced object, which is a normal behavior for memory recognition (Bello-Medina et al., 2019b). The effect observed in female NoTg mice was opposite in male 3xTg-AD mice ($p < 0.0001$). No differences were found in exploration time between Nov and Fam objects in female 3xTg-AD mice. This demonstrated that there is a deficit in NOL memory in female and male 3xTg-AD mice. On the other hand, the Bonferroni test showed statistical differences in exploration time of object displaced in female NoTg and female 3xTg-AD. This same effect was also observed in male NoTg in comparison with male 3xTg-AD (**Figure 3B**).

β-Amyloid Peptide Increases in the *Subiculum* and CA1 of the Dorsal Hippocampus in 3xTg-AD Mice

A two-way ANOVA showed significant differences in area occupied by β-amyloid peptide in the *subiculum* for genotype [$F_{(1,12)} = 45.94$; $p < 0.0001$], but neither for sex [$F_{(1,12)} = 0.44$; $p = 0.52$] nor interaction [$F_{(1,12)} = 0.42$; $p = 0.53$] (**Figure 4A**). The *post hoc* Bonferroni test showed that the β-amyloid area was larger in female 3xTg-AD (14.7%) than female NoTg mice (0.28%) ($p = 0.0002$); this same effect was observed in male 3xTg-AD (13.4%) with respect to male NoTg (0.27%) ($p = 0.014$) (**Figure 4A**).

A two-way ANOVA showed statistical differences in the area occupied for β-amyloid peptide in the CA1 for genotype [$F_{(1,12)} = 234.48$; $p < 0.0001$] but not for sex [$F_{(1,12)} = 0.29$; $p = 0.87$] nor interaction [$F_{(1,12)} = 0.29$; $p = 0.86$] factors (**Figure 4B**). The *post hoc* Bonferroni test showed that the β-amyloid area was larger in female 3xTg-AD (56,705.1 μm^2, which represent 10.9% of the total analyzed area of the *subiculum*) than female NoTg mice (344.9 μm^2, which represent 10.9% of the total analyzed area of the *subiculum* 0.24%) ($p = 0.0002$); this same effect was observed in male 3xTg-AD (46,748.1 μm^2, which represent 9.6% of the total analyzed area of the CA1) vs. male NoTg (257.77 μm^2, which represent 0.23% of the total analyzed area of the CA1) ($p = 0.014$) (**Figure 4B**). These results mean that female and 3xTg-AD mice at 5 months old present

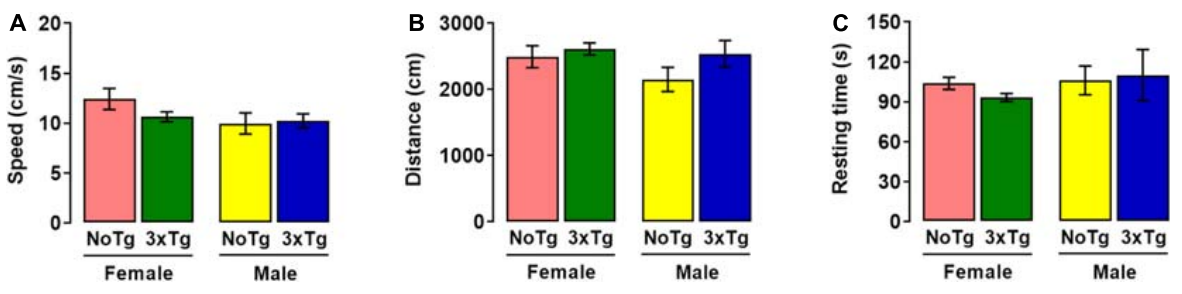

FIGURE 2 | Motor activity during habituation session of novel-object localization (NOL) task. Mean values (with standard error) of **(A)** speed, **(B)** distance traveled, and **(C)** resting time of female or male non-transgenic (NoTg) or Alzheimer's disease triple-transgenic (3xTg-AD) mice of 5 months old; $n = 10$ mice per group.

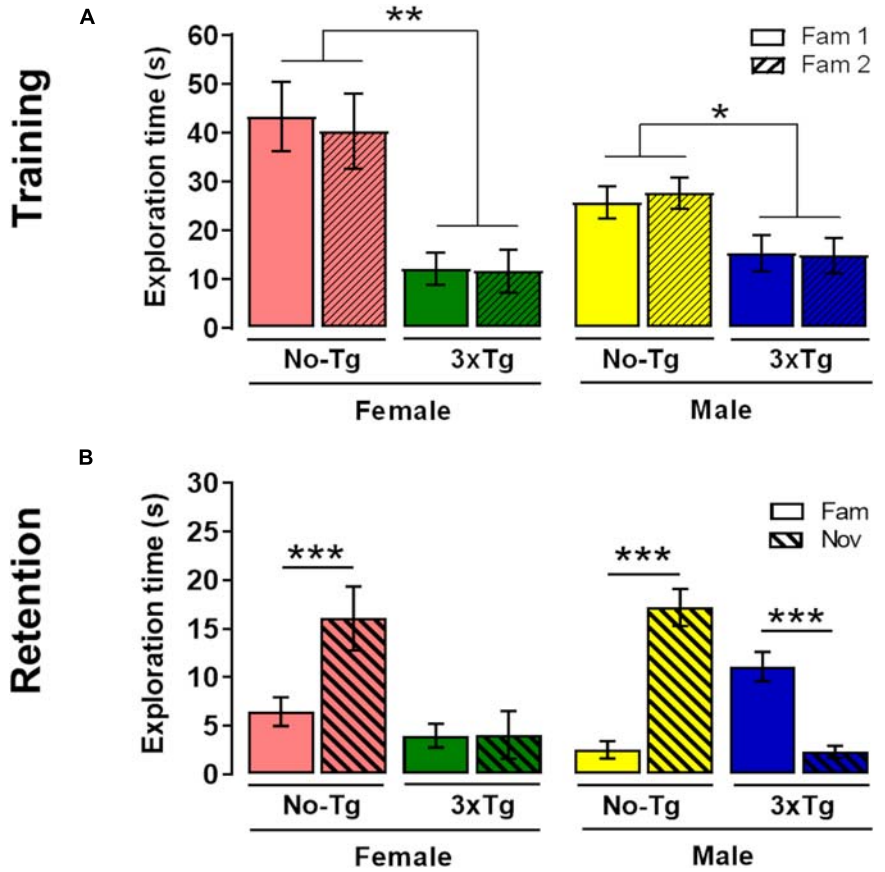

FIGURE 3 | Novel-object localization (NOL) task. Mean exploration time (with standard error) of familiar localization 1 (Fam 1) and familiar localization 2 (Fam 2) in **(A)** training and exploration time of familiar localization (Fam) and novel localization (Nov) in **(B)** retention test of female or male non-transgenic (NoTg) or Alzheimer's disease triple-transgenic (3xTg-AD) mice of 5 months old. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$; $n = 10$ mice per group.

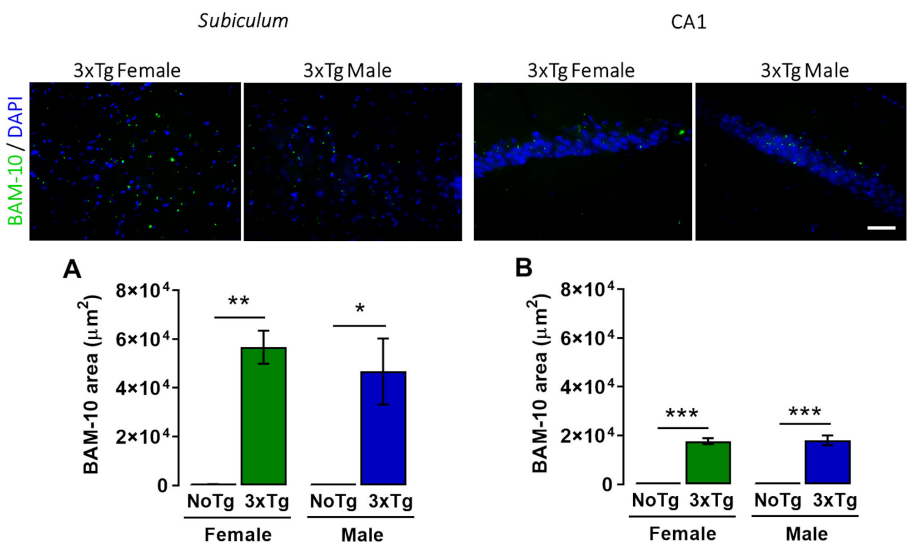

FIGURE 4 | Representative images of β-amyloid (green) immunohistochemistry and nuclei detection (blue) obtained with 40× objective in the *subiculum* and CA1 of the dorsal hippocampus of female and male 3xTg-AD at 5 months old. Mean BAM-10 area (μm^2) (with standard error) of female and male non-transgenic (NoTg) or Alzheimer's disease triple-transgenic (3xTg-AD) mice in the **(A)** *subiculum* and **(B)** CA1. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$; $n = 4$ mice per group. Scale bar = 20 μm.

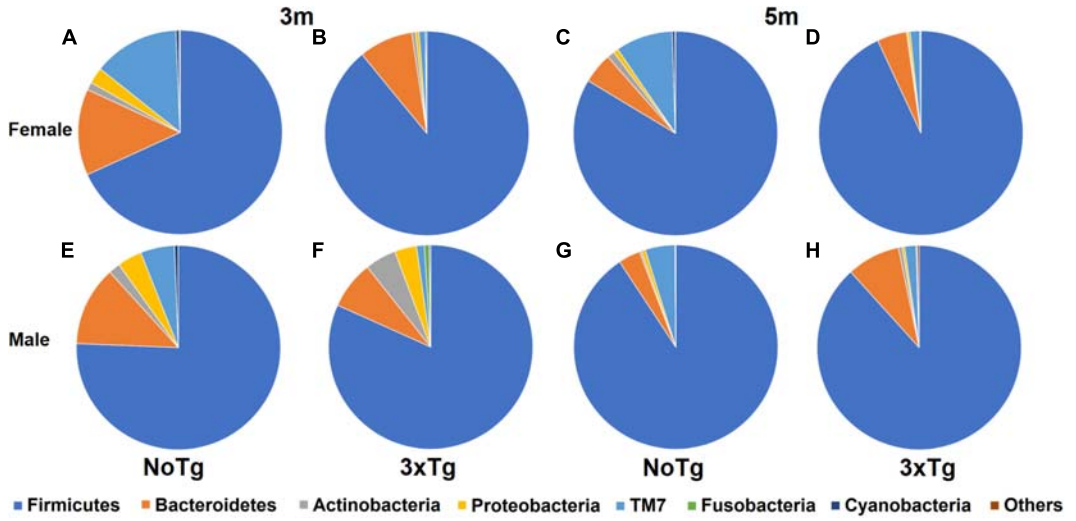

FIGURE 5 | Relative abundance of bacterial phyla in fecal samples of non-transgenic (NoTg) and Alzheimer's disease triple-transgenic (3xTg-AD) mice. Sectors in pie charts indicate Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, TM7, Fusobacteria, and Cyanobacteria phyla as shown by tag colors at the right side of the figure. Abundances of each phylum are shown as percentages beside each sector in the pie charts. "Others" groups phyla with <0.5% relative abundance (Verrucomicrobia, Acidobacteria, Thermotogae, Chloroflexi, Thermi, Gemmatimonadetes, Tenericutes, Euryarchaeota, FBP, Spirochaetes, and Synergistetes). The figure shows data for 3 and 5 months (m) old for NoTg female **(A,C)** and male mice **(E,G)** and for 3xTg-AD female **(B,D)** and male mice **(F,H)** (**Supplementary Tables 1, 2**).

β-amyloid accumulation in the *subiculum* and CA1 of the dorsal hippocampus at the early pre-symptomatic stage of AD.

Changes in the Fecal Bacterial Diversity Relative Abundance of Bacterial Phyla Are Associated With the Triple Transgenic Condition in the Mouse Model

The characterization of the relative abundance of phyla in the fecal samples showed significant changes when comparing by age (3 vs. 5 months old); in the male NoTg mice, there was an increase in the abundance of Firmicutes (p = 0.017) and Actinobacteria (p = 0.005), while a decrease was detected for Proteobacteria (p = 0.004), Fusobacteria (p = 0.011), Cyanobacteria (p = 0.001), and Bacteroidetes (p = 0.008). A decrease in Actinobacteria (p = 0.049) in female 3xTg-AD mice was observed (**Figure 5** and **Supplementary Table 1**). Concerning the genotype, the change in the abundance of TM7 was statistically significant. For instance, at 3 months, TM7 was more abundant in NoTg male mice vs. 3xTg-AD male (p = 0.003) and in NoTg female mice vs. 3xTg-AD female (p = 0.002). At 5 months, TM7 was more abundant in NoTg female mice vs. 3xTg-AD female (p = 0.007), and in males, this phylum was more abundant in NoTg male mice vs. 3xTg-AD male (p = 0.007). Fusobacteria were more abundant in 3xTg-AD male vs. NoTg male (p = 0.003), and Cyanobacteria were more abundant in NoTg male vs. 3xTg-AD male (p = 0.049) (**Figure 5** and **Supplementary Tables 1, 2**). Finally, when comparing by sex, Cyanobacteria were more abundant at 3 months in NoTg male vs. NoTg female (p = 0.007), and Fusobacteria were more abundant at 3 months in 3xTg-AD male vs. 3xTg-AD female at 3 months (p = 0.031) (**Figure 5** and **Supplementary Tables 1, 2**).

Alpha and Beta Diversity of Gut Microbiota

The estimation of alpha diversities for the same data showed only statistically significant changes for Shannon (p = 0.041) and Simpson (p = 0.035) indexes when comparing NoTg female vs. NoTg male mice at 3 months and NoTg female vs. NoTg male mice at 5 months (**Figures 6**, **7** and **Supplementary Table 4**).

The analysis of the significance of differences in the beta diversities by the ANOSIM similarity test was grouping genotype (**Figure 8**; NoTg female vs. 3xTg female at 3 months, **Figure 8A**; NoTg female vs. 3xTg female at 5 months, **Figure 8B**; NoTg male vs. 3xTg male at 3 months, **Figure 8C**; NoTg male vs. 3xTg male at 5 months, **Figure 8D**), age (**Figure 9**; NoTg female 3 months vs. 5 months, **Figure 9A**; 3xTg female 3 months vs. 5 months, **Figure 9B**; NoTg male 3 months vs. 5 months, **Figure 9C**; 3xTg male 3 months vs. 5 months, **Figure 9D**), and sex (**Figure 10**; NoTg female vs. NoTg male at 3 months, **Figure 10A**; NoTg female vs. NoTg male at 5 months, **Figure 10B**; 3xTg female vs. 3xTg male at 3 months, **Figure 10C**; 3xTg female vs. 3xTg male at 5 months, **Figure 10D**). The main differences were observed comparing by genotypes, showing differences for all comparisons: NoTg vs. 3xTg-AD females at 3 months old (R = 0.184, p = 0.018), NoTg vs. 3xTg-AD females at 5 months old (R = 0.124, p = 0.028), NoTg vs. 3xTg-AD males at 3 months old (R = 0.422, p = 0.001), and NoTg vs. 3xTg-AD male at 5 months old (R = 0.174, p = 0.022) (**Figure 8**); however, the comparison by age showed only differences for NoTg male mice 3 vs. 5 months old (R = 0.425, p = 0.002) (**Figure 9**). Finally, when comparing by

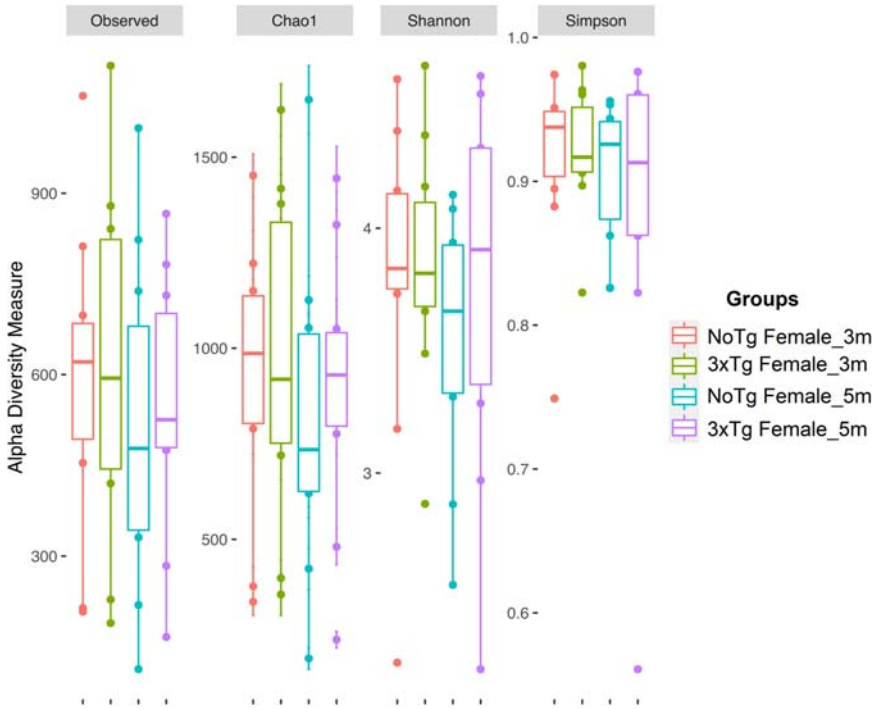

FIGURE 6 | Alpha diversity of bacteria in fecal samples collected from female mice. The figure shows data for non-transgenic (NoTg) (coral pink) and Alzheimer's disease triple-transgenic (3xTg-AD) mice at 3 months (m) old (light olive) and NoTg (light blue) and 3xTg-AD mice at 5 months old (light purple). The *Y*-axis indicates the values for the corresponding indexes: observed number of species (Observed), expected bacterial richness (Chao1), and Shannon and Simpson diversity (**Supplementary Tables 3, 4**).

sex, there was only a difference between NoTg female vs. NoTg male at 3 months old ($R = 0.225$, $p = 0.002$) (**Figure 10**).

The Genotype, Age, and Sex Are Associated With Changes in the Abundance of Bacteria Related to Neurodegenerative Diseases

The LEfSe analyses revealed bacteria with statistically significant changes in the abundance among all groups. At 3 months old, NoTg female mice had an increased abundance of members of the family F16 (phylum TM7) and the genus *Mycoplasma* (phylum Tenericutes); the 3xTg-AD female had an increase of genus *Dehalobacterium*, (phylum Firmicutes) and the genus *Desulfovibrio* (phylum Proteobacteria). At 5 months old, NoTg female had an increase in *Ruminococcus* (phylum Firmicutes), while 3xTg-AD females had an increase in *Gemella* (phylum Firmicutes). At 3 months old, NoTg male mice had an increase of the genus *AF12* (phylum Bacteroidetes), members of the family Mogibacteriaceae (phylum Firmicutes), and the family Sphingomonadaceae (phylum Proteobacteria). The 3xTg-AD male mice at 3 months old increased the family Koribacteraceae (phylum Acidobacteria); the family Streptomycetaceae; and the genera *Atopobium*, *Collinsella*, and *Microbacterium* (phylum Actinobacteria). There was also an increase in the genus *Pedobacter* (phylum Bacteroidetes); the family Clostridiaceae; genera *Allobaculum*, *Lactococcus*, *Selenomonas*, and *Veillonella* (the phylum Firmicutes); the families Erythrobacteraceae, Oxalobacteraceae, Xanthomonadaceae; and the genera *Bradyrhizobium*, *Campylobacter*, *Flexispira*, and *Neisseria* (phylum Proteobacteria); and the genus *S1* (phylum Thermotogae) (**Figure 11** and **Supplementary Table 5**).

A different profile in the abundances was observed at 3 months old only for NoTg mice. NoTg female had an increase in the abundance of members of the genera *Clostridium* (phylum Firmicutes) and *Mycoplasma* (phylum Tenericutes). NoTg male had an increase in the family Streptomycetaceae; genera *Actinomyces* and *Microbacterium* (phylum Actinobacteria); *Porphyromonas* and *Prevotella* (phylum Bacteroidetes); the families Clostridiaceae, Erysipelotrichaceae, Mogibacteriaceae; the genera *Allobaculum*, *Anaerococcus*, and *SMB53* (phylum Firmicutes); the families Erythrobacteraceae and Sphingomonadaceae; and the genera *Desulfovibrio*, *Kaistobacter*, *Methylobacterium*, *Paracoccus*, and *Sutterella* (phylum Proteobacteria) (**Figure 12** and **Supplementary Table 6**).

For the transgenic mice, at 3 months old, the abundances in the 3xTg-AD female showed an increase in the genus *Lactobacillus* (phylum Firmicutes), while at 5 months old, it showed an increase in the genera *Dorea*, *Gemella*, *Lachnobacterium*, *Peptoniphilus*, and *Ruminococcus* (phylum Firmicutes). The 3xTg-AD male mice at 3 months old showed an increase in the family Koribacteraceae (phylum Acidobacteria); the family Streptomycetaceae; the genera *Atopobium*, *Collinsella*, *Nesterenkonia*, and *Rothia* (phylum Actinobacteria); the genus

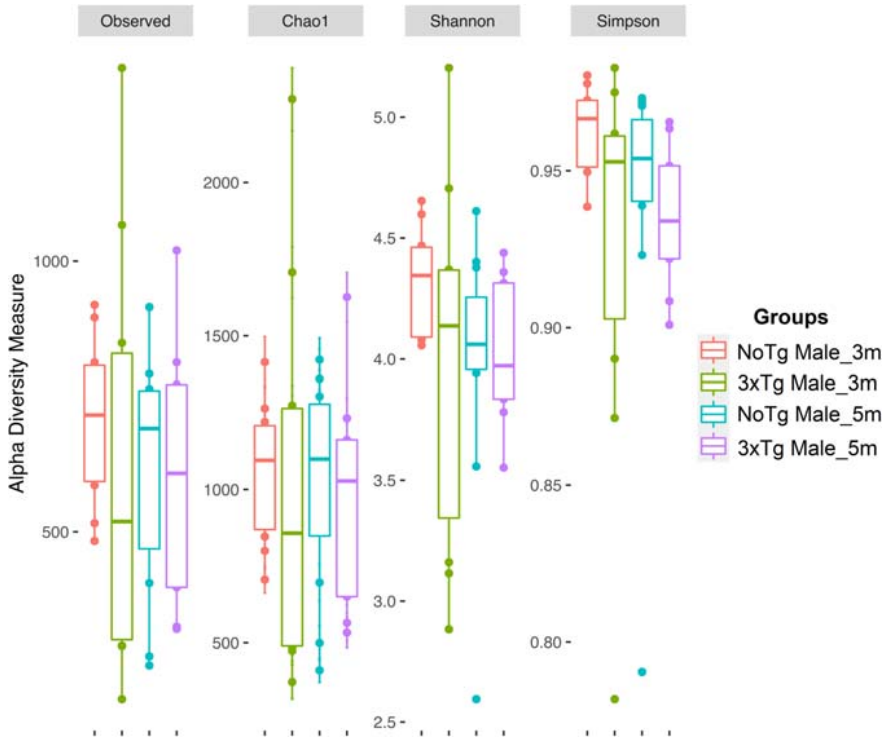

FIGURE 7 | Alpha diversity of bacteria in fecal samples collected from male mice. The figure shows data for non-transgenic (NoTg) (coral pink) and Alzheimer's disease triple-transgenic (3xTg-AD) mice at 3 months (m) old (light olive) and NoTg (light blue) and 3xTg-AD mice at 5 months old (light purple). The *Y*-axis indicates the values for the corresponding indexes: observed number of species (Observed), expected bacterial richness (Chao1), and Shannon and Simpson diversity (**Supplementary Tables 3, 4**).

Pedobacter (phylum Bacteroidetes); the genera *Allobaculum*, *Eubacterium*, *Lactococcus*, *Selenomonas*, and *Veillonella* (phylum Firmicutes); the families Beijerinckiaceae, Oxalobacteraceae, Phyllobacteriaceae, Rhodospirillaceae, Xanthomonadaceae; the genera *Aeromonas*, *Campylobacter*, *Erythrobacter*, *Flexispira*, and *Neisseria* (phylum Proteobacteria); and the genus *S1* (phylum Thermotogae). The 3xTg-AD male at 5 months old showed an increase in the family Christensenellaceae (phylum Firmicutes) (**Figure 13** and **Supplementary Table 7**).

DISCUSSION

We studied changes in the fecal microbiota associated with cognitive impairment in behavioral paradigm tests at an early adulthood of the AD transgenic mouse model. We found an impairment of memory retention of NOL in female and male 3xTg-AD mice at 5 months old, without motor activity alteration, demonstrating that such deficit in memory performance was cognitive specific. Furthermore, we observed an increase in the area occupied for β-amyloid peptide in the *subiculum* and CA1 of the dorsal hippocampus in female and male 3xTg-AD mice. Importantly, we found gut microbiota dysbiosis in female and male 3xTg-AD mice already at 3 and 5 months old, which until now are both considered within a pre-symptomatic stage of this pathology (Clark et al., 2015).

Two variants of object recognition paradigms have been reported: NOL and NOR (Denninger et al., 2018). In NOL, an object in a familiar localization is changed to a novel localization in the arena, which allows to evaluate spatial memory dependent on the dorsal hippocampus (Winters et al., 2008; Burke et al., 2012). For NOR, a familiar object is replaced by a novel object, which allows to evaluate recognition memory dependent on the perirhinal cortex (Burke et al., 2012). It is important to remark that as β-amyloid peptide accumulates in the subiculum and hippocampus in the early stage of AD (Clark et al., 2015), NOL is considered an appropriate measurement to evaluate early pathological symptomatology. Our results show that NOL learning was not affected in female and male from NoTg and 3xTg-AD genotypes. However, importantly, both sexes of 3xTg-AD mice showed a deficit in the NOL retention test at a pre-symptomatic stage of AD. These data appear to contradict the results reported by Guzmán-Ramos et al. (2012), in which they found that 3xTg-AD mice show a good performance in NOR at 5 months old; thus, the authors concluded that these mice do not show memory alterations at an early age. We consider that such a contradictory result is probably related to the fact that NOR is not a pure spatial task and therefore might not be affected by Aβ accumulation within the hippocampus. In the present study, we observed decreased exploration time of the two familiar-localization objects during NOL training session in female and male 3xTg-AD mice in comparison to NoTg

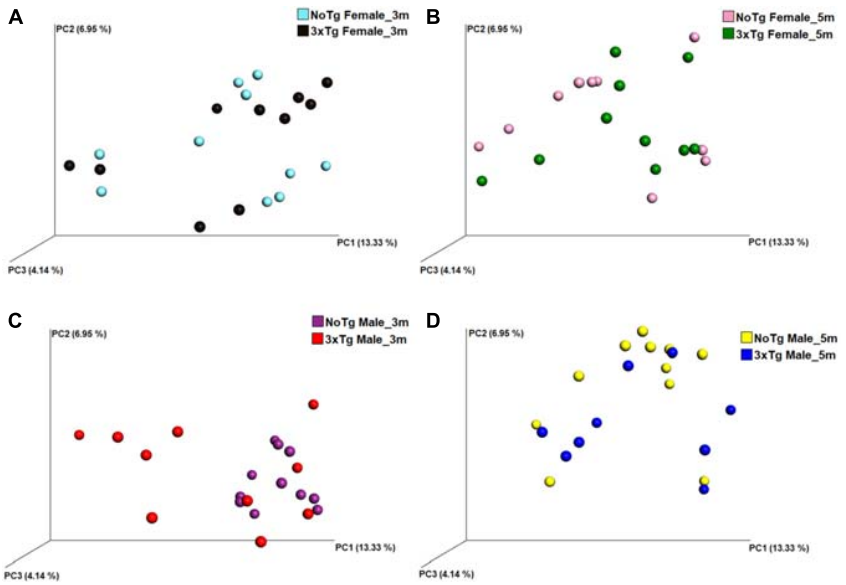

FIGURE 8 | Beta diversity of bacteria in fecal samples collected from non-transgenic (NoTg) and Alzheimer's disease triple-transgenic (3xTg-AD) mice. The graphics show beta diversity analyses calculated by dissimilarity metrics using operational taxonomic unit (OTU) tables and Unweighted UniFrac analyses. The analyses show the dissimilarity among mice by colors: NoTg female at 3 months (m) old (cyan), 3xTg-AD female at 3 months old (black), NoTg female at 5 months old (pink), 3xTg-AD female at 5 months old (green), NoTg male at 3 months old (purple), 3xTg-AD male at 3 months old (red), NoTg male at 5 months old (yellow), 3xTg-AD male at 3 months old (blue). Data comparisons by genotype and sex: NoTg vs. 3xTg-AD female mice at 3 months old **(A)**, NoTg vs. 3xTg-AD female mice at 5 months old **(B)**, NoTg vs. 3xTg-AD male mice at 3 months old **(C)**, and NoTg vs. 3xTg-AD male mice at 5 months old **(D)**. The three-dimensional scatter plots were generated using principal coordinates analyses (PCoA) in three different axis that show the percentage of total differences. There were significant differences on all comparisons according to ANOSIM similarity test (**(A)**, $R = 0.184$, $p = 0.018$; **(B)**, $R = 0.124$, $p = 0.028$; **(C)**, $R = 0.422$, $p = 0.001$; **(D)**, $R = 0.174$, $p = 0.022$).

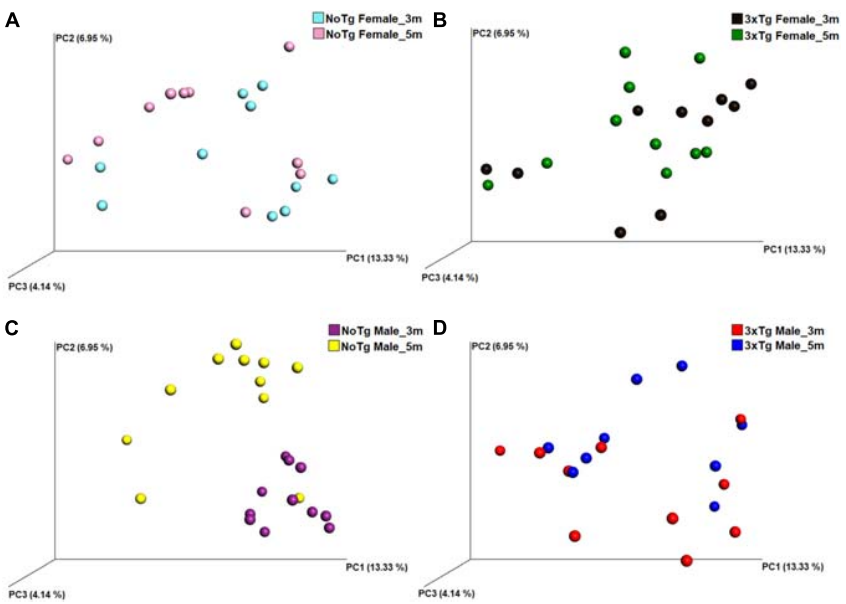

FIGURE 9 | Beta diversity of bacteria in fecal samples collected from non-transgenic (NoTg) and Alzheimer's disease triple-transgenic (3xTg-AD) mice. The graphics show beta diversity analyses calculated by dissimilarity metrics using operational taxonomic unit (OTU) tables and Unweighted UniFrac analyses. The analyses show the dissimilarity among mice by colors: NoTg female at 3 months (m) old (cyan), 3xTg-AD female at 3 months old (black), NoTg female at 5 months old (pink), 3xTg-AD female at 5 months old (green), NoTg male at 3 months old (purple), 3xTg-AD male at 3 months old (red), NoTg male at 5 months old (yellow), 3xTg-AD male at 3 months old (blue). Data comparisons by time in the same genotype: NoTg female mice 3 and 5 months old **(A)**, 3xTg-AD female mice 3 and 5 months old **(B)**, NoTg male mice 3 and 5 months old **(C)**, and 3xTg-AD male mice 3 and 5 months old **(D)**. The three-dimensional scatter plots were generated using principal coordinates analyses (PCoA) in three different axis that show the percentage of total differences. There is a significant difference in panel **(C)** according to ANOSIM similarity test ($R = 0.425$, $p = 0.002$) but not in panels **(A)** ($R = 0.032$, $p = 0.223$), **(B)** ($R = 0.020$, $p = 0.297$), and **(D)** ($R = 0.093$, $p = 0.076$).

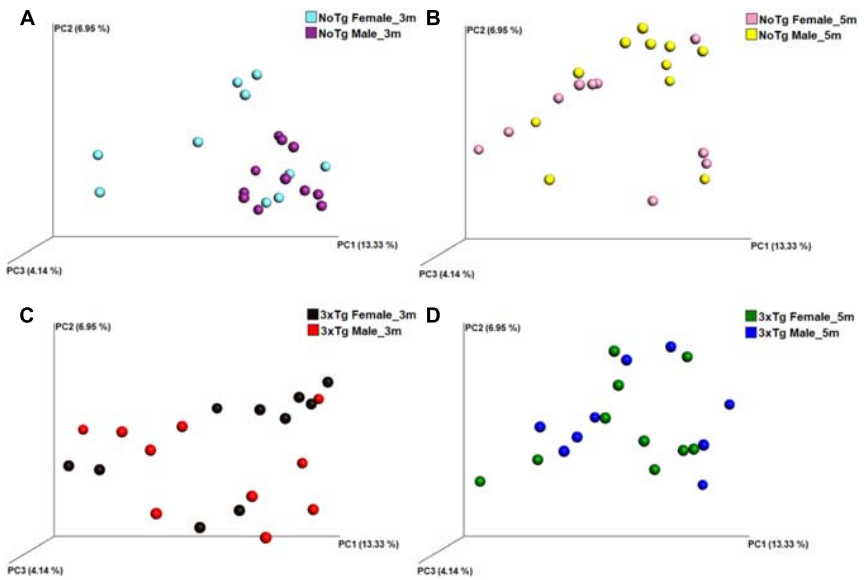

FIGURE 10 | Beta diversity of bacteria in fecal samples collected from non-transgenic (NoTg) and Alzheimer's disease triple-transgenic (3xTg-AD) mice. The graphics show beta diversity analyses calculated by dissimilarity metrics using operational taxonomic unit (OTU) tables and Unweighted UniFrac analyses. The analyses show the dissimilarity among mice by colors: NoTg female at 3 months (m) old (cyan), 3xTg-AD female at 3 months old (black), NoTg female at 5 months old (pink), 3xTg-AD female at 5 months old (green), NoTg male at 3 months old (purple), 3xTg-AD male at 3 months old (red), NoTg male at 5 months old (yellow), 3xTg-AD male at 3 months old (blue). The data is comparing by sex NoTg and 3xTg; NoTg female and male mice at 3 months **(A)**, NoTg female and male mice at 5 months **(B)**, 3xTg female and male mice at 3 months **(C)**, and 3xTg female and male mice at 5 months **(D)**. The three-dimensional scatter plots were generated using principal coordinates analyses (PCoA) in three different axes which shows the percentage of total differences. There is significant difference on **(A)** according to ANOSIM similarity test ($R = 0.225$, $p = 0.002$), but no for (**B**, $R = 0.082$, $p = 0.102$), (**C**, $R = 0.037$, $p = 0.231$), and (**D**, $R = 0.029$, $p = 0.275$).

mice. This result could be explained partially by impaired spatial memory information storage in 3xTg-AD mice. Previous works reported a relationship between the amount of exploration time of two familiar objects and the difference of score based on the difference in the familiar and novel object in the retention test of NOR and NOL (Albasser et al., 2009; Bello-Medina et al., 2013). Other factors suggested to impair memory consolidation of NOL task in 3xTg-AD mice are as follows: (a) low release of dopamine, norepinephrine, and glutamate (Guzmán-Ramos et al., 2012); (b) β-amyloid peptide accumulation (Clark et al., 2015); (c) tau phosphorylation related to changes in hippocampal theta oscillations and decrease in excitability in hippocampal neurons (Siddhartha et al., 2018); and (d) low dendritic spine density (Zou et al., 2015; Kommaddi et al., 2018). Importantly, in such previous studies that have not observed cognitive decline, they do report histopathological and synaptic plasticity alterations that could interfere with NOL consolidation memory. On the other hand, Billings et al. (2005) found earlier cognitive impairment already manifested at 4 months old, with deficits in long-term retention in Morris water maze, inhibitory avoidance task, as well as with concomitant accumulation of intraneuronal β-amyloid peptide in the hippocampus and amygdala. This work agrees with our results, where we found a NOL impairment in female and male 3xTg-AD at 5 months old that could be related to the β-amyloid accumulation observed at 5 months old and to gut microbiota dysbiosis already observed at 3 months and confirmed at 5 months old. In sum, our results strongly suggest that for 3xTg-AD mice, 5 months old cannot be any longer considered a pre-symptomatic stage of AD; we propose instead that this age should be conceptualized as an early symptomatic stage of AD.

Epidemiological and experimental findings suggest that AD has higher prevalence and incidence and symptoms are stronger in females than males in AD human patients as well as mouse models (Bachman et al., 1992; Ruitenberg et al., 2001; Carroll et al., 2010a; Creighton et al., 2019). In our results, we did not find any significant sex-related differences in motor activity, learning, NOL memory retention, or β-amyloid accumulation in the *subiculum* and CA1 in 3xTg-AD mice at the early symptomatic stage (5 months old). This lack of sex-related differences could be explained by previous reports that show that β-amyloid peptide levels did not differ between female and male until 9 months (Hirata-Fukae et al., 2008). Sex-related differences appear, however, at the symptomatic stage of AD due to loss of ovarian steroids related to menopause women aging (Carroll et al., 2007, 2010b; Carroll and Pike, 2008). Concomitantly, female 3xTg-AD mice at 5 months old are too young for showing estrogens and progesterone reduction. Furthermore, motor activities such as speed, traveled distance, and resting time alteration in female and male 3xTg-AD mice were similar. Previous studies have demonstrated that motor activity alteration occurs in the symptomatic stage of AD in female and male 3xTg-AD mice at 6 and 16 months old (Stover et al., 2015; Garvock-de Montbrun et al., 2019).

We characterized gut microbiota and evaluated its association with the trigger and development of AD. Our results documented that aging induces gut microbiota alterations; as male NoTg of

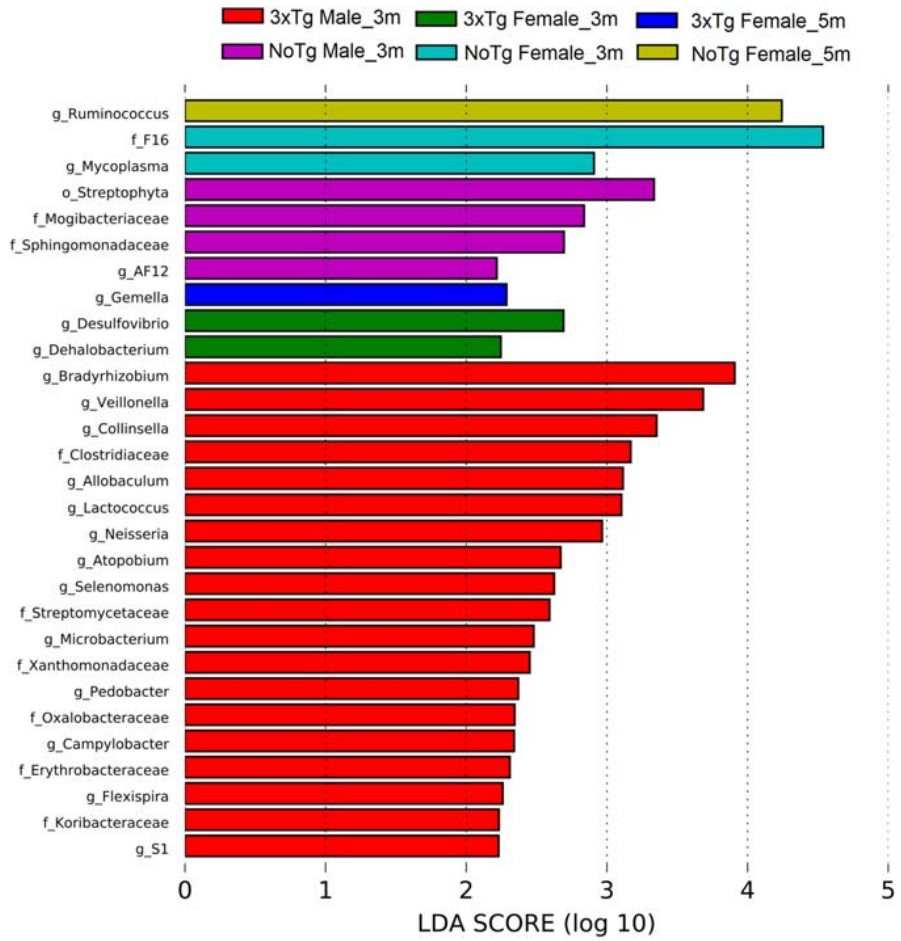

FIGURE 11 | Bacteria with a statistically significant change between non-transgenic (NoTg) and Alzheimer's disease triple-transgenic (3xTg-AD) mice at 3 and 5 months (m) old in male and female in fecal samples. The bacteria with statistical relevance are shown by colors: red color indicates 3xTg-AD male at 3 months old; green, 3xTg-AD female at 3 months old; blue, 3xTg-AD female at 5 months old; pink, NoTg male at 3 months old; cyan, NoTg female at 3 months old; light olive, NoTg female at 5 months old. Horizontal bars represent the effect size for each taxon. The length of the bars represents the log_{10} transformed linear discriminant analysis (LDA) score, indicated by vertical dotted lines. The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The name of bacteria with a statistically significant change in the relative abundance is written alongside the horizontal lines. Taxa names are abbreviated as "o," order; "f," family, and "g," genus. See **Supplementary Table 5** for full taxon description and LDA score and *p* values.

5 months old has an increase in Firmicutes and Actinobacteria and a decrease in Proteobacteria, Fusobacteria, Cyanobacteria, and Bacteroidetes phyla in comparison with male NoTg of 3 months old. A previous report indicates that Firmicutes, Proteobacteria, and Bacteroidetes are dominant phyla in $APP^{swe}/PS1^{\Delta}E9$ mice at 1 and 3 months old (Zhang et al., 2017). The decrease of Actinobacteria, Proteobacteria, Fusobacteria, and Cyanobacteria relative abundance has been reported as an indicator of aging in humans (Mariat et al., 2009) and male mice (Zhang et al., 2013) but not in female mice (Langille et al., 2014), as observed in our results. However, in female 3xTg-AD of 5 months old, a decrease in Actinobacteria phylum was observed in comparison with 3xTg-AD of 3 months old. This change could be related to the progression of AD due to that this decrease was not observed in NoTg mice of 3 and 5 months old. It is important to notice that the phylum Actinobacteria has been related to beneficial health effects like anti-inflammatory properties and decreased intestinal permeability (Vogt et al., 2017). Interestingly, the 3xTg-AD males have no differences between 3 and 5 months, which could be a sign of early aging even at an early age.

Furthermore, we observed a decrease in the relative abundance of TM7 in female and male 3xTg-AD mice at 3 and 5 months old in comparison to NoTg mice, while an increase was found in the relative abundance of Fusobacteria and Cyanobacteria in female and male 3xTg-AD mice at 5 months old in comparison to NoTg mice. This effect has been also reported in P301L mice, a tauopathy model at 3 and 6 months old (Sun et al., 2019), and in AD patients (Vogt et al., 2017). Also, a decrease in relative abundance in both TM7 and Cyanobacteria has been documented in female 3xTg-AD mice at 9 months old (Syeda et al., 2018). This evidence agrees with our results and suggests that 3xTg-AD mice at 3 and 5 months old may have similar alterations to that observed in the previously described symptomatic stage of AD.

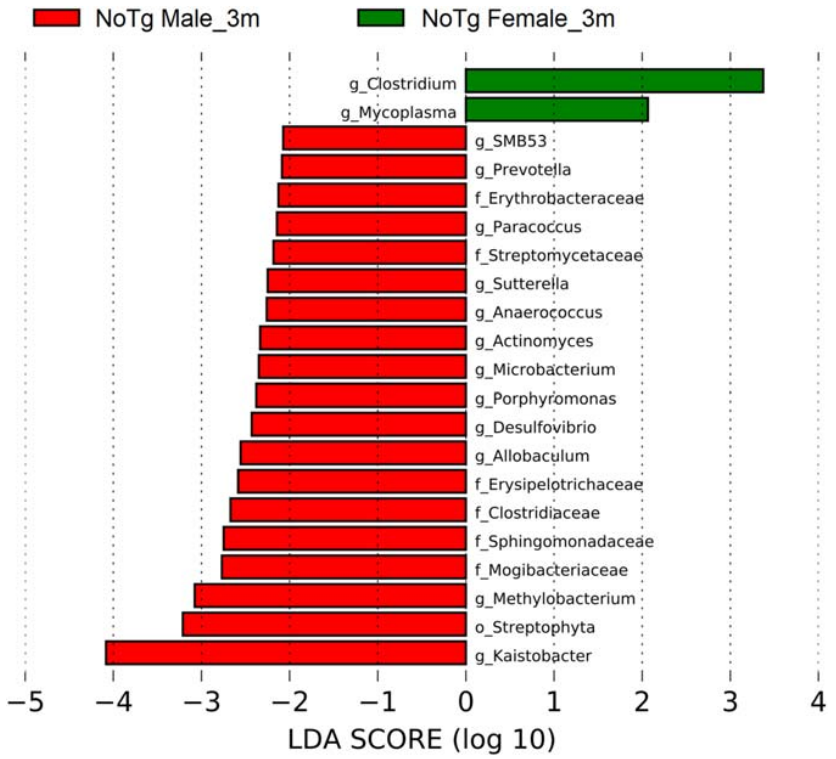

FIGURE 12 | Bacteria with statistically significant change on non-transgenic (NoTg) mice at 3 and 5 months (m) old for male and female in fecal samples. The bacteria with statistical relevance are shown by colors: red color indicates NoTg male at 3 months old; green, NoTg female at 3 months old. Horizontal bars represent the effect size for each taxon. The length of the bars represents the log_{10} transformed linear discriminant analysis (LDA) score, indicated by vertical dotted lines. The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The name of bacteria with a statistically significant change in the relative abundance is written alongside the horizontal lines. Taxa names are abbreviated as "c," class; "o," order; "f," family, and "g," genus. See **Supplementary Table 6** for full taxon description and LDA score and *p* values.

Diversity in gut microbiota is an indicator of richness and abundance of species of microorganisms present in a sample. We analyzed Shannon and Simpson indexes and found significant differences between male and female NoTg mice at 3 and 5 months old. These results suggest that bacterial diversity in the mice hybrid strain B6129SF1/J (NoTg) changes concerning sex. This agrees with other studies that show sexual differences in microbiota in other mouse strains (Org et al., 2016; Kozik et al., 2017; Elderman et al., 2018). However, until now, there are no reports that compare alpha diversity related to sex in the mouse hybrid strain B6129SF1/J used as the genetic background for the 3xTg-AD mice. It is important to note that, in our study, there are no differences between genotypes at any of the two ages analyzed. This could be because in such pre-symptomatic age of AD is too early to observe these subtle alterations. Our results agree with those reported by Bonfili et al. (2017), where they show no differences in alpha diversity in 3xTg-AD mice at 2, 3, 4.5, and 6 months old. This same effect was observed in P301L tau transgenic mice at 1, 3, and 6 months old (Sun et al., 2019). However, it has been demonstrated that 9 months old 3xTg-AD mice showed lower alpha diversity than NoTg mice (Syeda et al., 2018). This suggests that alpha diversity may not be a meaningful indicator of the development of AD at early or pre-symptomatic stages.

Now, regarding the beta diversity of gut microbiota, we found differences in female and male 3xTg-AD mice in comparison to NoTg mice at 3 and 5 months old. Male NoTg mice at 3 months old exhibited a statistically significant difference regarding 5 months old. The same effects were observed in 3xTg-AD mice at 2, 3, 4.5, and 6 months old (Bonfili et al., 2017) and at 9 months old (Syeda et al., 2018), including APPPS1 mice at 8 months old (Harach et al., 2017). These results suggest that beta diversity changes might be associated with the triggering and progression of AD. On the other hand, we found that beta diversity in female NoTg is different from male NoTg at 3 months old. This result was observed in a different strain of mice such as C57BL/6J, C3H/HeJ, and DBA/2J; this effect is dependent on sex hormone development during puberty and early adulthood; if female mice are gonadectomized, the gut microbiota change. This alteration was prevented by hormonal substitution (Org et al., 2016).

The present study contributes to the characterization of gut microbiota in NoTg and 3xTg-AD mice related to age and sex in the early symptomatic stage of AD. It is important to mention that a decrease in Actinobacteria phylum observed in 3xTg-AD mice is related to the triggering and progression of AD. *Bifidobacterium* is a principal bacterium of Actinobacteria phylum that participates in intestinal homeostasis, and the

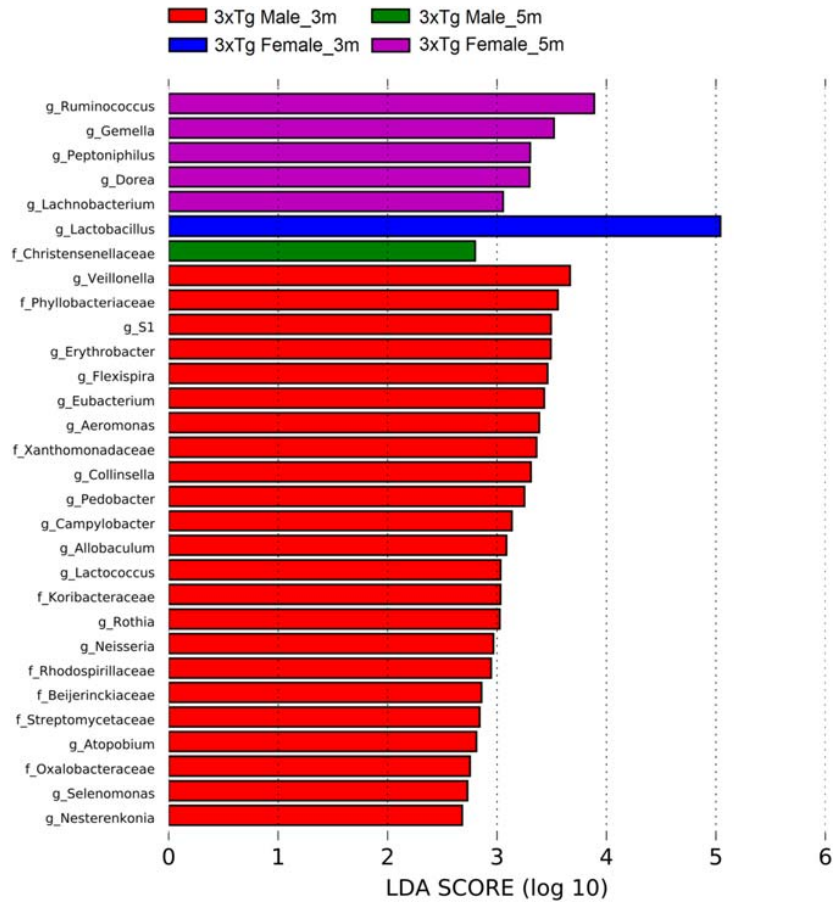

FIGURE 13 | Bacteria with statistically significant change on Alzheimer's disease triple-transgenic (3xTg-AD) mice at 3 and 5 months (m) old in male and female in fecal samples. The bacteria with statistical relevance are shown by colors: red indicates 3xTg-AD male at 3 months old, green indicates 3xTg-AD male at 5 months old, blue indicates 3xTg-AD female at 3 months old, and pink indicates 3xTg-AD female at 5 months old. Horizontal bars represent the effect size for each taxon. The length of the bars represents the log10 transformed linear discriminant analysis (LDA) score, indicated by vertical dotted lines. The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The name of bacteria with a statistically significant change in the relative abundance is written alongside the horizontal lines. Taxa names are abbreviated as "f," family, and "g," genus. See **Supplementary Table 7** for full taxon description and LDA score and *p* values.

gut–brain axis modulates the GABAergic system in the intestine (Binda et al., 2018). A decrease in Actinobacteria contributes to tau pathogenesis and cognitive decline (Sun et al., 2019). This loss in the relative abundance of Actinobacteria could be related to the memory retention deficit of NOL observed in this study. The TM7 phylum has been identified in a variety of natural habitats such as the skin, genital tract, and gastrointestinal tract. TM7 induced upregulation of many genes for biosynthesis of essential amino acids such as histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (He et al., 2015). Tryptophan, tyrosine, and phenylalanine are biosynthetic precursors for the neurotransmitters such as serotonin, dopamine, and norepinephrine (Fernstrom, 1994). A decrease in TM7 phylum could alter neuronal communication in 3xTg-AD mice, which affects information processing in memory retention of NOL.

Concerning the results of the LEfSe analysis in female and male NoTg at 3 and 5 months old in fecal samples, we found bacteria with a statistically significant change in LDA scores such as F16 (phylum TM7), genus *Mycoplasma* (phylum Tenericutes), Ruminococcus (phylum Firmicutes), AF12 (phylum Bacteroidetes), family Mogibacteriaceae (phylum Firmicutes), family *Sphingomonadaceae* (phylum Proteobacteria), genera *Clostridium* (phylum Firmicutes), *Mycoplasma* (phylum Tenericutes), family Streptomycetaceae, genera *Actinomyces* and *Microbacterium* (phylum Actinobacteria), *Porphyromonas* and *Prevotella* (phylum Bacteroidetes), families Clostridiaceae, Erysipelotrichaceae, Mogibacteriaceae, genera *Allobaculum*, *Anaerococcus*, *SMB53* (phylum Firmicutes), families Erythrobacteraceae, Sphingomonadaceae, genera *Desulfovibrio*, *Kaistobacter*, *Methylobacterium*, *Paracoccus*, and *Sutterella* (phylum Proteobacteria). These bacteria were found in the healthy gut in NoTg mice, maintaining homeostasis of microbiota with normal changes associated with aging and sex hormones dynamics (Zhang et al., 2013; Org et al., 2016). Furthermore, the results of the LEfSe analysis in female and male 3xTg-AD mice at 3 and 5 months

old in fecal samples, show bacteria with a statistically significant change in LDA scores such as *Xanthomonadaceae*, *Oxalobacteraceae*, *Streptomycetaceae*, *Koribacteraceae*, and *Streptomycetaceae* families, *Gemella*, *Dehalobacterium*, *Clostridium*, *Allobaculum*, *Selenomonas*, *Veillonella*, *Lactococcus*, *Desulfovibrio*, *Bradyrhizobium*, *Campylobacter*, *Erythrobacter*, *Neisseria*, *Flexispira*, *Microbacterium*, *Collinsella*, *Atopobium*, *Pedobacter*, and the S1 genera. These microorganisms have been associated with both pre-clinical models and patients who present AD mainly; however, other bacteria are related to aging, cognitive decline, cerebral damage, and inflammatory response in mice and humans (Thomas et al., 2012; Wang et al., 2016; Bonfili et al., 2017; Harach et al., 2017; Morris et al., 2017; Vogt et al., 2017; Zhang et al., 2017; Aguayo et al., 2018; Alonso et al., 2018; Antonets et al., 2018; Bäuerl et al., 2018; Dong et al., 2018; Zhuang et al., 2018; Haran et al., 2019; Li et al., 2019; Zhan et al., 2019; Beydoun et al., 2020; Na et al., 2020; Westfall et al., 2020). These results suggest that bacteria families and genera are representative microorganisms of gut microbiota of disease that could be considered a useful tool for diagnostic as well as a progression biomarker of AD.

Finally, it is important to mention some limitations of this study. Although some bacteria modifications have been reported in human AD patients, this, as other pre-clinical results, must be interpreted with caution because the results of this mouse transgenic model may not directly translate to the humans. Additionally, it is necessary to study the consequences and causalities when manipulating microbiota in the 3xTg-AD model. In this regard, it would be interesting to modulate gut microbiota during the first 5 months of age in 3xTg-AD and then evaluate the cognitive impairment associated with this neurodegenerative pathology in such early symptomatic stage of AD.

CONCLUSION

Our results suggest that 5 months old 3xTg-AD mice cannot be considered a the pre-symptomatic stage of AD. We propose that this age should be considered the early symptomatic stage of AD. Additionally, we found gut microbiota alterations at 3 months old in 3xTg-AD mice that could be considered and used as early biomarkers for the diagnostic and progression of AD. These biomarkers are Actinobacteria and TM7 phylum alterations as well as beta diversity significant changes and an increase in specific bacteria families and genera, i.e., *Gemella*, *Allobacullum*, and *Selenomonas*.

DATA AVAILABILITY STATEMENT

The corresponding FASTQ sequence files for all samples used in this study were deposited in the NCBI BioProject repository (Accession Number: PRJNA648144) link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA648144.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité de Bioética del Instituto de Neurobiología, Universidad Nacional Autónoma de México.

AUTHOR CONTRIBUTIONS

PB-M, JG-M, SD-C, and GP-L designed the research. PB-M and FH-Q performed the research. PB-M, FH-Q, and JG-M analyzed the data. PB-M, FH-Q, JG-M, SD-C, and GP-L wrote the manuscript. PB-M, FH-Q, MP-M, DG-F, GC-P, and GP-L revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.595583/full#supplementary-material

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