# IMPACT OF FETAL & EARLY POSTNATAL NUTRITION ON THE DEVELOPING BRAIN: IMPLICATION FOR ADULT DISEASE

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PUBLISHED IN: Frontiers in Neuroscience and Frontiers in Nutrition

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-724-8
DOI 10.3389/978-2-88971-724-8

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# IMPACT OF FETAL & EARLY POSTNATAL NUTRITION ON THE DEVELOPING BRAIN: IMPLICATION FOR ADULT DISEASE

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**Citation:** Page, K. C., Ozanne, S., Anday, E. K., eds. (2021). Impact of Fetal & Early Postnatal Nutrition on the Developing Brain: Implication for Adult Disease. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-724-8

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 21 August 2019
doi: 10.3389/fnins.2019.00826

# Neurodevelopment and Cognitive Impairment in Parents and Progeny of Perinatal Dietary Protein Deficiency Models

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There is an absolute dependence of the concept of development on supply of adequately balanced nutrients especially during the perinatal age which is critical to development. Therefore, an upgraded nutrition is specially required during gestation and lactation, as this is the critical period of neurodevelopment. This study sought to investigate the effect of protein deficiency during gestation ($F_0$) and lactation through to adolescence on neurological functions of subsequent ($F_1$ and $F_2$) generations, establishing the possible consequential mechanistic association. Rats in four groups were fed different rations of protein diets (PD) as formulated: 21% PD, 10% PD, 5% PD and control diet (standard rat chow, containing 16–18% protein), from adolescent through to gestation and lactation, next generations were weaned to the maternal diet group. Neurobehavioral studies (which include; Surface righting reflex, Negative geotaxis, Learning and Memory tests), brain oxidative stress and quantification of serotonin and dopamine levels in the brain were conducted. Result shows significantly altered neurobehavior, reflected in the reduction of reflex response and postural reaction score at $P \leq 0.05$. There was also a transgenerational cognitive impairment of brain function in the F-generations, following perinatal protein malnutrition as shown in the Y-maze result, measuring spatial memory and Morris water maze result (cognition), providing a background for the observed sensorimotor response. The significant increase in dopamine level, decrease in the antioxidant capacity of the protein deficient brain groups are consistent with significantly altered serotonin system, critical to neurodevelopment and functional activities of learning and memory. Therefore, persistent early life protein deficiency mediates dysfunction in neurodevelopment and this involves life-long changes in key neurotransmitters and the brain redox status underlying the neurobehavioral display.

**Keywords: neurobehavior, cognitive function, protein deficiency, neurotransmitter, transgeneration, perinatal**

OPEN ACCESS

***Edited by:***
*Kathleen C. Page,*
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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *14 May 2019*
***Accepted:*** *24 July 2019*
***Published:*** *21 August 2019*

***Citation:***
*Abey NO, Ebuehi OAT and Imaga NOA (2019) Neurodevelopment and Cognitive Impairment in Parents and Progeny of Perinatal Dietary Protein Deficiency Models. Front. Neurosci. 13:826. doi: 10.3389/fnins.2019.00826*

## INTRODUCTION

Epidemiological studies have indicated that perinatal age exposures; including diet and nutrition affects directly later life developments, due to a shift or programed conditions. One of the well reported consequences are highly induced integrated responses in the endocrine homeostasis. which results in later life to subsequent and persistent changes in developmental trajectory yielding

an altered phenotype which may appear as a physiological defect (Chan et al., 2011). Evidence of the dependence of brain development on nutrition is increasingly gaining interest. Nutritional status has been demonstrated to be associated with postnatal brain growth and maturation, subsequently impacting the neurodevelopment which may persist to adolescence (Black, 2018). Nutrition could also potentially protect against injury (Hurley et al., 2016). During the critical period of brain development (0–2 years), i.e., the first 1,000 days, brain development is rapid, with nutrition playing an important role as one of the environmental influences on the coding and expression of the genetic code for structural and functional impact. Subsequent cognitive and emotional process coupled with the entire brain development in animal and human subjects have recently illustrated that the duration of exposure, chronicity, and severity of nutritional deficiencies have differential effects on brain development (Colombo et al., 2018). Fetal and neonatal programing have been reported to be the underlying mechanism for the far-reaching adverse consequences in humans following inadequate protein intake during gestation and postnatal periods (Wu, 2016). This risk factor results in not only impaired growth of fetuses and infants, but also some other problems such as metabolic syndrome (including hypertension, obesity, and diabetes) and low quality of life as adults (Sosa-Larios et al., 2017). There have been quite a few focuses on unveiling nutraceutical significance in neurodevelopment, which has contributed immensely to disability in individuals who bear the burden. In developing/low-resource settings it may be associated with a significant risk of impoverishing the health reserves which threatens survival and deepens poverty. This study sought to investigate the effect of maternal protein deficiency during gestation ($F_0$) and lactation on neurological functions of subsequent ($F_1$ and $F_2$) generations, establishing the possible consequential mechanistic association.

# MATERIALS AND METHODS

## Ethics Statement

This study was carried out in accordance with the guidelines of health research Act 2004, for standard care and use of laboratory animal models. Ethical approval was given by the Health Research Ethics Committee (HREC) of College of Medicine University of Lagos (REC 11), Nigeria.

Forty virgin female Sprague Dawley rats (aged 6–8 weeks) were obtained from the animal house of the College of Medicine University of Lagos, Lagos Nigeria (**Figure 1**). Following the ethics of the standard care of animal models in research, rats were kept in groups in clean and capacious plastic cages (seven per cage) under standard laboratory conditions including well aerated room, good lighting, with suitable temperature (30° ± 2°C) in a neat environment and at a 12-h light/dark cycle. The animals were divided into four (4) groups and acclimatized for 2 weeks, where they had access to standard rat chow and water *ad libitum*.

## Grouping

The grouping was based on the diet received by the rat. Grouped rats received *ad libitum* formulated diets which include either of these diet formulations: 21% PD (protein diet), 10% PDD (Protein deficient diet 1), 5% PDD (Protein deficient diet 2), and CP (standard rat chow, containing 16–18% protein) throughout gestation and lactation, forming Groups 1 2, 3 and 4, respectively, and pups were weaned to maternal diet.

## Diet Formulation

The varying protein quality diet for rat was formulated using local materials such as: Maize, SBM, GNC, Fish meal cassava starch, oil etc., all in varying percentages toward the attainment of the desired percentage of protein in the diet. This feed was formulated by the help of some experts at federal institute of industrial research Oshodi (FIIRO). Formulated feeds were given to the rats.

## Prenatal Treatment

Vaginal cytology was evaluated 3 weeks pre-breed period, and the rats were time-mated with a certified reproductive male in each treatment group, the confirmed pregnant rats were separated to produce $F_1$ generation, this process was repeated for $F_1$ generation to produce $F_2$ generation, while the different diet group feeding continues. Presence of spermatozoa in vagina smear confirms day 1 of pregnancy. Weaning was done at postnatal day 21–28.

## Reflex and Sensorimotor Test

### Surface Righting Reflex (SRR)

The test was evaluated on postnatal day 2 through to 6. This test was carried out to measure the motor function and coordination by placing pups in a supine position and the time taken to adopt normal position was recorded, scored within cut-off time of 0–3 s (Naik et al., 2015).

### Negative Geotaxis Reflex

The vestibular and proprioceptive functions were evaluated in rats (postnatal day 2–14, every other day) were placed head down on an inclined board (about 45°), the time it took the animal to show a postural reaction by turning upright normally was recorded and scaled to score (Naik et al., 2015).

## Neurobehavioral Tests

At six (6 weeks), offspring were randomly selected ($n$ = 5) for different neurobehavioral trainings and trials.

### Y-Maze

The test was conducted as a modified method described by Yarube et al. (2016), The number and the sequence of arms entered were also recorded. The parameters were activity, defined as the number of arms entered, and percent alternation, calculated as the number of alternations or triads.

### Moriz Water Maze

A wide plastic cylindrical tank of about 120 cm width, that is surrounded by a wall (45 cm) and filled with water that was made opaque to reduce light penetration (25°C) was set up for

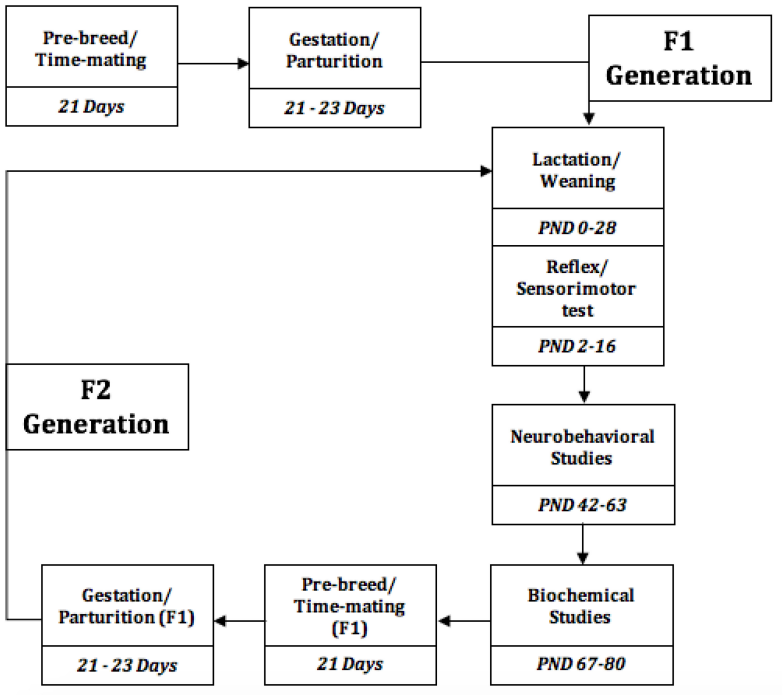


**FIGURE 1 |** Experimental Timeline of the events, from the study initiation to termination. PND, Postnatal days.

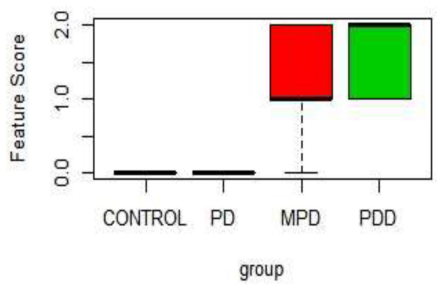


**FIGURE 2 |** Boxplot of Physical characteristic scores in the different diet group. PDD: 5% Protein Diet, Mild PD: 10% Protein Diet, PD: 21% Protein Diet, and CONTROL. Score Keys – Scale: 0–2: 0, No characteristic display; 1, Mild characteristics display; 2, Prominent Characteristics display. Features Scored: Paleness, Muscle wasting, Stunted growth, Alopecia, Edema, and lesion.

the experiment. A rescue platform, 10 cm high was hidden at the center of the tank, below water level. Rats were given three swimming sessions on each of five consecutive days. At trial, the time taken to locate the platform (escape latency) was measured (Wang et al., 2017).

## Serotonin and Dopamine Quantification

The brain serotonin and dopamine concentration in the rat were determined using high performance liquid chromatography (HPLC), Agilent 100 series with VWD detector degasser, Quat Pump, Col Com and a manual injector system. Striatum tissues in the brain were collected, homogenized, deproteinized by chilled acetonitrile and filtered using syringe filter in preparation for injection. Serotonin and dopamine contents were determined by comparing the peak height ratios of the standards (5-HT and dopamine) used as calibrators and the chromatogram of unknown (Ebuehi and Abey, 2016).

## Brain Antioxidant Assays

The brain antioxidant capacity was measured using the following biomarkers.

### Determination of Reduced Glutathione (GSH)

The reduced glutathione level was determined based on the yellow color formed after reacting with 5,5'dithiobis- 2-nitrobenzoic acid (DTNB), which is then read at 412 nm (Ellman, 1978).

### Determination of Catalase Activity (CAT)

The activity of the enzyme catalase was analyzed by measuring the initial rate of hydrogen peroxide (50 mM) decomposition, where one unit is the amount of enzyme that hydrolyses 1 mol. of $H_2O_2$ per minute, following method described by Cohen et al. (1996).

### Determination of Super Oxide Dismutase Activity

The super oxide dismutase (SOD) activity was determined according to the modified method of Sun and Zigman (1978), determined by the ability of SOD to inhibit the autoxidation of epinephrine, taken as the difference between superoxide anion production and decomposition, measured by the increase in absorbance at 320 nm.

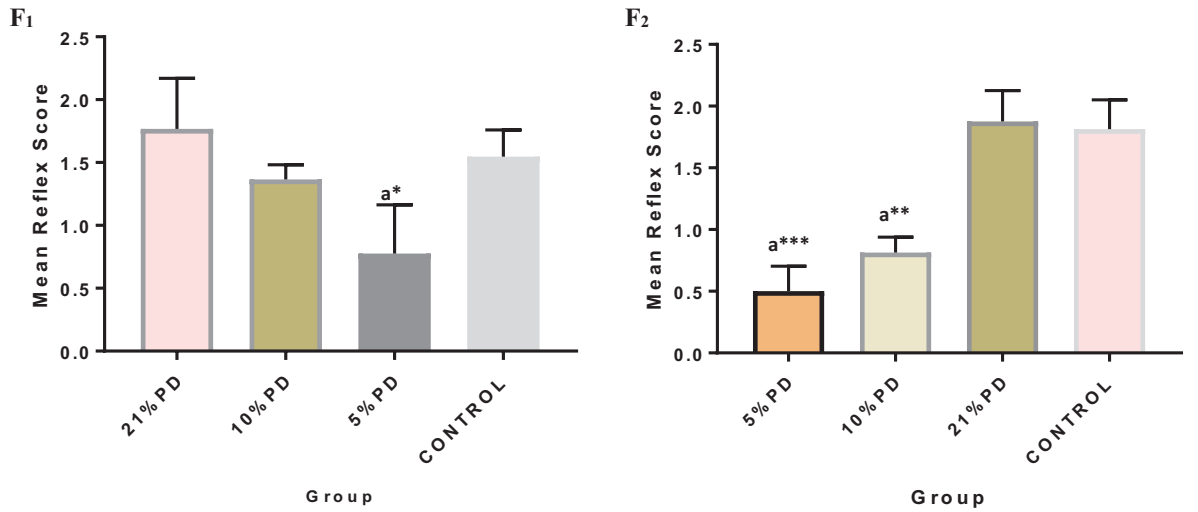


**FIGURE 3 |** Mean reflex response score in each of the diet groups of rats of $F_1$ and $F_2$-generations. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21% PD (Upgraded daily recommended intake), 5% (Protein deficient diet), and 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

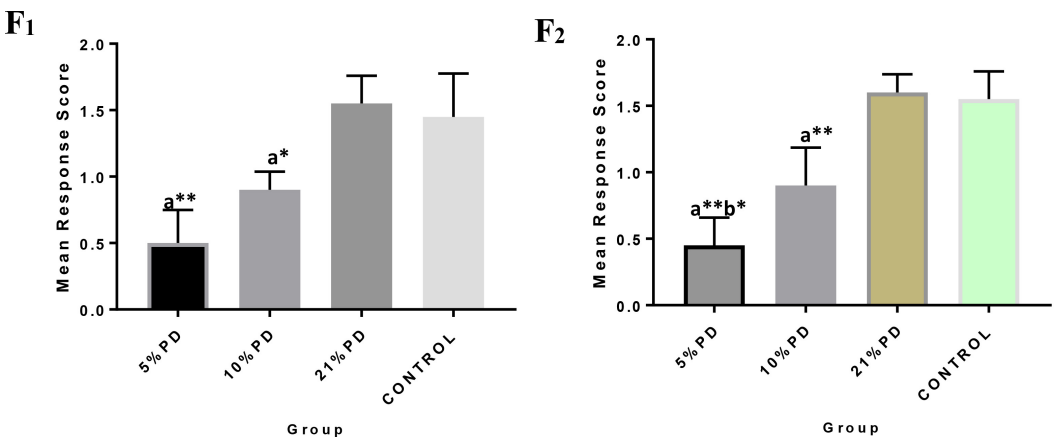


**FIGURE 4 |** $F_1$ and $F_2$ Mean response score of the postural reaction in each of the diet group of rats. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), and 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. [b]significantly different from the 10% diet group. $^*P < 0.05$, $^{**}P < 0.001$.

### Determination of Lipid Peroxidation Malondialdehyde (MDA)

Malondialdehyde is red specie absorbing at 535 nm (Buege and Aust, 1978). It is formed from the breakdown of polyunsaturated fatty acids, serving as a convenient index for determining the extent of lipid peroxidation that reacts with thiobarbituric acid. Absorbance taken at 535 nm, corresponds to the concentration of MDA per mg protein.

## Determination of Concentration of Nitric Oxide

Nitric Oxide was assayed by the Griess reaction method (Bryan and Grisham, 2007). 0.2 ml of the Griess reagent mixed with 3 ml of the sample and incubated at room temperature for 10 min. Absorbance was read using the spectrophotometer at 540 nm. This procedure is not stoichiometric and thus, a standard curve of sodium nitrite was plotted in order to extrapolate the values for unknown samples.

### Statistical Analysis

All data were analyzed with maternal and weaning diets as factors. Results are presented as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 7.0 for analysis of variance (ANOVA) followed by *post hoc* Turkey's test, when appropriate; $P \leq 0.05$ is considered significant.

## RESULTS

**Table 1** shows the score on a scale of 0–2 based on prominence/severity of characteristic displayed. Control and

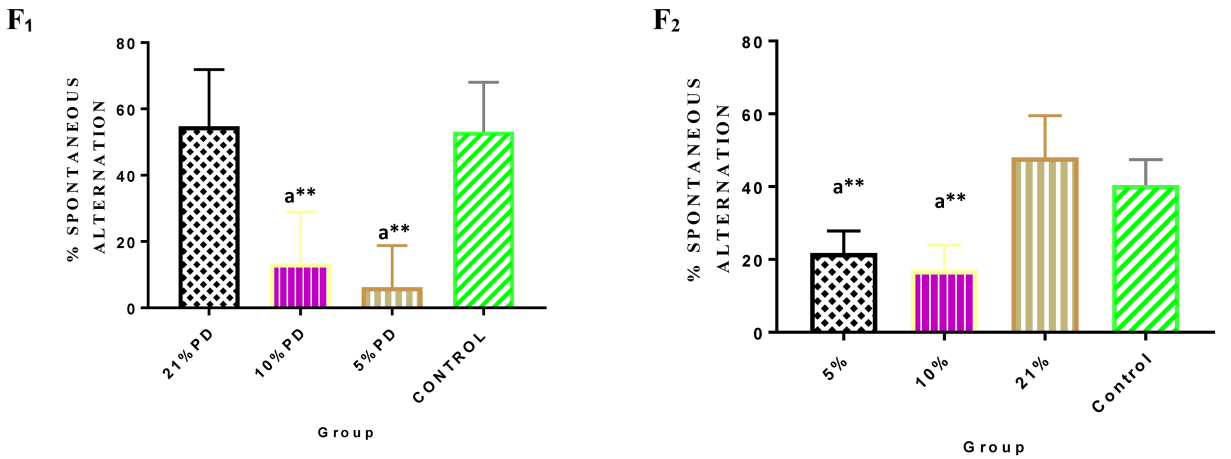


**FIGURE 5 |** Y-maze Percentage Spontaneous Alternation in each of the diet group of $F_1$ and $F_2$-generation rats. Data are expressed as mean ± SEM. Statistically significant difference between individual group and the hypothetical value of 50% at $P < 0.05$. Spontaneous alteration % $= \frac{\#\ spontanuos\ alternation}{total\ number\ of\ arm\ entries - 2} \times 100$ Control (standard rat chow containing 16–18% Protein), 21% PD (Upgraded daily recommended intake), 5% (Protein deficient diet), and 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. $^{**}P < 0.001$.

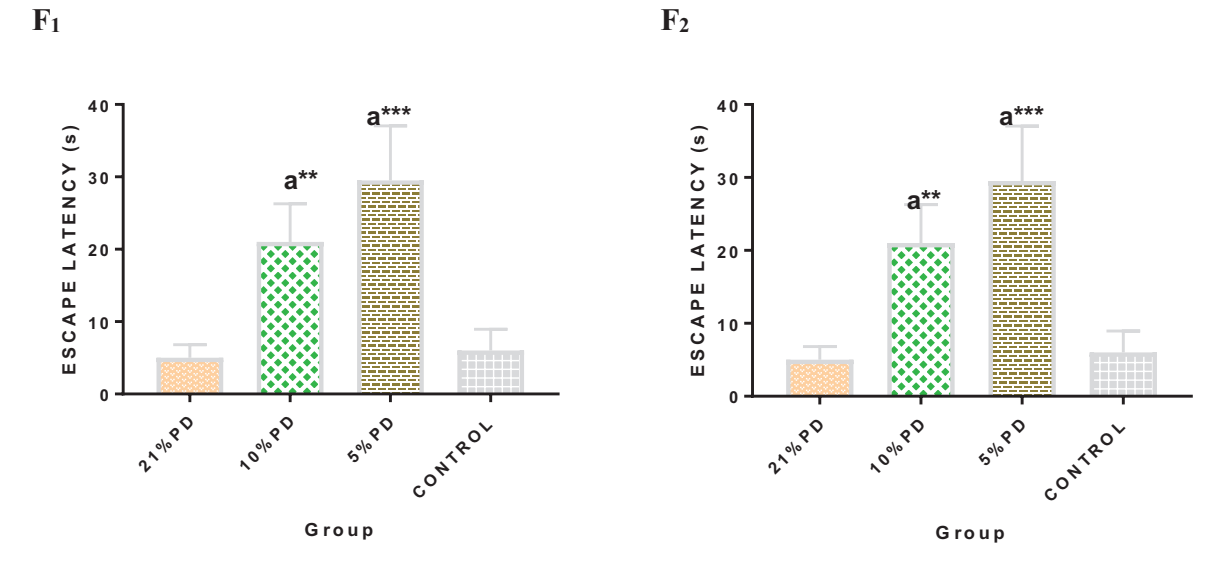


**FIGURE 6 |** Mean Moriz Water Maze Experiment showing the Escape Latency demonstrated by each group in $F_2$-generations. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), and 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. $^{**}P < 0.001$, $^{***}P < 0.0001$.

21%PD had no display of the scored features, this relationship is represented on the box-plot (**Figure 2**).

In **Figure 2**, the 5% PD and 10% PD had characteristic score skewed toward the positive, reflecting a higher display, while control and 21% PD skewed toward the negative, with no visible display. These distinctive features of perinatal protein deficiency compared to control and 21% protein diet group include; prominent paleness, muscle wasting, hair loss and stunted growth.

Significantly higher response time in the protein deficient models (**Figure 3**) as well as a negative postural score (**Figure 4**) in the negative geotaxis test at the early postnatal days of the protein deficient diet groups. In **Figure 5**, Y-maze% spontaneous alternation result shows significant deficit in the 5% protein diet group in the two (2) generations ($F_1$ and $F_2$) while 10% was only significant in $F_1$-generation. In addition, escape latency observed in **Figure 6** shows that the malnourished groups spent longer time to locate the rescue platform during trial sessions, a display of cognitive deficit. With significant dietary factor influence, at $P < 0.001$.

Serotoninergic and dopaminergic neurotransmission, important in neurobehavioral control and cognition was

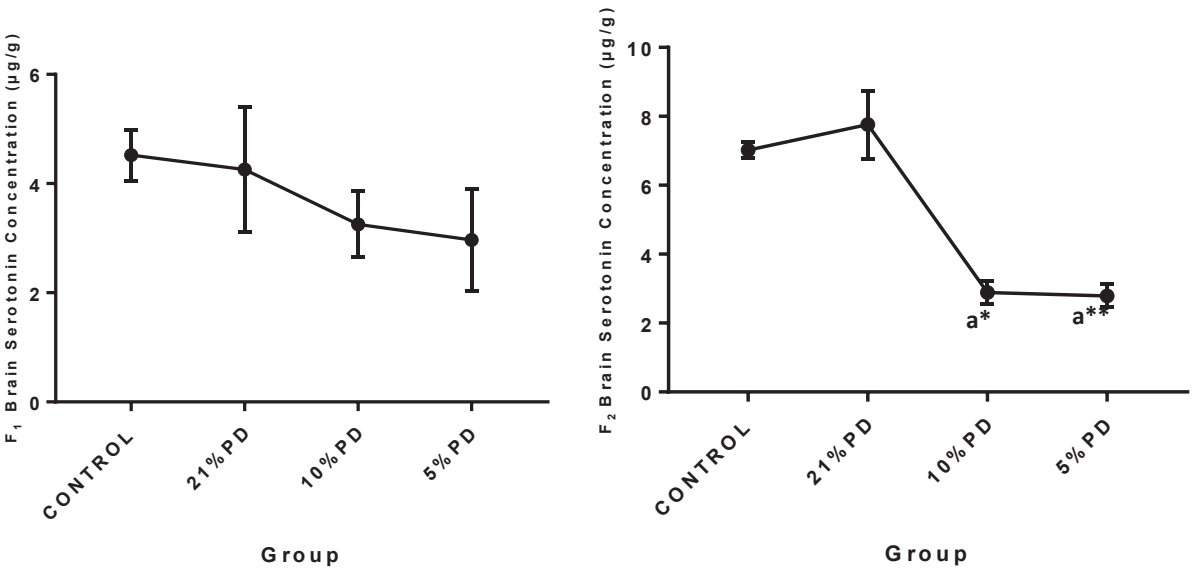


**FIGURE 7 |** Concentration of Brain Serotonin (µg/g) in the $F_1$ and $F_2$-generations of rat in each of the different diet groups. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. $^*P < 0.05$, $^{**}P < 0.001$.

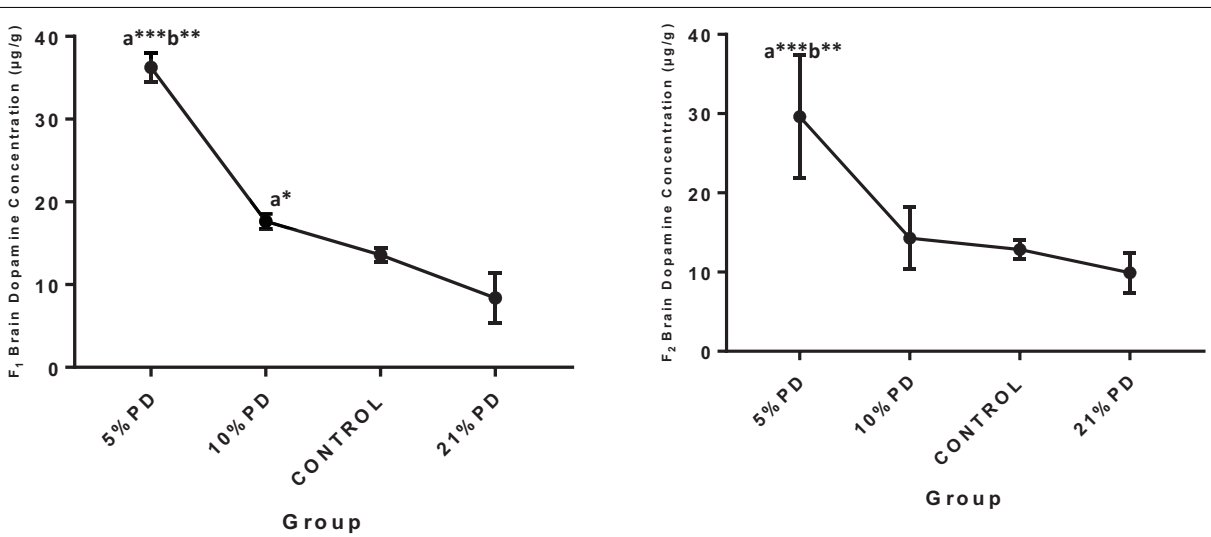


**FIGURE 8 |** Concentrations of Brain Dopamine (µg/g) in the $F_1$ and $F_2$-generations in each of the different diet group of rats. Data are expressed as mean ± SEM, Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. [b]significantly different from the 10% diet group. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

perturbed in the nutritionally stressed models (**Figures 7**, **8**) which persists to subsequent generation. Endogenous antioxidant status of the malnourished groups were suppressed. **Tables 2** and **3**, with significant increase in the Malondialdehyde level.

## DISCUSSION

Neurodevelopment is peculiar to early life and this stage is critically dependent on maternal protein intake. Dietary protein deficiency and its corresponding effect on neurodevelopment critical to survival and quality of life that subsequently follows, perturbs the neurotransmission system and cognition programing. The brain in an attempt to compensate for the nutritional inadequacies, may change its functional activation, as an important signature for the reorganization, providing explanation for the various chemical alterations and behavioral display reported in the result.

As earlier stated by Guo and Katta (2017), protein is crucial to the health of hair follicles, and hence the alopecia observed in

**TABLE 1 |** Physical features of rats fed different protein diets in the $F_1$-generation.

| Characteristics | Control | 21% PD | 10% PD | 5% PD |
|---|---|---|---|---|
| Paleness | 0 | 0 | 0 | 2 |
| Muscle wasting | 0 | 0 | 1 | 2 |
| Stunted growth | 0 | 0 | 1 | 2 |
| Alopecia (hair thinning and loss) | 0 | 0 | 1 | 2 |
| Edema | 0 | 0 | 2 | 1 |
| Lesion | 0 | 0 | 2 | 1 |

*Features Scored: Paleness, Muscle wasting, Stunted growth, Alopecia, Edema, and lesion. Score Keys – Scale: 0–2: 0, No characteristic display; 1, Mild characteristics display; 2, Prominent Characteristics display.*

the protein deficient groups, also in accordance with the previous research (Belluscio et al., 2014), stunted growth is attributed to the suboptimal intrauterine condition of the nutritionally challenged group.

Protein deficiency caused perturbations in brain development observed as delayed neurological reflex and postural response. Therefore, the neuronal response of these deficient models has been negatively impacted leading to the weakening of the nerves and deterring communications and potentiation essential for learning (Naik et al., 2015). The working memory and cognition of the protein deficient group lagged significantly; taking more time to perform learned task, this suggests a fundamental defect in the neurodevelopmental process and degeneration in brain function, which persists in both parent and progeny.

Malnutrition in early life as earlier reported (Chertoff, 2015), affects the neurochemistry and morphology of the hippocampal structure, which is known to be involved in learning and memory. One of the key factors on which brain function depends is the availability of neurotransmitter molecule for release into synapse, this alteration in the neurotransmitter scale may therefore underlie the functional deficit displayed by the protein malnourished groups. In addition, dopamine alteration perturbs neural encoding, this suggests a clinical association with other dysfunctions such as anxiety and psychosis (Ebuehi et al., 2001, 2009; Kelly et al., 2017). In malnutrition condition, free radicals mediate tissue damage, due to the inadequate protective and repair mechanisms in the protein-deficient models (Feoli et al., 2006). Therefore, maternal undernutrition may contribute to the transgenerational imbalance in the brain redox status of the following generation. The developmental behavior, and cognitive deficits observed on perinatal malnourished models might derive their effects from mechanisms which include the perturbed redox status and neurotransmission as an insult to the Brain.

## CONCLUSION

Perinatal protein deficiency altered the brain development in parent and offspring. Increased oxidative damage and altered neurotransmission in the brain of $F_1$ generation of rats, underlie the perturbed neurobehavioral display and cognitive deficit in the nutritionally challenged groups. Therefore, persistent perinatal exposure to protein malnutrition increases the risk of cognitive defect and other brain disorders in subsequent generations. This study affirms the importance of dietary protein at the early developmental life process laying emphasis on the mechanism associated with the resultant transgenerational neurodevelopment and cognitive impairment following persistent perinatal protein deficiency. More work

**TABLE 2 |** Oxidative stress biomarkers in different diet groups of $F_1$-generation of rat models.

| Group | Total protein (mg/ml) | Catalase (U/L/min) | GSH (μmol/L) | SOD (U/L/min) | MDA (μmol/L) | Nitric oxide (mg/ml) |
|---|---|---|---|---|---|---|
| Control | 31.43 ± 6.10 | 20.72 ± 4.00 | 45 ± 3.80 | 0.0249 ± 0.01 | 0.29 ± 0.09 | 6.989 ± 1.20 |
| 21% PD | 41.35 ± 8.00 | 15.92 ± 3.99 | 53.14 ± 4.00 | 0.0438 ± 0.01 | 1.02 ± 0.9 | 7.147 ± 0.40 |
| 10% PD | 34.16 ± 1.41 | 21.29 ± 1.96 | 45.89 ± 0.98 | 0.1989 ± 0.03 | 2.5 ± 0.7$^{a**}$ | 1.585 ± 0.70$^{a**b**}$ |
| 5% PD | 20.23 ± 2.98$^{a*}$ | 34.89 ± 5.00$^{a**b*c*}$ | 37.74 ± 4.70$^{a*}$ | 0.00334$^{a**}$ ± 0.0 | 24.55 ± 4.6$^{a**b**}$ | 1.6 ± 1.10$^{a**b**}$ |

*Data are expressed as mean ± SEM of $F_1$ antioxidant status significantly difference from 21% Protein and control Diet group. Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), and 10% (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. [b]significantly different from the 10% diet group. *P < 0.05, **P < 0.001.*

**TABLE 3 |** Oxidative stress biomarkers in different diet groups of $F_2$-generation of rat models.

| Group | Total protein (mg/ml) | Catalase (U/L/min) | GSH (μmol/L) | SOD (U/L/min) | MDA (μmol/L) | Nitric oxide (mg/ml) |
|---|---|---|---|---|---|---|
| Control | 1.9166 ± 0.19 | 5.9259 ± 0.40 | 13.051 ± 1.5 | 0.0116 ± 0.002 | 0.0673 ± 0.01 | 9.40 ± 0.94 |
| 21% PD | 1.965 ± 0.27 | 8.3951 ± 0.56 | 16.219 ± 1.3 | 0.0261 ± 0.001 | 0.0289 ± 0.01 | 11.82 ± 0.09 |
| 10% PD | 1.0224 ± 0.07$^{a*b*}$ | 60.25 ± 6.90$^{a**b**}$ | 11.46024 ± 1.1 | 0.050435$^{b*}$ ± 0.001 | 0.279 ± 0.1$^{a**b**}$ | 8.08 ± 1.34 |
| 5% PD | 0.878 ± 0.01$^{a*b*}$ | 75.062a$^{**b**}$ | 9.065 ± 0.7$^{a*}$ | 0.0809 ± 0.0001$^{a**b**c*}$ | 0.356 ± 0.04$^{a**b**}$ | 3.264 ± 1.0$^{a**b*c*}$ |

*Data are expressed as mean ± SEM of $F_2$ antioxidant status significantly difference from 21% Protein and control Diet group. Control (standard rat chow containing 16–18% Protein), 21% PD (upgraded daily recommended intake), 5% (Protein deficient diet), and 10 (Mild protein deficient diet). [a]significantly different from 21% diet group and or control. [b]significantly different from the 10% diet group. *P < 0.05, **P < 0.001.*

needs to be done to establish a molecular mechanism for these effects which could be linked to neurodegenerative disorders.

## DATA AVAILABILITY

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

## AUTHOR CONTRIBUTIONS

NA, OE, and NI designed the study. OE and NI constructively supervised the analyses of the study. NA managed the analyses, statistical analysis, the literature search, and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 29 August 2019
doi: 10.3389/fnut.2019.00142

# Effects of Phytoestrogens on the Developing Brain, Gut Microbiota, and Risk for Neurobehavioral Disorders

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**Specialty section:**
This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Nutrition

**Received:** 08 June 2019
**Accepted:** 14 August 2019
**Published:** 29 August 2019

**Citation:**
Rosenfeld CS (2019) Effects of Phytoestrogens on the Developing Brain, Gut Microbiota, and Risk for Neurobehavioral Disorders. Front. Nutr. 6:142. doi: 10.3389/fnut.2019.00142

Many pregnant and nursing women consume high amounts of soy and other plant products that contain phytoestrogens, such as genistein (GEN) and daidzein. Infants may also be provided soy based formulas. With their ability to bind and activate estrogen receptors (ESR) in the brain, such compounds can disrupt normal brain programming and lead to later neurobehavioral disruptions. However, other studies suggest that maternal consumption of soy and soy based formulas containing such phytoestrogens might lead to beneficial behavioral effects. Select gut microbes might also convert daidzein and to a lesser extent genistein to even more potent forms, e.g., equol derivatives. Thus, infant exposure to phytoestrogens may result in contrasting effects dependent upon the gut flora. It is also becoming apparent that consumption or exposure to these xenoestrogens may lead to gut dysbiosis. Phytoestrogen-induced changes in gut bacteria might in turn affect the brain through various mechanisms. This review will consider the evidence to date in rodent and other animal models and human epidemiological data as to whether developmental exposure to phytoestrogens, in particular genistein and daidzein, adversely or beneficially impact offspring neurobehavioral programming. Consideration will be given to potential mechanisms by which such compounds might affect neurobehavioral responses. A better understanding of effects perinatal exposure to phytoestrogen can exert on brain programming will permit pregnant women and those seeking to become pregnant to make better-educated choices. If phytoestrogen-induced gut dysbiosis contributes to neurobehavioral disruptions, remediation strategies may be designed to prevent such gut microbiota alterations and thereby improve neurobehavioral outcomes.

**Keywords: genistein, daidzein, microbiome, cognition, anxiety, autism, soy formula**

## INTRODUCTION

Fetuses and infants are exposed to phytoestrogens through the maternal diet. Neonates can also be exposed to such compounds through soy milk, which has shown in a surgence in usage. With their structural similarity to 17β-estradiol, these chemicals may bind and activate estrogen receptors (ESR) within the brain during the perinatal and adult periods. Consequently, phytoestrogens might disrupt normal organizational-activational programming of the brain that is regulated by testosterone or neural conversion of testosterone to estrogen (1–4). With this primary concern in mind, animal model and human epidemiological studies have been initiated to examine how such class of chemicals affect neurobehavioral programming (5).

Phytoestrogens may be metabolized by gut microbes to bioactive forms with even greater potency (6). For instance, S-equol is one metabolite that results from intestinal bacterial metabolism of daidzein/daidzin (7). Population differences exist though in those possessing gut bacteria capable of this conversion compared to those who lack such resident flora with 60% of Asian individuals but only 25% of Western individuals harboring such gut bacteria able to synthesize S-equol (8). A study with 15 adult German individuals consuming a typical German diet revealed half of these individuals excreted S-equol at concentrations ranging from 8 to 128 ng/mL (9). After consumption of soy protein, elevated concentrations of GEN (820 ng/mL), daidzein (960 ng/mL), and S-equol (1,740 ng/mL) were detected. As reviewed by Yuan et al (10), with only 1/3 to ½ of the population as a whole able to metabolize daidzein to equol, not all individuals may reap the potential beneficial effects of soy isoflavones, such as prevention of cardiovascular diseases, osteoporosis, and select cancers. Those individuals lacking equol-generating bacteria tend to instead produce O-desmethylangolensin (ODMA). ODMA-producers have a higher predilection for obesity than equol-producers (11). In this way, gut bacteria can influence the how phytoestrogens might affect host systems, including neurobehavioral and metabolic responses.

Phytoestrogen consumption or exposure might also influence the gut microbe composition. Within Asian populations, increased consumption of soy is associated with increased number of equol-generating bacteria within the intestine (12). A similar study found that ingestion of daidzein positively correlated with greater abundance of *Asaccharobacter celatus* and *Slackia isoflavoniconvertens*, two equol producing bacteria (13). Other rodent and human studies suggest that genistein (GEN) or soy based foods impacts gut microorganisms (14–22). Alterations in bacteria residing in the gut or skewing to pathobionts (otherwise considered gut dysbiosis) may result in neurobehavioral changes, giving rise to the term the microbiome-gut-brain axis (23–26). Germ-free (GF) mice, which lack a gut microbiome, provide the first solid evidence of a connection between gut bacteria and host neurobehavioral responses. Impairment of the hypothalamic-pituitary-adrenal gland (HPA) axis results with the absence of normal gut bacteria, but bacterial reconstitution or fecal transplantation early on mitigates such effects (27). Moreover, GF mice are more anxious, less exploratory, and show cognitive and social deficits to provide a few examples of behavioral deficits characterized in this mouse model (25, 28–31). The microbiome-gut-brain axis term has been coined based on these above results (32, 33). It suggests that there is crosstalk between the gut microbiota and brain with bacteria within the intestines able to modulate neurobehavioral responses and in turn through the vagal nerve and other potential mechanisms, neural responses can influence the composition of gastrointestinal bacteria and the gut itself. Thus, phytoestrogen-induced changes in gut bacteria might be another way in which exposure to such compounds lead to later neurobehavioral disruptions.

This review with thus consider the evidence to date that pre- and/or post-natal exposure to phytoestrogens with an emphasis on genistein, may cause later neurobehavioral deficits. Such effects might be due to direct binding of such chemicals to neural ESR, metabolism by gut microbes to even more potent compounds, or through phytoestrogen stimulated changes on gut bacterial populations. In relation to the latter possibility, there are several mechanisms by which gut bacterial shifts may affect host neural circuitry (23). Examples include shifts to pathobionts might lead to intestinal pathologies, such as increased gut leakiness or inflammation that could permit even more virulent organisms to penetrate through the gut barrier and induce systemic effects. Bacterial metabolites, including those that mimic host neurotransmitters, may be transmitted to the brain via the circulation or vagal nerve where they can disrupt normal homeostatic mechanisms. Should the bacteria enter into the circulation, they may then penetrate through the blood brain barrier and thereby gain direct access to neurons and glial cells. Phytoestrogen compounds and bacterial metabolites can also stimulate epigenetic changes in the brain, resulting in potential disruptive gene expression changes, which will be delved into further. These potential mechanisms by which developmental exposure to phytoestrogens can affect later neurobehavioral responses are summarized in **Figure 1**. Finally, open-ended questions and future directions in this area will be explored.

## ANIMAL MODEL STUDIES REPORTING NEUROBEHAVIORAL EFFECTS FOLLOWING GESTATIONAL OR POSTNATAL EXPOSURE TO GEN OR OTHER PHYTOESTROGENS

Rodent model studies reveal pre- and post-natal exposure to GEN can lead to reproductive (34, 35) and behavioral abnormalities such as increased defensive behaviors and demasculinization in male mice (36–38). Administration of GEN (either through diet-500 μg/g diet or gavage—20 to 75 mg/kg gavage) to pregnant Sprague-Dawley rats results in similar concentration of GEN aglycone in the fetal brain as identified in the maternal brain (39), strongly supporting the notion of relatively efficient placental transfer of this phytoestrogen. Another study with Sprague-Dawley rats also showed that ingested phytoestrogens can be transferred across the placenta and are concentrated in maternal milk, as evidenced by high infant plasma concentrations of these compounds (40). Thus, during pre- and post-natal

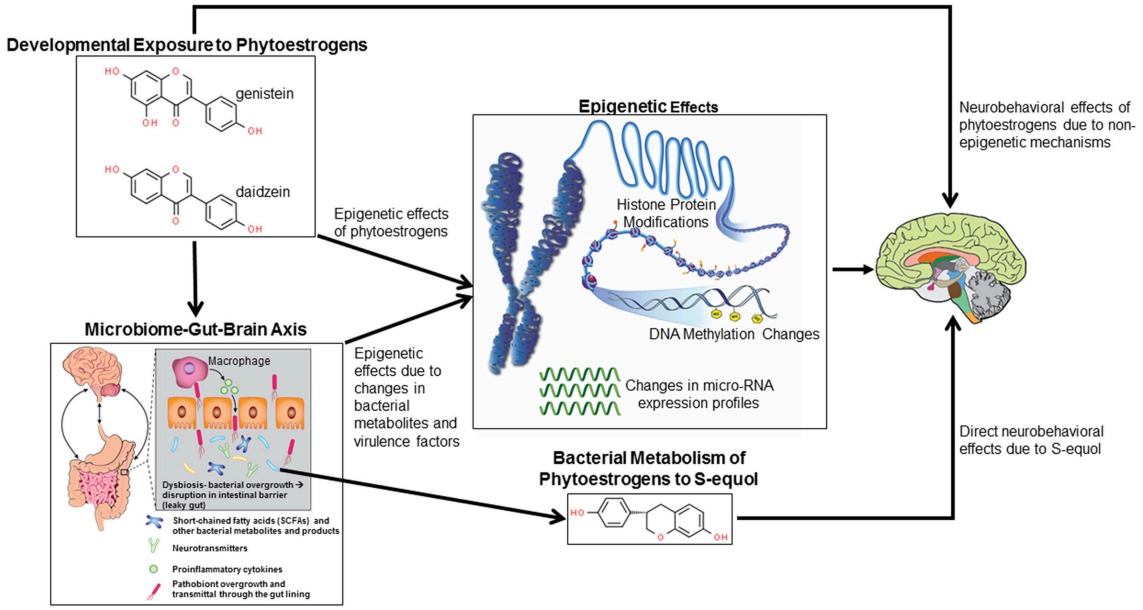


**FIGURE 1 |** Potential mechanisms by which developmental exposure to GEN and other phytoestrogens might alter neurobehavioral programming. Such compounds might induce epigenetic and non-epigenetic changes in the brain, leading to later behavioral alterations. Phytoestrogens might alter gut microbial species leading to disruptions of the microbiome-gut-brain axis. Bacterial metabolites and virulence factors may induce host epigenetic modifications that alter neurobehavioral responses. Select bacteria may metabolize phytoestrogens to more potent metabolites, in particular S-equol, that can cause neurobehavioral alterations.

life, GEN can pass through the blood brain barrier where it can result in direct neurobehavioral effects, as shown in **Figure 1**.

Male offspring derived from C57BL/6 dams supplemented with GEN (5 or 300 mg/kg) throughout gestation and lactation exhibit reduced anogenital distance, suggestive of outward feminization and decreased body mass with effects most pronounced in those receiving the low dose of GEN. Moreover, this group of males later showed reduced aggressive but increased defensive behaviors (36). CD1 dams exposed to GEN at 100 μg/g body weight from gestational day (GD) 11 to day 8 post-partum give rise to male offspring who as adult show increased anxiety and aggressive behaviors (37). These males had reduced number of positively stained cells in the amygdala neuronal cells staining positive for nitric-oxide synthase. A similar GEN exposure dose and period to Wistar rats resulted in sex-specific offspring effects with females displaying reduced novelty preference and exploration in an open field maze (41). On the other hand, developmental exposure to GEN (soy based diet, Purina 5001) from gestation through puberty has been suggested to abolish bisphenol A (BPA)-induced effects on anxiogenic behaviors in juvenile Wistar rats and restore normal sex differences in exploratory behavior in these animals (42). Using aromatase knockout (ArKO) mice, another study indicated that pre- and post-natal exposure to a phytoestrogen rich diet (containing 600 μg total dietary phytoestrogens, Harlan Teklad Global Diet 8604) is needed for normal brain sexual differentiation, namely sex differences in progestin receptor expression in the medal amygdala (43).

Exposure of Wistar rats from postnatal day (PND 0-3) to GEN (10 mg/kg body weight) advanced vaginal opening but reduced the number of kisspeptin immunoreactive fibers in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC), two regions of the brain associated with the timing of pubertal onset and estrous cyclicity (44). This group also showed persistence in the number of multiple oocyte follicles. GEN (250 micrograms) administration on PND 1 and 2 to female and male rats resulted in females demonstrating similar number of AVPV neurons co-expressing tyrosine hydroxylase (TH) and estrogen receptor 1 (ESR1) as males, suggestive that this compound abolished normal sex differences in this brain region that would otherwise exist or defeminized these females (45).

In a recent study published by my laboratory, we showed that pre- and post-natal exposure of California mice (*Peromyscus californicus*), who are monogamous and biparental, to GEN (250 mg/kg feed weight) results in socio-communicative deficits when animals are weaned at 30 days of age (46). As detailed below, this recent paper also linked social and vocalization behaviors with GEN-induced gut microbiome and metabolome changes.

A study testing the effects of perinatal exposure to daidzein placed female Balb/cJ mice on a daidzein supplemented diet (200 mg/kg diet) throughout gestation and lactation and then examined anxiety-like and social behaviors and spatial learning and memory of adult male mice (47). Male mice developmentally exposed to daidzein had suppressed exploration but increased anxiogenic and aggressive behaviors. These males also exhibited increased social investigation of females and some improvements in spatial learning and memory. *Esr1* expression was increased in

the bed nucleus of the stria terminalis, medial preoptic area, ARC, and central amygdala region but reduced in the lateral septum of these males. Collectively, these data suggest that early daidzein exposure masculinizes male-typical behaviors, which might be due to shifts in neural *Esr1* expression.

With their rapid developmental cycle, zebrafish (*Danio rerio*) have also been useful models in teasing apart the effects of early phytoestrogen exposure on brain development. Exposure of zebrafish embryos to GEN (10 μm) triggers apoptosis in the hindbrain and anterior spinal cord, which is not inhibited by co-administration with ICI 182,780 (48). *In vitro*, GEN can bind and activate ESR1, ESR2-A, and ESR2-B, and testing of transgenic ERE-luciferase fish reveals that this compound can activate estrogen pathways during the larval stages. Additionally, GEN stimulates ectopic expression of the aromatase-B gene in an ESR-dependent manner in the anterior brain. The findings from this study suggest that early GEN exposure of zebrafish may result in ESR-dependent and ESR-independent effects within the brain. Another study testing a range of GEN concentrations (0.25 to $1 \times 10^{-4}$ M) found that this phytoestrogen stimulated EGFP expression in the brain of mosaic ESR zebrafish embryos (49). GEN-induced embryos displayed several pathological changes, including neural apoptosis, reduced heart rates, suppressed hatching times, reduced body length, and increased mortality in a dose-dependent manner. In those that survived, the higher doses of GEN also resulted in pericardial edema, yolk sac edema, and spinal kyphosis.

To test the effects of soy formula in a non-human primate, infant male rhesus macaques (*Macaca mulatta*) were provided cow's milk based formula, soy based formula, or soy formula with added manganese from birth to 4 months of age (50). Those reared on soy or soy + Mn based formula engaged in reduced play behavior and showed heightened affiliative clinging behavior in social interactions. Both groups also showed blunted wake cycles and shorter durations of daytime inactivity. The findings suggest that soy and Mn might interact to affect brain development.

## GESTATIONAL EXPOSURE TO PHYTOESTROGENS AND FEEDING INFANT SOY FORMULA INCREASE THE RISK OF NEUROBEHAVIORAL DISORDERS IN CHILDREN

Infants provided soy formula can have circulating concentrations of isoflavones (genistein, daidzein, and their glycosides) that are up to 13,000 to 22,000 higher than plasma estradiol, whereas, infants provided breast milk or cow milk have neglible amounts of these chemicals (51).

A study with 3,664 boys and 3,412 girls as part of the Avon Longitudinal Study of Parents and Children revealed that girls exposed during infancy to soy formula show reduced female-typical play behavior, but comparable disruptions were absent in boys (52). This same cohort revealed that girls fed soy products during infancy have an increased risk of early menarche, but this study only included a limited number of soy-exposed individuals and did not test specific mechanisms (53).

Based on medical records analyzed from the Simons Foundation Autism Research Initiative Simplex Collection that included data in infant formula and autism diagnostic score for 1,949 autistic children, a potential linkage between feeding infant soy formula and subsequent risk for autistic behaviors was found (54). Additional data from this cohort revealed that soy based formulas might contribute to febrile seizures in autistic boys and girls and a concurrent increased risk of epilepsy in these boys (55).

Other studies have found no differences in cognitive function and infants fed soy or breast milk. For instance, event-related potentials to speech sounds and behavioral measures were similar in infants fed either soy based formula or breast milk (56). While another study found differences in the electroencephalographic (EEG) activity in formula-fed vs. breast-fed infants, no differences were detected between those provided soy vs. milk formula (57). A similar study by this research group also found no differences in EEG spectral development between infants fed soy vs. milk-based formula (58). In a double-blind study, lactose free milk or lactose free soy based formulas did not improve difficulties in infant behaviors, such as fussiness, crying, or need for attention as reported by caregivers (59). Mental and psychomotor development indices also do not seem to differ between infants provided milk-based formula compared to soy based formula (60), but this study did find that infants who were breastfed showed improved cognitive development relative to both formula-fed groups.

## EFFECTS OF PHYTOESTROGENS ON THE GUT MICROBIOME

Consumption of soy or exposure to phytoestrogens alters the gut microbiota denizens (14–22). Most current studies have focused on whether direct or adult exposure affects the gut microbiome, although there is every reason to believe that developmental exposure to such chemicals influences infant gut bacteria populations, which may be even more vulnerable during this initial colonization period than those of adults. This section will consider whether phytoestrogen exposure or soy consumption at any point in the lifespan affects gut microbial profiles.

Soy-fed neonatal White Dutch Landrace pigs show correlations between diet-responsive intestinal metabolites and gut microbes (61). Specifically, bacteria within the duodenum of sow-fed pigs have greater α-diversity (overall species diversity). In this intestinal region, soy-fed pigs possessed a greater percentage of Cyanobacteria. Extending from the duodenum to the jejunum and ileum, 77, 48, and 19 genera, respectively, were altered by diet. Associations existed with ileum metabolites, such as acylcarnitines and 3-aminoisobutyric acid, and diet-induced microbial shifts. Another study with White Dutch Landrace Duroc piglets showed that a soy formula diet affected the intestinal epithelial lining, microbial populations, and intestinal epithelial barrier as well as anti-inflammatory markers (62). The Peyer's patches within the ileum of sow-fed

piglets possess larger lymphoid nodule size and second lymphatic nodules with prominent germinal centers, indicative of enhanced immune reaction. Soy fed piglets have increased expression of VE-cadherin and putrescine in the ileum. *Lactobacillaceae* spp. and *Clostria* spp. are significantly elevated but *Enterobacteriaceae* spp. decreased in soy-fed piglets.

The plasma metabolome of vegans consuming a soy-rich diet differs from that of omnivores, but the gut microbial profile between the two groups of individuals is surprisingly quite similar (16). Within the plasma metabolome, vegans had higher amounts of vitamins and other plant-derived compounds, including ascorbate, xanthine metabolites and derivatives of benzoate metabolism. This group also showed increased amount of metabolites associated with chlorogenic acid bacterial metabolism, such as hippurate, catechol sulfate and 3-hydroxyhippurate. While daidzein and genistein were predictably elevated in vegans relative to omnivores, equol concentrations did not differ between the two groups of individuals. The findings of this study suggest that a vegan or soy-rich diet might alter gut bacteria-derived metabolism but not necessarily the gut microbiota themselves.

Three particular studies in mice have shown that direct consumption of GEN and exposure during pre- or post-natal life is associated with gut microbiota changes, which may result in metabolic and cognitive changes (63–65). Firstly, in the study by Huang et al. (65), non-obese diabetic male and female mice were exposed orally to GEN (20 mg/kg body weight) from GD 7 to PND 21. Female GEN offspring had hyperglycemia (suggestive of type 1 diabetes), which was linked with decreased serum concentrations of inflammatory markers, interleukin (IL) 10, IgG2a, and IgM. Male GEN offspring only had decreased concentrations of IgG1. Contrasting sex differences were evident for other inflammatory markers with GEN females showing an overall increased in pro-inflammatory splenic cell counts, whereas T cell indices were decreased in males. By PND 90. GEN females demonstrated increased levels of Enterobacteriales, a bacteria associated with a pro-inflammatory response, and intestinal mRNA expression of the anti-inflammatory marker, α-defensin, was reduced at this timepoint. Taken together, this study suggests that perinatal exposure to GEN induces later sex-dependent differences in gut microbiota and associated host changes with females showing increased incidence of metabolic disorders and a heightened pro-inflammatory response but exposed males tending to have anti-inflammatory responses. This study, however, did not measure any indices of neurobehavioral function in these mice.

In another mouse study, adult C57Bl6 mice were provided a high fat diet (HFD) or a HFD containing GEN for 6 months (64). Those fed the latter diet gained less weight, had reduced serum triglycerides, and showed improve glucose tolerance relative to those fed the HFD alone. Correspondingly, mice consuming the HFD with GEN had modifications in the gut microbiota that were shifted to those producing less amounts of lipopolysaccharide (LPS, virulent factor typical of gram negative bacteria) and decreased expression of hepatic pro-inflammatory cytokines. Supplementation of GEN to those on the HFD also resulted in improved cognitive function. While the findings suggest that chronic exposure to GEN may reduce intestinal pathobionts, suppress hepatic inflammation, and improve cognitive ability, only potential correlations can be drawn between GEN-induced microbiota shifts and later host changes. This study also did not explore for potential sex differences or how microbiota shifts may result in beneficial host effects, such as through bacterial metabolite changes.

C57BL6 mice females were placed 3 week prior to breeding and throughout gestation and lactation one of four diets dies: (1) HFD, (2) HFD with low dose GEN (0.25 g/kg diet), (3) HFD with high dose GEN (0.6 g/kg diet), or (4) control diet (63). At weaning, female offspring derived from the HFD groups had reduced birth weights, exhibited glucose intolerance, and had elevated serum insulin, triacylglycerol (TG), and total cholesterol (TC) relative to the control group. In contrast, those derived from HFD + low GEN showed increased birth weight, improved glucose tolerance, and reduced fasting insulin concentrations compared to HFD offspring. TG and TC levels were reduced in HFD + high GEN group relative to HFD. Offspring derived from dams fed the HFD + low GEN diet also showed enrichment for *Bacteroides* and *Akkermansia*, whereas, *Rikenella* and *Rikenellaceae_RC9_ gut_group* were elevated in HFD + high GEN offspring. Taken together, the findings from this study suggest that maternal GEN might mitigate some of the negative HFD-induced metabolic effects, and changes in gut microbes might at least partially mediate such effects.

Metabolites produced by gut microbes may influence host function (23, 66–70). However, these above studies examining effects of GEN on the gut microbiome did not examine for changes in host or microbial metabolites.

A recent study with Southern white rhinoceros (SWR, *Ceratotherium simum simum)* suggested that dietary phytoestrogens might transform the gut microbiota composition, which may be another mechanism resulting in increased infertility in captive populations fed a diet enriched with such compounds (71). The bacterial profiles of SWR were compared to that of the greater one-horned rhinoceros (GOHR [*Rhinoceros unicornis*]), who consume a similar phytoestrogen rich diet but show high levels of fertility in captivity. Microbial communities differed between the two groups of rhinos with SWR possessing greater relative numbers of Bacteroidetes, while Firmicutes was elevated in GOHR. While the findings in this study suggest that consumption of a phytoestrogen-rich diet changes microbial populations and thereby affects host reproductive function, the small sample size used in this study population, especially for GOHR, could be of potential concern. Additionally, each rhinoceros species may have unique signature pattern of resident gut bacteria, phytoestrogen metabolism, and fecundity. This study also did not determine whether the infertility issues in SWR are central (hypothalamus or pituitary gland) vs. gonadal in origin. Notwithstanding, the findings provide further evidence in an endangered species that a phytoestrogen-rich diet can alter gut microbial composition with potential pathophysiological changes resulting in the host.

In recent work from my laboratory, we discovered that developmental exposure of California mice to GEN (250 mg/kg feed weight) results in gut microbiome and metabolome changes, along with the neurobehavioral disruptions detailed above (46). By using mixOmics analyses (72), an R based program that allows for multiple data integration, we found several interactions between GEN-induced gut microbiome, metabolome, and socio-communicative behaviors (46). For instance, comparison of GEN males to AIN (control) males revealed that fraction of calls in the ultrasonic range (> 20 kHZ) was positively associated with daidzein, α-tocopherol, *Flexispira* spp., and *Odoribacter* spp. (**Figure 2**). Comparison of GEN exposed females to AIN females showed that average vocalization power positively correlated with *Rikenaceae* and *Ruminococcaceae* (**Figure 3**), both of these bacteria were elevated in females exposed to GEN. While findings with this informatics approach are interesting, they can only suggest correlation not causation. Even so, this informatics approach is likely to be useful in elucidating linkages between gut microbiome, gut metabolome, and neurobehavioral responses. Some follow-up studies can then be envisioned based on these results include treating control animals with the bacteria or metabolite that is linked with neurobehavioral deficits. Conversely, the effects of early supplementation of bacteria or metabolites that are reduced in GEN exposed animals might be tested to determine if such remediation strategies mitigate later neurobehavioral deficits.

The collective studies reveal the complexity of GEN and other phytoestrogens effects on gut microbiota communities, bacterial metabolites, and host responses. By altering the gut microbes and bacterial metabolite, GEN and other phytoestrogens might disrupt the microbiome-gut-brain axis, as shown in **Figure 1**. The net outcome of whether such changes lead to beneficial or detrimental effects is assumingly driven by a multitude of factors, including species, sex, when during the lifespan the exposure occurs, duration of exposure, other concurrent dietary factors, e.g., HFD, and host response being analyzed. Moreover, all of these current studies only reveal potential associations between gut microbiota/bacterial metabolite change and host effects. Actual causation studies would require transplanting the fecal microbiome from GEN or phytoestrogen exposed individuals into GF mice and then assessing whether similar phenotypic outcomes occur in these mice as identified in donors directly exposed to these xenoestrogens.

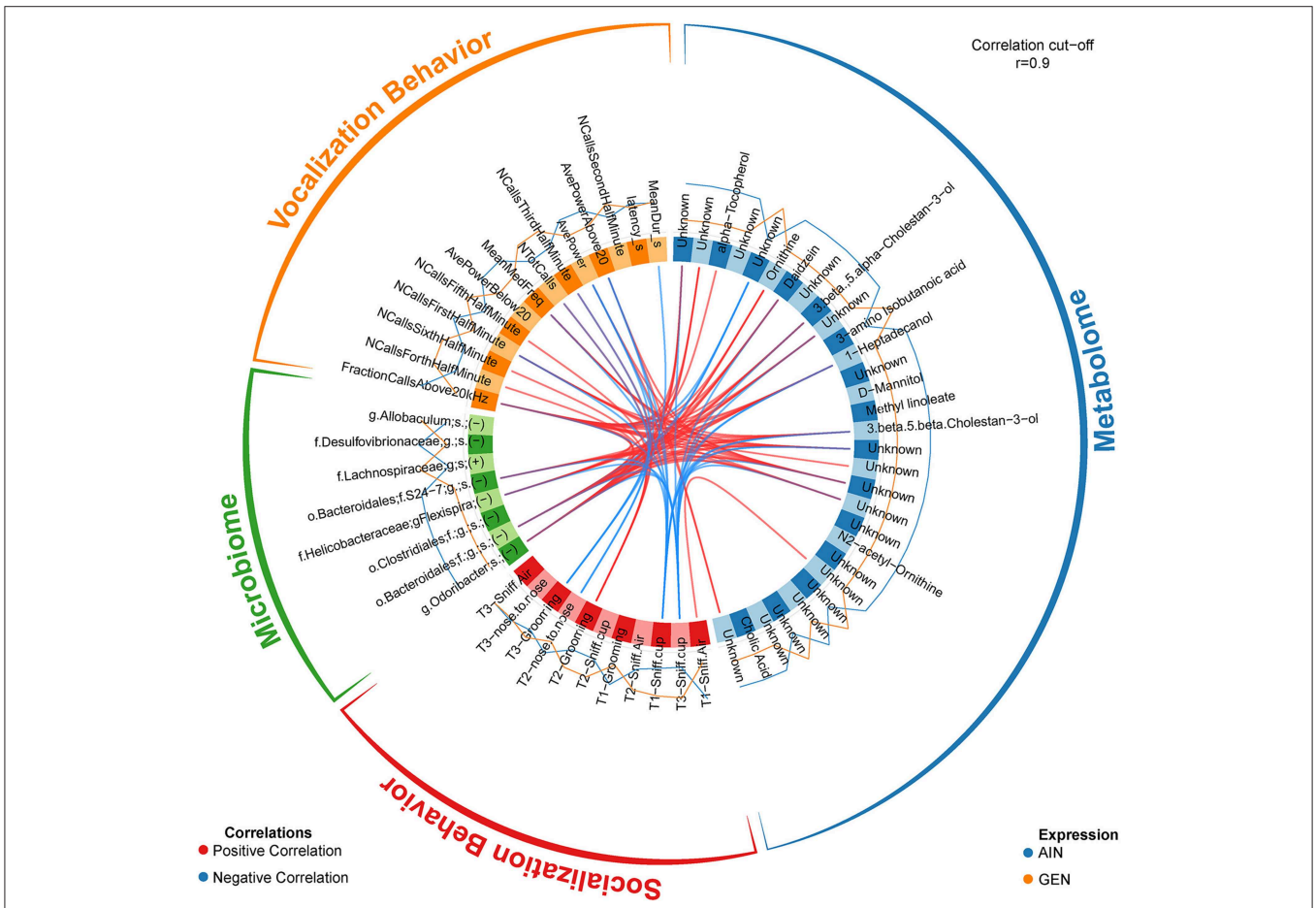


**FIGURE 2 |** Circos correlations as determined with mixOmics analyses (72) for behavioral responses, gut microbiome, and gut metabolome changes in GEN exposed males vs. AIN (control) male California mice. Adapted from Marshall et al. (46) with permission from BioScientifica Limited.

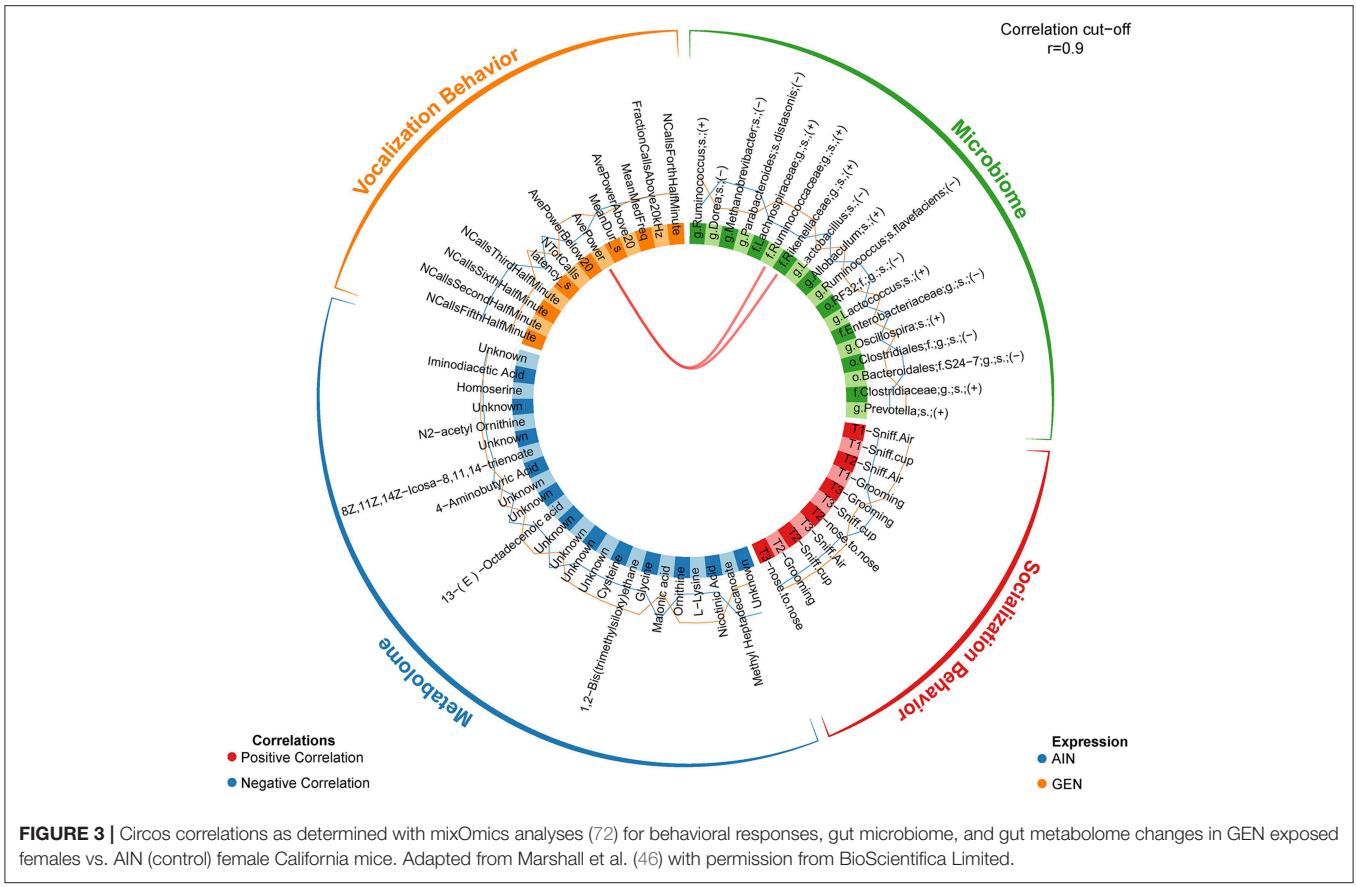


**FIGURE 3 |** Circos correlations as determined with mixOmics analyses (72) for behavioral responses, gut microbiome, and gut metabolome changes in GEN exposed females vs. AIN (control) female California mice. Adapted from Marshall et al. (46) with permission from BioScientifica Limited.

## NEUROBEHAVIORAL EFFECTS OF EQUOL IN RODENT MODELS AND HUMAN STUDIES

As reviewed in Setchell and Clerici (73), equol was first isolated in equine urine in 1932, and it was identified 50 years later in human urine as a metabolite of soy isoflavones, daidzin and daidzein. Equols are metabolites formed gut microorganism reduction of soy isoflavones (74, 75). In fact, many isoflavones must be metabolized in the gut by microbes in order to reach their full biological activity (76). Equol exists as a diasteroisomer with certain intestinal bacteria being enantiospecific in synthesizing exclusively the S-(-)equol enantiomer form (77, 78). This form demonstrates selective affinity for estrogen receptor beta (ESR2) (79).

The role of intestinal bacteria in converted isoflavones to equol is shown by the fact that GF animals provided a soy diet do not have detectable levels of equol in their urine (80). In humans who have had an ileostomy, where the ileum is attached to the abdominal wall to bypass the large intestine, have reduced production of microbial metabolites, including equol, dihydrogenistein, and ODMA in the urine (81). However, such patients are able to effectively deglycosylate isoflavonoid glucosides in the small intestine and have normal absorbtion of aglycone metabolites.

In humans, there is genetic heterogenity in equol production following soy isoflavone consumption (8). Importantly, only 30–50% of Western individuals produce equol, thus suggesting that only these individuals experience full metabolic benefits of dietary soy (8). While most equol-producing bacteria belong to the *Coriobacteriaceae* family, other bacteria can convert daidzein/daidzin (and to a lesser extent, GEN) to S-equol (75, 82, 83). While no current studies have examined the effects of developmental exposure on later neurobehavioral responses, S-equol has been associated, not only with postive metabolic effects, but also with neuroprotection based on *in vitro* and *in vivo* models (84–88). However, one study with ovariectomized rats indicated that daily treatment with S-equol did not positively affect learning and memory (89). In our recent study that tested the effects of S-equol (10 mg/kg body weight) in male and female C57BL6J mice provided a HFD, we found that this supplementation worsened aspects of HFD-induced metabolic disorders, as indicated by reduced physical activity in male and females, decraesed energy expenditure in males, and hyperglycemia/hyerinsulinemia (90). On the other hand, S-equol individuals had decreased anxiogenic and depression-like behaviors. Potentially analogous beneficial neural findings have been reported in women with equol producers and post-menopausal individuals consuming soy showing improved cognitive performance and emotional responses (91, 92).

Ishikawata et al. (92) concludes that equol supplementation might be a useful adjuvant for menopausal women who are non-equol producers. However, additional studies are needed to determine the potential effects on mood-related symptoms and underpinning neural mechanisms. Thus, conversion of phytoestrogens to S-equol may result in neurobehavioral changes, as shown in **Figure 1**.

## EPIGENETIC CHANGES INDUCED BY PHYTOESTROGENS OR BACTERIAL METABOLITES

Phytoestrogens and bacterial changes induced by exposure to such compounds can lead to epigenetic changes within the host, as shown in **Figure 1**. While there is scant evidence showing phytoestrogens mediate epigenetic change in the brain, a wealth of data exists reveals these compounds alter the epigenome, especially DNA methylation changes, in other non-cancerous and cancerous cells and tissues. One of the earliest studies to show such effects placed male mice on a casein-based or GEN (300 mg/kg) diet for varying lengths of time (93). GEN treatment positively correlated with changes in prostate DNA methylation at CpG islands (93). Hypermethylation and subsequent silencing of the proto-oncogene H-Ras occurred in rats treated with the phytoestrogenic compounds, coumestrol or equol (94). One study suggested genistein may reverse hypermethylation of various cancer-promoting genes, including $p26^{INK4a}$, *RARb*, and *MGMT* (95). However, this study was *in vitro* based with human esophageal squamous cell carcinoma, KYSE, and prostate carcinoma (LNCaP and PC3) cell lines.

Supplementation of GEN (250 mg/kg diet) to pregnant *a/a* dams carrying $A^{vy}/a$ (viable yellow mice) offspring resulted in a shift to more pseudoagouti offspring, who had increased methylation of CpG island sites within the $A^{vy}$ IAP promoter site (96). Mice with the $A^{vy}$ allele have an IAP inserted in the pseudoexon 1A region (97), resulting in *agouti* expression being controlled by the long terminal repeat (ltr) of the IAP proximal to the agouti gene, which is correlated with the degree of IAP methylation. Hence, $A^{vy}/a$ mice have coat colors ranging from yellow (hypomethylation of the $A^{vy}$ IAP and who are prone to obesity and diabetes) to mottled (yellow with varying degrees of agouti patches) to completely pseudoagouti (full hypermethylation of the $A^{vy}$ IAP who will be overall healthy). This same group also showed that concurrent developmental exposure to BPA and the same dose of GEN resulted in GEN mitigating the negative epigenetic effects of BPA on the $A^{vy}$ gene with a subsequent reduction in the number of yellow coat-color $A^{vy}/a$ mice prone to develop metabolic disorders (98). Using a similar approach, however, we were not able to replicate these findings (99). Instead, we found that co-exposure during gestation to both BPA and GEN favored the birth of *a/a* compared to $A^{vy}/a$ mice. Besides altering the uterine transcriptome profile, neonatal exposure of female C57Bl6 mice to GEN (50 mg/kg from PND 1-5) caused dysrgegulation of genes involved in chromatin remodeling and altered transcriptional responses to subsequent glucocorticoid treatment (Dexamethasone 1 mg/kg from PND 5-21) (100).

GEN stimulates various epigenetic changes in human breast cancer cell lines. One study reported that in MDA-MB-231 human breast cancer cells, GEN resulted in hypomethylation in the promoter region of several tumor suppressor genes with concomitant increased expression of these beneficial genes (101). Similarly, in MCF-7 breast cancer cells, GEN suppressed methylation of BRCA1 and correspondingly inhibited cell proliferation (102). In MCF-7 and MDA-MB 231 breast cancer cell lines, GEN, daidzein, and equol caused a decrease in number of trimethylated histone protein marks, which tend to be associated with suppression of transcription, but an increase in number of acetylating histone protein marks, which remove histone proteins bound to DNA and thus increase gene transcription. The gene involved include *EZH2*, *BRCA1*, *ESR1*, *ESR2*, *SRC3*, and *P300* (103).

Comparison of genes that are epigenetically altered in the rat mammary gland to predict breast cancer patient survival using the Cancer Genomic Atlas (TCGA) revealed that two genes, *HPSE* and *RPS9*, were hypomethylated in the mammary gland of rats prepurtertally treated with GEN or GEN + BPA and were also associated with increased patient survival (104). These findings and the *in vitro* studies above provide strong evidence that at least in the mammary gland, GEN may induce beneficial DNA methylation and other epigenetic changes. Similarly, GEN and soy protein isolate (SPI) repress WNT signaling in the rat colon, which is associated with a reduction in pre-neoplastic lesions (105). Follow-up studies reveal that GEN and SPI appear to inhibit WNT signaling by methylating defined regions within *Sfrp2*, *Sfrp5*, and *Wnt5a* and by altering histone protein binding to DNA, as judged from an increase in histone deacetylase 3 (HDAC3), suppressing trimethylation of histone 3 lysine 9 (H3K9me3). Additionally, both treatments inhibit phosphorylation of histone H3 Serine 10 (H3S10P) (106).

A multitude of host effects can result from gut microbial changes. Bacterial metabolites and other products, such as LPS in Gram negative bacteria and peptidoglycans from Gram positive bacteria, can induce a variety of host epigenetic changes, as reviewed in Rosenfeld and Kapila (23), Bhat et al. (107), Alam et al. (108), Eshraghi et al. (109). Herein, we will consider a few examples.

Histone proteins that bind to DNA are subject to various epigenetic modifications, such as methylation, acetylation, sumoylation, and ubiquitination, and such post-translational modifications alter the affinity to which these proteins bind to DNA. Acetylation tends to pull the histone proteins away from DNA, resulting in increased gene activation, whereas, deacetylation of histone proteins is associated with increased histone protein binding and decreased gene transcription. Acetylation of histone proteins is regulated by two primary class of enzymes, histone aceytltransferases (HAT) that transfer acetyl groups to histone proteins and histone deacetylases (HDAC) that remove acetyl groups from these proteins. Several bacterial products can affect these two enzymes. Bacterial-derived short chain fatty acids (SCFA), mainly butyric acid (BA), propionic acid (PPA), and acetic acid act as HDAC inhibitors with BA being the

most potent (110, 111). Other SCFA, such as lactate and pyruvate, may induce weak HDAC inhibitory responses (112–114).

In contrast, acetate, a byproduct of microbial fermentation of dietary fiber, up-regulates HAT substrate availability (26). Acetate supplementation appears to mitigate neuroglial activation and loss of cholinergic cells in rats with neuro-inflammation due to LPS treatment (115). These beneficial effects are likely due to increased acetylation of neural H3K9, H4K8, and H4K16 and acetate suppression of HDAC2. The metabolite, epigallocatechin-3-gallate (EGCG), primary component of green tea, also can increase acetylation of H3 and H4 and simultaneously inhibit HDAC and DNA methyltrasferase (DNMT) (116). Conversely, fisetin, a flavonoid found in fruits and vegetables activates HDAC but suppresses HAT (117).

Several gut microbiota synthesize a variety of water soluble B-vitamins, including folate or folic acid, biotin, vitamin B2, B6, and B12, that can promote methylation of DNA and histone proteins and induce other epigenetic changes (118). Folate, vitamins B2, B6, and B12 enter at various points into one-carbon metabolism, which leads to the synthesis of the common methyl donor, S-adenosyl methionine (SAM), which also acts as a substrate for DNMT (119). Thus, bacterial production of B- vitamins can alter host DNA methylation and potentially histone protein methylation profiles.

Human and animal studies provide further evidence that gut bacterial shifts lead to DNA methylation and subsequent gene expression changes in various host cells and tissues. A linkage exists in pregnant women between gut microbial profiles, namely for Firmicutes and Bacteroidetes, and leukocyte DNA methylation patterns for genes regulating lipid metabolism and obesity (120). Feeding mice a maternal methyl supplemented diet causes gut dysbiosis and associated changes in colonic mucosal DNA methylation and transcriptomic patterns, and colitis in resulting offspring (121).The presence of gut bacteria stimulates hypermethylation of CpG motifs in the *Tlr4*/*TLR4* gene, whereas in the percentage of methylation of CpG motifs in the 5′ region of the *Tlr4* gene becomes reduced within the large intestine of GF mice (122).

Other epimutations may result due to changes in gut flora, such as chromatin rearrangements, alterations in non-coding or micro (mi)RNAs, and RNA splicing factors (123). Colonization of GF mice with commensal gut microbes alters the miRNA expression profile within the ileum and colon, resulting in gene expression changes in these regions (124). Several miRNA, such as miR-143, miR-148a, miR-200b, miR-200c, and miR-378 are downregulated in the intestines of mice infected with *Listeria monocytogenes* (125). In contrast, commensal gut microbes, *Escherichia coli* and A4 bacteria inhibit the expression of miR-10a in antigen-presenting dendritic cells through toll-like receptors that induce an MyD88-dependent pathway (126). This miRNA is associated with induction of a variety of cancers and inflammatory bowel disease. Therefore, suppression of this gene by beneficial gut microbes may be a useful adjuvant treatment for both of these disorders.

Gut pathogens might induce widespread effects on host gene expression profiles by suppressing RNA polymerase II, an enzyme required for synthesis of coding and non-coding RNAs (127). Intriguingly, select endosymbiotic bacteria seem to produce small non-coding RNAs with the potential to exert cross-kingdom communication and affect host processes (128). As we delve deeper into the complex relationships that have evolved between host and resident microorganisms, more such complex inter-relationships with the potential to affect the host epigenome and gene profiles are likely to be discovered.

## CONCLUSIONS

The current animal and humans studies reveal that developmental exposure to GEN and other phytoestrogens can induce mixed effects on later neurobehavioral responses that are likely mediated by a variety of processes (summarized in **Figure 1**). Offspring sex, timing and duration of exposure, and phytoestrogen examined are assumingly a few variable that determines whether the effects of these compounds is beneficial or detrimental to neurobehavioral responses, as well as the types of behaviors that are affected by early exposure to such compounds. In male rodents, exposure to phytoestrogens alone tends to induce an increase in anxiogenic behaviors but reduces exploratory behaviors (37, 47). Thus, it is not clear how to interpret the finding that co-exposure GEN and BPA suppresses anxiety-like behaviors that occur due to BPA exposure alone (42). The findings though suggest that other dietary components need to be considered when assessing potential neurobehavioral disturbances due to phytoestrogens. Such is the case also when mice are co-exposed to GEN and a HFD in that former seems to alleviate the disease-promoting effects of the latter (63, 64).

Similarly, the human studies have yielded vastly different findings with some reports indicating play behavior in girls is affected by early phytoestrogen exposure (52). Other studies implicate early exposure to such compounds in affecting risk that sons will develop autism and other behavioral disorders (54, 55), although it is clear that boys are already at a least 3:1 greater risk for these disorders than girls (129). Additional reports indicate babies provide soy formula do not show different behavioral patterns that those provide other types of formula (56–60). Taken together, the current human data do not provide a clear picture as to whether phytoestrogens or soy formula or consumption of such products by expecting mothers jeopardizes offspring brain development and risk for neurobehavioral disorders. Larger cohort studies are needed that also screen for maternal intake of soy and other products containing phytoestrogens and then link such maternal intake and/or feeding of soy formula with various behavioral trajectories in sons and daughters. The current studies though provide some ideas as to which behaviors should be considered in these additional epidemiological studies.

Such behavioral changes might be due to direct effects on the brain. Developmental exposure to GEN results in vacuolization of oxytocin neurons in the neonatal mouse hypothalamus (130). Brain nitrergic and vasopressinergic circuitry systems in rodents and Japanese quail might also be vulnerable to GEN (131–133). GEN exposure might also impede the gonadotropin releasing hormone network and hypothalamic-pituitary-adrenal axis (134–139).

Besides inducing direct effects on brain programming, early exposure to phytoestrogens, namely GEN, may alter resident gut flora populations that could in turn affect neurobehavioral functions through the microbiome-gut-brain axis. As with direct effects on the brain, gut microbiome alterations likely depend upon type of phytoestrogen exposure, host species examined, and time and duration of exposure to phytoestrogens (14–21, 61, 62). GEN-induced changes have been previously linked to changes in host cardiometabolic responses (17). Yet, studies examining the effects of phytoestrogens on gut microbial profiles and host responses can only establish correlation between these types of changes. To establish that such gut microbe changes actually cause host alterations, fecal microbial transfer from phytoestrogen-exposed individuals into GF mice that lack resident gut microbiota and who have not been exposed to such chemicals is required. If the gut flora alterations due to phytoestrogens lead to neurobehavioral disruptions, then GF mice transplanted with fecal material from exposed mice should demonstrate similar phenotypic changes. Such was the case when samples from offspring exposed to maternal HFD were transferred to GF mice who went on to display similar social deficits as the donor mice derived from dams on the HFD (140).

The resident gut microorganisms may also convert ingested or exposed phytoestrogens to even more potent forms, such as conversion of daidzein/daidzin to equol derivatives. While most rodent models have bacteria capable of this metabolism, human populations tend to be segrated into equol-producers, mainly from Asian countries, and non-equol producers, other geographical regions (8). Individuals lacking equol-generating bacteria tend to instead produce ODMA, and these individuals have a higher predilection for obesity than equol-producers (11). The current animal and human studies suggest that direct exposure to equol may yield beneficial cognitive and emotional effects. However, it remains to be ascertained how early exposure to this bacterial metabolite affects later neurobehavioral outcomes.

Phytoestrogen-induced changes in gut bacteria may affect other gut bacterial metabolites. As detailed above, soy-fed neonatal White Dutch Landrace pigs show linkages between diet-responsive intestinal metabolites and gut microbes (61). Such metabolite changes may be transmitted through the vagal nerve or systemic circulation to affect neural function. To establish actual causation between such bacterial metabolites and neurobehavioral changes, metabolites identified to be enriched due to phytoestrogen exposure would need to be administered to control dams and offspring neurobehavioral responses examined throughout the lifespan. This approach was done with 4-ethylphenylsulfate (4-EPS), a metabolite shown to be elevated in maternal immune activation mouse model for ASD, which resulted in autistic-like behaviors in control mice (141).

Another mechanism to dissect neurobehavioral effects directly due to phytoestrogen exposure vs. those mediated by the gut microbiome-metabolome, intracerebroventricular (ICV) injection with radioactive labeled phytoestrogens might be performed in neonatal animals, as has been done for other estrogenic compounds (142–144). This method, however, is likely not feasible in fetal mice.

The current studies also reveal that phytoestrogens might alter neurobehavioral responses through epigenetic changes, including DNA methylation, histone protein modifications, and alteration in miRNA profiles. Further work is thus needed to examine whether such compounds directly affect the enzymes mediating these epigenetic modifications, including DNMT, putative DNA demethylases, HDAC, and HAT. Phytoestrogen-induced gut dysbiosis and resulting changes in bacterial metabolites and virulence factors may also impact the host epigenome. This could be due to alteration in substrates, such as acetate or B-vitamins, required for such epigenetic modifications and/or by affecting host enzymes catalyzing these reactions. Of these, bacterial-derived SCFA, in particular BA, can significantly suppress HDAC, which would result in acetyl groups remaining on the histone proteins and separated away from their associated DNA strand with their promoter region being available for transcription factor binding and increasing gene transcription. Direct supplementation of such bacterial metabolites or challenging the host with bacterial virulence factors, such as LPS, will be useful in teasing apart how bacterial fluctuations affects the host epigenome and downstream CNS dysfunction and risk for other host diseases.

In conclusion, developmental exposure to phytoestrogens and potential effects on neurobehavioral function may be thought of as a see-saw with the ultimate outcome dependent on other contributory factors that can from the other side push the board and ultimate responses in a downward or upward direction. Such factors include offspring sex, type, duration, and timing of phytoestrogen exposure, other dietary components or endocrine disrupting chemicals, species examined, and behavioral changes considered to list just a few examples. In the middle of the board rests the gut microbiome, dramatically influencing in a variety of ways the ultimate outcome.

## AUTHOR CONTRIBUTIONS

CR researched the area, wrote the manuscript, and revised it based on the Editor and Reviewers suggestions.

## FUNDING

The studies were supported by NIEHS 1R01ES025547 (to CR).

## ACKNOWLEDGMENTS

I am grateful to all coauthors who assisted in work published from my laboratory that is discussed in the current manuscript.

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 13 September 2019
doi: 10.3389/fnins.2019.00962

# Maternal Obesity Alters Neurotrophin-Associated MAPK Signaling in the Hypothalamus of Male Mouse Offspring

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**Specialty section:**
This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience

**Received:** 29 May 2019
**Accepted:** 28 August 2019
**Published:** 13 September 2019

**Citation:**
Bae-Gartz I, Janoschek R, Breuer S, Schmitz L, Hoffmann T, Ferrari N, Branik L, Oberthuer A, Kloppe C-S, Appel S, Vohlen C, Dötsch J and Hucklenbruch-Rother E (2019) Maternal Obesity Alters Neurotrophin-Associated MAPK Signaling in the Hypothalamus of Male Mouse Offspring. Front. Neurosci. 13:962. doi: 10.3389/fnins.2019.00962

**Purpose:** Maternal obesity has emerged as an important risk factor for the development of metabolic disorders in the offspring. The hypothalamus as the center of energy homeostasis regulation is known to function based on complex neuronal networks that evolve during fetal and early postnatal development and maintain their plasticity into adulthood. Development of hypothalamic feeding networks and their functional plasticity can be modulated by various metabolic cues, especially in early stages of development. Here, we aimed at determining the underlying molecular mechanisms that contribute to disturbed hypothalamic network formation in offspring of obese mouse dams.

**Methods:** Female mice were fed either a control diet (CO) or a high-fat diet (HFD) after weaning until mating and during pregnancy and gestation. Male offspring was sacrificed at postnatal day (P) 21. The hypothalamus was subjected to gene array analysis, quantitative PCR and western blot analysis.

**Results:** P21 HFD offspring displayed increased body weight, circulating insulin levels, and strongly increased activation of the hypothalamic insulin signaling cascade with a concomitant increase in ionized calcium binding adapter molecule 1 (IBA1) expression. At the same time, the global gene expression profile in CO and HFD offspring differed significantly. More specifically, manifest influences on several key pathways of hypothalamic neurogenesis, axogenesis, and regulation of synaptic transmission and plasticity were detectable. Target gene expression analysis revealed significantly decreased mRNA expression of several neurotrophic factors and co-factors and their receptors, accompanied by decreased activation of their respective intracellular signal transduction.

**Conclusion:** Taken together, these results suggest a potential role for disturbed neurotrophin signaling and thus impaired neurogenesis, axogenesis, and synaptic plasticity in the pathogenesis of the offspring's hypothalamic feeding network dysfunction due to maternal obesity.

**Keywords: synaptic plasticity, BDNF, HFD, microarray, neurogenesis, CAMKII phosphorylation, IBA1, vGLUT2**

## INTRODUCTION

The rising incidence of maternal obesity is a worldwide phenomenon and the relationship between maternal obesity and the offspring's long-term predisposition for metabolic disorders, such as obesity and type-2-diabetes, is undeniable (Howie et al., 2009; Plagemann, 2011; O'Reilly and Reynolds, 2013). Human and rodent studies have led to the description of novel interactive pathways between the maternal environment and the fetus (Poston, 2012). In particular, there is a clear link between maternal adiposity and expression and function of important modulators of energy homeostasis in adipose tissue, liver and the brain of the offspring (Bouret, 2009; Catalano et al., 2009; McCurdy et al., 2009; Li et al., 2011; Maric-Bilkan et al., 2011; Rooney and Ozanne, 2011; Zhang et al., 2011).

The central control of energy homeostasis is a complex process regulated by a collection of neurons primarily located in the mediobasal hypothalamus. Specifically, the melanocortin system of the arcuate nucleus of the hypothalamus (ARC) has been shown to convey anorexigenic and orexigenic signals in response to circulating hormones and nutrients (Elias et al., 1999; Cowley et al., 2001; Aponte et al., 2011; Krashes et al., 2011). While early studies in rodents focused on analyzing the effects of hormones or nutrients on anorexigenic or orexigenic neuropeptide gene expression in the ARC, research interest shifted toward a deeper functional understanding of acute effects of substances like insulin, leptin, ghrelin, or glucose on electrophysiological properties of neuropeptide releasing ARC producing cells (Cowley et al., 2001, 2003a,b; Jobst et al., 2004; Pinto et al., 2004; van den Top et al., 2004; Dhillon et al., 2006; Konner et al., 2007). A number of studies described plasticity of synaptic input patterns as an important component of such changes in neuronal activity (Horvath and Diano, 2004; Pinto et al., 2004; Horvath and Gao, 2005; Sternson et al., 2005; Gao et al., 2007; Benani et al., 2012). For instance, leptin was shown to have a major influence on synaptic organization of orexin neurons in the lateral hypothalamus (Horvath and Gao, 2005), whereas ghrelin was found to impact synapse formation in various extra-hypothalamic brain regions (Abizaid et al., 2006; Diano et al., 2006). Furthermore, high-fat feeding in rodents was also linked to changes in the synaptic input pattern of hypothalamic feeding neurons (Benani et al., 2012), and it was found that rats prone to diet-induced obesity display a completely different pattern of synaptic inputs in the ARC compared to diet-resistant littermates before even being exposed to high-fat feeding (Horvath et al., 2010). In conclusion, there is accumulating evidence that synaptic plasticity induced by metabolic cues plays a pivotal role in determining the function of hypothalamic neuronal circuits regulating appetite.

Neurotrophins are a family of ubiquitously expressed growth factors controlling development, survival, and function of neurons (Hempstead, 2006; Reichardt, 2006) that consist of six members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5), ciliary neurotrophic factor (CNTF), and the glia cell derived neurotrophic factor (GDNF) group of ligands. Upon secretion, which is usually triggered by membrane depolarization (Blochl and Thoenen, 1996; Lessmann et al., 2003), they are capable of mediating many activity-dependent central processes, including neuronal differentiation and growth, synapse formation and plasticity of synaptic input patterns (Park and Poo, 2013). There are two distinct classes of neurotrophin receptors (NTR) that can bind to neurotrophins: p75NTR, which binds to all neurotrophins, and various subtypes of tropomyosin receptor kinase (Trk) receptors, which are each specific for different neurotrophins. Among the neurotrophins, BDNF has gained much attention in the context of energy homeostasis regulation – not for its well described positive effects on synaptogenesis and neuronal plasticity (Unger et al., 2007), but its anorexigenic effect (Rask-Andersen et al., 2011). In humans, a polymorphism in the BDNF gene has been linked to obesity (Hotta et al., 2009; Thorleifsson et al., 2009). Furthermore, BDNF-deficient mice exhibit obesity due to overfeeding (Kernie et al., 2000), a phenotype that is mirrored in mice deficient for the high-affinity BDNF receptor TrkB (Xu et al., 2003). However, to what extent BDNF's anorexigenic effects are mediated by its action on synaptic plasticity or neuronal differentiation remains to be determined. Given the fact that an increasing number of studies indicate that synaptic plasticity in the hypothalamus plays a crucial role in the control of energy homeostasis (Pinto et al., 2004; Yang et al., 2011; Liu et al., 2012), it is very likely that BDNF and other less well characterized neurotrophins might influence hypothalamic feeding network formation and function (Vanevski and Xu, 2013).

In the context of maternal obesity and the offspring's predisposition to obesity and diabetes in later life, the hypothalamus has emerged as a particularly vulnerable organ (Bouret, 2009). Hypothalamic dysfunction in the offspring of overfed or obese mothers has been suggested by several animal studies, predominantly showing the effect of maternal adiposity and diet on hypothalamic neuropeptide and hormone receptor expression or, more recently, reactive inflammation-associated hypothalamic neuronal dysfunction (Cottrell and Ozanne, 2007; Cottrell et al., 2009; Alfaradhi and Ozanne, 2011; Poston, 2012;

**Abbreviations:** (p)AKT, (phosphorylated) RAC-alpha serine/threonine-protein kinase; (p)AMPK, (phosphorylated) 5′-adenosine monophosphate-activated protein kinase; (p)CAMKII, (phosphorylated) $Ca^{2+}$/calmodulin-dependent protein kinase II; (p)ERK, (phosphorylated) extracellular-signal regulated kinases; (p)GSK3beta, (phosphorylated) glycogen synthase kinase 3 beta; (p)JNK1, (phosphorylated) c-Jun N-terminal kinase 1; (p)JNK2, (phosphorylated) c-Jun N-terminal kinase 2; (p)JNK3, (phosphorylated) c-Jun N-terminal kinase 3; (p)p38, (phosphorylated) p38 mitogen-activated protein kinase; ARC, arcuate nucleus of the hypothalamus; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CO, control group; DCX, doublecortin; G, gestational day; GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase; GDNF, glial cell derived neurotrophic factor; GO-terms, gene ontology terms; GTT, glucose tolerance test; HFD, high fat diet; HRP, horseradish peroxidase; IBA1, ionized calcium-binding adapter molecule 1; InsR, insulin receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; LepR, leptin receptor; MAPK, mitogen activated protein kinase; NENF, neuron-derived neurotrophic factor; NGF, nerve growth factor; NPY, neuropeptide Y; NT3, neurotrophin 3; NT4/5, neurotrophin 4/5; P, postnatal day; p75NTR, p75 neurotrophin receptor; PCLgamma, phospholipase C gamma; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3 kinase; POMC, propriomelanocortin; PVN, paraventricular nucleus; TrkA, tropomyosin receptor kinase A; TrkB, tropomyosin receptor kinase B; TrkC, Tropomyosin receptor kinase C; vGAT, vesicular gamma-aminobutyric acid (GABA) transportervGlut2 Vesicular glutamate transporter 2.

Rother et al., 2012; Velloso, 2012). Furthermore, a number of studies suggested stable wiring of hypothalamic feeding networks as an important pathomechanism in hypothalamic dysfunction resulting from increased circulating hormone levels in maternal obesity (Bouret et al., 2004a,b; Pinto et al., 2004; Bouret and Simerly, 2006; Bouret, 2009). Especially, insulin and leptin have been shown to permanently change hypothalamic network formation during the critical time window of perinatal development (Bouret and Simerly, 2006; Ozanne, 2011). However, the role of neurotrophins as a potential link between a metabolically disturbed intrauterine milieu and altered formation of hypothalamic neuronal networks responsible for the regulation of feeding behavior and energy balance has not been addressed to date.

# MATERIALS AND METHODS

## Animal Care

All animal procedures were conducted in compliance with protocols approved by the Committee on the Ethics of Animal Experiments of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Permit number: 37.09.292, RDA number: 84-02.04.2014.A057) and were in accordance with National Institutes of Health guidelines. Mice (C57BL/6N) were bred locally at a designated animal unit of the University Hospital of Cologne (Cologne, Germany). All animals were housed individually and were maintained at 22°C on a 12 h light, 12 h dark cycle. As bedding, spruce granulate (Lignocel FS 14; Rettenmaier & Söhne GmbH, Germany) was provided. Nestlets, mouse smart home and aspen bricks served as enrichment (Plexx B.V., The Netherlands). Animal care and use was performed by qualified individuals and supervised by a veterinarian. The manuscript complies with the Animals in Research: Reporting *in vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010).

Three weeks old female mice were fed a standard chow (#.R/M-H SSniff, Soest containing 41.2% carbohydrates, 19% protein, and 3.3% fat; 9% of calories from fat) or high-fat diet (#C1057 modified containing 26.9% carbohydrates, 21% protein, and 35.1% fat; 60% of calories from fat) for 9–10 weeks preconception and during gestation and lactation, yielding sedentary control (CO; $n = 20$) and high-fat diet (HFD; $n = 21$) group. Male breeders were standard chow-fed and had access to the HFD during mating process (48 h). Male breeders that were in contact with HFD for two times were excluded for further mating. Water and food were available *ad libitum*. Body weight of the dams was monitored daily from G0 to G18. Blood samples for serum analyses were collected 1 week before mating (named G0) and at G15. In order to minimize stress-induced side effects, dams and offspring were not fasted and were collected between 9 and 10o'clock in the morning. After delivery, body weight of the pups was monitored daily starting within 24 h after birth. On P2, litter size was adjusted to six for each litter. Smaller litters were excluded from the experiment (CO $n = 2$, HFD $n = 2$). On P21, male pups were sacrificed non-fasted and blood samples were collected via intracardial puncture for further analyses. Organs were excised. The hypothalamus was cut immediately caudal to the optic chiasm. The dissection was limited laterally by the hypothalamic sulci and dorsally by the mammillothalamic tract (Rother et al., 2012). The remaining brain tissue was also preserved, both epigonadal fat pads were harvested, the weight was determined and all tissues were immediately frozen at −80°C for further analysis. A maximum of two offspring per dam were analyzed to minimize litter-dependent bias. All studies were performed using male offspring. Cohorts used for this study represent a subset of animals that were also used in another study by our group, see Bae-Gartz et al. (2016).

## Analytical Procedures

Serum levels of insulin and leptin were measured by ELISA using mouse standards according to the manufacturer's guidelines (mouse insulin ELISA (EZRMI-13K and EZML-82K) with a sensitivity of 0.01 ng/ml and intra-assay variation of 1.06%; Millipore CorpBillerica, MA, United States).

## Intraperitoneal Glucose Tolerance Test

Glucose tolerance tests (GTT) were performed as previously described (Bae-Gartz et al., 2016). Briefly, animals were fasted for 16 h (18:00–10:00 h). After determination of fasted blood glucose levels, each animal received an intraperitoneal (ip) injection of 20% glucose (10 ml/kg body weight = 2 g glucose/kg body weight). Blood glucose levels were measured after 15, 30, 60, and 120 min.

## Quantitative PCR

Total RNA was isolated from hypothalamus of CO and HFD animals using TRI-Reagent® (Sigma-Aldrich) according to the manufacturer's guidelines. RNA quantity and purity were determined by measuring UV absorption with a Tecan spectrophotometer (Tecan, Nano Quant infinite M200 Pro). Quantitative changes in mRNA expression for genes encoding *Bdnf, Ngf, Nt3, Nt4/5, Cntf, Gdnf, TrkA, TrkB, TrkC, p75Ntr, Prr7, Arc, Egr, Syt1, Homer* were assessed by quantitative real-time PCR as described previously using the 7500 Real-time PCR system (Applied Biosystem, Foster City, CA, United States) or the IQ TM SYBR-Green© Supermix and a BioRad iQ5-Cycler (Bio-Rad Laboratories, Hercules, CA, United States) (Plank et al., 2006; Rother et al., 2012). In all samples, the relative amount of specific mRNA was normalized to at least four ubiquitously expressed housekeeping genes (β-Actin, hypoxanthin-guanin-phosphoribosyltransferase (HPRT), glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH), and β-glucuronidase (GUSB). Oligonucleotides were designed with Primer Express software (Perkin-Elmer, Foster City, CA, United States). Primer pairs and Taqman probes are listed in **Supplementary Table 1**.

## Microarray Analyses of the Hypothalamus

Microarray experiments of mice hypothalamic RNA were performed as single-color experiments using 4_44K Mouse (v2)

Whole Genome Arrays from Agilent Technologies (Santa Clara, CA, United States) as described before (Rother et al., 2012). First, differentially expressed genes between the CO group and HFD group, were identified using the Rank Product test (Breitling et al., 2004). Genes were called significant with a percentage false positive below 0.001. All statistical calculations were performed using R version 3.1.2 and Bioconductor. Functional Annotation Clustering was performed by DAVID Bioinformatics Resources[1].

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE135830[2].

## Western Blot Analysis

Frozen dissected hypothalamic tissue of CO and HFD offspring was homogenized in lysis buffer as previously described (Alejandre-Alcazar et al., 2007). Protein concentration was determined with a BCA-Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). Lysates resolved on a 10% reducing SDS-PAGE gel were transferred to a nitrocellulose membrane. Blots were probed with the following antibodies, see **Supplementary Table 2**. Monoclonal mouse anti-mouse-β-Actin (Cell Signaling, # 3700, 1:1000) and anti-mouse GAPDH (Cell Signaling, # 2118, 1:1000) served as a loading control. Anti-mouse IgG, horseradish peroxidase (HRP)-linked (Cell Signaling, # 7076, 1:2000), and anti-rabbit IgG, HRP-linked (Cell Signaling, # 7074, 1:2000) were used as secondary antibodies.

## Statistical Analysis

Values are shown as means ± standard error of the mean (SEM). The results of realtime RT-PCR were calculated based on the ΔΔCt– method and expressed as fold induction of mRNA expression compared to the corresponding control group (1.0-fold induction). Densitometric analysis of protein bands was performed using Bio-Rad ImageLab software (Bio-Rad, Munich, Germany). Two tailed Mann-Whitney-test was used to test significance of differences between HFD and CO animals at given time points. A $p$-value less than 0.05 was considered significant. The calculations were performed according to a previous agreement with the Institute of Medical Statistics, Informatics and Epidemiology, University of Cologne.

# RESULTS

## Metabolic Phenotype

To assess the metabolic consequences of maternal diet-induced obesity in the offspring, we first determined body weight gain and non-fasted insulin and leptin levels of the dams. Before mating, females on HFD were markedly heavier than controls (**Supplementary Figure 1A**). Maternal diet affected the body weight gain of the dams but at G18 the absolute body weight was similar in both groups (**Supplementary Figure 1A**). Serum insulin levels did not differ on a significant level between CO and HFD dams, but there was a strong insulin increase between G0 and G15 in HFD dams (**Supplementary Figure 1B**). Serum leptin levels revealed a marked increase in HFD dams at G0 and G15 (**Supplementary Figure 1C**). Litter size and sex ratio did not differ between groups (**Supplementary Figures 1D,E**). Portions of dams and offspring characteristics of this cohort of mice have been reported previously (Bae-Gartz et al., 2016; Schmitz et al., 2018). Offspring of obese mouse dams displayed significantly increased body weight and epigonadal fat pad weight at P21 (**Figures 1A,B**). Interestingly, HFD offspring had lower body weights at P1 (**Figure 1A**). Additionally, HFD offspring showed significantly increased non-fasted serum insulin and leptin concentrations at P21 compared to controls (**Figures 1C,D**). To assess the effects of maternal obesity to the offspring's glucose metabolism, GTTs were performed. HFD offspring showed increased blood glucose levels compared with CO offspring 15 min after glucose injection in the intraperitoneal GTT (**Figure 1E**). At all other time points, there were no significant differences detectable.

## Hypothalamic Phenotype

To examine whether maternal obesity also affects hypothalamic insulin and leptin signal transduction, we first quantified hypothalamic insulin and leptin receptor expression. Both, insulin and leptin receptor levels were significantly increased in HFD offspring at P21 (**Figures 2A,B**). Furthermore, we found significantly increased levels of phosphorylated RAC-alpha serine/threonine-protein kinase (pAKT) as well as increased protein levels of glycogen synthase kinase 3ß (pGSK3ß) in HFD offspring indicating robust activation of hypothalamic PI3K signaling at P21 following maternal obesity (**Figures 2C,D**). Consequently, hypothalamic 5′-Adenosine monophosphate-activated protein kinase (AMPK) phosphorylation was significantly decreased in HFD offspring (**Figure 2E**).

Additionally, we assessed hypothalamic protein levels of Ionized calcium-binding adapter molecule 1 (IBA1), a marker for microglial activation and promotion of reactive gliosis, that has been associated with diet-induced obesity and hypothalamic dysfunction in mice with a just recently described role in enhancing orexigenic AgRP expression upon insulin action (Valdearcos et al., 2017; Winkler et al., 2019). Interestingly, hypothalamic IBA1 protein levels were significantly increased in HFD offspring at P21 (**Figure 2F**). POMC and NPY mRNA levels revealed no significant difference between CO and HFD offspring (**Supplementary Figure 2**).

## Hypothalamic Transcriptome Analysis

To evaluate the global hypothalamic gene expression profile accompanying the observed increase in insulin and leptin signaling and microglial activation at P21, we next performed genome-wide gene expression arrays with hypothalamic tissue of CO and HFD offspring (CO $n$ = 5, HFD $n$ = 6). Unsupervised principal component analysis revealed that global gene expression information differed markedly between CO

[1]https://david-d.ncifcrf.gov/

[2]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135830

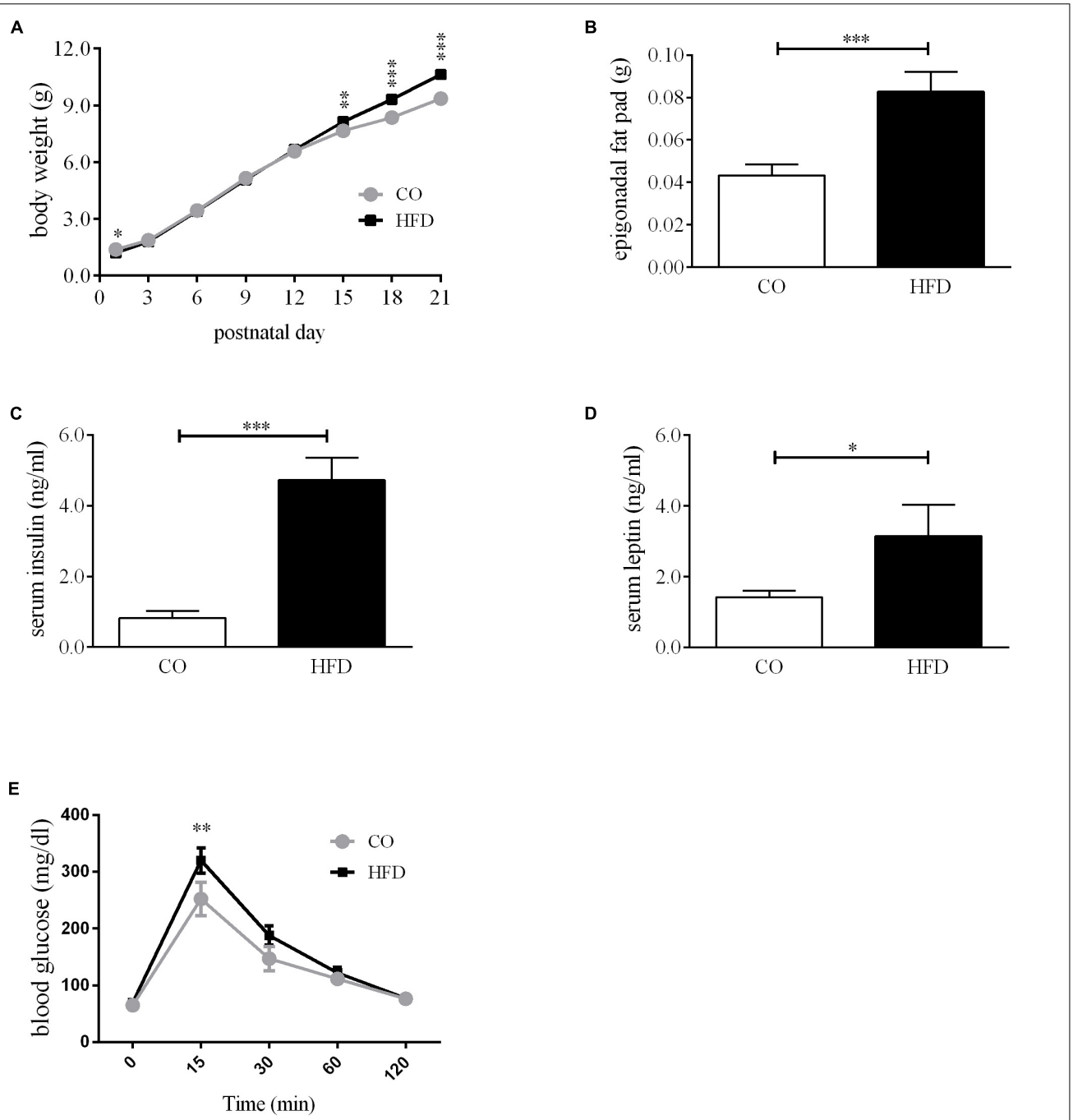


**FIGURE 1 |** Offspring metabolic phenotype at P21. **(A)** Bodyweight gain offspring, $n$ = 49–73. **(B)** Epigonadal fat pad weight, CO $n$ = 35, HFD $n$ = 22. **(C)** Serum insulin levels at P21, CO $n$ = 8, HFD $n$ = 6. **(D)** Serum leptin levels at P21, CO $n$ = 7, HFD $n$ = 6. **(E)** Intraperitoneal glucose tolerance test (GTT) at P21, CO $n$ = 8 HFD $n$ = 7. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; CO, control; HFD, high fat diet; g, gram; p, postnatal day.

and HFD offspring (**Figure 3**). To determine the affected biological processes, we next performed Functional Annotation Clustering of the differentially expressed genes via DAVID analysis (**Supplementary Tables 3, 4**, gene order and probes for differentially expressed genes of hypothalamic tissue at P21 upregulated and downregulated). Interestingly, numerous GO-Terms and KEGG-Pathways related to the field of synaptic plasticity and neuron development ranged among the most prominently enriched functionally related gene groups when comparing HFD and CO offspring (**Table 1**).

## Hypothalamic Neurotrophin Expression

With regard to the microarray results in the hypothalamus of HFD and CO offspring at P21, we first determined the

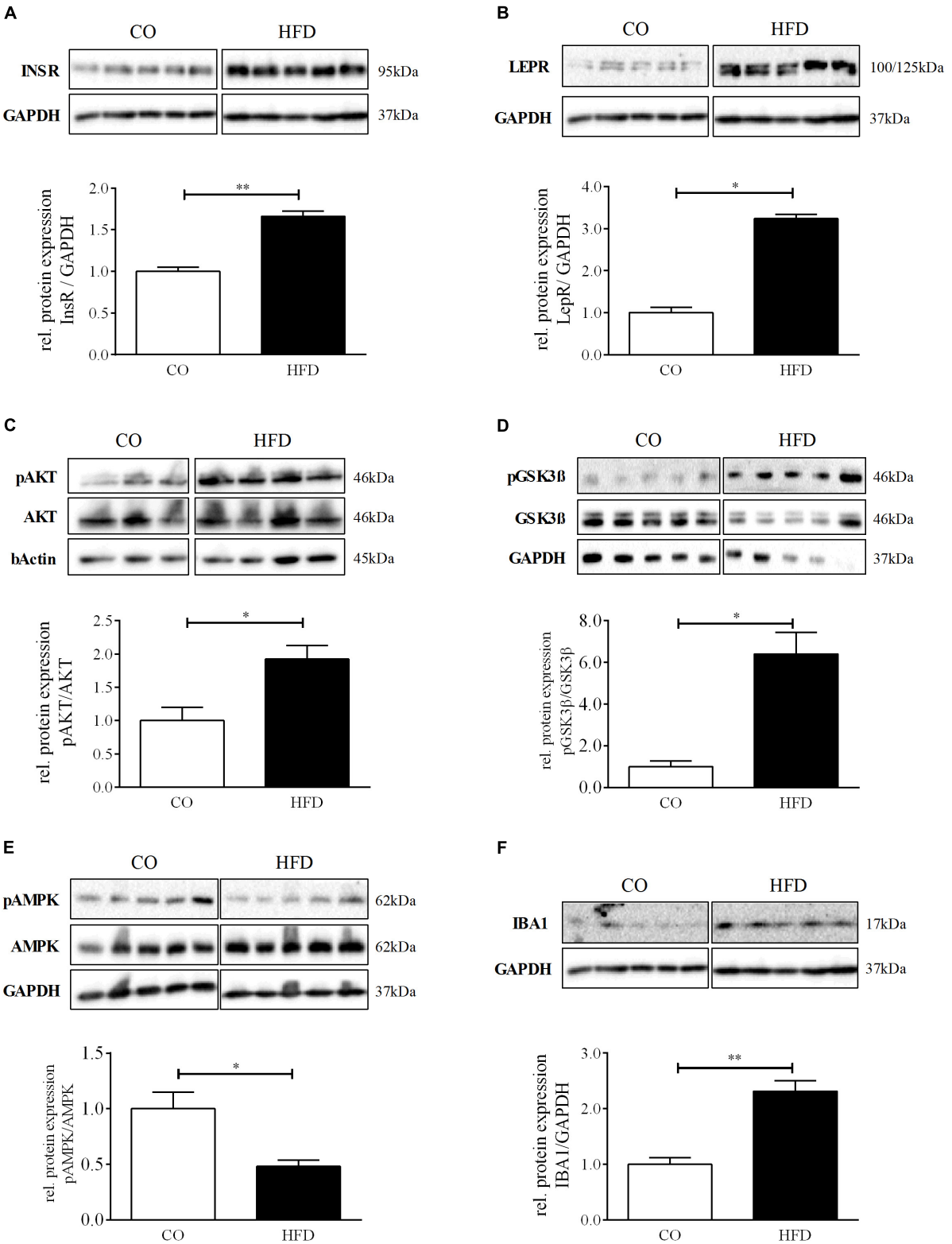


**FIGURE 2 |** Hypothalamic phenotype at P21. **(A–F)** Representative Western Blots of INSR, LEPR, pAKT, pGSK3β, pAMPK, and IBA1 in hypothalamic tissue at P21. **(A–F)** Densitometric analysis of protein expression at P21: **(A)** INSR/GAPDH, CO $n = 5$, HFD $n = 5$. **(B)** LEPR/GAPDH, CO $n = 5$, HFD $n = 5$. **(C)** pAKT/AKT, CO $n = 4$, HFD $n = 4$. **(D)** pGSK3β/GSK3β, CO $n = 5$, HFD $n = 4$. **(E)** pAMPK/AMPK. **(F)** IBA1/GAPDH, CO $n = 5$, HFD $n = 5$. Data are presented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; CO, control; HFD, high fat diet.

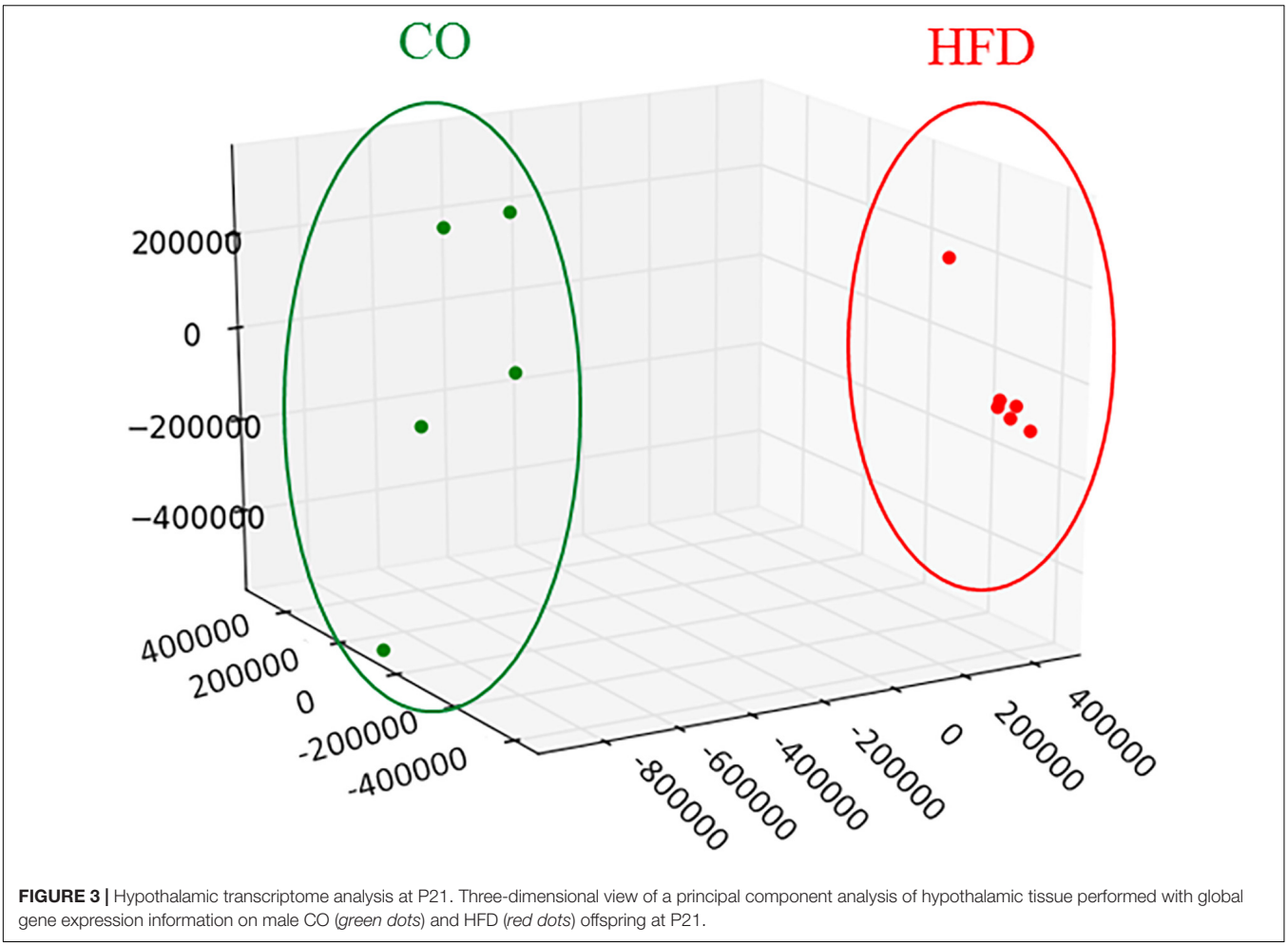


**FIGURE 3 |** Hypothalamic transcriptome analysis at P21. Three-dimensional view of a principal component analysis of hypothalamic tissue performed with global gene expression information on male CO (*green dots*) and HFD (*red dots*) offspring at P21.

mRNA expression of several neurotrophic growth factors (*Bdnf*, *Ngf*, *Nt3*, *Nt4/5*, *Cntf*, and *Gndf*) in the hypothalamus of HFD and CO offspring at P21. Interestingly, we found a significant reduction in gene expression of *Bdnf* (66.7% of control; $p < 0.05$), *Ngf* (56.3% of control; $p < 0.01$) and *Nt4/5* (41.0% of control; $p < 0.01$) in HFD offspring compared to controls (**Figure 4A**). Moreover, there was also a trend toward reduced mRNA expression detectable for Nt3 and Gdnf, with 56.8 and 61.9% of control expression, respectively ($p = 0.07$). In contrast, for Cntf we detected a tendency toward increased gene expression as a result of maternal obesity ($p = 0.10$) (**Figure 4A**).

To determine whether altered hypothalamic gene expression as a result of maternal obesity is not only found for neurotrophic growth factors but also for their receptors, we quantified gene expression of *TrkA*, *TrkB*, *TrkC,* and *p75Ntr* in the hypothalamus of HFD and CO offspring at P21. We found no change in mRNA expression levels of *TrkC*, but significantly reduced gene expression for *TrkA*, *TrkB*, and *p75Ntr* (**Figure 4B**).

mRNA expression patterns of both, neurotrophins and neurotrophin receptors, in the rest of the brain did not reflect the pattern that we found in the hypothalamus (**Figures 4C,D**). However, mRNA expression of *Bdnf* was also found to be significantly reduced ($p = 0.045$) (**Figure 4C**) and *p75Ntr* significantly increased in the rest of the brain (0.023) (**Figure 4D**).

## Hypothalamic Neurogenesis and Synaptic Plasticity

To next evaluate the effects of maternal obesity on hypothalamic neuronal network plasticity, we first determined protein expression of doublecortin (DCX), a specific marker for newborn neurons, in the hypothalamus of HFD and CO offspring at P21. Hypothalamic DCX protein expression was significantly increased in HFD offspring compared to controls indicating enhanced hypothalamic neurogenesis (**Figure 4E**). Supporting this finding, also hypothalamic protein levels of proliferating cell nuclear antigen (PCNA), a marker for DNA synthesis but also DNA repair (Frade and Ovejero-Benito, 2015), was significantly increased in HFD offspring (**Figure 4F**).

We additionally quantified phosphorylation of synapsin, which is known to facilitate the transport of neurotransmitter-filled vesicles to the synapse during an action potential and thereby promotes synaptic plasticity. There was no statistically significant difference between groups (**Figure 5A**). However, we detected significantly reduced protein levels of phosphorylated

**TABLE 1 |** Functional Annotation Clustering for differentially expressed genes between CO and HFD offspring.

| Category | Term | *P*-value | FE |
|---|---|---|---|
| KEGG_PATHWAY | mmu04080:Neuroactive ligand-receptor interaction | 0.002 | 2,323 |
| GOTERM_CC_FAT | GO:0045211~postsynaptic membrane | 0.005 | 2,873 |
| GOTERM_CC_FAT | GO:0045211~postsynaptic membrane | 0.005 | 2,873 |
| GOTERM_CC_FAT | GO:0032589~neuron projection membrane | 0.017 | 14,102 |
| GOTERM_CC_FAT | GO:0031252~cell leading edge | 0.020 | 2,644 |
| GOTERM_BP_FAT | GO:0048812~neuron projection morphogenesis | 0.022 | 2,095 |
| GOTERM_BP_FAT | GO:0048858~cell projection morphogenesis | 0.026 | 1,966 |
| GOTERM_CC_FAT | GO:0044456~synapse part | 0.028 | 2,018 |
| GOTERM_CC_FAT | GO:0030054~cell junction | 0.029 | 1,610 |
| GOTERM_BP_FAT | GO:0048666~neuron development | 0.029 | 1,749 |
| GOTERM_BP_FAT | GO:0060080~regulation of inhib. postsynaptic membrane potential | 0.030 | 10,638 |
| SP_PIR_KEYWORDS | postsynaptic cell membrane | 0.032 | 2,439 |
| GOTERM_BP_FAT | GO:0032990~cell part morphogenesis | 0.037 | 1,873 |
| INTERPRO | IPR006028:Gamma-aminobutyric acid A receptor | 0.040 | 5,183 |
| GOTERM_CC_FAT | GO:0045202~synapse | 0.042 | 1,650 |
| GOTERM_CC_FAT | GO:0031256~leading edge membrane | 0.042 | 8,974 |
| GOTERM_CC_FAT | GO:0042995~cell projection | 0.044 | 1,488 |
| GOTERM_BP_FAT | GO:0060081~membrane hyperpolarization | 0.046 | 8,510 |
| GOTERM_BP_FAT | GO:0007409~axonogenesis | 0.048 | 1,914 |

*Enriched GO-Terms, Interpro and KEGG Pathways after Functional Annotation Clustering by DAVID Analysis. To simplify representation, only key nodes of the GO biological process tree found are shown. CO n = 5, HFD n = 6. GO, Gene Ontology; FE, fold enrichement; BP, Biological Process.*

$Ca^{2+}$/calmodulin-dependent protein kinase II (CAMKII) in HFD offspring at P21, indicating changes in hypothalamic synaptic plasticity and efficacy as a result of maternal obesity (**Figure 5B**). To next evaluate the excitatory and inhibitory synaptic tone in the hypothalamus at P21, we measured hypothalamic protein expression levels of the vesicular glutamate transporter 2 (vGLUT2), a marker for excitatory synapses, and vesicular GABA transporter (vGat), a marker for inhibitory synapses. HFD offspring displayed significantly increased levels of vGLUT2 compared to controls, while vGAT remained unaltered (**Figures 5C,D**), indicating an overall increased excitatory tone in the hypothalamus of HFD offspring.

## Hypothalamic MAPK Signaling

Neurotrophin-associated signaling has been shown to converge on the levels of mitogen activated protein kinase (MAPK) signaling (Thomas and Huganir, 2004), an intracellular cascade closely tied to synaptic plasticity in several parts of the brain (Borrie et al., 2017). To investigate whether intracellular MAPK signaling in the hypothalamus of the offspring might be affected by maternal obesity, we determined total protein amount and phosphorylation of the MAP kinases extracellular signal regulated kinases 1/2 (ERK1/2), p38, c-Jun N-terminal kinase 1 (JNK1), and JNK2/3 in the hypothalamus of offspring of obese and lean mouse dams on P21. The ratio between phosphorylated and total amount of ERK1/2 was significantly reduced to 32.9% ($p = 0.041$) (**Figure 6A**) in the hypothalamus of HFD offspring indicating a decreased hypothalamic activation of the ERK1/2 pathway following maternal obesity. Moreover, for p38 we revealed significant reduction of pp38 in relation to total p38 to 19.8% ($p = 0.002$), indicating reduced activation of the p38 pathway in the hypothalamus of HFD offspring (**Figure 6B**). There were no significant changes in phosphorylation of JNK1 and JNK2/3 in the hypothalamus at P21 (**Figures 6C,D**).

# DISCUSSION

In the present study, we determined the effects of maternal obesity on the offspring's hypothalamic glucose sensing and insulin signaling mechanisms, assessed the global gene expression profile of the hypothalamus, and addressed hypothalamic neurotrophin expression and associated mechanisms of synaptic plasticity at P21.

We were able to describe numerous novel hypothalamic changes in HFD offspring: (a) increased IBA1 expression, (b) a global gene expression pattern indicative of major changes in neurogenesis and plasticity, and (c) reduced neurotrophin expression, changes in neurogenesis and plasticity marker expression as well as reduced MAPK signaling (ERK1/2 and p38).

In detail, we showed that maternal obesity resulted in a massive increase in hypothalamic insulin signaling at P21 and we found increased expression of hypothalamic IBA1, a marker for activated microglia. Activated microglia has been shown to promote reactive gliosis in the hypothalamus (Valdearcos et al., 2017), a mechanism directly affecting hypothalamic connectivity (Dorfman and Thaler, 2015). Just recently, acute insulin administration in mice was shown to induce both, hypothalamic AgRP expression and microglia activation (Winkler et al., 2019) suggesting a role for microglial cells in close vicinity to orexigenic neurons in facilitating the release of hunger signals to counteract hypoglycemia. In light of this hypothesis, the strong and long-lasting increase in hypothalamic insulin signaling in HFD offspring at P21 might be one reason for the observed activation of microglia in order to prevent potential hypoglycemia. This would result in increased sensation of hunger and the promotion of reactive hypothalamic gliosis with all its consequences for formation and function of hypothalamic feeding networks (Thaler et al., 2012; Valdearcos et al., 2017). This interpretation is supported by the finding of Vogt et al., who could show that blockade of insulin signaling in hypothalamic anorexigenic POMC neurons protects the offspring from maternal HFD-induced malformation of POMC neuron projections (Vogt et al., 2014). It is important to note that increased hypothalamic insulin signaling at P21 in our model of maternal obesity seems to precede insulin resistance in the brain and peripheral organs in adult offspring as shown by previous work of our group (Schmitz et al., 2018) and others (Samuelsson et al., 2008; Akyol et al., 2012;

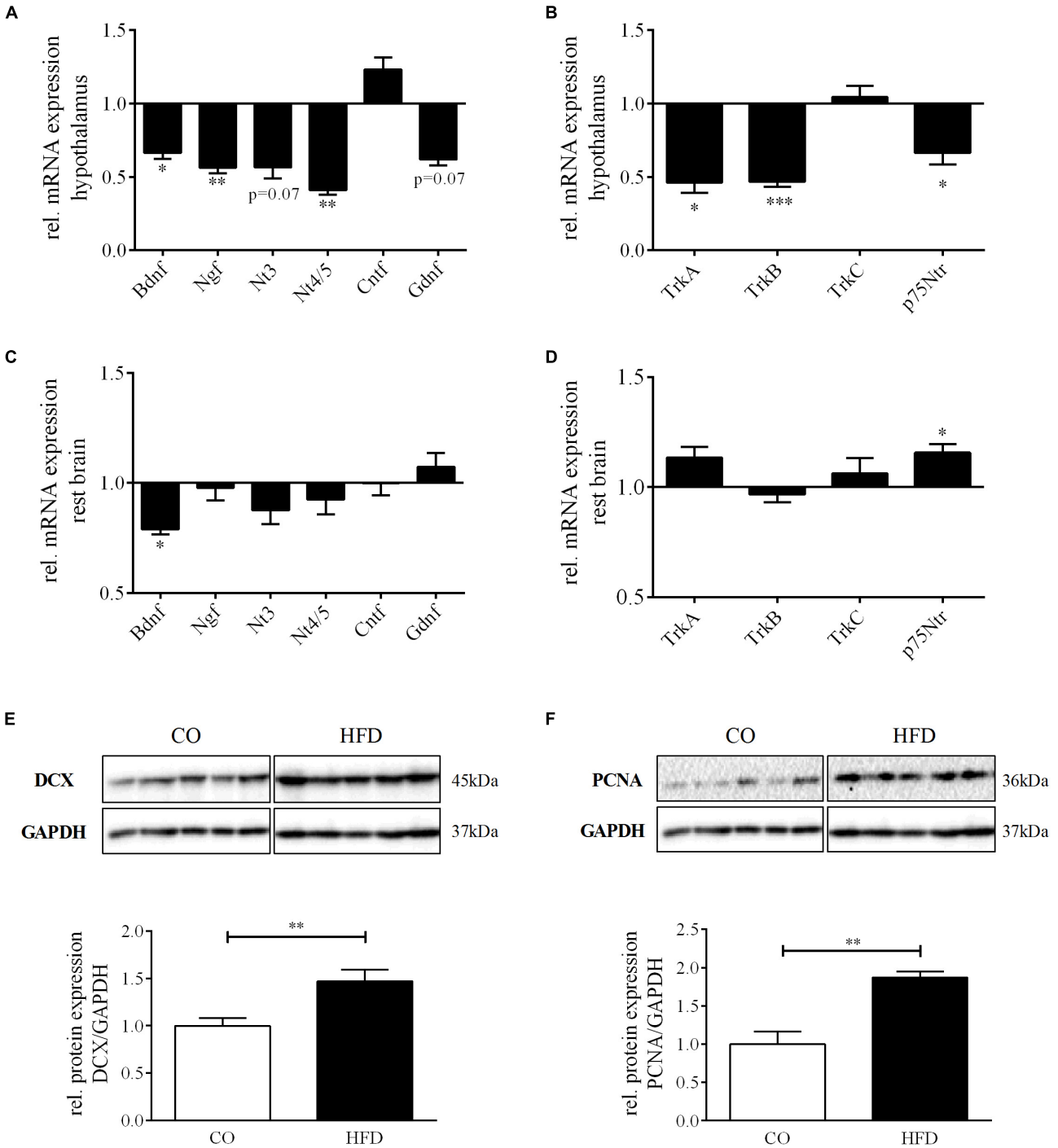


**FIGURE 4 |** Neurotrophin expression and neurogenesis at P21. Relative gene expression compared to controls (= 1.0) at P21, CO $n = 8$, HFD $n = 8$. **(A)** Hypothalamic neurotrophins Bdnf, Ngf, Nt3, Nt4/5, Cntf, and Gdnf and **(B)** hypothalamic neurotrophin receptors TrkA, TrkB, TrkC, and p75Ntr. **(C)** Rest brain neurotrophins Bdnf, Ngf, Nt3, Nt4/5, Cntf, and Gdnf and **(D)** rest brain neurotrophin receptors TrkA, TrkB, TrkC, and p75Ntr. **(E,F),** Representative Western Blots of DCX and PCNA in hypothalamic tissue at P21. **(E,F)**, Densitometric analysis of protein expression at P21: **(E)** DCX/GAPDH, CO $n = 5$, HFD $n = 5$. **(F)** PCNA/GAPDH, CO $n = 5$, HFD $n = 5$. Data are presented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; CO, control; HFD, high fat diet.

Gomes et al., 2018; George et al., 2019). Thus, it is tempting to speculate whether adult-onset hypothalamic insulin resistance might be a functional consequence of increased hypothalamic insulin signaling during early life. With regard to leptin's effect at P21, it is worth mentioning that leptin is known to inhibit AMPK in the arcuate nucleus and reduce food intake (Hardie, 2015).

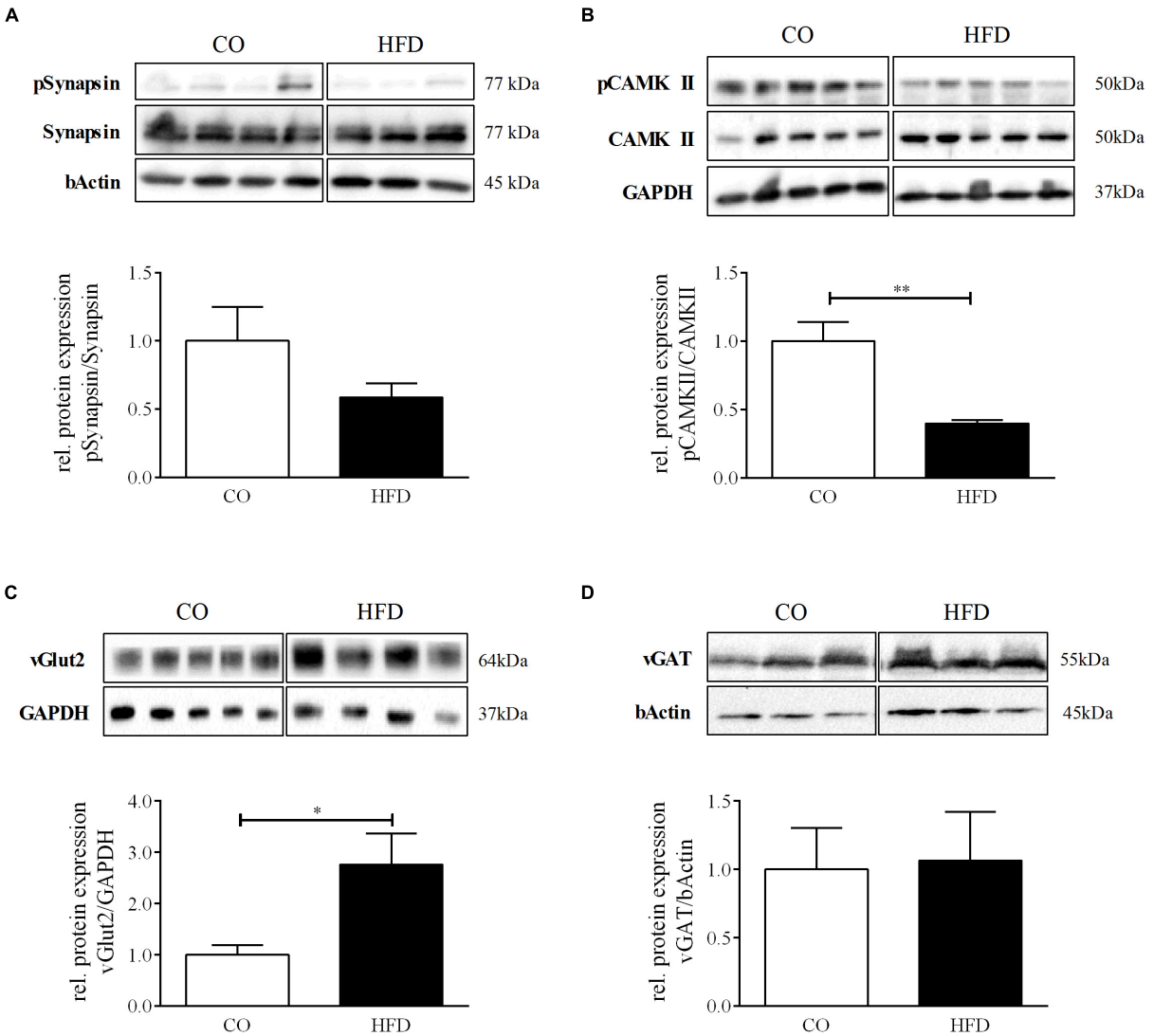


**FIGURE 5 |** Hypothalamic synaptic plasticity at P21. **(A–D)** Representative Western Blots of pSynapsin, pCAMKII, vGlut2, and vGAT in hypothalamic tissue at P21. **(A–D)** Densitometric analysis of protein expression at P21: **(A)** pSynapsin/Synapsin, CO $n = 4$, HFD $n = 4$. **(B)** pCAMKII/CAMKII, CO $n = 4$, HFD $n = 4$. **(C)** vGLUT2/GAPDH, CO $n = 5$, HFD $n = 4$. **(D)** vGAT/GAPDH, CO $n = 3$, HFD $n = 3$. Data are presented as mean ± SEM. $^*p < 0.05$; CO, control; HFD, high fat diet.

Thus, it seems plausible that increased hypothalamic leptin action at P21 reflects the overfed state in HFD offspring. It would, however, be of great interest to attribute the reduction in phosphorylated hypothalamic AMPK found in our model at P21 to a specific cell type to further understand leptin's role in hypothalamic AMPK signaling.

Furthermore, we performed microarray analysis of hypothalamic tissue of CO and HFD offspring at P21. These data revealed significantly different gene expression signatures as a result of maternal obesity. Here we present all differentially expressed genes arising from the comparison of CO and HFD offspring (**Supplementary Tables 3, 4**). Among the most upregulated genes in HFD offspring we found multiple zinc finger proteins. Analyzing the most prominently downregulated genes in HFD offspring, we found amongst others *Map3k10* and *Cntfr*, a member of the MAP kinase family and a neurotrophin receptor. Furthermore, among the affected biological processes by maternal HFD, many GO-Terms referring to neurogenesis, axogenesis, and synaptic plasticity peaked out. By qPCR measurements in hypothalamic tissue, we confirmed that, indeed, HFD offspring displays significantly reduced hypothalamic mRNA expression levels for various neurotrophins and neurotrophin receptors, including *Bdnf* and its receptor *TrkB*. This finding is interesting in several ways. Besides its direct effect on synaptogenesis and neuronal plasticity (Unger et al., 2007), BDNF has been shown to have a clear anorexigenic effect (Molteni et al., 2004; Rask-Andersen et al., 2011). Heterozygous BDNF-knockout mice display hyperphagia with significantly increased body weight and elevated serum leptin and insulin levels along with decreased hypothalamic

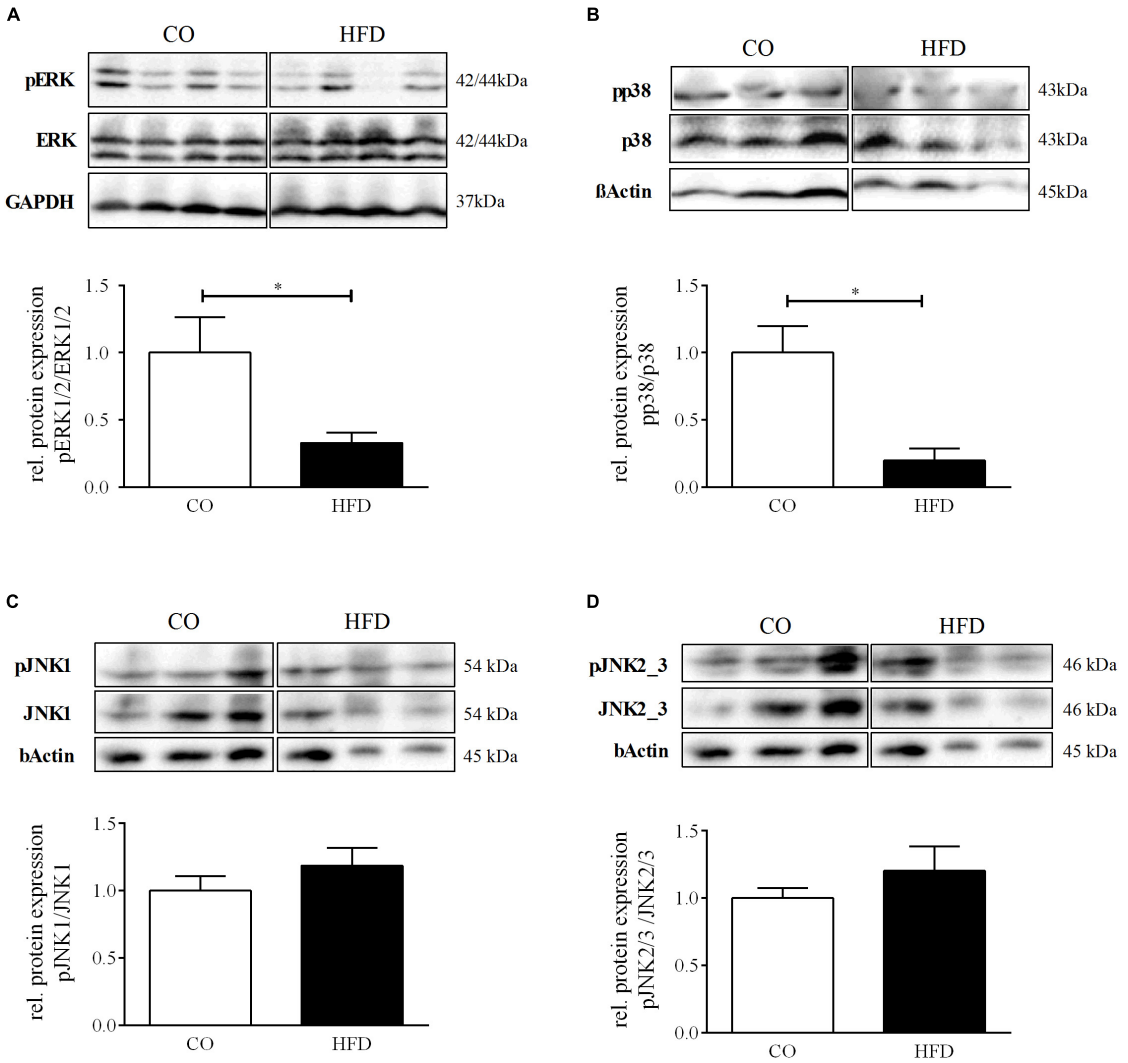


**FIGURE 6 |** Hypothalamic MAP kinases signaling. **(A–D)** Representative Western Blots of pERK, pp38, pJNK1, pJNK2/3 in hypothalamic tissue at P21. **(A–D)** Densitometric analysis of protein expression at P21: **(A)** pERK/ERK, CO $n = 4$, HFD $n = 4$. **(B)** pp38/p38, CO $n = 4$, HFD $n = 4$. **(C)** pJNK1/JNK1, CO $n = 4$, HFD $n = 4$. **(D)** pJNK2/3/JNK2/3, CO $n = 4$, HFD $n = 4$. Data are presented as mean ± SEM. *$p < 0.05$; CO, control; HFD, high fat diet.

*Bdnf* mRNA expression (Kernie et al., 2000). Furthermore, intra-cerebroventricular application of BDNF or other TrkB ligands can reverse this effect and cause significant weight loss (Kernie et al., 2000; Burns et al., 2010; Fox et al., 2013), and BDNF injection specifically into the ventromedial nucleus of the hypothalamus leads to reduced food intake and an increase in energy expenditure (Wang et al., 2007). Moreover, also reduced TrkB receptor expression in the brain of mice leads to increased body weight and food intake (Xu et al., 2003). Keeping all that in mind, it is well conceivable that reduced hypothalamic BDNF signal transduction in offspring of obese mouse dams as indicated by reduced gene expression of both, *Bdnf* and TrkB, might contribute to their predisposition for obesity on P21 and permanently shape hypothalamic feeding networks to metabolic dysfunction. Future studies are needed to prove this hypothesis.

Information on the role of other members of the neurotrophin family and their receptors in energy homeostasis regulation is scarce. However, it is known that intra-cerebroventricular injection of NGF or NT4/5 also causes weight loss in mice (Kernie et al., 2000). NGF acts mainly through the TrkA receptor, whereas NT4/5 has been shown to activate TrkB receptor signaling (Glebova and Ginty, 2005). Thus, the hypothalamic gene expression pattern in the offspring of obese mouse dams in our experiment suggests an overall reduction in hypothalamic TrkA and B signaling following maternal obesity. This could contribute to the significantly reduced activation of the receptor associated MAP kinases ERK1/2 and p38 (Chaves et al., 2013; Wuhanqimuge et al., 2013). Both, ERK1/2 and p38, are sensitive to feeding status and show increased activation upon fasting in the hypothalamus of mice (Ueyama et al., 2004). At the same time, ERK1/2 signaling

plays an important role in synapse formation (Huang and Reichardt, 2001; Alonso et al., 2004; Hans et al., 2004) and participates in long-term synaptic plasticity in hippocampus and sensory neurons (Martin et al., 1997). Various studies indicate that MAPKs are located in synaptic terminals influencing short- and long-term plasticity by phosphorylation of synaptic targets such as synapsin (Jovanovic et al., 2000; Sweatt, 2004; Boggio et al., 2007; Giachello et al., 2010). Thus, a reduction in Trk-mediated MAPK activation along with a reduction in synapsin phosphorylation to approximately 50% following maternal obesity (although not statistically significant) would suggest a link between maternal body weight and both, the offspring's hypothalamic neuronal plasticity and their metabolic phenotype on P21. However, it is important to note that we did only observe a trend toward reduced synapsin phosphorylation in this study.

In addition to the above mentioned MAPK signaling pathways, neurotrophins also activate the phospholipase C gamma (PLCgamma) and the phosphatidylinositol 3-kinase (PI3K) pathways. While we did not address the role of PLCgamma signaling in this study, we determined hypothalamic AKT phosphorylation, an important downstream messenger of PI3K in HFD and CO offspring at P21. In contrast to the reduction in MAPK signaling, we found a massive increase in AKT phosphorylation in the hypothalamus of HFD offspring accompanied by increased phosphorylation of the downstream target GSK3ß. This finding cannot be explained by the observed changes in neurotrophin expression. As mentioned above, however, we found a strong increase in hypothalamic insulin signaling in HFD offspring. Thus, one could speculate that a potential effect of altered neurotrophin signaling is masked by the strong insulin action on hypothalamic cells at this time point. Being aware of the fact that reduced BDNF and NGF serum and tissue levels have been associated with hyperinsulinemic states like obesity and type-2-diabetes (Molteni et al., 2002; Geroldi et al., 2006; Suwa et al., 2006; Bullo et al., 2007; Krabbe et al., 2007; Fujinami et al., 2008; Yamanaka et al., 2008; Golden et al., 2010), even a causal relationship between overactive insulin signaling and decreased neurotrophin levels as part of a negative feedback mechanism is imaginable (Ding et al., 2006). Of course, further experiments will be needed to prove this hypothesis including deeper analysis of functional insulin sensitivity at P21.

The overlap in effects of neurotrophins and other metabolic cues on MAPKs and PI3K might also explain the effects of maternal obesity on expression of markers of synaptic plasticity in the hypothalamus of the offspring. Phosphorylation of synapsin in the hypothalamus shows a tendency toward reduction following maternal obesity along with significantly reduced pCAMKII protein levels. CAMKII is a protein involved in synaptic plasticity, memory and learning and its phosphorylated form is considered constitutively active. CAMKII activity is required for induction of long-term potentiation in the hippocampus and the associated structural plasticity of dendritic spines (Murakoshi et al., 2017). To our knowledge, CAMKII has so far not been assessed in the hypothalamus with regard to network formation of feeding circuits. However, reports on leptin modulating CAMKII activity in the hippocampus *in vitro* in a dose-dependent manner (Vogt et al., 2014) and altered hypothalamic CAMKII activity in diabetic rats (Ramakrishnan et al., 2005b) support our hypothesis that hormones or other metabolic factors might affect hypothalamic spine formation and synaptic activity via CAMKII-mediated processes. Interestingly, reduced hypothalamic CAMKII activity *in vitro* goes along with reduced p38 levels (Ramakrishnan et al., 2005a). And also, ERK1/2 has been functionally linked to synapsin expression *in vitro* (Chen et al., 2018). Both findings might suggest a possible direct connection between reduced MAP kinase signaling and the reduction of expression of both plasticity markers found in the hypothalamus of HFD offspring.

To investigate the overall synaptic tone in the hypothalamus, we set out to quantify vGLUT2 and vGAT protein expression representing excitatory and inhibitory synapses, respectively. While hypothalamic vGAT protein expression did not differ between groups, hypothalamic vGLUT2 protein levels were significantly increased in HFD offspring, indicating an overall increase in excitatory synapses. The ratio of excitatory and inhibitory synaptic inputs to hypothalamic feeding neurons is known to change in response to metabolic hormones, such as leptin and ghrelin (He et al., 2018). Within the hypothalamus, vGlut2 expressing neurons are most abundant in the ventromedial hypothalamic nucleus (VMH) (Tong et al., 2007). Interestingly, mice lacking vGlut2 in the VMH lack the ability to counteract hypoglycemia caused by insulin administration (Tong et al., 2007). Thus, elevated hypothalamic vGLUT2 expression in HFD offspring might directly result from sustained insulin signaling as described before. However, only recently, vGlut2 neurons in the nucleus arcuatus were found to be fast-acting anorexigenic neuronal populations that interconnect peripheral signals like CCK or leptin with other ARC neurons or directly project to the PVN (Guillebaud, 2019). Hypothalamic vGlut2 neurons were shown to be leptin sensitive (Guillebaud, 2019). Thus, increased leptin serum levels, increased hypothalamic leptin receptor protein expression and increased hypothalamic vGLUT2 expression in HFD offspring of our model confirm this recently described functional relationship. As leptin and vGlut2 are both involved in promoting satiety, upregulation of vGlut2 might be a compensatory mechanism of HFD offspring to counteract elevated body weight and fat pad weight at P21. Only future experiments that address the localization of vGlut2 within the different nuclear regions of the hypothalamus can answer the question which of these two conflicting interpretations is adequate or which effect might outweigh the other.

Interestingly, and in contrast to the impression of reduced hypothalamic plasticity marker expression in HFD offspring at P21, hypothalamic markers for proliferation and neurogenesis (DCX and PCNA) were significantly increased. This is in accordance with the only other study that addressed hypothalamic neurogenesis in the context of maternal HFD feeding so far (Chang et al., 2008). Chang et al. elegantly assessed neurogenesis in different hypothalamic nuclei at several time points during lactation and revealed that the effects on

neurogenesis differ significantly between the distinct nuclei, with the most prominent increase in neurogenesis in the periventricular nucleus and the lateral hypothalamus. However, rat dams in their study received HFD only during part of the gestation and did not display an obese phenotype. In contrast, offspring exposure to gestational diabetes was shown to result in the malformation of medio-basal hypothalamic nuclei, which was suggested to be secondary to reduced neuron formation (Harder et al., 2001; Fahrenkrog et al., 2004; Franke et al., 2005; Dearden and Ozanne, 2015). Neurogenesis is generally thought to have a positive effect on network plasticity. However, recent studies reveal that in the hypothalamus, neuronal precursor cells are capable of differentiating into orexigenic or anorexigenic neurons in response to nutritional signals. Hypothalamic neurogenesis may thus act as an adaptive mechanism in order to respond to changes in food supply (Recabal et al., 2017). So, together with insulin's postulated effect on IBA1 and vGlut2 expression in our model, increased neurogenesis (of orexigenic neurons?) in the hypothalamus of HFD offspring might thus also be interpreted as part of the physiologic response to prevent of hypoglycemia. Co-localization of DCX and orexigenic and anorexigenic cell markers would be helpful to test this hypothesis and evaluate whether, indeed, the HFD offspring's neuronal precursor cells are more prone to differentiate into orexigenic neurons.

During pregnancy and lactation, the offspring of obese mothers is inevitably exposed to a variety of altered circulating hormonal and nutritional signals. Especially, the quality of ingested fatty acids seems to be of important matter for brain development and function of the offspring, as ingestion of increased amounts of saturated or trans fatty acids by the mother even in the absence of obesity has been shown to cause hypothalamic dysfunction in mice and rat offspring (Pimentel et al., 2011; Rother et al., 2012). We found reduced hypothalamic neurotrophin signaling following maternal obesity that was induced by ingestion of a diet rich in saturated fatty acids. In a very similar animal model, maternal obesity has been shown to cause BDNF deficiency in the hippocampus and deficits in spatial learning in the offspring (Tozuka et al., 2010). Also, omega-3-fatty acid deficiency during pregnancy and lactation has been shown to alter BDNF-TrkB-signaling in the hypothalamus and cause anxiety-like behavior in the rat (Bhatia et al., 2011). Taken together, hypothalamic neurotrophin signaling might be influenced by the quality and quantity of dietary fatty acids ingested by the dam. This hypothesis is further supported by the fact that also in adult rats, high-fat feeding causes reduced BDNF-signaling in the hippocampus, that can be restored upon reduced dietary fatty acid intake (Woo et al., 2013).

The timeliness of linking diet-induced changes in hypothalamic neurotrophin signaling to alterations in energy homeostasis is further substantiated by a report of Byerly et al. (2013) who identified neuron-derived neurotrophic factor (NENF) as a novel secreted protein in the hypothalamus regulating appetite by interacting with melanocortin signaling. Thus, neurotrophin action in the hypothalamus is gaining more and more attention as an important modulator of hypothalamic control of energy balance – either by directly modulating synaptic network formation and function or by interacting with established pathways of energy homeostasis regulation like the melanocortin system.

We aimed to shed light on the molecular mechanisms responsible for hypothalamic energy balance regulation from a completely new perspective. There are several limitations to our study – including the fact that we were not able to attribute the observed effects to specific nuclear regions of the hypothalamus and that we are lacking a direct mechanistic link between our observations and hypothalamic network formation in our animals. Yet, taken together, there is clear evidence that body weight and feeding status of the mother has an impact on the offspring's hypothalamic neurotrophin signaling and expression of proteins involved in synaptogenesis and neurotransmitter release. Future experiments will have to show whether the observed changes are the underlying mechanism for altered wiring of hypothalamic feeding circuits and might therefore explain the predisposition for disturbed energy homeostasis as a consequence of maternal obesity. Another limitation of the study is that only male offspring were analyzed in the animal model. Some effects of developmental programming on long-term offspring health are gender-specific (Howie et al., 2009; Carter et al., 2012; de Souza et al., 2015). De Souza et al. who recently published studies examining the effects of maternal obesity on the offspring, used only male offspring. Our data is comparable with those studies (Alfaradhi et al., 2016; Frihauf et al., 2016; Liang et al., 2016; Beeson et al., 2018; Berends et al., 2018; Loche et al., 2018).

## CONCLUSION

Taken together, we detected a global gene expression pattern in HFD offspring at P21 that was indicative of major changes in hypothalamic neurogenesis and plasticity. Targeted analysis of hypothalamic neurotrophin expression, neurogenesis and plasticity marker expression, and MAPK signaling (ERK1/2 and p38) confirmed our hypothesis that hypothalamic neurotrophin associated MAPK signaling might contribute to the pathogenesis of the offspring's hypothalamic feeding network dysfunction in maternal obesity. Thereby, we hope to add a puzzle piece to the ongoing search for targets for the prevention and treatment of adult metabolic diseases.

## DATA AVAILABILITY

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE135830[3]. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation,

[3]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135830

to any qualified researcher. Additionally, the raw data is included in the **Supplementary Files**.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Permit Number: 37.09.292).

## AUTHOR CONTRIBUTIONS

Each author has made an important scientific contribution to the study. IB-G, RJ, JD, and EH-R participated in the study design. IB-G and RJ carried out the animal experiments. SB, C-SK, LB, RJ, IB-G, and CV performed the molecular analyses. IB-G, RJ, and EH-R analyzed and interpreted the data. AO performed the microarray analysis. LS, NF, SB, TH, SA, and JD carefully revised the manuscript. EH-R was the guarantor of this work.

## FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (RO 4109/2-1 to EH-R and KFO 2722 to EH-R), the University Hospital of Cologne Rotationsstellen-Pool (Grant No. 9/2015 to IB-G), and the Boll Foundation (Project No. 210-03-17 to EH-R and IB-G).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00962/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 01 October 2019
doi: 10.3389/fnins.2019.01035

# Increased Maternal Prenatal Adiposity, Inflammation, and Lower Omega-3 Fatty Acid Levels Influence Child Negative Affect

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**Specialty section:**
This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience

**Received:** 04 July 2019
**Accepted:** 12 September 2019
**Published:** 01 October 2019

**Citation:**
Gustafsson HC, Holton KF, Anderson AN, Nousen EK, Sullivan CA, Loftis JM, Nigg JT and Sullivan EL (2019) Increased Maternal Prenatal Adiposity, Inflammation, and Lower Omega-3 Fatty Acid Levels Influence Child Negative Affect. Front. Neurosci. 13:1035. doi: 10.3389/fnins.2019.01035

**Objective:** Increased maternal adiposity during pregnancy is associated with offspring risk for psychiatric disorders. Inflammation secondary to adiposity is believed to be an important mechanism through which this effect occurs. Although increased adiposity introduces risk, not all children of overweight mothers develop these problems. Gestational factors that modify this risk are not well-understood. If maternal increased adiposity exerts its effects on offspring outcomes by increasing inflammation in the gestational environment, then anti-inflammatory inputs such as omega-3 fatty acids may be one protective factor. The goal of this study was to investigate whether maternal pre-pregnancy body mass index (BMI) and omega-3 fatty acid levels independently and/or interactively predicted offspring infant negative affect, an early life marker of risk for psychopathology.

**Methods:** Data came from a prospective study of women recruited during pregnancy and their 6 month old infants ($N$ = 62; 40% female). Maternal pre-pregnancy BMI was pulled from medical charts and third trimester omega-3 fatty acid concentrations were assessed in plasma. Child negative affect was assessed using observer- and maternal-ratings at 6 months of age. Maternal inflammation was indexed by third trimester plasma levels of interleukin-6, tumor necrosis factor-alpha, and monocyte chemoattractant protein-1.

**Results:** Maternal pre-pregnancy BMI was associated with increased infant negative affect whereas eicosapentaenoic acid was associated with less infant negative affect. Maternal omega-3 fatty acid levels moderated the effect of BMI on infant negative affect, such that omega-3 fatty acids buffered children against the negative consequences of

increased adiposity. Supporting the role of maternal inflammation in these associations, maternal BMI and omega-3 fatty acid levels interacted to predict maternal third trimester inflammation. Further, maternal inflammation was associated with increased infant negative affect.

**Conclusion:** Results suggest that omega-3 supplementation during pregnancy may protect against offspring behavioral risk associated with increased maternal adiposity.

**Keywords: pre-pregnancy body mass index, omega-3 fatty acids, DHA, EPA, infant temperament, negative affect, inflammation**

## INTRODUCTION

Increased maternal adiposity in the prenatal period introduces risks to maternal and obstetric health (Schmatz et al., 2010; Marshall and Spong, 2012) but also to the developing child (Catalano and Ehrenberg, 2006; Poston, 2012; Menting et al., 2018). The potential public health importance of these risks is exceptional because a significant proportion of pregnant women in the United States are overweight or obese (King, 2006; Huda et al., 2010). Inflammation secondary to adiposity is believed to be an important mechanism through which this effect occurs. Increased adipocyte mass releases a host of inflammatory cytokines, which increase the *in utero* inflammatory profile and alter fetal brain development in ways that contribute to the offspring's long-term mood and behavior (Bilbo and Tsang, 2010; Schmatz et al., 2010). Indeed, higher maternal pre-pregnancy body mass index (BMI) is associated with risk for neurodevelopmental and psychiatric disorders in offspring, including attention-deficit/hyperactivity disorder (ADHD), anxiety, and depression (Rivera et al., 2015; Edlow, 2016). These disorders are thought to be rooted in early development. Their risk appears to be detectable in part by increased behavioral and emotional dysregulation in infancy, for example by increased infant negative affect (NA) (the propensity to feel and express negative emotions, i.e., increased crying, fearfulness, and fear reactivity) (Nigg, 2006; Rothbart, 2007). Few studies, however, have examined early markers of behavioral risk in relation to maternal adiposity. Doing so holds promise to (a) inform our understanding of the developmental mechanisms through which increased maternal BMI shapes offspring risk for future neurodevelopmental and psychological disorders, and (b) provide clarity as to when in development this risk appears and thus can be intervened upon.

Over the past decade, members of our research team have developed a non-human primate model to study the effects of maternal obesity on offspring behaviors that serve as analogs for psychiatric disorders. In this model, adult female macaques are fed either a western-style diet (WSD), comparable in fat content to the average contemporary American diet, or a control diet for at least 2 years prior to pregnancy, throughout pregnancy, and during the lactation period. As expected, the WSD promoted weight gain and increased adiposity in most animals. We discovered a series of long-lasting alterations in the behavior of offspring from mothers consuming the WSD (Sullivan et al., 2010; Thompson et al., 2017). These behavioral alterations, which were primarily in the domain of negative valence systems, were due to both the WSD and to increased maternal adiposity associated with that diet (Thompson et al., 2018). Both male and female offspring showed increased anxiety-like behaviors (Sullivan et al., 2010; Thompson et al., 2017). Supporting the hypothesis that increased inflammation may be part of the mechanism through which these effects occur, we recently reported that maternal pre-pregnancy adiposity was associated with increased gestational inflammation, which in turn was associated with offspring behaviors indicative of anxiety (Thompson et al., 2018).

These results are noteworthy for two reasons. First, they offer proof-of-concept that the effects of maternal obesity on offspring behavioral and emotional dysregulation can be detected early in development. Second, they demonstrate that maternal diet and weight can exert independent effects on offspring behavioral development. The latter is commonly assumed, but not often tested, particularly with regard to offspring behavioral outcomes. Whether these novel components of this research translate to human populations remains relatively untested. The role that specific nutrients such as individual fatty acids (FAs) play in programing offspring behavior is also unclear.

Though maternal obesity appears to place children at risk for psychological and behavioral problems, not all children of overweight or obese mothers develop such difficulties. Given how common maternal obesity currently is, the determination of gestational factors that protect offspring from alterations in neurodevelopment programed by maternal obesity is crucial. If maternal obesity exerts its effects on offspring outcomes by increasing inflammation in the gestational environment (Bilbo and Tsang, 2010; Segovia et al., 2014) then anti-inflammatory inputs, such as circulating omega-3 (n-3) FAs, may ameliorate these effects. For example, the fetus of a woman who is overweight (a pro-inflammatory state) but who has high levels of n-3 FAs (an anti-inflammatory input) may be exposed to less gestational inflammation than one whose mother is overweight and has fewer circulating n-3 FAs. These differences in exposure to inflammation may underlie differences in offspring neurobehavioral outcomes.

Omega-3 FA supplementation effectively reduces inflammation and inflammation-associated sequelae in the context of autoimmune diseases (Simopoulos, 2002; Calder, 2007) as well as several psychiatric conditions (Freeman et al., 2006; McNamara and Carlson, 2006); whether these anti-inflammatory effects extend to inflammation secondary to adiposity has not yet been tested, but is theoretically

plausible. Preliminary research with rodents prenatally exposed to maternal immune activation supports the idea that n-3 supplementation may buffer offspring from the behavioral consequences of such exposure. Offspring exposed to gestational inflammation (induced via maternal immune activation) fed an n-3 supplemented diet exhibited less anxiety than those who did not receive such supplementation (Li et al., 2015).

The goal of the current study was to examine the independent and interactive effects of maternal pre-pregnancy BMI and circulating n-3 FAs on infant NA, an early life risk factor for psychopathology. To align our human study with those we previously conducted in non-human primates, we focus on indicators of infant fear and sadness (dimensions of NA thought to underlie the development of anxiety) assessed at 6 months of age (to align with the non-human primate studies).

Our research questions were: (1) *Are maternal pre-pregnancy BMI and maternal third trimester circulating n-3 FAs associated with infant NA at 6 months of age?* (2) *Are these effects independent of one another?* (3) *Do maternal pre-pregnancy BMI and maternal circulating n-3 FAs interact to predict infant NA?* We hypothesized that maternal pre-pregnancy BMI and gestational n-3 FAs would be independently associated with infant NA (such that increased BMI would be associated with more NA and higher levels of gestational n-3 FAs with less NA). Additionally, we hypothesized that BMI and n-3 FAs would interact to predict infant NA, with higher n-3 FAs serving as a protective factor.

As described, maternal BMI and FAs are purported to be linked with infant NA via alterations in maternal systemic inflammation during pregnancy. An explicit test of this mechanism was outside of the scope of the current study (largely due to this study's relatively small sample size and its incompatibility with the complexity of the statistical model that would be needed to test this mechanism explicitly). However, we did conduct initial analyses, to provide additional context for our findings and preliminary proof of concept.

# MATERIALS AND METHODS

## Procedure

Data used in this study came from an ongoing longitudinal pilot study described previously (Sullivan et al., 2015). Pregnant women were recruited in the second trimester and followed into the postpartum period. Data used in the current analyses came from laboratory visits that occurred at 37 weeks gestation and at 6 months postpartum. During the 37-week visit, women provided a blood sample and completed questionnaire measures of their mood and demographic information. When their child was 6 months old, mother-infant dyads completed a laboratory visit that included several behavioral assessments. Women also completed questionnaires about their infant's behavior. This laboratory visit was approximately 90 min and was videotaped for later coding. The testing room was equipped with a high chair, a chair for the mother, and two cameras (one focused on the infant, one on the mother). Participants' medical records were reviewed for information about maternal health, including pre-pregnancy BMI. Oregon Health and Science University's (OHSU) Institutional Review Board approved all procedures, and written informed consent was obtained from all participants.

## Participants

### Recruitment

Pregnant women were recruited from OHSU during their second trimester. In enrolling participants, an effort was made to over-select families likely to have offspring with a full range of emotion and behavioral regulation difficulties to enable study of offspring regulation in relation to multiple dimensions relevant to risk. This was done by over-selecting families where one or more of the biological parents had a past/current diagnosis or current elevated symptoms of ADHD, defined as $>80^{th}$ percentile on the Barkley Adult ADHD Rating scale (BAARS-IV) Quick Screen (Barkley, 2011) (79% of families met these criteria). ADHD symptoms were chosen as a psychopathology proxy for dysregulation liability because ADHD has very high heritability (Larsson et al., 2014) and in addition to evidence of offspring experiencing symptoms such as impulsivity and hyperactivity, they are also likely to have difficulties in emotion regulation (Sullivan et al., 2015). We enrolled 62 women who were followed through 68 pregnancies. After enrolling in the study with the first child, six women conceived a second child and completed the same assessments for their second pregnancy, resulting in complete data on six sibling pairs. The nesting of children within families was handled in our statistical analyses (described in *Analytic Strategy*).

### Exclusion Criteria

Exclusion criteria included high-risk or medically complicated pregnancy, extreme life circumstances (specifically, homelessness), being <18 years old, and active substance use (including alcohol, tobacco, marijuana, opioids, cocaine). Six women enrolled in the study were taking psychotropic medication during pregnancy and were excluded from the current analyses because of *a priori* concern that this medication use might confound results. In *post hoc* sensitivity analyses, we re-ran our models with these women included; the same pattern of findings emerged.

## Measures

### Maternal Pre-pregnancy BMI

Participants' medical records were reviewed for maternal pre-pregnancy BMI ($kg/m^2$).

### Maternal Psychological Distress

A potential alternative explanation for any inflammation related findings would be elevated maternal stress or her own NA, which could be an alternative source of inflammation or indicate potential genetic transmission of NA proneness from mother to child. At the third trimester visit, mothers completed the Center for Epidemiologic Studies Depression Scale (CES-D) (Radloff, 1977) ($\alpha = 0.92$) and the Perceived Stress Scale (PSS) (Ezzati et al., 2014) ($\alpha = 0.91$). Maternal scores on the CES-D and the PSS were highly correlated ($r = 0.84$), so they were converted to z scores

and then averaged to create a composite variable. This variable was used as a covariate in our multivariate models. Mothers also completed the CES-D at 6 months ($\alpha = 0.94$); these scores were included in sensitivity analyses.

### Maternal Circulating FAs

Maternal circulating FAs were assessed using blood samples collected in the third trimester. Blood was drawn by venipuncture, centrifuged, and plasma was separated, aliquoted, and frozen at −80°C until assay. Plasma FAs were analyzed by direct transesterification using a Trace GC coupled to a DSQ mass spectrometer (ThermoElectron) as described previously (Lagerstedt et al., 2001). In addition to examining the total n-3 score [the sum of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid concentrations], we also separately examined the two most bioactive FAs: DHA and EPA. These two FAs are particularly important for fetal brain development (Innis, 2008; Coletta et al., 2010).

### Maternal Ratings of Infant NA

When children were 6 months old, mothers completed the Revised Infant Behavior Questionnaire (IBQ-R) (Gartstein and Rothbart, 2003). Mothers were asked to rate how often their child completed various behaviors over the previous week on a seven-point scale (1 = *never* to 7 = *always*). In our models, we examined the Sadness ($\alpha = 0.86$) and Fear ($\alpha = 0.84$) subscales.

### Observational Measures of Infant NA

The well-established still face paradigm (Tronick et al., 1979; Moore et al., 2009) was used to measure the infant's reaction to the mother's lack of emotional response. The mother placed the infant in a high chair and sat in a chair an arm's length away. The mother was instructed to play with the infant for 2 min as she normally would. The mother was then instructed to turn her face away from the infant for 15 s and then turn back to face the infant for the still face period (2 min), during which the mother maintained a neutral and expressionless face and did not interact with the child. The mother was then instructed to look away again for 15 s and then return to interacting and playing with the infant as she normally would for an additional 2 min. If the infant became persistently upset, the still face period was stopped early, and the pair moved into the reunion period. Pacifiers and other toys were not allowed during this task. Five infants' data were coded as missing because the mother interacted with the infant during the still face period; individuals who interacted with their infant during this task did not differ from the rest of the sample on any of the variables included in the current study.

Two observers, blind to the study hypotheses, coded each behavioral assessment video using a modified version of a published coding scheme (Moore et al., 2009). Infant behavior was coded in increments of 5 s for four mutually exclusive behavioral categories: affective expression, gaze, vocalization, and reactive/regulatory behavior. Infant behaviors were coded through four separate viewings of the video: one for each of the behavioral categories. In each 5 s task epoch, affective expression was coded as Positive, Negative, Neutral, or Obscure. The number of epochs rated as Negative was divided by the total number of task epochs to derive a score which captured the percentage of time the child expressed NA. Vocalization was coded as None/Not Negative or Negative. Again, the number of task epochs rated as containing negative vocalizations was divided by the total number of task epochs to derive a percentage of negative vocalizations score. **Table 1** provides operational definitions for each of these behavioral categories. Infant behaviors were coded through four separate viewings of the video: one for each of the behavioral categories.

Inter-observer reliability was determined by analyzing percentage agreement. All of the videos were assessed for agreement between the two independent coders. The mean percentage agreement between coders was greater than 85% for all behaviors.

### Maternal Gestational Inflammation

Maternal inflammation was assessed using blood samples collected during the third trimester of pregnancy. Plasma samples were assayed for concentrations of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). These cytokines were selected because they index the nuclear factor-κappa B signaling pathway (Liu et al., 2017) which has been associated with obesity (Baker et al., 2011). Sample collection and assay followed published procedures (Gustafsson et al., 2018), as follows.

Blood was drawn by one-time venipuncture into $K_2$ EDTA tubes (BD Vacutainer Systems, Franklin Lakes, NJ, United States). The blood was then centrifuged at 2800 rpm for 15 min at 4°C. Plasma was separated, collected, aliquoted, and frozen at −80°C until assayed.

Plasma concentrations of IL-6 were measured by enzyme-linked immunosorbent assays (Human IL-6 quantikine HS ELISA kits (HS600B; assay range: 0.2–10 pg/ml, sensitivity: 0.11 pg/ml), R&D Systems, Inc., Minneapolis, MN, United States) according to the manufacturer's instructions. All standards and samples were run in duplicate. Plasma samples were initially diluted 1:2 using Assay Diluent RD1-75 and incubated for 2 h at room temperature on a horizontal orbital microplate

**TABLE 1 |** Operational definitions for behavioral coding.

| Measure | Behavior | Description |
|---|---|---|
| Affective expression | Positive | Infant's corners of mouth raised and/or cheeks raised. |
| | Negative | Infant's brows may be sharply lowered and eyes may be tightly closed. This code includes anger, sadness, and frustration. |
| | Obscure | Infant's mouth or face hidden from view for the entire interval. |
| | Neutral | Infant displaying a relaxed face with no obvious muscle tension. |
| Vocalization | None/not negative | Infant is not vocalizing or vocalizations are not indicative of being fussy or upset. This code includes silence, cooing, laughing, babbling, coughing, and sneezing. |
| | Negative | Infant is displaying negative communication such as fussing, crying, screaming (i.e., if upset or angry), and other expressions of mild fussiness. |

shaker. Following standard wash procedures, human IL-6 HS Conjugate was added to each well, and plates were incubated as described above. Plates were then washed and incubated with the Substrate Solution (60 min), Amplifier Solution (30 min), and Stop Solution. Plates were read within 30 min of adding the Stop Solution using a microplate spectrophotometer (Benchmark Plus microplate, Bio-Rad Laboratories, Inc., Hercules, CA, United States). Plasma concentrations of TNF-α (assay range: 5.3–3,900 pg/ml, sensitivity: 1.5 pg/ml) and MCP-1 (assay range: 32.0–23,500 pg/ml, sensitivity: 0.47 pg/ml) were assayed using Luminex polystyrene bead-based multiplex immunoassays (customized Luminex Performance Human Obesity Panel, FCST08-05; R&D Systems) according to the manufacturer's instructions. All standards and samples were run in duplicate. Plasma samples were initially diluted 1:2 using the matrix solution provided, and samples were incubated overnight at 4°C with color-coded beads that were pre-coated with cytokine-specific capture antibodies. The plates were then washed by vacuum filtration, incubated with biotinylated detection antibodies (1 h, room temperature), washed, and incubated with phycoerythrin-conjugated streptavidin (30 min, room temperature). Plates were read on the dual-laser, flow-based Luminex 100 Analyzer (Luminex, Austin, TX, United States). For both the ELISAs and multiplex assays, sample values were determined based on standard curves calculated using computer software to generate four- and five-parameter curve-fits, respectively (Prism 7 for Windows, GraphPad Software, Inc., La Jolla, CA, United States). Cytokine values were log transformed to correct for non-normality prior to analysis, although raw cytokine values (in pg/ml) are presented in **Table 2** for ease of interpretation.

Prior to conducting our sensitivity analyses, we conducted a confirmatory factor analysis (CFA) to examine whether it was appropriate to consider IL-6, TNF-α, and MCP-1 values together as a latent variable (simultaneously considering multiple indices of maternal inflammation—and in this case multiple indices of the NF-κB signaling pathway–in this manner is intended to yield a more reliable and comprehensive assessment of maternal systemic inflammation than any single cytokine value). Results from this CFA (results available by request from the authors) supported the construction of a latent variable indicated by the three pro-inflammatory cytokines, IL-6, TNF-α, and MCP-1; this latent variable was used in all supplemental inflammation models.

**TABLE 2 |** Sample demographics and descriptive statistics ($N$ = 62).

| | Mean (SD) or% | Median (IQR) | Range |
|---|---|---|---|
| Maternal age, years | 30.4 (5.0) | 30.0 (27.1–34.0) | 18–41.3 |
| Paternal age, years | 33.3 (6.3) | 32.0 (28.8–37.0) | 23–50 |
| Maternal education[a] | 6.8 (1.2) | 7.0 (5.0–8.0) | 5–9 |
| Paternal education[a] | 6.6 (1.6) | 7.0 (5.0–8.0) | 3–9 |
| Child sex,% female | 40% | | |
| % Taking omega-3 fatty acid supplement | 40% | | |
| % Breastfeeding at 6 months | 94.7% | | |
| *Child race* | | | |
| European American | 76.8% | | |
| Asian | 5.4% | | |
| African American | 5.4% | | |
| Hispanic | 5.4% | | |
| Native American | 3.6% | | |
| Biracial | 3.6% | | |
| Maternal pre-pregnancy BMI | 27.2 (7.1) | 25.0 (23.0–30.4) | 17–49 |
| *Maternal omega–3 fatty acids* | | | |
| Total Omega-3 fatty acids (nmol/ml) | 518.6 (170.9) | 524.0 (385.1–632.6) | 204.6–859.1 |
| DHA (nmol/ml) | 331.0 (116.5) | 316.7 (243.8–425.9) | 131.2–559.7 |
| EPA (nmol/ml) | 50.0 (44.7) | 37.7 (25.9–54.1) | 16.9–250 |
| *Maternal psychological distress* | | | |
| Maternal perceived stress | 15.6 (7.5) | 15.5 (8.0–22.0) | 3–31 |
| Maternal depressive symptoms | 15.7 (11.2) | 13.0 (7.4–21.0) | 0–53 |
| *Infant negative affect* | | | |
| Still face paradigm negative behavior | 21.2 (26.2) | 10.0 (0–44.5) | 0–88 |
| Still face paradigm negative vocalizations | 22.8 (32.8) | 8.0 (0–39.8) | 0–100 |
| IBQ–R sadness | 3.7 (0.9) | 3.6 (3.0–4.5) | 2.1–5.2 |
| IBQ-R fear | 2.4 (0.7) | 2.4 (1.8–2.9) | 1.3–3.8 |
| *Maternal inflammation* | | | |
| IL-6 (pg/ml) | 1.6 (0.8) | 1.5 (1.1–2.0) | 0.4–3.6 |
| TNF-α (pg/ml) | 11.3 (3.2) | 10.8 (8.9–13.5) | 5.7–17.9 |
| MCP-1 (pg/ml) | 93.0 (26.5) | 88.4 (78.0–113.0) | 26.4–171.5 |

[a] *1 = Grade School, 2 = Some High School, 3 = High School Equivalent, 4 = High School degree, 5 = Some College but no degree, 6 = Associates degree, 7 = Bachelor's degree, 8 = Masters, Law, 2–3 years degree, 9 = Doctorate, Ph.D., Medical degree. BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IBQ-R, Revised Infant Behavior Questionnaire. IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; MCP-1, monocyte chemoattractant protein-1. At the 3rd trimester visit, women self-reported all supplements that they were consuming, including supplements that contain omega-3 fatty acids. At 6 months postpartum, women reported whether they were currently breastfeeding their child.*

## Analytic Strategy

Our research questions were tested using M*plus* 7.4 (Muthén and Muthén, 1998–2017) and the robust maximum likelihood estimator which can accommodate non-normal data. Missing data were handled using full information maximum likelihood, a missing data technique that uses all available information to produce estimates that are less biased and more efficient than those produced via other methods of handling data that are missing at random (Collins et al., 2001). Auxiliary variables were included to maximize precision of the missing data matrix (Graham, 2003). These auxiliary variables were maternal and paternal ADHD symptoms (assessed using the BAARS-IV Quick Screen), (Barkley, 2011) age (years), and education (highest completed; 1 = *Grade School*, 9 = *Doctorate*). Non-independence of observations (i.e., the nesting of children within families) was handled using the M*plus Cluster* command.

We first estimated a series of univariate models wherein each infant behavioral measure was regressed on (a) maternal

pre-pregnancy BMI, (b) total n-3s, (c) DHA, and (d) EPA. Each infant behavior measure was considered in its own model. Uncorrected *p*-values are reported for ease of comparison with other studies and because corrected *p*-values elevate Type II error in this small study; however, family-wise error was controlled and adjusted *p*-values reported also. A Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) was applied to account for multiple comparisons due to moderate correlation among outcomes tested.

Next, we estimated a series of regression analyses where maternal pre-pregnancy BMI and each measure of circulating FAs were considered in the same model, while simultaneously controlling for maternal third trimester psychological distress.

To test whether maternal n-3 FAs moderated the effect of maternal pre-pregnancy BMI on infant NA, we added an interaction term which captured the interaction between maternal BMI and the particular measure of FAs to the previous model. Based on our theoretical interest in factors that might ameliorate the effect of BMI on infant outcomes we *a priori* decided to examine moderation only for infant behavioral measures that were associated with BMI in the univariate models. In these models, each infant behavior measure was regressed on maternal pre-pregnancy BMI, the particular measure of maternal FAs, maternal psychological distress, and the interaction between maternal BMI and circulating FAs. All continuous variables were mean-centered prior to model estimation.

Omega-3 fatty acid levels were treated as a continuous variable in all analyses. However, to visualize significant interaction effects, we employed the pick-a-point method and probed this interaction using methods described by Preacher et al. (2006). The pick-a-point approach involves selecting three values of your moderator and plotting the model-implied regression lines for these three values, as a way to visually illustrate the nature of the interaction.

## Analyses Examining Inflammation

As described in the "Introduction," section an explicit test of inflammation as a mechanism through which BMI and FA interactively influence infant NA was outside of the scope of this study. However, we did conduct the following analyses, to provide additional context for our findings and preliminary proof of concept.

First, we examined whether, in this sample, maternal BMI and FA levels were associated with maternal inflammation during pregnancy. In separate models, we regressed the maternal third trimester inflammation latent variable on (a) maternal pre-pregnancy BMI, (b) total n-3s, (c) DHA, and (d) EPA. Next, we examined whether maternal BMI and fatty acid levels interacted to predict maternal inflammation. Specifically, we regressed maternal inflammation on maternal BMI, fatty acid levels, and an interaction term that captured the interaction between the particular measure of FAs (again, total n-3s, DHA, and EPA were examined in separate models). All continuous variables were mean-centered prior to model estimation. Significant interactions were probed as above.

In a separate set of univariate models, aimed at examining whether maternal inflammation was related to infant NA, we regressed each infant behavioral measure on maternal inflammation.

# RESULTS

## Descriptive Statistics

Demographic data about this sample as well as means (SDs) and medians (interquartile range) for the study variables appear in **Table 2**. None of the demographic variables presented in this table were significantly correlated with infant NA ($ps > 0.06$) and thus were not included as covariates in analyses.

## Relating BMI, Fatty Acids, and Infant NA

**Table 3** presents the results from the univariate models in which each measure of infant NA was successively regressed on maternal pre-pregnancy BMI and each measure of circulating FAs. Increased maternal pre-pregnancy BMI was associated with greater infant negative behavior during the still face paradigm ($\beta = 0.45$, corrected $p = 0.016$). Additionally, EPA was associated with less mother-reported sadness ($\beta = -0.56$, corrected $p < 0.001$) and fear ($\beta = -0.47$, corrected $p < 0.001$), as well as less negative behavior ($\beta = -0.38$, corrected $p = 0.004$) and negative vocalizations ($\beta = -0.36$, corrected $p = 0.004$) during the still face paradigm. DHA and the total n-3 FA score were not significantly associated with infant NA.

## Independent Effects of BMI and FAs, Covarying for Maternal Psychological Distress

These associations remained similar when maternal pre-pregnancy BMI was considered in a model with each measure of circulating FAs and with maternal psychological distress during the third trimester (**Table 4**). Maternal pre-pregnancy BMI remained significantly associated with infant negative behavior during the still face after covarying for maternal psychological distress and total n-3 FAs ($\beta = 0.39$, $p = 0.027$) as well as psychological distress and DHA ($\beta = 0.36$, $p = 0.036$) but was no longer significant when considered in a model with EPA and psychological distress ($\beta = 0.31$, $p = 0.140$), though the effect size was similar in magnitude. Maternal EPA was associated with maternal rated infant sadness ($\beta = -0.61$, $p < 0.001$) and fear ($\beta = -0.53$, $p < 0.001$) as well as infant negative vocalizations during the still face paradigm ($\beta = -0.30$, $p = 0.037$).

In sensitivity analyses, we examined whether these effects survived controlling for maternal depressive symptoms at 6 months (included in the model in place of maternal third trimester psychological distress). Each of these effects survived this control.

## Moderation Analyses

The results from the moderation analyses appear in **Table 5**. Based on our *a priori* decision to only investigate moderation for infant outcomes that were associated with maternal BMI in bivariate analyses, these models were focused on infant negative behavior during the still face paradigm.

**TABLE 3 |** Results of univariate models where each infant negative affect measure was regressed on maternal pre-pregnancy BMI and omega-3 fatty acid concentrations.

| | IBQ-R sadness | | | IBQ-R fear | | | Still face paradigm negative behavior | | | Still face paradigm negative vocalizations | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | β(SE) | *p*-value | B-H corrected *p*-value | β(SE) | *p*-value | B-H corrected *p*-value | β(SE) | *p*-value | B-H corrected *p* value | β(SE) | *p*-value | B-H corrected *p*-value |
| Pre-pregnancy BMI | 0.06 (0.15) | 0.682 | 0.712 | −0.10 (0.17) | 0.566 | 0.669 | 0.45 (0.16) | 0.004 | 0.016* | 0.34 (0.20) | 0.095 | 0.222 |
| Omega-3 fatty acids | −0.36 (0.19) | 0.064 | 0.170 | −0.28 (0.18) | 0.111 | 0.222 | −0.17 (0.16) | 0.282 | 0.404 | −0.18 (0.17) | 0.286 | 0.404 |
| DHA | −0.32 (0.17) | 0.052 | 0.156 | −0.21 (0.17) | 0.216 | 0.370 | −0.09 (0.17) | 0.585 | 0.669 | −0.08 (0.19) | 0.670 | 0.712 |
| EPA | −0.56 (0.12) | 0.000 | < 0.001** | −0.47 (0.12) | 0.000 | < 0.001** | −0.38 (0.12) | 0.001 | 0.004** | −0.36 (0.11) | 0.001 | 0.004** |

*$p < 0.05$, **$p < 0.01$. B-H corrected p-value = Benjamini-Hochberg corrected p-value. BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IBQ-R, Revised Infant Behavior Questionnaire.*

**TABLE 4 |** Results from multivariate models that consider both pre-pregnancy BMI and Omega-3 fatty acid concentrations, and covary for maternal psychological distress.

| | IBQ-R sadness | | IBQ-R fear | | Still face paradigm negative behavior | | Still face paradigm negative vocalizations | |
|---|---|---|---|---|---|---|---|---|
| | β(SE) | *p*-value | β(SE) | *p*-value | β(SE) | *p*-value | β(SE) | *p*-value |
| Pre-pregnancy BMI | −0.13 (0.15) | 0.401 | −0.28 (0.19) | 0.130 | 0.39 (0.14) | 0.027* | 0.40 (0.22) | 0.065 |
| Omega-3 fatty acids | −0.32 (0.20) | 0.107 | −0.30 (0.17) | 0.081 | 0.02 (0.16) | 0.883 | −0.09 (0.18) | 0.599 |
| Maternal psychological distress | 0.27 (0.13) | 0.041 | 0.29 (0.15) | 0.048 | 0.24 (0.14) | 0.084 | −0.22 (0.15) | 0.123 |
| Pre-pregnancy BMI | −0.05 (0.14) | 0.722 | −0.22 (0.17) | 0.187 | 0.36 (0.17) | 0.036* | 0.39 (0.21) | 0.068 |
| DHA | −0.29 (0.17) | 0.091 | −0.19 (0.16) | 0.225 | 0.04 (0.18) | 0.815 | −0.04 (0.20) | 0.831 |
| Maternal psychological distress | 0.25 (0.13) | 0.056 | 0.30 (0.15) | 0.041 | 0.26 (0.14) | 0.066 | −0.20 (0.15) | 0.167 |
| Pre-pregnancy BMI | −0.20 (0.13) | 0.114 | −0.33 (0.15) | 0.026 | 0.31 (0.19) | 0.140 | 0.33 (0.22) | 0.142 |
| EPA | −0.61 (0.13) | < 0.001** | −0.53 (0.12) | < 0.001** | −0.21 (0.14) | 0.093 | −0.30 (0.14) | 0.037* |
| Maternal psychological distress | 0.27 (0.12) | 0.025 | 0.28 (0.13) | 0.031 | 0.21 (0.13) | 0.110 | −0.22 (0.14) | 0.118 |

*$p < 0.05$, **$p < 0.01$. BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IBQ-R, Revised Infant Behavior Questionnaire.*

Results suggest that n-3 FAs moderate the effect of maternal pre-pregnancy BMI on infant negative behavior during the still face paradigm. As is visually depicted in **Figure 1**, for individuals who had average levels of circulating n-3 FAs (defined as our sample mean), there was a positive association between increased maternal pre-pregnancy BMI and greater infant NA ($\beta_{\text{Maternal Pre-pregnancy BMI}} = 0.33$, $p = 0.02$). However, the BMI-infant NA association was weaker for individuals who had higher circulating levels of n-3 FAs ($\beta_{\text{interaction}} = -0.41$, $p = 0.007$). This is supportive of our hypothesis that n-3 FAs may buffer children against the negative impact of increased maternal pre-pregnancy BMI. See **Figure 1** for a visual depiction of this finding. The models that considered EPA and DHA separately yielded the same pattern of results, suggesting that this moderation effect was not specific to only one of these n-3 FAs.

**TABLE 5 |** Results of moderation analyses investigating the interaction between maternal pre-pregnancy BMI and omega-3 fatty acids in the prediction of infant negative affect.

| | Still face paradigm negative behavior | |
|---|---|---|
| | β(SE) | *p*-value |
| Pre-pregnancy BMI | 0.33 (0.14) | 0.018* |
| Omega-3 fatty acids | −0.13 (0.14) | 0.380 |
| Maternal psychological distress | 0.07 (0.17) | 0.663 |
| BMI × omega-3 fatty acids | −0.41 (0.15) | 0.007** |
| Pre-pregnancy BMI | 0.29 (0.11) | 0.009** |
| Third trimester DHA | −0.09 (0.15) | 0.557 |
| Maternal psychological distress | 0.16 (0.14) | 0.259 |
| BMI × DHA | −0.42 (0.14) | 0.002** |
| Pre-pregnancy BMI | −0.02 (0.23) | 0.016* |
| Third trimester EPA | −0.80 (0.33) | 0.922 |
| Maternal psychological distress | 0.17 (0.13) | 0.183 |
| BMI × EPA | −0.56 (0.22) | 0.012* |

*$p < 0.05$, **$p < 0.01$. BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.*

## Inflammation Analyses: Preliminary Proof of Concept

As expected, increased maternal pre-pregnancy BMI was associated with greater maternal inflammation during the third trimester of pregnancy (β = 0.88, $p < 0.001$), while total n-3

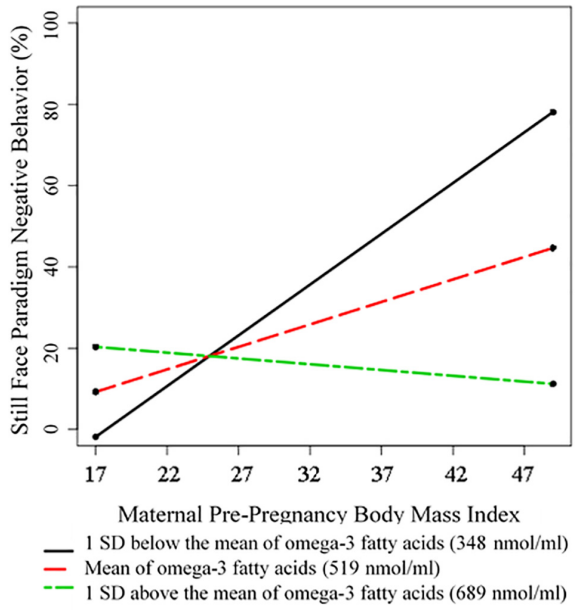


**FIGURE 1 |** Visual Depiction of the Interaction between Maternal Pre-Pregnancy BMI and Omega-3 Fatty Acids in Predicting Infant Negative Behavior during the Still Face Paradigm. Omega-3 fatty acid levels were treated as a continuous variable in all analyses. However, for the purposes of visualization, we employed the pick-a-point method and probed this interaction following the direction of Preacher et al. (2006). The pick-a-point approach involves selecting three values of your moderator and plotting the model-implied regression lines for these three values, as a way to visually illustrate the nature of the interaction (it does not affect the moderation analyses, where levels of omega-3 fatty acid concentrations were treated as a continuous variable). Following convention, in this figure we have plotted the model-implied slopes that correspond to our sample mean, one standard deviation above our sample mean, and one standard deviation below our sample mean of circulating omega-3 fatty acid concentrations.

FAs ($\beta = -0.48$, $p = 0.021$), EPA ($\beta = -0.32$, $p = 0.011$), and DHA ($\beta = -0.47$, $p = 0.008$) were associated with less maternal inflammation. Further, maternal pre-pregnancy BMI and total n-3 FA levels interacted to predict maternal inflammation ($\beta = -0.39$, $p = 0.026$); the positive association between increased maternal pre-pregnancy BMI and maternal inflammation is strongest for women who have low n-3 FA levels, and is weaker for those who have higher levels. The maternal BMI by DHA interaction term ($p = 0.529$) and the BMI by EPA interaction term ($p = 0.270$) were not statistically significant (these were considered in separate models), suggesting that total n-3 FAs are more influential in this context.

Maternal inflammation was significantly associated with maternal report of infant sadness ($\beta = 0.88$, $p < 0.001$), but was not significantly associated with the other measures of infant NA ($ps > 0.411$).

## DISCUSSION

The goal of the current study was to examine whether maternal pre-pregnancy BMI and circulating n-3 FAs during the third trimester of pregnancy independently and/or interactively predicted infant NA, an early risk factor for psychopathology. Though increased maternal prenatal adiposity and n-3 FA intake have each been linked with psychopathology in older children, this study's focus on an infant marker of such risk is novel and has potential to inform our understanding of the developmental pathways through which gestational factors influence long-term risk. Study results suggest that there are both independent and interactive associations. To our knowledge, this is the first human study to report evidence that n-3 FAs may modify the effect of increased adiposity on infant outcomes.

Results from our univariate models suggest that greater maternal pre-pregnancy BMI is associated with greater observer ratings of infant NA, a finding that is consistent with our research in non-human primates (Thompson et al., 2017, 2018). Additionally, we found that greater EPA concentrations are associated with less NA, as rated by both mothers and independent observers. This same general pattern of findings emerged when pre-pregnancy BMI and n-3 FAs were included in the same model along with maternal third trimester psychological distress, a potential confounder of this association. However, several of these effects did not survive correction for multiple comparisons. The protective effect of EPA on maternal report of infant NA (both fear and sadness) appears to be the most robust of these associations. Omega-3 FAs are important during pregnancy, as they support optimal placental functioning and are required for healthy fetal growth and brain development, particularly during the third trimester (Greenberg et al., 2008; Chavan-Gautam et al., 2018). Their protective effects on fetal growth and child cognitive development have been well-documented (Innis, 2007; Greenberg et al., 2008), though this is the first study to link maternal n-3 FA concentrations during pregnancy with measures of infant emotional development.

Interestingly, EPA emerged as a particularly consistent predictor of infant NA, as compared to either DHA or total n-3 FAs. Specifically, mothers who had high levels of circulating EPA tended to have children who cried less and were less fearful and sad. Much of the existing literature examining the effects of n-3 FAs on offspring development has focused on the effects of DHA; though, as mentioned above, they have largely investigated cognitive, rather than emotional, correlates of maternal nutrition. Previous research examining the efficacy of n-3 supplementation for the treatment of adult depression suggests that EPA may be more efficacious for improving depressive symptoms than DHA (Parker et al., 2006; Martins, 2009). Infant NA is an early life risk factor for depression; thus maternal EPA consumption may be an important protective factor for offspring emotional health.

The current study also found evidence of interactive effects, such that the effect of maternal pre-pregnancy BMI on observer ratings of infant NA was moderated by maternal n-3 FA concentrations during pregnancy. Specifically, we found that the association between increased maternal pre-pregnancy BMI and increased observer ratings of infant NA was weaker for women who had higher circulating n-3 FA concentrations. That is, higher n-3 FAs appear to buffer children against the negative consequences of increased maternal pre-pregnancy

BMI. These effects do not appear to be specific to a particular type of n-3 FA as we observed the same pattern of findings when examining DHA and EPA. In our preliminary proof of concept analyses, we found evidence that this reduction in risk may be due to the interactive effect of maternal BMI and n-3 FAs on maternal inflammation during the third trimester. Specifically, we found that the association between increased maternal BMI and increased infant NA was weaker when women had higher n-3 FA levels; greater maternal prenatal inflammation, in turn, was association with greater infant NA. These findings, if replicated, suggest that n-3 supplementation during pregnancy may help reduce children's NA early in life (and by extension, reduce their risk for later psychopathology), particularly among children whose mothers are overweight or obese. Omega-3 supplementation is a promising intervention target as it is safe and easier to achieve adherence with than weight loss or dietary modifications, which are often ineffective (Jeffery et al., 2000; Mann et al., 2007). Moreover, weight loss is not typically recommended during pregnancy even among obese women (Kapadia et al., 2015), making this a less promising avenue for intervention.

This study had several strengths. First, utilizing prospective longitudinal data, this is one of the first human studies to examine the effects of increased maternal adiposity on infant emotional development. Though previous research has linked increased maternal prenatal adiposity with risk for psychopathology in older children, few studies have considered these factors in relation to infant markers of risk. Additionally, as described above, the existing literature examining the effects of maternal n-3 FAs on child development has focused on child cognition or motor development, making our assessment of infant emotional development unique. Methodological strengths included consideration of multiple assessment methods for infant NA as well as our inclusion of a measure of circulating n-3 FAs obtained from plasma samples (which yields a more objective measure of maternal dietary intake than self-report measures, and more accurately reflects fetal exposure to n-3 FAs).

This study also had limitations. The present findings are preliminary due to the small sample size. Though our use of longitudinal data was a strength of this study, we only examined three time points of data. Future research should incorporate additional prenatal and postnatal assessments. Additionally, we utilized data from a unique sample of women that was over-represented for women prone to emotional dysregulation (i.e., those with a diagnosis of ADHD or endorsing high levels of ADHD symptoms were over-sampled), and although this increased our power to detect effects, it also leaves us unable to generalize these findings to a general population sample. However, this study does justify larger studies. Although we have strong reason to believe that increased maternal adiposity and n-3 FAs may influence the developing child via their effect on the *in utero* inflammatory profile, the current study did not explicitly test for mediation or moderated-mediation (as would be necessary to test this hypothesis), nor did it assess inflammation in amniotic fluid, which might yield a more direct measure of child cytokine exposure. This study's relatively small sample size limited our ability to test such a complicated model, though our supplemental inflammation analyses provide preliminary proof of concept and support that this may be the mechanism of influence. Specifically, we found that the effect of maternal pre-pregnancy BMI on maternal inflammation was strongest for women who had lower levels of n-3 FAs and was weaker for women who had higher levels of n-3 FA levels. Additionally, we found that maternal inflammation was associated with maternal report of infant sadness, though it was not related to the other measures of infant NA. These null findings may reflect a lack of power to detect such an effect, differences in the context and the types of NA-related behaviors that maternal and laboratory observations capture, or the numerous potential contributors by which maternal obesity influences infant neurobehavioral development including alterations in cytokines not examined in the current study, exposure to increased nutrients such as glucose and saturated fatty acids, and alterations in metabolic hormones (Rivera et al., 2015). Though preliminary, we believe that these results complement the results of previous research that has shown that fatty acids can, directly and indirectly, influence the expression of adipokines (cytokines secreted by adipose tissue), as well as the production of cytokines from other cell types (Drevon, 2005). Further, these results are in alignment with *in vitro* experiments with human macrophages and hepatocytes which indicate that EPA can reduce cytokine expression in the context of induced inflammatory response (Hao et al., 2010). Future research will need to investigate and clarify such biological mechanisms as they relate to the current findings.

In summary, the current study is the first human study to investigate the independent and interactive effects of maternal pre-pregnancy BMI and circulating n-3 FAs during pregnancy on infant NA, an early life risk factor for psychopathology. Results indicate that EPA has a particularly robust association with decreased infant fear and sadness according to maternal report. Further, we provide novel evidence that n-3 FAs during pregnancy may modify the effects of increased maternal pre-pregnancy BMI on observer ratings of infant NA. Specifically, we found that n-3 FAs buffer infants against the negative consequences of increased maternal adiposity.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Oregon Health & Science University Institutional Review Board. Written informed consent to

participate in this study was provided by the participants and/or the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

ES and JN conceived the project. HG, ES, JN, KH, and JL designed the research. AA, EN, and CS performed the experiments. HG, JN, and ES analyzed the data. All authors discussed the data. HG and ES wrote the manuscript, with contributions from all other authors.

## FUNDING

Research reported in this publication was supported by the Abracadabra Foundation, the Bob and Charlee Moore Institute for Nutrition & Wellness, and by the National Institutes of Health under National Institute of Mental Health award numbers R3759105 (JN) and R01 MH117177 (ES and JN) and the National Center for Advancing Translational Sciences award number TL1TR002371 (HG). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## ACKNOWLEDGMENTS

This material is the result of work supported with resources and the use of facilities at the VA Portland Health Care System and OHSU, Portland, OR, United States. JL is an employee of the U.S. Department of Veterans Affairs. Contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 02 October 2019
doi: 10.3389/fnins.2019.01045

# Pre- and Post-natal High Fat Feeding Differentially Affects the Structure and Integrity of the Neurovascular Unit of 16-Month Old Male and Female Mice

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**Specialty section:**
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

**Received:** *28 June 2019*
**Accepted:** *17 September 2019*
**Published:** *02 October 2019*

**Citation:**
*Contu L, Nizari S, Heath CJ and Hawkes CA (2019) Pre- and Post-natal High Fat Feeding Differentially Affects the Structure and Integrity of the Neurovascular Unit of 16-Month Old Male and Female Mice. Front. Neurosci. 13:1045. doi: 10.3389/fnins.2019.01045*

Compelling experimental and clinical evidence supports a role for maternal obesity in offspring health. Adult children of obese mothers are at greater risk of obesity, diabetes, coronary heart disease and stroke. These offspring may also be at greater risk of age-related neurodegenerative diseases for which mid-life obesity is a risk factor. Rodent diet-induced obesity models have shown that high fat (HF) diet consumption damages the integrity of the blood–brain barrier (BBB) in the adult brain. However, there is currently little information about the effect of chronic HF feeding on the BBB of aged animals. Moreover, the long-term consequences of maternal obesity on the cerebrovasculature of aged offspring are not known. This study determined the impact of pre- and post-natal HF diet on the structure and integrity of cerebral blood vessels in aged male and female mice. Female C57Bl/6 mice were fed either a 10% fat control (C) or 45% HF diet before mating and during gestation and lactation. At weaning, male and female offspring were fed the C or HF diet until sacrifice at 16-months of age. Both dams and offspring fed the HF diet weighed significantly more than mice fed the C diet. Post-natal HF diet exposure increased hippocampal BBB leakiness in female offspring, in association with loss of astrocyte endfoot coverage of arteries. Markers of tight junctions, pericytes or smooth muscle cells were not altered by pre- or post-natal HF diet. Male offspring born to HF-fed mothers showed decreased parenchymal GFAP expression compared to offspring of mothers fed C diet, while microglial and macrophage markers were higher in the same female diet group. In addition, female offspring exposed to the HF diet for their entire lifespan showed more significant changes in vessel structure, BBB permeability and inflammation compared to male animals. These results suggest that the long-term impact of prenatal HF diet on the integrity of cerebral blood vessels differs between male and female offspring depending on the post-natal diet. This may have implications for the prevention and management of age- and obesity-related cerebrovascular diseases that differentially affect men and women.

**Keywords: maternal obesity, aged, high fat diet, cerebrovascular, sex difference**

## INTRODUCTION

Rates of obesity have risen consistently over the past three decades (Ng et al., 2014) in association with increasingly sedentary lifestyles and consumption of diets that are high in saturated fat (Corella et al., 2011; Phillips et al., 2012). Global obesity of women aged 20 years and older is currently estimated to be around 30%, with prevalence close to or over 60% in some countries (Ng et al., 2014). Rates of maternal obesity are also on the rise (Kim et al., 2007; Heslehurst et al., 2010; Gregor et al., 2016), due to both greater numbers of obese pregnant women and excess weight gain during pregnancy (Siega-Riz and Gray, 2013; Lindberg et al., 2016).

The developmental origins of health and disease (DoHAD) hypothesis posits that fetal adaptations in response to the early life environment have long-term consequences on health and alter the relative risk of developing diseases in later life. In particular, it is suggested that alterations made during the prenatal period to promote fetal wellbeing can become maladaptive if there is a mismatch between the pre- and post-natal environments (Wadhwa et al., 2009). There is now a significant body of clinical and experimental evidence supporting an influence of maternal obesity on the health of adult offspring, including higher body-mass index (Hochner et al., 2012; Eriksson et al., 2015) and increased risk of coronary heart disease, diabetes, stroke, asthma and premature death (Reynolds et al., 2013; Parlee and MacDougald, 2014; Godfrey et al., 2017). However, few studies have examined the impact of maternal obesity on offspring health beyond middle age.

Age is a major risk factor for the development of neurodegenerative diseases in which there is cerebrovascular dysfunction, including stroke, vascular dementia and Alzheimer's disease (AD) (Sweeney et al., 2018). Modifiable conditions such as diabetes, hypertension and obesity also increase the risk of developing these diseases (Kivipelto et al., 2002; Reitz and Mayeux, 2014). The prevalence of stroke and AD is higher in aged women than men and the risk is increased after the onset of menopause (Persky et al., 2010; Lisabeth and Bushnell, 2012).

Cerebral blood vessels are composed of endothelial cells, basement membrane proteins, pericytes, smooth muscle cells, astrocytes and neurons that are collectively referred to as the neurovascular unit (NVU). The NVU is characterized by the expression of the blood–brain barrier (BBB) which is formed and maintained by tight junctions, pericytes and astrocytes and acts as a barrier to the unregulated entry of peripheral components into the brain (Daneman and Prat, 2015). Breakdown of the BBB is a major complication of cerebrovascular accidents and may contribute to the pathophysiology of AD (Daneman and Prat, 2015).

Reports from animal studies suggest that high fat diet consumption causes damage to the BBB, including increased leakiness, downregulation of tight junctions and cytoskeletal proteins and loss of pericyte coverage in brain areas such as the hypothalamus and hippocampus (Kanoski et al., 2010; Davidson et al., 2012; Pallebage-Gamarallage et al., 2012; Hargrave et al., 2016; Hajiluian et al., 2017; Mamo et al., 2019; Salameh et al., 2019). Loss of BBB integrity in late life has also been associated with mid-life obesity in humans (Gustafson et al., 2007). There is also evidence to suggest that BBB damage is exacerbated in aged mice fed a HF diet (Tucsek et al., 2014a,b). However, no studies have examined the effect of chronic (e.g., >1 year) high fat feeding on the cerebrovasculature of aged animals. The purpose of this study was to determine the long-term impact of pre- and post-natal high fat feeding on the structure and integrity of the NVU and BBB in 16-month old male and female mice.

## MATERIALS AND METHODS

### Animal Model

Proven female C57Bl/6 breeders were fed either a control (C, 10% kcal fat, 20% kcal protein, 70% kcal carbohydrate, $n$ = 11) or high fat (HF, 45% kcal fat, 20% kcal protein, 35% kcal carbohydrate; Special Diet Services, United Kingdom, $n$ = 11) diet for 4 weeks before mating and during gestation and lactation. Diets were isocaloric and matched for amino acid, macro minerals and vitamin composition (**Supplementary Table S1**). Studs were maintained on the C diet. At weaning, male and female offspring were assigned either the C or HF diet, generating four experimental groups ($n$ = 9/group/sex): C/C, C/HF, HF/C, HF/HF representing the pre- and post-weaning diet. All offspring were maintained on the diet until sacrifice at 16 months of age, but underwent food restriction (to approximately 90% free feeding weight) for 3 months at 6- and 12-months of age as part of a separate behavioral study (**Supplementary Figure S1A**). At sacrifice, weight-to-length ratio was calculated by dividing body weight (g) by nasal-anal distance (cm). Gonadal fat pad weight was also recorded for the offspring. All experiments were reviewed and approved by the Open University Animal Welfare and Ethics Review Board and the Home Office as per the UK Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012 (PPL 70/8507).

### Western Blotting

Mice were deeply anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 0.01 M phosphate buffered saline (PBS). Brains were removed immediately, dissected into individual regions and snap frozen. Hippocampal tissues from C/C, C/HF, HF/C and HF/HF mice ($n$ = 4/group/sex) were homogenized in Ripa lysis buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Igepal, 50 mM NaF, 1 mM NaVO3] containing a protease inhibitor cocktail (Merck Millipore, Watford, United Kingdom), spun down (13,000 g, 10 min, 4°C) and supernatants were frozen at –80°C until further use. Proteins (10–45 μg) were separated by gel electrophoresis on 4–20% Tris–glycine or 10% Tris–tricine gels (Fisher Scientific, Loughborough, United Kingdom) and transferred onto a nitrocellulose membrane. Membranes were incubated overnight at 4°C with primary antibodies against markers of the NVU (**Table 1**). Blots were stripped and re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:50,000, Sigma-Aldrich) antibody to ensure equal protein loading. Two blots were replicated for each antibody. Immunoblots were quantified by densitometry using ImageJ

**TABLE 1 |** Antibodies used in Western blotting (WB) or immunohistochemistry (IH) to label the NVU.

| NVU Protein | Antibody | Dilution | Source |
|---|---|---|---|
| Endothelial cell | Anti-CD31 | 1:100 | BD Biosciences, Wokingham, United Kingdom |
| Tight junction | Anti- zona occludens-1 (ZO-1) Anti-claudin 5 | 1:300 1:500 | Abcam, Cambridge, United Kingdom Fisher Scientific, Loughborough, United Kingdom |
| Pericyte | Anti-platelet-derived growth factor receptor-β (PDGFRβ) | 1:200 | R&D Systems, Abingdon, United Kingdom |
| Smooth muscle cell | Anti-α smooth muscle actin (α-SMA) Anti-α-SMA-FITC | 1:350 (WB) 1:350 (IH) | Sigma-Aldrich, Poole, United Kingdom Sigma-Aldrich, Poole, United Kingdom |
| Basement membrane | Anti-laminin | 1:500 (WB) 1:350 (IH) | Sigma-Aldrich, Poole, United Kingdom |
| Astrocyte | Anti-glial fibrillary acidic protein (GFAP) | 1:5000 (WB) 1:1000 (IH) | Agilent Technologies, Stockport, United Kingdom Abcam, Cambridge, United Kingdom |

(NIH, MD, United States) and calculated as an optical density ratio of protein levels normalized to GAPDH levels.

## Immunohistochemistry and Staining

Mice were perfused intracardially with 0.01 M PBS followed by 4% paraformaldehyde ($n$ = 5/group/sex). Brains were sectioned on a cryostat (20 μm thickness), collected in a free-floating manner and stored at −20°C. For single-labeling immunohistochemistry, tissue sections were washed in 0.01 M PBS, blocked with 3% hydrogen peroxide and 15% normal goat serum (Sigma-Aldrich) and incubated with anti-ionized calcium binding adaptor molecule 1 (Iba1, 1:500, Alpha Labs, Eastleigh, United Kingdom), potato lectin (*Solanum tuberosum*, 1:500, Vector Labs, Peterborough, United Kingdom) or biotinylated anti-mouse (1:500, Vector Labs). Sections were incubated with anti-rabbit (1:400, Vector Labs) and/or avidin-biotin complex (1:200, Vector Labs) and developed using glucose oxidase enhancement with DAB as the chromogen.

For Iba1 and CD68 co-localization, sections were treated with boiling sodium citrate buffer (10 mM containing 0.1% Triton X-100) and incubated overnight with anti-Iba1 (1:350) and anti-CD68 (1:500, Biorad, Watford, United Kingdom). Sections were then developed with anti-rabbit AlexaFluor 555 (1:200, Fisher Scientific) and anti-rat AlexaFluor 633 (1:200, Fisher Scientific). For NVU labeling, sections were treated with pepsin (1 mg/mL in 0.2N HCl, 30 s at 37°C) and incubated overnight with anti-laminin and anti-GFAP (**Table 1**). The next day the sections were washed in PBS and incubated with FITC-conjugated anti-α-SMA (1:350, Sigma-Aldrich), anti-mouse AlexaFluor 405 (1:200, Fisher Scientific), anti-rabbit AlexaFluor 555 (1:200, Fisher Scientific) and anti-chicken AlexaFluor 633 (1:200, Fisher Scientific). Sections were coverslipped using Mowiol® mounting media (Sigma-Aldrich) containing 0.1% v/v Citifluor (Citifluor Ltd., London, United Kingdom).

## Image Acquisition and Analysis

Non-overlapping DAB images were captured across the entire hippocampal formation or median eminence using a ×10 objective on a Nikon Eclipse 80 Brightfield Microscope. Non-overlapping images of fluorescent immunohistochemistry were taken using a Leica SP5 scanning laser confocal microscope. Brightfield images and individual channels from confocal images were quantified by calculating the percentage area covered by staining, total cell count and cell size using Fiji (NIH, MD, United States). For quantification of CD68 + microglia, the rolling ball radius was set at 2–15 $\mu m^2$, while quantification of CD68 + macrophages were detected using a rolling ball radius of 16 $\mu m^2$-infinity. For 3D reconstruction, confocal images were deconvolved using AutoQuant X3 (MediaCybernetics Inc., Rockville, MD). Deconvolved images were processed using Imaris (Bitplane®) and surfaces were created for laminin and GFAP. To quantify the amount of astrocyte endfoot contact with laminin at hippocampal arteries (defined as positive for α-SMA and >10 μm diameter), the total area of contact between GFAP and laminin ($\mu m^2$) was calculated using the Imaris Xtension "Surface to Surface Contact Area" (Imaris V8.31, ImarisXT Bitplane Inc., created by Matthew J. Gastinger, Bitplane) and standardized to vessel diameter and length (μm). Only surfaces that made direct contact with each other (i.e., 0 μm distance) were quantified. GFAP-to-laminin contact was calculated for three randomly selected arteries for each mouse ($n$ = 5/group/sex) and the average values per mouse were used for statistical analysis.

## Statistical Analysis

Data were confirmed to be normally distributed using the Kolmogorov–Smirnov test. The ROUT test was used to identify and exclude outliers. All analyses were carried out using two-way ANOVA with Holm–Sidak multiple comparisons *post hoc* test (GraphPad Prism, San Diego, CA, United States). Data represent mean ± SEM and $p < 0.05$ was considered to be statistically significant. Significant differences between C/C vs. HF/HF and C/HF vs. HF/C groups were considered biologically irrelevant and were not reported.

# RESULTS

## High Fat Diet Results in Weight Gain in Mothers and Aged Offspring

Consumption of the HF diet resulted in a significantly greater weight and weight-to-length ratio of dams at both mating and weaning compared to dams fed the C diet (**Figures 1A,B**). 16-month old male and female C/HF and HF/HF offspring weighed significantly more than C/C and HF/C mice, respectively (**Figure 1C**). Weight-to-length ratio was also significantly greater

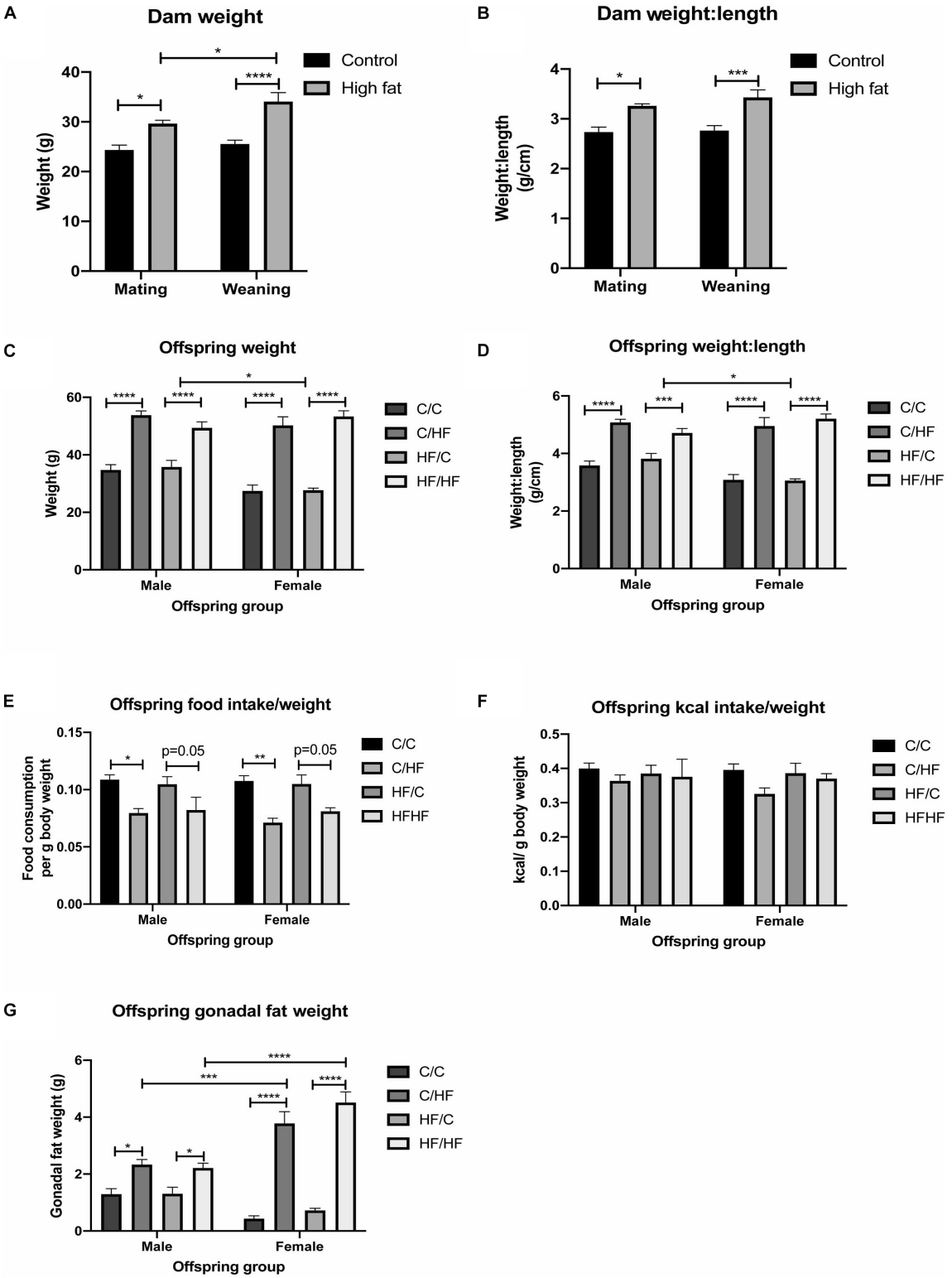


**FIGURE 1 |** Effect of pre- and post-natal high fat feeding on food intake, body weight and gonadal fat. **(A,B)** Body weight **(A)** and weight-to-length ratio **(B)** of dams fed the control (C) or high fat (HF) diet for 4 weeks before mating and at weaning. **(C,D)** Body weight **(C)** and weight-to-length ratio **(D)** of 16-month old male and female C/C, C/HF, HF/C and HF/HF offspring. **(E,F)** Average daily food **(E)** and kcal **(F)** intake per gram body weight of 16-month old offspring diet groups. **(G)** Weight of gonadal fat for male and female C/C, C/HF, HF/C and HF/HF mice. Data represent mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, two-way ANOVA with Sidak–Holm *post hoc* test.

in offspring fed the HF diet compared to those fed the C diet postnatally (**Figure 1D**). Changes in body weight were equivalent between diet groups during periods of food restriction and *ad libitum* consumption (**Supplementary Figures S1B,C**), although female HF/C mice gained significantly more weight after restriction than their male counterparts (**Supplementary Figure S1C**). Male and female HF-fed offspring weighed significantly more than C-fed mice throughout the 16-month period (**Supplementary Figures S1D,E**). 16-month old male HF/C offspring weighed significantly more than their female counterparts and there was a non-significant trend ($p = 0.05$) toward lower body weight in C/C female compared to C/C males (**Figure 1C**). However, both groups of female offspring fed the HF diet (i.e., C/HF and HF/HF) weighed the same and had the same weight-to-length ratio as males in the comparable diet groups (**Figures 1C,D**). Proportional to their body weight, HF-fed mice consumed less food than C-fed mice (**Figure 1E**), but kcal consumption was similar between diet groups (**Figure 1F**). Gonadal fat weight, which is related to total body fat (Rogers and Webb, 1980), was significantly higher in male and female C/HF and HF/HF groups compared to C/C and HF/C mice, respectively (**Figure 1G**). No differences in weight, weight-to-length ratio or gonadal fat were observed between offspring that were maintained on the same postnatal diet (i.e., C/C vs. HF/C or C/HF vs. HF/HF). Female C/HF and HF/HF mice also showed greater fat content than corresponding male mice (**Figure 1G**).

## Pre- and Post-natal Diet Does Not Affect the Level of NVU Components in Aged Offspring

To evaluate the long-term effect of pre- and post-natal high fat feeding on NVU components, hippocampal tissues from offspring were processed by Western blotting for markers of endothelial cells (CD31), tight junction proteins (ZO-1 and claudin 5), pericytes (PDGFRβ), cerebrovascular basement membranes (laminin), smooth muscle cells (α-SMA) and astrocytes (GFAP). As shown in **Figure 2A**, no differences were noted in the levels of CD31 between any diet group or between males and females. A trend toward increased expression of ZO-1 and claudin 5 was observed in female vs. male mice, but this did not reach statistical significance ($p = 0.06$ and $p = 0.05$, respectively) and no differences were noted between diet groups for either protein (**Figures 2B,C**). No effects of diet or sex were observed in the expression of PDGFRβ (**Figure 2D**), laminin (**Figure 2E**) or α-SMA (**Figure 2F**). Two-way ANOVA revealed a significant effect of sex on GFAP expression ($p = 0.02$), however, *post hoc* comparisons between individual male and female offspring groups did not reveal any significant differences (**Figure 2G**).

## Pre- and Post-natal High Fat Feeding Differentially Affects the Leakiness of the Hippocampal BBB Between Male and Female Offspring

To determine if BBB integrity was differentially affected between the offspring groups, tissue sections were processed using antibodies against laminin, α-SMA, GFAP and mouse IgG as markers of blood vessels, arteries, astrocytes and extravasated plasma proteins, respectively. Within the male offspring groups, BBB integrity appeared relatively intact in the C/C group, with little IgG expression detected in the parenchyma (**Figures 3A–D**). Diffuse IgG staining was detected around capillaries in the hippocampi of both C/HF and HF/C mice (**Figures 3E–L**), although the degree of IgG detection was variable between animals within both groups. Interestingly, HF/HF mice appeared to have less IgG staining than the C/HF and HF/C mice (**Figures 3M–P**). However, no statistically significant differences in the percent of hippocampal area positive for IgG were observed between diet groups (**Figure 3Q**). A similar pattern of detection was observed in tissues processed with biotinylated anti-mouse alone (**Supplementary Figure S2A**), confirming that the pattern of staining was not due to non-specific binding of IgG to laminin, α-SMA or GFAP. Analysis of laminin expression indicated increased expression in offspring of mothers fed the HF diet, however, this did not differ significantly from the other diet groups when analyzed by two-way ANOVA (**Figure 3R**). By contrast, GFAP expression as measured by cell count and area coverage was significantly lower in the hippocampus of HF/C and HF/HF mice compared to C/C and C/HF mice, respectively (**Figure 3S** and **Supplementary Figure S3A**). Average size of GFAP-positive astrocytes did not differ between diet groups (**Supplementary Figure S3B**).

Within the female offspring groups, IgG detection was minimal in the C/C group (**Figures 4A–D**) and appeared to be more prominent in the C/HF group (**Figures 4E–H**), although there was no statistically significant difference between the groups. The pattern of staining in HF/C mice was similar to that of C/C mice (**Figures 4I–L**). A large amount of IgG staining was observed in the hippocampi of HF/HF mice although intra-group variability was high (**Figures 4M–P**). Compared to HF/C mice, IgG coverage in HF/HF mice bordered statistical significance in quadruple-stained sections ($p = 0.06$) and was significantly higher in DAB-processed sections (**Supplementary Figure S2A**). IgG values did not differ significantly between HF/HF mice and the other diet groups (**Figure 4Q**). Laminin expression was significantly higher in the hippocampus of HF/HF mice compared to C/HF animals (**Figure 4R**). No significant differences in the coverage (**Figure 4S**), number (**Supplementary Figure S3A**) or size (**Supplementary Figure S3B**) of GFAP-positive astrocytes were noted between female offspring diet groups.

Comparisons between male and female offspring showed a trend toward increased IgG staining in female mice, which was significantly higher in HF/HF females compared to HF/HF males (**Figure 5A** and **Supplementary Figure S2B**). The expression of laminin followed a similar pattern but no statistically significant differences were noted between the sexes (**Figure 5B**). Both hippocampal coverage and cell counts of GFAP-positive astrocytes were significantly higher in male C/C and C/HF groups relative to the comparable female groups, while no differences were noted between males and females born to mothers fed the HF diet (**Figure 5C** and **Supplementary Figure S3C**). Average size of GFAP-positive astrocytes did not differ

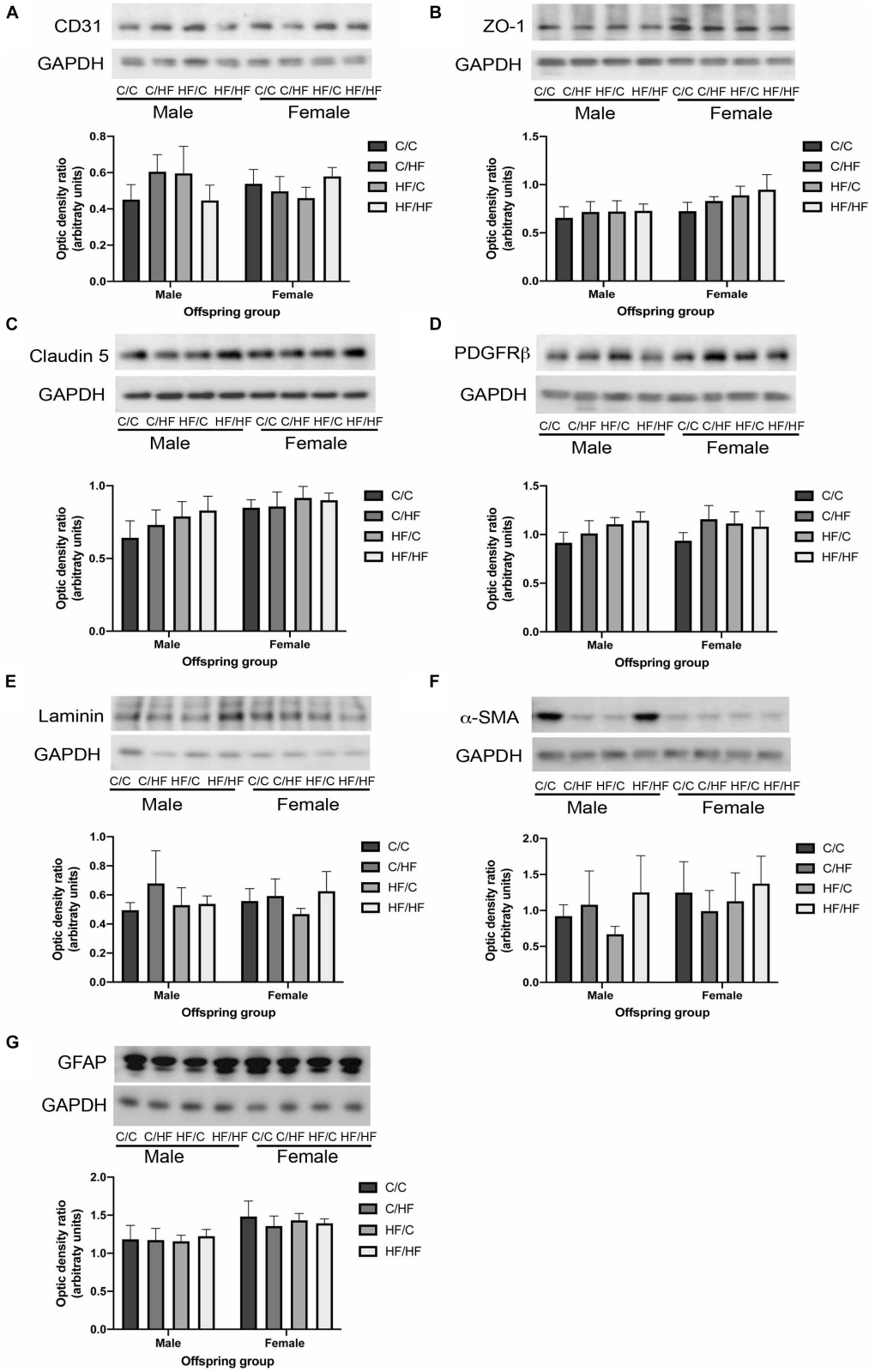


**FIGURE 2 |** Expression of neurovascular unit components in aged offspring. **(A–G)** Hippocampal homogenates from 16-month old male and female C/C, C/HF, HF/C and HF/HF mice were assessed by Western blot to determine the expression of CD31 **(A)**, zona occludens-1 (ZO-1, **B**), claudin 5 **(C)**, platelet-derived growth factor receptor-β (PDGFRβ, **D**), laminin **(E)**, α-smooth muscle actin (α-SMA, **F**) and glial fibrillary acidic protein (GFAP, **G**). Data represent mean ± SEM.

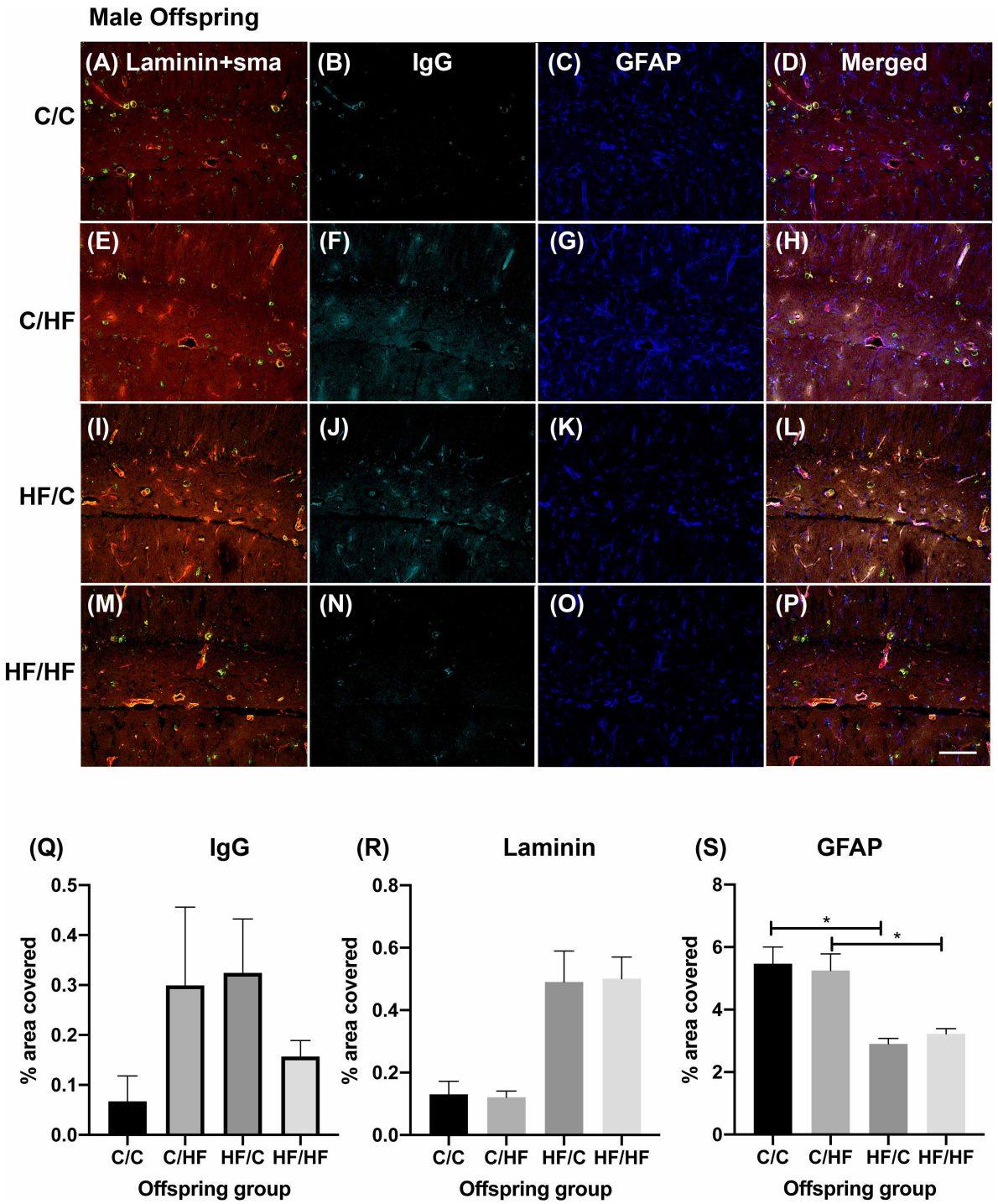


**FIGURE 3 |** Effect of pre- and post-natal high fat diet on hippocampal blood–brain barrier integrity in male offspring. **(A–P)** Photomicrographs of hippocampal tissue sections stained for laminin (red, **A,E,I,M**), smooth muscle actin (green, **A,E,I,M**), IgG (turquoise, **B,F,J,N**) and GFAP (blue, **C,G,K,O**) in 16-month old male C/C **(A–D)**, C/HF **(E–H)**, HF/C **(I–L)** and HF/HF mice **(M–P)**. **(Q–S)** Quantification of hippocampal area positive for IgG extravasation **(Q)**, laminin **(R)** and GFAP **(S)**. Data represent mean ± SEM. $^*p < 0.05$, two-way ANOVA with Holm–Sidak *post hoc* test. Scale bar = 100 μm.

between male and female mice in any diet group (**Supplementary Figure S3D**). The density of potato lectin staining was similar between all diet groups and between male and female offspring (**Figure 5D**), suggesting that the observed differences in BBB leakiness and NVU expression were not due to differences in blood vessel density.

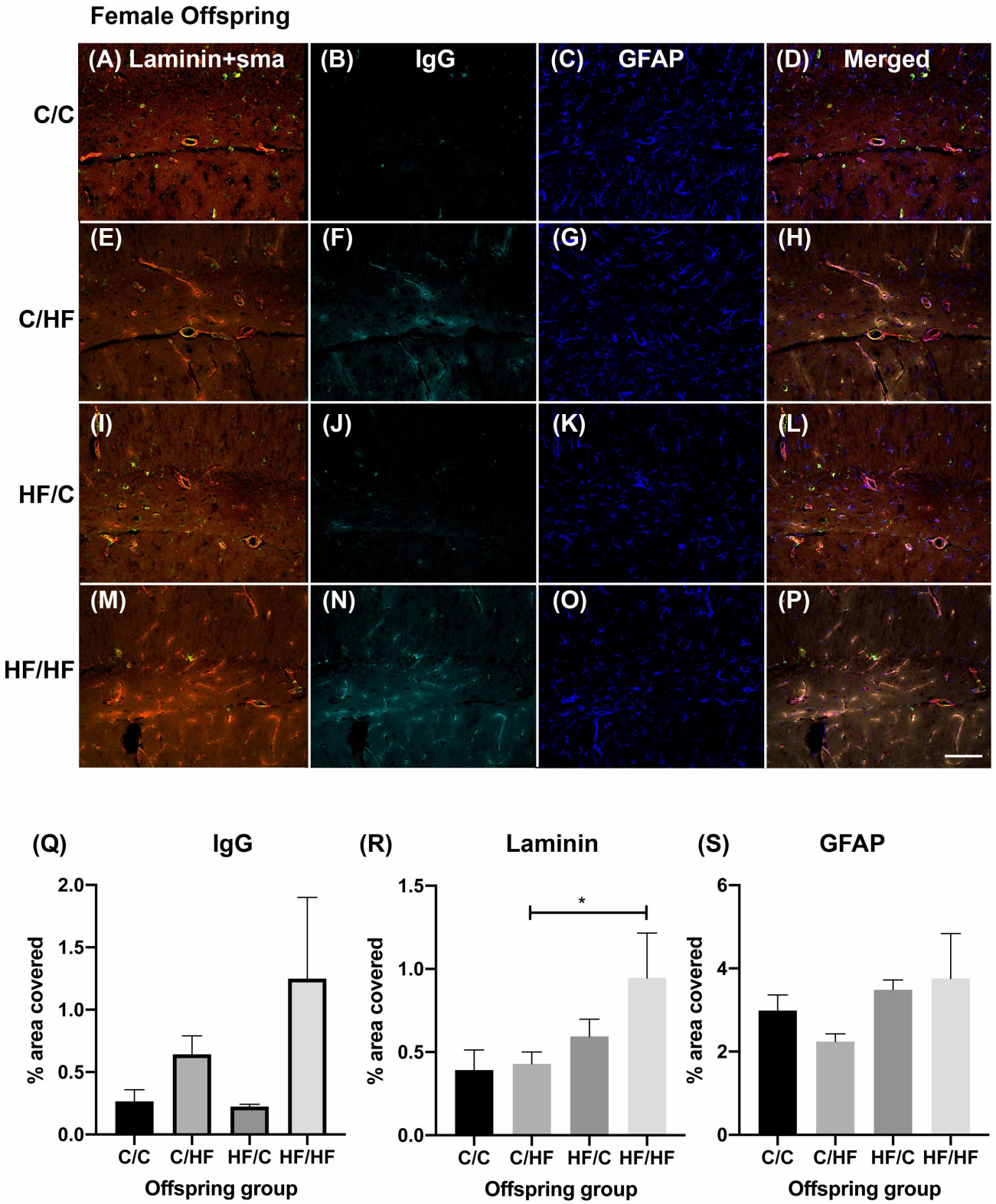


**FIGURE 4 |** Effect of pre- and post-natal high fat diet on hippocampal blood–brain barrier integrity in female offspring. **(A)** Photomicrographs of hippocampal tissue sections stained for laminin (red, **A,E,I,M**), smooth muscle actin (green, **A,E,I,M**), IgG (turquoise, **B,F,J,N**) and GFAP (blue, **C,G,K,O**) in 16-month old male C/C **(A–D)**, C/HF **(E–H)**, HF/C **(I–L)** and HF/HF mice **(M–P)**. **(Q–S)** Quantification of hippocampal area positive for IgG extravasation **(Q)**, laminin **(R)** and GFAP **(S)**. Data represent mean ± SEM. *$p < 0.05$, two-way ANOVA with Holm–Sidak *post hoc* test. Scale bar = 100 μm.

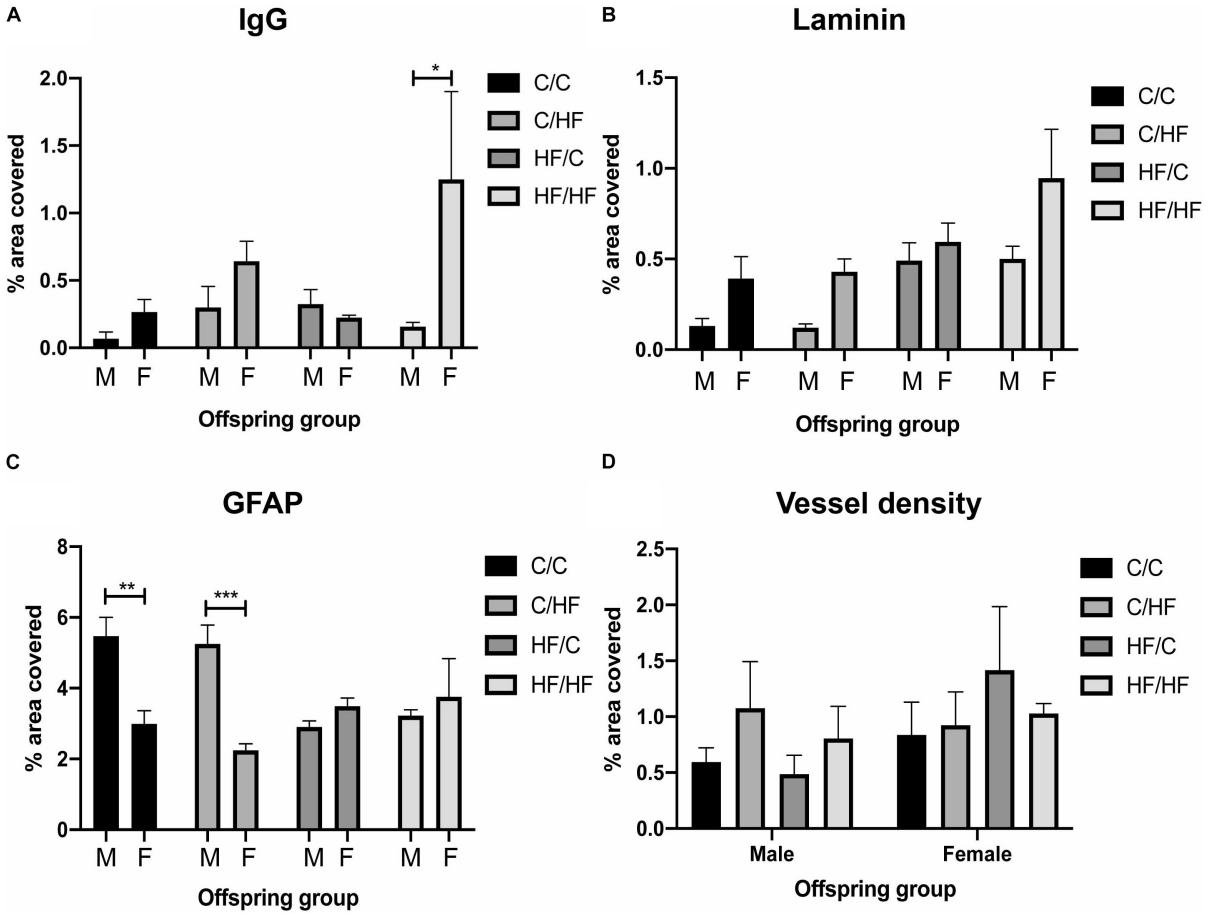


**FIGURE 5 |** Comparison of blood–brain barrier integrity and vessel density between male and female offspring. **(A–C)** Quantification of hippocampal area positive for IgG extravasation **(A)**, laminin **(B)** and GFAP **(C)** in 16-month old male and female C/C, C/HF, HF/C and HF/HF mice. **(D)** Vessel density in hippocampus of male and female offspring. Data represent mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-way ANOVA with Holm–Sidak *post hoc* test.

## Leakiness of the Median Eminence Is Not Affected by Pre- and Post-natal High Fat Feeding

To determine if HF feeding also affected the NVU in an area with endogenous BBB leakiness, expression of IgG, GFAP and laminin was determined in the median eminence (ME). IgG staining was observed in the ME in male and female mice in all diet groups (**Figures 6A–H**). IgG coverage was similar between C and HF-fed mice and between males and females (**Figure 6I**). Hypothalamic areas surrounding the ME and arcuate nucleus did not show the presence of IgG, suggesting that stable IgG expression was not due to diffusion of IgG from the ME. Similarly, expression of GFAP (**Figure 6J**) and laminin (**Figure 6K**) did not differ significantly between diet groups.

## Prenatal High Fat Feeding Increases Inflammatory Markers in Aged Female Offspring

To evaluate if BBB leakiness and/or HF diet was associated with increased inflammation, sections were stained for the microglial marker Iba1. Two-way ANOVA showed a significant effect of diet ($p = 0.03$) and sex ($p = 0.04$) on% area coverage by Iba1, but *post hoc* tests were not significant between any groups, although comparison of female C/HF vs. HF/HF bordered significance ($p = 0.05$) (**Figure 7A**). Microglia number and average size did not differ between diet groups, however, microglia size was significantly higher in female HF/C mice compared to HF/C males (**Figures 7B,C**). To determine if microglia activity was altered, sections were double labeled with Iba1 and CD68 (**Figures 7D–K**), a macrophage marker that is also expressed by phagocytic microglia (Zotova et al., 2013). Quantification of the percentage of Iba1-positive microglia that were also positive for CD68 showed that C/HF male offspring expressed significantly more phagocytic microglia than male C/C and HF/HF mice (**Figure 7L**). The percent of CD68-positive microglia was also significantly higher in female HF/HF vs. C/HF mice (**Figure 7L**). Comparisons between male and female offspring showed that male C/HF mice had significantly higher expression of phagocytic microglia than C/HF females, however, CD68 expression in microglia was significantly higher in HF/C and HF/HF female mice compared to the comparable male diet groups (**Figure 7M**).

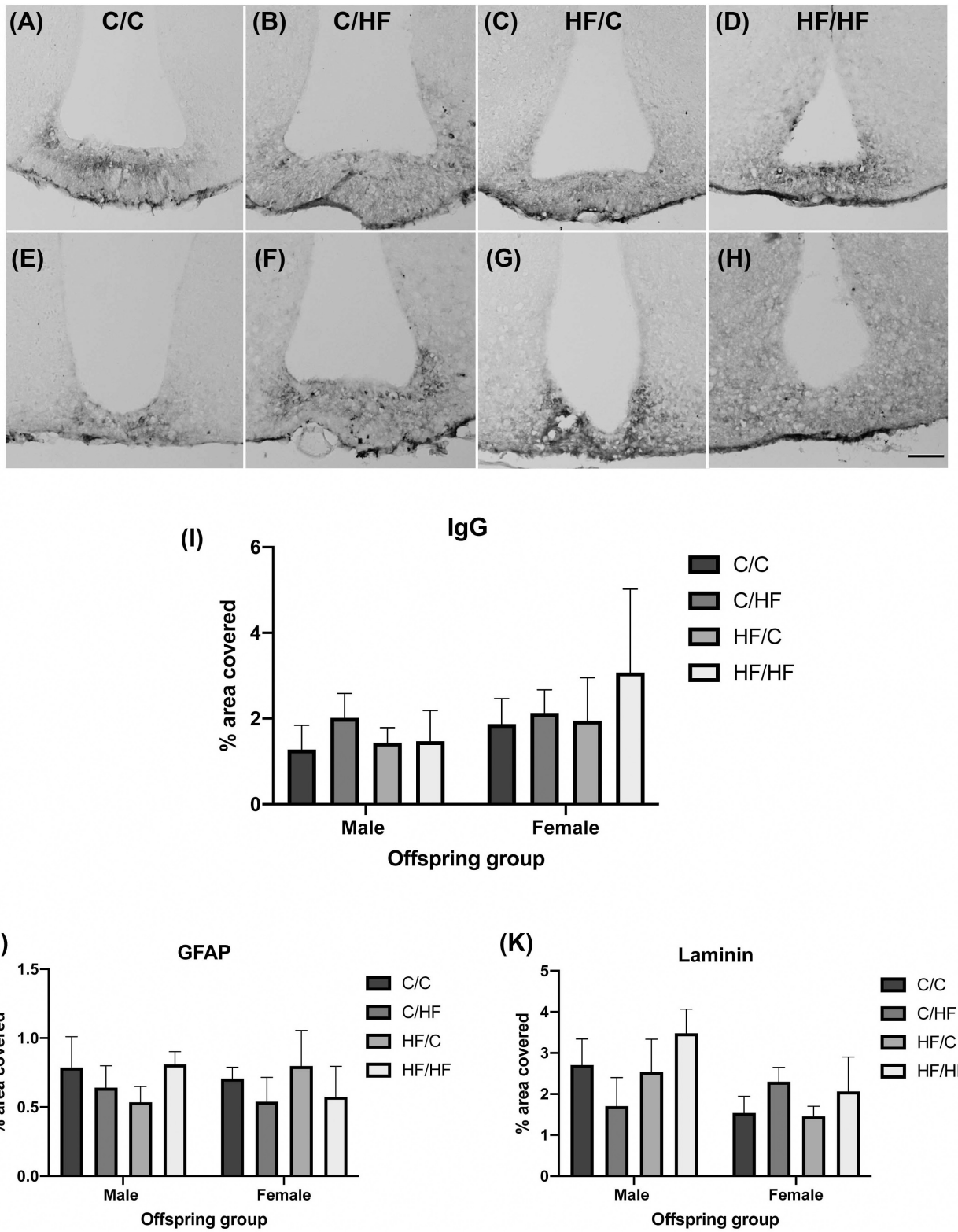


**FIGURE 6 |** Effect of pre- and post-natal high fat diet on hypothalamic blood–brain barrier integrity in male and female offspring. **(A–H)** Photomicrographs of the median eminence stained for IgG in 16-month old male **(A–D)** and female **(E–H)** C/C **(A,E)**, C/HF **(B,F)**, HF/C **(C,G)** and HF/HF **(D,H)** mice. **(I)** Quantification of IgG extravasation in the median eminence. **(J,K)** Quantification of median eminence area positive for GFAP **(J)** and laminin **(K)**. Data represent mean ± SEM. Scale bar = 200 μm.

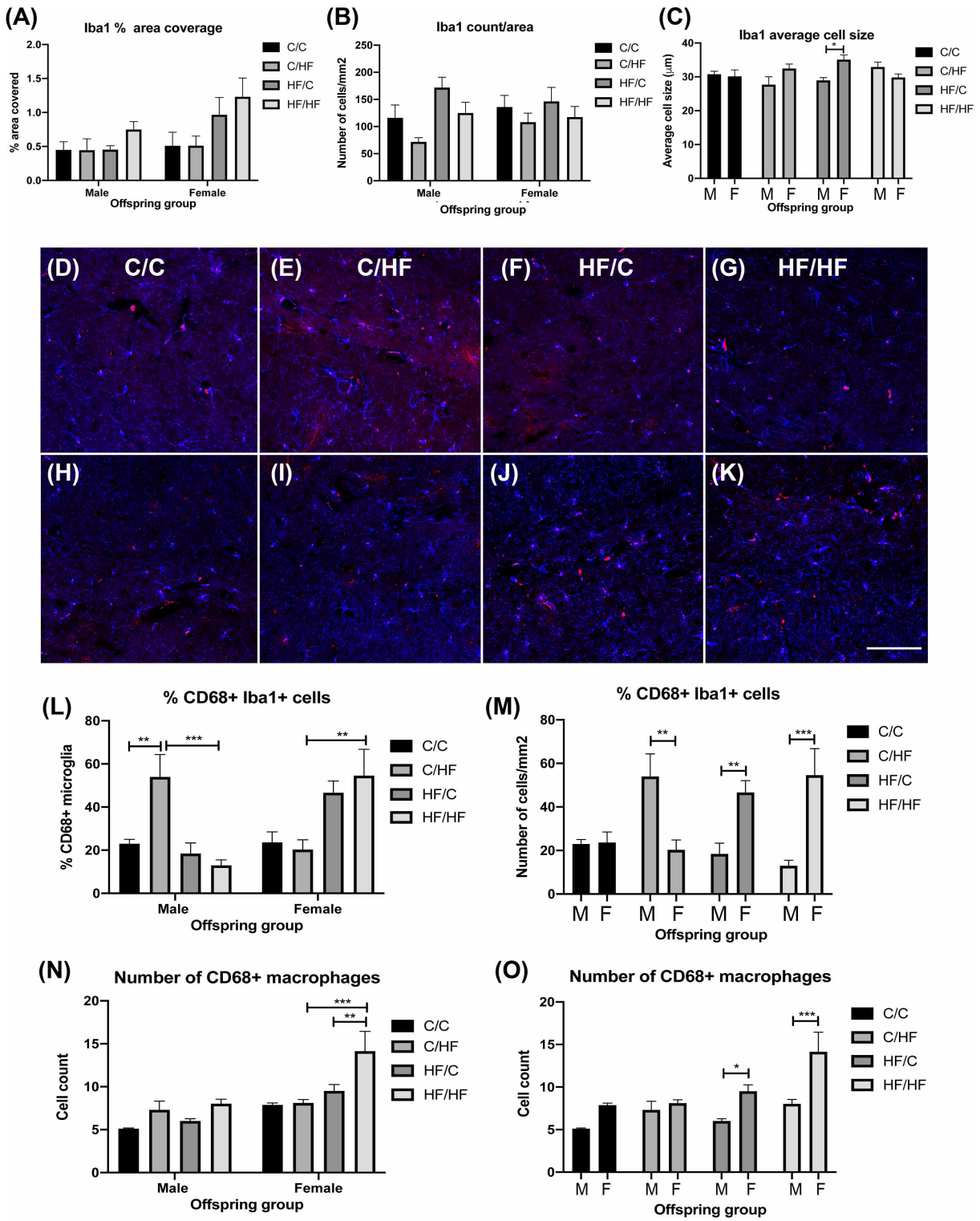


**FIGURE 7 |** Evaluation of microglia and macrophage expression in the hippocampus of aged male and female offspring. **(A–C)** Quantification of the percent of hippocampal coverage **(A)**, cell count **(B)** and average size **(C)** of Iba1-positive microglia in 16-month old C/C, C/HF, HF/C and HF/HF mice. **(D–K)** Photomicrographs of hippocampal tissue sections stained for Iba1 (blue) and CD68 (red) in 16-month old male **(D–G)** and female **(H–K)** C/C **(D,H)**, C/HF **(E,I)**, HF/C **(F,J)** and HF/HF **(G,K)** mice. **(L,M)** Quantification of the percentage of Iba1-positive microglia that are also positive for CD68 within each diet group **(L)** and between male and female offspring **(M)**. **(N,O)** Quantification of the number of CD68-positive macrophages within each diet group **(N)** and between male and female offspring **(O)**. Data represent mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-way ANOVA with Holm–Sidak *post hoc* test. Scale bar = 100 μm.

The number of CD68-positive macrophages, which were located primarily in the perivascular spaces, was also significantly higher in HF/HF females compared to both female C/HF and HF/C mice (**Figure 7N**). Significantly more macrophages were also observed in female HF/C and HF/HF females compared to males in the same diet groups (**Figure 7O**).

## Perivascular Coverage by Astrocytes Is Decreased by Post-natal High Fat Feeding in Female but Not Male Offspring

As astrocytes contribute to the maintenance of the BBB (Daneman and Prat, 2015), we assessed whether the observed differences in parenchymal GFAP expression were also observed at the NVU. The amount of direct surface area contact between astrocyte endfeet and laminin was analyzed using 3D reconstructions of hippocampal arteries from male (**Figures 8A–D**) and female (**Figures 8E–H**) offspring. Male offspring showed a pattern of increased astrocyte coverage in post-natal HF groups, with the highest amount of perivascular contact in the HF/HF group, although these differences did not differ significantly from the C groups (**Figure 8I**). Interestingly, the opposite pattern was detected in the female offspring, where astrocyte-to-laminin contact was significantly lower in HF/HF mice compared to HF/C animals (**Figure 8I**). Comparison between male and female offspring revealed that astrocyte endfoot coverage was significantly higher in C/C females compared to C/C males and significantly lower in HF/HF females versus the HF/HF male group.

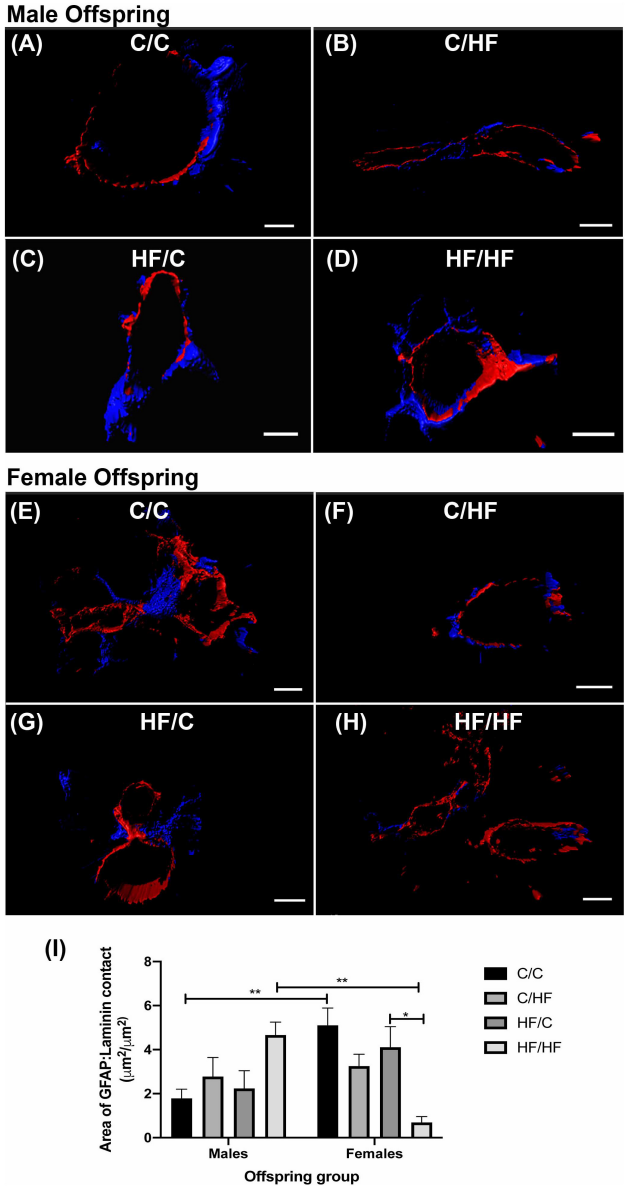


**FIGURE 8 |** Astrocyte endfoot coverage of hippocampal arteries in male and female offspring. **(A–H)** Representative images of the 3D reconstruction of GFAP (blue) and laminin (red) staining of hippocampal arteries in male **(A–D)** and female **(E–H)** C/C **(A,E)**, C/HF **(B,F)**, HF/C **(C,G)** and HF/HF **(D,H)** mice. **(I)** Quantification of the surface area of contact between GFAP-positive astrocytes and laminin in arteries of male and female offspring. Data represent mean ± SEM. $*p < 0.05$, $**p < 0.01$, two-way ANOVA with Holm–Sidak *post hoc* test. Scale bars: **(A)** = 7 μm; **(B,D–F,H)** = 10 μm; **(C)** = 5 μm; **(G)** = 8 μm.

## DISCUSSION

Previous studies using HF feeding paradigms have typically assessed BBB structure in young adult animals after relatively short periods of feeding and none have to our knowledge evaluated the long-term effect of maternal obesity on the cerebrovasculature of aged offspring. The purpose of this study was to examine the impact of diet-induced maternal obesity on the structure and integrity of the NVU and BBB in aged offspring in the presence or absence of chronic HF feeding. We found that post-natal exposure to a HF diet resulted in increased leakiness of the BBB in the hippocampus of female offspring in association with loss of astrocyte endfoot coverage of hippocampal vessels. Male, but not female offspring born to mothers fed a HF diet also showed a trend toward increased BBB permeability and significantly decreased parenchymal GFAP expression compared to mice born to lean mothers. By contrast, female offspring exposed to the HF diet for their entire lifespan (i.e., HF/HF) exhibited more significant changes in NVU structure, BBB permeability and inflammation compared to their male counterparts.

Previous studies examining the effects of HF diet on the hippocampal BBB have reported increased permeability to circulating compounds (e.g., fluorescein, Evans blue, albumin, IgG) in rodents fed the diet over a 2–4 months period (Kanoski et al., 2010; Davidson et al., 2012; Pallebage-Gamarallage et al., 2012; Hargrave et al., 2016; Hajiluian et al., 2017; Mamo et al., 2019; Salameh et al., 2019). These findings are in agreement with our observations that chronic post-natal high fat

feeding alone (i.e., C/HF) resulted in a trend toward increased extravasation of IgG into the hippocampus in both male and female offspring relative to C/C mice and significantly higher IgG content in HF/HF vs. HF/C females. We chose to use IgG extravasation as a proxy of BBB integrity because it is endogenously expressed and has a relatively large molecular weight (Saunders et al., 2015), suggesting that its presence in the parenchyma represents substantial damage to the BBB. However, this approach may have underestimated smaller changes in the tightness of the BBB and contributed to the intra-group variation that was observed.

Evaluation of the staining pattern of IgG in the median eminence, an area of endogenous BBB leakiness, revealed no effect of pre- or post-natal HF exposure on IgG content in the aged offspring. These findings are in contrast to previous reports of increased permeability of the hypothalamic BBB to albumin and glucose after 16 and 36 weeks of HF feeding (Salameh et al., 2019). Yi et al. (2012) reported increased IgG in the arcuate nucleus of mice fed a HF diet for 16 weeks, which was due to selective permeability to IgG1. The reasons for the discrepancy between the current findings and previous reports are unclear. It may be that chronic exposure to a HF diet from weaning induces compensatory changes in the hypothalamic BBB that counteract diet-induced damage or that the hypothalamic BBB of C/HF and HF/HF mice may be leakier to small molecular weight compounds (e.g., albumin) that were not evaluated in the current study. Alternatively, quantification of IgG staining using a general anti-mouse IgG that recognizes both heavy and light chains may have masked differences in selective permeability to specific IgG isotypes. Therefore, whether the observed changes in BBB permeability in the current study are specific to the hippocampus or other brain areas with an endogenously tight BBB requires further investigation.

While some BBB studies have reported decreased tight junction protein expression (Kanoski et al., 2010; de Aquino et al., 2018; Mamo et al., 2019) and reduced capillary density in the CA1 region of the hippocampus of HF-fed animals (Tucsek et al., 2014b), we did not observe differences in total levels of ZO-1 or claudin-5 or in vessel density between C/C and C/HF mice. As tight junction proteins are also expressed by neurons, astrocytes and oligodendrocytes (Bauer et al., 1999; Romanitan et al., 2010; Freeman and Granholm, 2012), it may be that subtle changes in the distribution or expression of tight junction proteins were masked in the analysis of whole hippocampal homogenates. Similarly, by analyzing vessel density across the entire hippocampus, region-specific changes between diet groups may have been overlooked. However, a previous study comparing BBB permeability in 24-month old mice fed a C or HF diet for 5 months reported increased levels of IgG in the hippocampus in HF-fed mice in the absence of differences in the expression of occludin or claudin-5 (Tucsek et al., 2014a). This suggests that alternative mechanism(s) may contribute to BBB leakiness in HF-fed aged mice. In support of this, we found that astrocyte endfoot contact with laminin in hippocampal arteries mirrored the pattern of IgG staining across the diet groups, in that areas of low astrocyte coverage were associated with higher parenchymal IgG and vice versa. Given that astrocytes contribute to the maintenance of the BBB (Daneman and Prat, 2015), this suggests that HF feeding may also contribute to BBB damage by directly influencing the degree of perivascular contact of astrocytes at the NVU, although whether this precedes or results from BBB dysfunction remains to be determined.

To date, few studies have examined the impact of prenatal high fat feeding or maternal obesity on the structure and function of the offspring NVU. A study by Kim et al. (2016a) found that fenestrations, endothelial transporters and tight junctions were significantly altered in the hypothalamic blood vessels of fetal and neonatal mice born to mothers fed a HF diet. We did not observe differences in IgG staining in the median eminence between 16-month old C/C and HF/C mice. As discussed above, whether this is due to post-natal adaptations during brain maturation or insensitivity of the current methods to detect small or selective changes in BBB permeability remains unknown. Endothelial denudation and thickening of the middle cerebral artery has been reported in 6-month old rat offspring of obese mothers (Lin et al., 2018) and neonatal and adult HF/C offspring display larger infarct volumes and poorer functional deficits after stroke compared to C/C rats (Lin et al., 2016; Teo et al., 2017). We have previously reported that vessel morphology and thickness and GFAP expression were altered in the hippocampus of 5-month old HF/C male mice compared to C/C offspring (Hawkes et al., 2015). In the present study, only parenchymal GFAP expression differed significantly between male C/C and HF/C mice, although there was also a trend toward increased expression of laminin and IgG extravasation in male HF/C offspring. Although previous studies have reported increased GFAP expression in the hypothalamus of fetal and neonatal mice born to obese mothers (Kim et al., 2016b), our findings are more consistent with the observation that astrocyte expression in male offspring born to mothers fed a hypercaloric drink was lower than those born to un-supplemented mothers (Molina et al., 2018). These findings, in combination with the stable expression of Iba1 as well as CD68 + microglia and macrophages between C/C and HF/C males, suggest that prenatal HF exposure does not induce a general gliosis in aged male offspring. Whether this results from an early adaptive mechanism against a pro-inflammatory developmental milieu (Molina et al., 2018) or is an aging-related phenomenon remains to be determined.

Differences between male and female mice were observed in almost every parameter of this study. Female offspring had a greater fat mass, a higher degree of IgG extravasation, lower expression of GFAP and higher expression of Iba1 and CD68 than male counterparts. The greater accumulation of visceral fat and increased BBB permeability in female mice are consistent with the effects of estrogen loss during reproductive senescence (Wilson et al., 2008; Bake et al., 2009; Shi and Clegg, 2009), which begins around 11 months

of age in the C57Bl/6 strain (Nelson et al., 1981, 1982). In women and female rodents, estrogen appears to have an age-dependent effect on the health of the NVU and BBB function. In young rats, estrogen replacement improves BBB leakiness after ovariectomization but further exacerbates dye extravasation in reproductively senescent females (Bake and Sohrabji, 2004). Results from the Women's Health Initiative study found that hormone replacement therapy in postmenopausal women increased the risk of stroke and the incidence of mild cognitive impairment and all cause dementia (Shumaker et al., 2004; Henderson and Lobo, 2012). Although levels of testosterone, the main source of estradiol in men, also decrease with age, estrogen levels remain constant in the aging male (Vermeulen et al., 2002). Thus, although increased levels of estradiol have been reported in both obese men and women (Schneider et al., 1979; Vermeulen et al., 2002), it is possible that the persistent elevation in estrogen in obese, postmenopausal women exacerbates age-related vascular damage to a greater extent than in males. Astrocyte expression is also sensitive to fluctuations in estrogen (Fuente-Martin et al., 2013) and greater numbers of GFAP-positive astrocytes have been previously reported in the hippocampus and the posterodorsal portion of the medial amygdala of adult male versus female mice (Conejo et al., 2005; Johnson et al., 2008). Although these reports are consistent with our findings, additional experiments are needed to understand why opposite patterns of perivascular astrocyte expression were observed between male and female offspring.

The relatively weak impact of prenatal HF exposure alone (i.e., HF/C) in female offspring in our study supports previous reports that male offspring are more likely than female offspring to develop behavioral, cognitive and epigenetic brain changes following exposure to a perinatal HF diet (Bilbo and Tsang, 2010; Vucetic et al., 2010; Edlow et al., 2016; Graf et al., 2016; Deardorff et al., 2017; Glendining and Jasoni, 2019). Notably however, female HF/C mice demonstrated larger microglia and higher numbers of phagocytic microglia and macrophages than HF/C male offspring. Sexual dimorphism in microglia number and activation has been described, with microglia in adult female animals reported to have a more inflammatory profile than males (Nissen, 2017), which has been hypothesized to contribute to the increased prevalence of neurodegenerative diseases such as Multiple Sclerosis and AD in women (Nissen, 2017). However, previous studies have reported lower expression of pro-inflammatory and higher levels of anti-inflammatory cytokines in the brains of P21 and young adult female offspring born to HF-fed mothers compared to male (Bilbo and Tsang, 2010; Graf et al., 2016). Therefore, further investigation is needed to determine how perinatal HF exposure impacts on the inflammatory profile across the lifespan of male and female offspring.

We also find it notable that the degree of IgG extravasation, loss of perivascular astrocyte contact and degree of inflammation was significantly greater in female HF/HF mice compared to HF/HF males. Moreover, the difference in astrocyte endfoot contact, parenchymal IgG coverage and Iba1 expression was also higher between C/HF and HF/HF females than males. These data suggest that possible compensations made by male mice during early life exposure to a HF diet are advantageous in the context of a chronic, postnatal obesogenic environment, but that these same adaptations are either not made or are deleterious in the same context in aged females.

Although the current study did not include behavioral analyses, a range of cognitive and pathological effects have been reported in both prenatal and postnatal HF feeding studies. In murine models, HF-diet-induced obesity is associated with impaired cognitive performance and reduced plasticity in both young adult and middle-aged animals (Winocur et al., 2005; Farr et al., 2008; Stranahan et al., 2008). Obesity also accelerates cognitive dysfunction and pathology in AD mouse models (Moser and Pike, 2017; Rollins et al., 2019). In humans, childhood, adolescent and adult obesity is associated with reduced executive function and lower global cognition (Wang et al., 2016), while mid-life obesity is associated with more rapid deterioration in cognitive function and increased risk of developing AD (Kivipelto et al., 2002). However, the association between body-mass index and cognitive function is weaker in old age (Aslan et al., 2015) and the relative risk for obesity-related stroke is lower in older individuals (Emerging Risk Factors Collaboration Wormser et al., 2011). These findings support the influence of early life programing on the long-term health of the brain. To that end, adult offspring exposed to a HF diet during the perinatal period have been reported to have higher levels of anxiety (Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012; Sasaki et al., 2013; Kang et al., 2014) and depression (Lin et al., 2015; Balsevich et al., 2016) and impaired memory performance (White et al., 2009; Can et al., 2012; Page et al., 2014; Lepinay et al., 2015), although opposite findings have also been reported (Clouard et al., 2016; Johnson et al., 2017; Val-Laillet et al., 2017). Impairments in memory acquisition and retention and increased pathology have also been noted in two mouse models of AD in 12–13 months offspring exposed to a high fat diet during gestation and lactation (Martin et al., 2014; Nizari et al., 2016), suggesting that perinatal HF exposure may also contribute to the pathogenesis of AD. As suggested above, the impact of maternal obesity appears to differentially affect male and female offspring. Male, but not female offspring born to HF-fed dams have been reported to show impairments on the novel object recognition test (Graf et al., 2016), while young adult female, but not male, macaques born to mothers fed a high fat diet showed increased anxiety to novel and threatening objects (Sullivan et al., 2010). Recently, a multi-generational study reported that second and third generation female, but not male offspring born to HF-fed mothers showed impairments in executive function and memory (Sarker and Peleg-Raibstein, 2018). As rates of maternal obesity continue to rise, the current findings may have implications for the future incidence and management of age-related neurovascular diseases, such as stroke and AD, that are more common in obese, post-menopausal women (Lisabeth and Bushnell, 2012; Christensen and Pike, 2015).

## 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 Open University Animal Welfare and Ethics Review Board.

## AUTHOR CONTRIBUTIONS

LC, CJH and CAH designed the experiments which were carried out by LC, SN, and CAH. CJH and CAH wrote and edited the manuscript.

## FUNDING

This work was funded by Alzheimer's Research UK.

## ACKNOWLEDGMENTS

The authors wish to thank Iwona Loza, Agata Stramek and Karen Evans for technical support.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.01045/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

SYSTEMATIC REVIEW
published: 25 October 2019
doi: 10.3389/fnins.2019.01108

# Examining Adolescence as a Sensitive Period for High-Fat, High-Sugar Diet Exposure: A Systematic Review of the Animal Literature

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Animal studies suggest that poor nutrition (e.g., high-fat, high-sugar diets) may lead to impairments in cognitive functioning. Accumulating evidence suggests that the deleterious effects of these diets appear more pronounced in animals maintained on this diet early in life, consistent with the notion that the developing brain may be especially vulnerable to environmental insults. The current paper provides the first systematic review of studies comparing the effects of high-fat, high-sugar diet exposure during adolescence and adulthood on memory performance. The majority of studies (7/8) identified here report diet-induced memory problems when diet exposure began in adolescence but not adulthood. These findings lend support to the hypothesis that adolescence is a sensitive period during which palatable diets may contribute to negative neurocognitive effects. The current review explores putative mechanisms involved in diet-induced cognitive dysfunction and highlights promising areas for further research.

**Keywords: fat, sugar, adolescence, memory, hippocampus**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *11 June 2019*
***Accepted:*** *01 October 2019*
***Published:*** *25 October 2019*

***Citation:***
*Murray S and Chen EY (2019) Examining Adolescence as a Sensitive Period for High-Fat, High-Sugar Diet Exposure: A Systematic Review of the Animal Literature. Front. Neurosci. 13:1108. doi: 10.3389/fnins.2019.01108*

## INTRODUCTION

Dessert, pizza, and soda are the three primary sources of daily calorie intake for children and adolescents in the United States (Reedy and Krebs-Smith, 2010). Though the negative health risks associated with poor diet (e.g., diabetes and cardiovascular disease) are widely known, there is considerably less public awareness regarding the link between diet quality and cognitive functioning. Evidence from animal and human studies suggest that diets high in saturated fats and refined sugars are associated with impaired memory performance and hippocampal dysfunction (Greenwood and Winocur, 1990; Kanoski et al., 2007; Francis and Stevenson, 2011; Baym et al., 2014). Emerging evidence from the animal literature suggests that the negative effects of these diets may be more pronounced when exposure occurs during the adolescence, consistent with the notion that the developing brain is especially vulnerable to environmental insults (Schneider, 2013). Recent reviews (Yeomans, 2017; Davidson et al., 2019) have synthesized the literature regarding the effects of palatable diets on cognition, including reviews with a focus on the effects of diet exposure during adolescent development in particular (Reichelt, 2016; Reichelt and Rank, 2017; Del Olmo and Ruiz-Gayo, 2018). The current paper seeks to extend this literature by conducting the first systematic review of animal studies including groups exposed to high-fat, high-sugar diet during adolescence and adulthood and tested for memory.

## METHODS

Relevant studies up to 5/12/2019 were identified through PubMed and Web of Science database searches using the terms "juvenile," "adolescent," "adolescence," "diet," "fat," "sugar," and "memory." These search terms yielded a total of 613 results and an additional 5 articles were identified from reference lists (**Figure 1**). After removing duplicates ($n = 353$), 265 abstracts were screened and 208/265 excluded. Papers were excluded on the basis of the type of publication (i.e., reviews or abstracts were not included), if the study was conducted in humans, and aspects of study design. For example, diet exposure occurred outside of adolescence (approximately postnatal day 28–60 Spear, 2000), there was no diet manipulation or a different type of diet (e.g., protein restriction), or there was no memory task administered or the study assessed emotional memory which is thought to rely on the amygdala. This left a total of 57 full-text articles assessed for eligibility. Of these, 49 studies were excluded, leaving 8 eligible studies. Eligible studies included both adolescent and adult groups of rodents exposed to high-fat or high-sugar diets and tested for memory performance. Though calculating total N is complicated by the fact that some studies used separate cohorts of rodents to test different outcomes (e.g., to avoid the confound of exposure to behavioral testing on brain tissue), the eight studies included a minimum total of 309 rodents.

## RESULTS

A total of eight studies including both adolescent and adult groups were identified (**Table 1**; Privitera et al., 2011; Boitard et al., 2012, 2014; Kendig et al., 2013; Valladolid-Acebes et al., 2013; Hsu et al., 2015; Klein et al., 2016; Labouesse et al.,

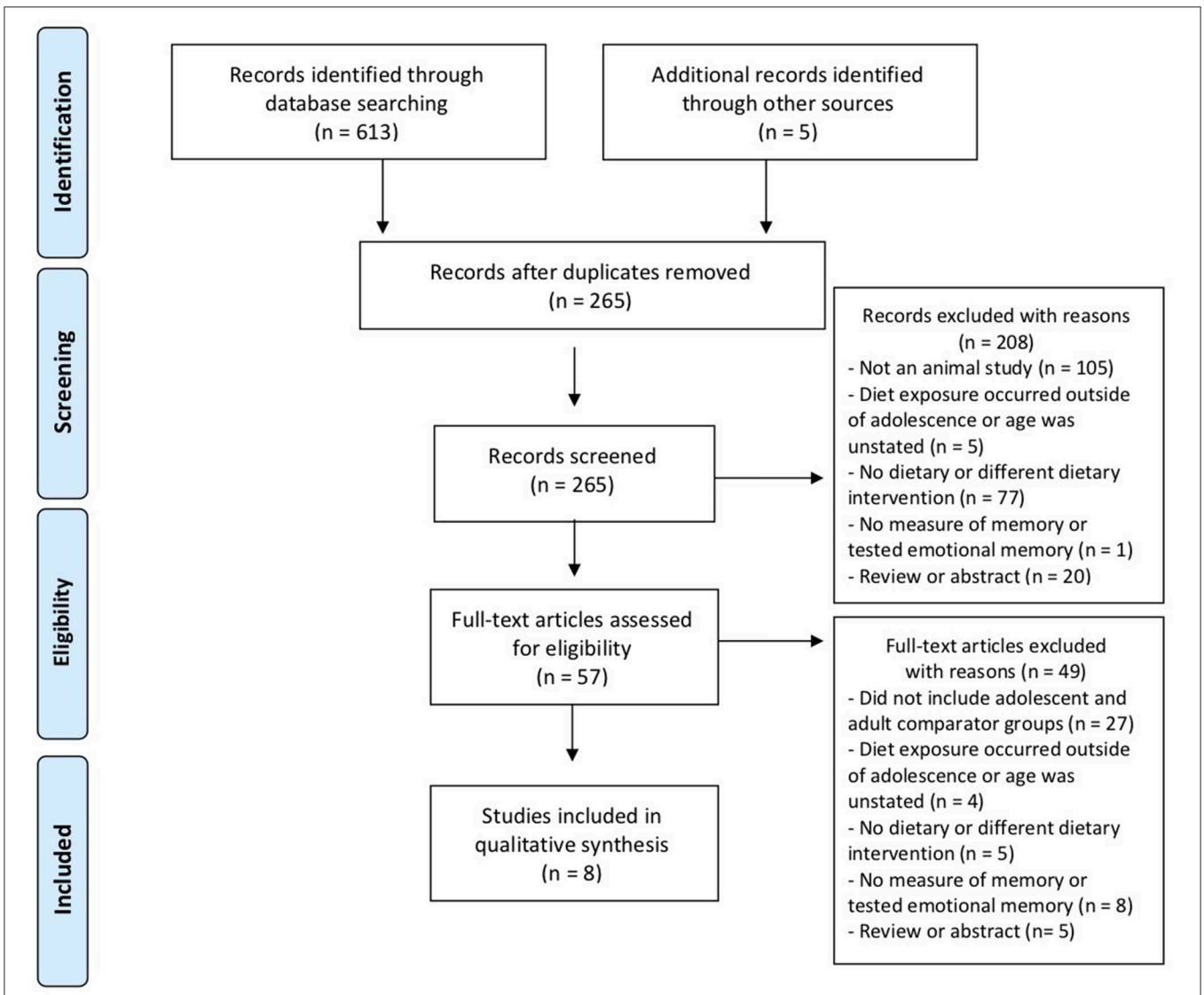


**FIGURE 1 |** PRISMA flow diagram illustrating the process by which relevant studies were identified.

**TABLE 1 |** Description of the eight animal studies identified in the current review.

| | Species | Sex | Groups (at least 4 groups per study) | Diet duration | Age at testing | Behavioral measure | Behavioral findings | Neural findings |
|---|---|---|---|---|---|---|---|---|
| Privitera et al. (2011) | Sprague Dawley rats | Male | Adol (3 wks) or Adult (8 wks) X HFD (60%) or Ctl (10%) $n = 6$ per group | 2–3 wks | 8 or 14 wks | CPP | CPP was not seen in adolescent HFD-fed groups when tested immediately or after several wks; CPP was seen in adolescent Ctl and adult HFD-fed groups | NA |
| Boitard et al. (2012) | C57BL/6J mice | Male | Adol (3 wks) or Adult (12 wks) X HFD (45%) or Ctl $n = 27$ Adol and 26 Adult | 11 wks | 14 or 23 wks | Radial maze | Mice fed a HFD in adolescence showed impaired performance on the recombination task but not the standard spatial discrimination task with no effect observed in adult rats | Adolescent mice fed a HFD showed reduced neurogenesis in the HP which was not seen in adult HFD-fed mice |
| Kendig et al. (2013) | Hooded Wistar rats | Male | Adol (4 wks) or Adult (9 wks) X Standard chow plus 10% sucrose solution 2 h daily or standard chow plus. 1% sodium saccharin 2 h daily $n = 10$ per group | 4 wks | 8 and 13 wks | MWM | Sucrose groups did not show different escape latencies during training; Sucrose groups showed impaired performance on probe trials; No effect of age | NA |
| Valladolid-Acebes et al. (2013) | C57BL/6J mice | Male | Adol (5 wks) or Adult (8 wks) X HFD (45%) or Ctl $n = 12$–15 per group | 8 wks | 13, 16, and 18 wks | NLR | Decreased discrimination ratios for NRL task in adolescent HFD groups even after calorie restriction; no effect in adults | Increased dendritic spine density in HP of adolescent HFD group but not adult HFD group |
| Boitard et al. (2014) | Wistar rats | Male | Adol (3 wks) or Adult (12 wks) X HFD (45%) or Ctl $n = 10$–14 per group | 4, 8, or 12 wks | Varies | MWMR | No difference during learning or short term (2 h delay) trial of MWM; Adolescent HFD-fed animals showed poorer performance on the long-term (4 days) and reversal learning trials; No differences in adult groups | No evidence of increased inflammation following a HFD; after an immune challenge, adolescent HFD rats showed greater inflammation in the HP; not seen in adults |
| Hsu et al. (2015) | Sprague Dawley rats | Male | Adol (4 wks) or Adult (9 wks) X Chow plus *ad libitum* 11% sucrose or chow plus *ad libitum* 11% high-fructose corn syrup-55 or Ctl $n = 12$–13 per group | 4 wks | 8 or 13 wks | Barnes maze; Y maze | Adolescent HFCS group showed impaired acquisition and performance on the Barnes Maze Task whereas no effect was seen in adult groups; No effect of HFCS on Y maze performance in adolescent groups | Increased HP inflammation in adolescent HFCS group compared to age-matched controls with no differences seen between adult diet groups |
| Klein et al. (2016) | C57BL/6J mice | Female | Adol (6 wks) or Adult (10 wks) X HFD (60%) or Ctl $n = 5$–10 per group | 12 or 14 wks | 18 or 24 wks | MWMR | Adolescent sedentary rats fed a HFD during adolescence showed impaired flexible memory performance; Exercise prevented flexible memory impairments; HFD during adulthood did not lead to flexible memory impairments | Sedentary rats fed a HFD during adolescence showed decreased immature neurons in the HP; HFD was also associated with newborn neurons and survival of proliferating cells in the HP of adults |
| Labouesse et al. (2017) | C57BL/6J mice | Male | Adol (4 wks) or Adult (10 wks) X HFD (63%) or Ctl $n = 9$–10 per group | 4 wks | 10–17 or 15–21 wks | Y maze; MWM; T maze with reversal learning | Adolescent HFD group showed poorer performance on spatial working memory tasks (Y-maze and adapted MWM) and reversal learning in a T maze with no difference in learning T maze | Reduced in RELN cells in the mPFC of adolescent HFD group and impaired synaptic functioning; HFD adults showed reduced RELN in the HP |

*Adol, adolescence; Wks, weeks; HFD, high fat diet; Ctl, control; CPP, conditioned place preference; NA, not applicable; HP, hippocampus; MWM, Morris Water Maze; NLR, novel location recognition; MWMR, reversal learning with MWM; RELN, Reelin; mPFC, medial prefrontal cortex.*

2017). Six of these examined the effects of a high fat diet (HFD). Two assessed the effects of sucrose and one of these two also tested the effects of high fructose corn syrup (HFCS). Six of the eight studies included analysis of possible neural mechanisms. Adolescent diet exposure began between 3 and 6 weeks of age and adult diet exposure began between 8 and 12 weeks of age. Diet duration ranged significantly across studies, from 2 to 14 weeks. Only one of the eight studies included

female subjects. Five of the eight studies included additional measures to eliminate potential confounds, such as locomotor activity, distance traveled, swimming speed, and time spent exploring. Of the eight studies identified, seven reported poorer performance across a range of cognitive tasks (**Table 2**) following diet exposure during adolescence but not adulthood (Privitera et al., 2011; Boitard et al., 2012, 2014; Valladolid-Acebes et al., 2013; Hsu et al., 2015; Klein et al., 2016; Labouesse et al., 2017). The one study that did not observe an age-dependent effect reported poorer cognitive performance following sucrose access regardless of age of exposure (Kendig et al., 2013). All animals were tested in adulthood including groups with diet exposure during adolescence.

In a seminal study by Privitera et al. (2011), rats fed a HFD during adolescence failed to develop conditioned place preference compared to rats fed a control diet during adolescence and adult rats fed a HFD (Privitera et al., 2011). Conditioned place preference, mediated in part by the hippocampus (Hitchcock and Lattal, 2018), is assessed by exposing animals to two locations, one of which contains a reinforcer (in this case, Cheetos®) and measuring the time spent in the location previously associated with a reinforcer. Boitard et al. (2012) also reported impaired memory flexibility in animals fed a HFD in adolescence but not adulthood. Impaired memory flexibility, or the ability to update memory by learning novel contingencies after learning a different set of relationships, relies in part on hippocampus (Rossato et al., 2006). Indeed, this study reported a 23% reduction in hippocampal neurogenesis in mice fed a HFD in adolescence but not adulthood. In another study by Boitard et al. (2014), rats fed a HFD in adolescence also exhibited impaired memory flexibility as well as impaired long-term (4 days delay), but not short-term (2 h delay) spatial memory, suggesting possible deficits in memory consolidation. The results of this study also point to a possible role of neuroinflammation, as adolescent HFD animals exhibited elevated inflammatory cytokines within the hippocampus in response to an immune challenge. Both the behavioral and inflammation findings were not replicated in HFD-fed adult rats.

Given the role of the prefrontal cortex in cognitive flexibility (Kim et al., 2011) and the well-characterized maturation of this region during adolescence (Arain et al., 2013), research has also begun to explore the role of the prefrontal cortex in HFD-induced deficits. Labouesse et al. (2017) demonstrate impaired spatial working memory along with cognitive flexibility, assessed via reversal learning, in animals fed a HFD in adolescence. Briefly, reversal learning was assessed by training animals to associate either the right or left arm of a T-shaped maze in water with an escape platform. After acquisition, the location of the escape arm was switched. These behavioral findings were accompanied by a significant reduction in reelin, a regulator of synaptic plasticity, within the medial prefrontal cortex. Neither effect was observed when diet exposure was initiated adulthood, though adult HFD-exposed mice did exhibit a downregulation of reelin in the hippocampus (Labouesse et al., 2017). Given that the adult HFD group also showed significant increases in body weight compared to age-matched controls, these deficits appear to be driven by age of diet exposure and not obesity.

Impaired spatial memory, assessed using a novel location recognition paradigm with 1 and 24 h delays, has also been observed in adolescent mice fed a HFD, with no effects in an adult HFD group (Valladolid-Acebes et al., 2013). Moreover, though elevated leptin levels were observed in HFD-fed mice of both age groups, only HFD-fed adolescent mice showed evidence of leptin resistance. This is of particular relevance as leptin has been shown to influence synaptic plasticity within the hippocampus and improve memory (Harvey et al., 2006). Interestingly, this study also found increased dendritic spine density of hippocampal neurons in HFD-fed adolescent mice which was hypothesized to reflect a potential compensatory reorganization of neuronal architecture.

In an effort to determine whether physical activity might protect against or ameliorate diet-induced memory deficits, Klein et al. (2016) compared memory performance between HFD-fed adolescent animals with and without simultaneous access to a running wheel. This study corroborates earlier findings showing that HFD during adolescence is associated with poorer memory flexibility and fewer immature neurons in the hippocampus. Exercise was shown to prevent HFD-related impairments in memory flexibility and increase the survival of proliferating cells and newborn mature neurons in the hippocampus (Klein et al., 2016). Exercise introduced after

**TABLE 2 |** Brief description of behavioral tests used to assess memory function in the studies identified via systematic review.

| Task | Brief description |
|---|---|
| Morris Water Maze | Animals are trained to learn and remember visual cues to successfully locate a platform submerged in a pool of water |
| Barnes maze | Animals are trained to learn and remember visual cues to successfully locate a hidden escape under one of many holes surrounding a circular platform |
| Novel Location Recognition | Animals are presented with two objects, one of which has been moved from its previous location. If animals spend more time exploring the novel location, they are thought to have remembered that the other object has maintained its original location |
| Y-maze | Animals are allowed to explore the arms of a Y-shaped. If animals spend more time exploring one arm after another, they are thought to have remembered that they already explored the other† |
| Conditioned Place Preference | Animals are trained to associate a location with a rewarding or neutral stimuli and condition place preference is thought to be established when animals tend to prefer the location associated with reward in its absence |
| Radial Maze | Animals learn to associate one of eight arms in the radial-maze with a food reward |
| Water T-maze | Animals learn to associate one arm of the T-maze with escape |
| Reversal training* | New contingencies, such as a new (1) location for the hidden platform after training in the MWM or (2) arm of the radial-maze associated with a reward or (3) arm of the water T-maze associated with escape |

† *The Y-maze used in Hsu et al. (2015) relied on animals learning to associate one arm of the maze with an escape, similar to the T-maze paradigm. *Other tasks can be used to memory or cognitive flexibility.*

HFD access did not improve memory flexibility in animals with HFD exposure in adulthood though adult mice did not show impairments in diet-related spatial learning or memory flexibility. This study did not include an adult comparator group with access to both exercise and a HFD, making it difficult to compare results directly. Of note, adult HFD exposure did affect the survival of proliferating cells and newborn neurons in the hippocampus; in the absence of a behavioral effect, the authors propose that this may indicate "an age-dependent utility of neurogenesis." This echoes previously described findings of lower reelin concentrations in the hippocampus of adult HFD mice, as here too neuroadaptations were not associated with cognitive performance as it was assessed.

Findings regarding an age-dependent vulnerability for the neurocognitive effects of sugar appear limited and mixed. One study found that compared to age-matched rats fed a standard chow diet, both adolescent and young adult rats given 2 h of sucrose access daily showed poorer performance on the Morris Water Maze (MWM) (Kendig et al., 2013), indicating an effect of sucrose access but not age of exposure. The MWM is often used as a spatial memory task and entails that animals learn to use visual cues in their surrounding environment to locate a hidden platform in a pool of water. Notably, this study also reported impaired memory performance among sucrose-exposed animals after over 6 weeks of abstinence. In another study, exposure to a HFCS solution during adolescence was associated with impaired performance on the Barnes maze task, which is similar to the MWM in that animals must learn to use spatial cues to locate an escape box hidden beneath one of 18 holes surrounding a circular platform. Unlike the MWM, this task is not administered in water; instead, animals are motivated to find an escape with the addition of bright light and white noise. In addition to differences in memory performance, animals that consumed a HFCS in adolescence exhibited increased IL-1β and IL-6 relative to adolescent sucrose- and chow-fed controls, whereas no differences in memory or inflammation were observed between adult diet groups (Hsu et al., 2015). This study found no effect of adolescent HFCS exposure on performance on the Y maze task, which the authors argue suggests a hippocampal-specific effect of HFCS. Performance on the Y maze is sometimes assessed by recording the number of alternations between the right and left arms of a Y-shaped maze which are taken as indications that the animal remembers which arm it last traveled down. However, in this study, animals were trained to associate one of two arms with an escape box (much like the T-maze mentioned earlier but without a reversal training component). In contrast to Kendig et al. (2013), Hsu et al. (2015) did not find sucrose to significantly affect memory performance in either adolescent or adult groups despite similar concentrations of sucrose, ages at diet initiation, and diet exposure periods. However, rodents were provided limited sugar access in the study by Kendig et al. (2013) and limited access models have been shown to promote greater sucrose intake (Eikelboom and Hewitt, 2016). These two studies also included different animal strains which may contribute to differences in memory performance (Jonasson, 2005). These experiments also operationalized memory using different tasks. Taken together, these findings suggest that consumption of HFCS, but not sucrose, may exert a more negative effect on memory and neuroinflammation during adolescence than adulthood.

## DISCUSSION

The current findings suggest that adolescence represents a window of sensitivity to the deleterious neurocognitive effects of HFD and HFCS. This finding was observed rather consistently and despite between-study variability in diet composition, age at diet exposure, duration of diet exposure, age at testing, and the use of different measures of memory.

Proposed mechanisms for adolescent diet-induced cognitive deficits include reduced neurogenesis, altered synaptic plasticity, neuroinflammation, and dysfunction of appetite-regulating hormones, such as leptin. The current findings are consistent with multiple studies showing a reduction in neurogenesis following HFD access during adolescence (Boitard et al., 2012; Klein et al., 2016; Vinuesa et al., 2016, 2018). Studies also show reductions in hippocampal brain derived neurotropic factor (BDNF), a key regulator of neurogenesis, and increased markers of apoptosis (i.e., cell death) in adolescent HFD-fed mice (Wu et al., 2018). Such findings may help to explain neuroimaging data showing brain atrophy, including reduced hippocampal gray matter, among rats fed a HFD in adolescence or young adulthood (Kalyan-Masih et al., 2016; Rollins et al., 2019). Adolescent sucrose access has been associated with reductions in neurogenesis or neuroproliferation in some (Gueye et al., 2018) but not all studies (Ferreira et al., 2018; Xu and Reichelt, 2018). Sucrose exposure during this developmental period has also been shown to result in reductions in hippocampal parvalbumin-containing ϒ-aminobutyric acid (GABA)-ergic interneurons, thought to be critical for memory processes (Fuchs et al., 2007; Ognjanovski et al., 2017) and implicated in Alzheimer's disease pathology (Zallo et al., 2018).

Neuroinflammation has been proposed as another potential mediator of HFD-induced memory disturbance in adolescent animals though findings are mixed. As mentioned earlier, Boitard et al. (2014) report elevated hippocampal inflammation in response to an immune challenge in adolescent HFD-fed animals but no difference in basal markers of inflammation (Boitard et al., 2014). Mice fed a HFD in adolescence show evidence of microglial activation along with increased inflammation in the hippocampus (Vinuesa et al., 2018; Wu et al., 2018). In contrast, some studies have not observed significant differences in inflammatory biomarkers following adolescent HFD access (Kaczmarczyk et al., 2013; Wang et al., 2015). Recent evidence suggests that aged animals may be more susceptible to neuroinflammation following access to a cafeteria diet (Teixeira et al., 2019), suggesting an additional developmental period of vulnerability to high fat, high sugar diets.

Changes in synaptic plasticity, as reported by Labouesse et al. (2017), have also been documented previously following adolescent exposure to HFD. Hippocampal slices from adolescent mice fed a HFD for only 48 h showed partial inhibition of LTP, with particular impairment in LTP maintenance (Contreras et al., 2017). Moreover, adolescent HFD exposure has also been shown to result in synaptic remodeling and a 50% decrease in the Shank2 protein involved in spine morphogenesis

(Vinuesa et al., 2018). Though the effects of a HFD on the hippocampus have been well-studied, Labouesse et al. (2017) also points to a role for the medial prefrontal cortex in diet-induced memory deficits. Given that these regions are known to form a functional system (Bizon et al., 2012), future research exploring how palatable diets affect cognitive development at the circuit level using a systems neuroscience approach may be insightful.

Many of the behavioral and neural outcomes described here have been reported in adult animals following access to palatable diets. For example, impaired reversal learning and reduced BDNF have been reported in adult animals fed a HFD (Kanoski et al., 2007) and signs of leptin resistance in diet-induced obesity have been observed independently of age (Sainz et al., 2015). Moreover, studies in the current review show evidence of neural changes in animals with HFD access beginning in adulthood. Therefore, it has been proposed that adult animals may have a higher threshold for sensitivity to diet-induced cognitive deficits such that higher fat content or longer diet duration may be necessary to observe behavioral differences (Boitard et al., 2012; Valladolid-Acebes et al., 2013).

Though Klein et al. (2016) suggest that exercise may help to prevent the deleterious effects of HFD exposure during adolescence, another promising intervention is switching animals to a lower fat diet (Kaczmarczyk et al., 2013; Boitard et al., 2016; Sims-Robinson et al., 2016). However, some data suggest long-term neurocognitive effects of adolescent exposure to palatable diets even after prolonged periods (e.g., up to 61 weeks) of diet abstinence (Kendig et al., 2013; Reichelt et al., 2015; Wang et al., 2015; Noble et al., 2019). Two of these studies examined the effects of sucrose (Kendig et al., 2013; Reichelt et al., 2015), one examined the effects of HFCS (Noble et al., 2019), and one examined the effects of a high fat diet (Wang et al., 2015). Additional research is needed to better understand potential long-term effects of adolescent consumption of fat and sugar on risk for cognitive decline in late life as well as to identify effective approaches prevention and intervention. One candidate for study may be working memory training as this has been shown to improve reference memory, reversal learning, and synaptic plasticity in rodents (Ioakeimidis et al., 2018).

The current set of findings are not without limitations. First, with a few exceptions (Boitard et al., 2012; Hsu et al., 2015), the studies described here did not directly compare outcomes between adolescent and adult groups and instead compared diet groups with their age-matched counterparts. In addition, age at testing varied between adolescent and adult groups; if diet duration is the constant for both groups and testing occurs directly following diet exposure, adolescent animals will be tested earlier in development relative to adults. However, typically, younger cohorts show superior memory performance relative to older cohorts (Lindner, 1997; Mizoguchi et al., 2010; Johnson and Wilbrecht, 2011), making it unlikely that this age discrepancy at testing would influence these findings. It is also worth noting that reversal learning tasks may reflect a number of cognitive functions, including inhibition, attention, information processing, and decision-making regarding search strategies, that may contribute to poorer task performance separate from or because of their contribution to memory processes. An additional limitation of the current research is the paucity of studies including female mice; future research examining sex differences in adolescent exposure to high-fat, high-sugar diets is warranted given evidence of sex differences in memory in rodent models (Jonasson, 2005). It should also be noted that some HFD formulations derive a sizeable proportion of the carbohydrate content from sucrose, which may confound findings. Moreover, fat source appears to differentially affect memory outcomes, with research showing that polyunsaturated fats improve memory and increase BDNF in adolescent animals (Dos Santos et al., 2018).

As pointed out by Hooijmans et al. (2014), systematic reviews of laboratory animal experiments are fairly uncommon and assessing risk of bias using the guidelines this group of authors set forth is somewhat challenging as certain information is often unstated in animal studies. For example, few studies included here stipulated that group assignment was based on random allocation and that assessments were conducted by investigators blind to experimental conditions. Future studies should include such information to assist in study evaluation. At the review-level, it is possible that the current search strategy did not capture all relevant studies, though two search databases were used. It is also quite possible that publication bias resulted in a skewed set of findings presented here, though one study included here did not observe an effect of age.

## CONCLUSIONS AND FUTURE DIRECTIONS

Current dietary recommendations encourage limiting intake of added sugars and saturated fat throughout the lifespan. The findings reviewed here suggest that additional awareness could be raised about the intake of these diets during adolescence in particular. However, efforts to translate animal findings are needed to inform public policy initiatives. While available data are generally consistent with the hypothesis that diets high in fat and sugar may be associated with poorer neuropsychological performance in youth (Junger and van Kampen, 2010; Francis and Stevenson, 2011; Gibson et al., 2013; Baym et al., 2014), more studies are needed. Memory function is essential for supporting learning and has implications for academic achievement and everyday functioning; therefore, understanding the impact of high-fat, high-sugar diets on cognitive development throughout the lifespan remains an important area for further study.

## AUTHOR CONTRIBUTIONS

SM conducted the systematic review of the literature. SM and EC contributed to the final version of the manuscript. EC supervised the project.

## FUNDING

This research was supported by a National Science Foundation Graduate Research Fellowship (SM) and publishing was made possible by Temple University Libraries Open Access Publishing Fund.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 17 December 2019
doi: 10.3389/fnins.2019.01335

# Significant Effects of Maternal Diet During Pregnancy on the Murine Fetal Brain Transcriptome and Offspring Behavior

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**Specialty section:**
This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience

**Received:** 29 June 2019
**Accepted:** 27 November 2019
**Published:** 17 December 2019

**Citation:**
Edlow AG, Guedj F, Sverdlov D, Pennings JLA and Bianchi DW (2019) Significant Effects of Maternal Diet During Pregnancy on the Murine Fetal Brain Transcriptome and Offspring Behavior. Front. Neurosci. 13:1335. doi: 10.3389/fnins.2019.01335

**Background:** Maternal over- and undernutrition in pregnancy plays a critical role in fetal brain development and function. The effects of different maternal diet compositions on intrauterine programing of the fetal brain is a lesser-explored area. The goal of this study was to investigate the impact of two chowmaternal diets on fetal brain gene expression signatures, fetal/neonatal growth, and neonatal and adult behavior in a mouse model.

**Methods:** Throughout pregnancy and lactation, female C57Bl/6J mice were fed one of two standard, commercially available chow diets (pellet versus powder). The powdered chow diet was relatively deficient in micronutrients and enriched for carbohydrates and n-3 long-chain polyunsaturated fatty acids compared to the pelleted chow. RNA was extracted from embryonic day 15.5 forebrains and hybridized to whole genome expression microarrays ($N$ = 5/maternal diet group). Functional analyses of significantly differentially expressed fetal brain genes were performed using Ingenuity Pathways Analysis and Gene Set Enrichment Analysis. Neonatal behavior was assessed using a validated scale ($N$ = 62 pellet-exposed and 31 powder-exposed). Hippocampal learning, locomotor behavior, and motor coordination were assessed in a subset of adults using fear conditioning, open field testing, and Rotarod tests ($N$ = 16 pellet-exposed, 14 powder-exposed).

**Results:** Comparing powdered to pelleted chow diets, neither maternal weight trajectory in pregnancy nor embryo size differed. Maternal powdered chow diet was associated with 1647 differentially expressed fetal brain genes. Functional analyses identified significant upregulation of canonical pathways and upstream regulators involved in cell cycle regulation, synaptic plasticity, and sensory nervous system development in the fetal brain, and significant downregulation of pathways related to cell and embryo death. Pathways related to DNA damage response, brain immune response, amino acid and fatty acid transport, and dopaminergic signaling were significantly dysregulated. Powdered chow-exposed neonates were significantly longer but not heavier than pelleted chow-exposed counterparts. On neonatal behavioral

testing, powdered chow-exposed neonates achieved coordination- and strength-related milestones significantly earlier, but sensory maturation reflexes significantly later. On adult behavioral testing, powdered chow-exposed offspring exhibited hyperactivity and hippocampal learning deficits.

**Conclusion:** In wild-type offspring, two diets that differed primarily with respect to micronutrient composition had significant effects on the fetal brain transcriptome, neonatal and adult behavior. These effects did not appear to be mediated by alterations in gross maternal nutritional status nor fetal/neonatal weight. Maternal dietary content is an important variable to consider for investigators evaluating fetal brain development and offspring behavior.

**Keywords: maternal diet, fetal brain, transcriptome, strength, coordination, sensory, micronutrient, fatty acid**

## INTRODUCTION

In both human epidemiologic and animal model studies, maternal under- and overnutrition in pregnancy has been well-demonstrated to have a deleterious impact on fetal brain development and offspring behavior (Tozuka et al., 2009, 2010; White et al., 2009; Antonow-Schlorke et al., 2011; Brion et al., 2011; Krakowiak et al., 2012; Kang et al., 2014; Edlow et al., 2016a,b; Li et al., 2016; van der Burg et al., 2016; Veena et al., 2016; Edlow, 2017; Winther et al., 2018). The impact of maternal dietary micronutrient composition on the developing fetal brain has not been as well-characterized. Dietary micronutrient deficiency may have broader relevance to conditions such as maternal obesity, in which a relative micronutrient deficiency has been proposed due to poor maternal dietary quality (Pinhas-Hamiel et al., 2003; Kimmons et al., 2006; Laraia et al., 2007; Darnton-Hill and Mkparu, 2015; Jones et al., 2016; Scholing et al., 2018). The relative contribution of maternal micronutrient deficiency compared to maternal pre-pregnancy obesity or maternal high-fat diet in mediating some of the deleterious effects of maternal obesity on the developing brain is unknown. Similarly, to what extent the deleterious impact of maternal undernutrition in pregnancy on the developing fetal brain may be mediated by micronutrient versus macronutrient deficiency in the diet remains to be elucidated.

While the impact of maternal macronutrient and micronutrient intake in pregnancy has been examined in several human observational studies, these focus primarily on the impact of maternal intake on pregnancy outcomes such as preeclampsia and preterm birth, on fetal growth trajectory, and on neonatal outcomes such as small- and large-for-gestational age, and incidence of congenital anomalies (Mousa et al., 2019). Fewer studies have focused directly on the impact of maternal pregnancy and lactational nutrition on fetal brain development and offspring behavior (Prado and Dewey, 2014; Li et al., 2019). There remains a knowledge gap regarding the impact of maternal micro- and macronutrient intake specifically on fetal brain development and offspring behavior.

We sought to address this knowledge gap by evaluating the impact of maternal dietary composition in pregnancy and lactation on fetal brain development and offspring behavior, in the absence of maternal pre-pregnancy obesity or maternal over- or under-nutrition, using two standard commercially available chow diets that differed significantly with respect to their micronutrient content (including vitamins and minerals), and differed in macronutrient content only with respect to carbohydrate. Our objective was to evaluate the impact of maternal pregnancy diet on fetal brain gene expression, neonatal behavior, neonatal growth trajectory, and adult offspring behavior. If standard maternal chow diets themselves have an impact on fetal brain development, the choice of chow diet may be an important variable to consider for neuroscience researchers investigating the impact of maternal exposures on the developing brain.

## MATERIALS AND METHODS

### Mouse Strain, Breeding, Pregnancy and Lactation Diets

This study was part of a larger research program examining the impact of maternal nutrient supplementation in pregnancy on fetal and offspring brain development in mouse models of Down syndrome compared to wild-type (Guedj et al., 2018). Female C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME, United States) were crossed with Ts1Cje males [B6 T(12:16)1Cje/CjeDnJ], to generate pregnancies in which approximately half of the fetuses were affected with Down syndrome and half were wild-type. Only outcomes from wild-type fetuses and offspring (exposed to the intrauterine environment of a wild-type/C57Bl/6J dam) were examined. Breeder pairs received either a standard commercially available pelleted chow (Teklad 2918), or a standard commercially available purified powdered chow diet (Bioserv F3197). The content of each diet is depicted in **Table 1**.

The rationale was to determine the effects of different chow diets on wild-type fetal brain development and offspring behavior, in order to select an optimal control diet for a subsequent set of experiments focused on the impact of isoflavone supplementation during pregnancy on fetal brain development and offspring behavior in Down syndrome (Guedj et al., 2018). The powdered chow was specifically selected for

**TABLE 1 |** Maternal diet composition.

| Class | Component | Powder chow Bioserv F3197 | Regular chow Teklad 2918 |
|---|---|---|---|
| **Isoflavone** | Daidzein, genistein | Not present | 150–250 mg/kg |
| **macronutrients** | Crude protein | 18.1% | 18.6% |
| | Fat | 7.1% | 6.2% |
| | Carbohydrate | 59.3% | 44.2% |
| | Crude fiber | 4.8% | 3.5% |
| | Nutral detergent fiber | Not present | 14.7% |
| | Ash | 2.2% | 5.3% |
| **Caloric profile** | Protein | 0.72 Kcal/g | 0.74 Kcal/g |
| | Fat | 0.64 Kcal/g | 0.56 Kcal/g |
| | Carbohydrates | 2.37 Kcal/g | 1.20 Kcal/g |
| | Total | 3.74 Kcal/g | 3.1 Kcal/g |
| **Micronutrients-Minerals** | Calcium | 5.1 g/kg | 10 g/kg |
| | Phosphorus | 2.8 g/kg | 7 g/kg |
| | Sodium | 1.03 g/kg | 2 g/kg |
| | Potassium | 3.6 g/kg | 6 g/kg |
| | Chloride | 1.6 g/kg | 4 g/kg |
| | Magnesium | 0.51 g/kg | 2 g/kg |
| | Zinc | 37.7 mg/kg | 70 mg/kg |
| | Manganese | 10.5 mg/kg | 100 mg/kg |
| | Copper | 6.0 mg/kg | 15 mg/kg |
| | Iodine | 0.21 mg/kg | 15 mg/kg |
| | Iron | 37.2 mg/kg | 200 mg/kg |
| | Selenium | 0.17 mg/kg | 0.23 mg/kg |
| | Chromium | 1 mg/Kg | Not present |
| | Fluoride | 1 mg/kg | Not present |
| | Sulfur | 301 mg/kg | Not present |
| **Micronutrients-Vitamins** | Vit A | 4.14 IU/g | 15 IU/g |
| | Vit D3 | 1 IU/g | 1.5 IU/g |
| | Vit E | 0.083 IU/g | 110 IU/g |
| | Vit K3 | Not present | 50 mg/kg |
| | Vit B1 | 6 mg/kg | 17 mg/kg |
| | Vit B2 | 6 mg/kg | 15 mg/kg |
| | Niacin | 30 mg/kg | 70 mg/kg |
| | Vit B6 | 5.8 mg/kg | 18 mg/kg |
| | Pantothenic acid | 14.7 mg/kg | 33 mg/kg |
| | Vit B12 | 0.025 mg/kg | 0.08 mg/kg |
| | Biotin | 0.2 mg/kg | 0.4 mg/kg |
| | Folate | 2 mg/kg | 4 mg/kg |
| | Choline | 1028 mg/kg | 1200 mg/kg |
| | Vit K1 | 0.88 mg/kg | Not present |
| **Amino acids** | Alanine | 4.6 g/kg | 11 g/kg |
| | Arginine | 6.4 g/kg | 10 g/kg |
| | Aspartic acid | 11.2 g/kg | 14 g/kg |
| | Cystine | 3.5 g/kg | 3 g/kg |
| | Glutamic acid | 35.6 g/kg | 34 g/kg |
| | Glycine | 4.3 g/kg | 8 g/kg |
| | Histidine | 4.8 g/kg | 4 g/kg |
| | Isoleucine | 9.6 g/kg | 8 g/kg |
| | Leucine | 14.6 g/kg | 18 g/kg |
| | Lysine | 13.0 g/kg | 9 g/kg |
| | Methionine | 4.5 g/kg | 4 g/kg |
| | Phenylalanine | 7.8 g/kg | 10 g/kg |
| | Proline | 18.0 g/kg | 16 g/kg |
| | Serine | 10.0 g/kg | 11 g/kg |
| | Threonine | 7.7 g/kg | 7 g/kg |
| | Tryptophan | 2 g/kg | 2 g/kg |
| | Tyrosine | 10 g/kg | 6 g/kg |
| | Valine | 11.4 g/kg | 9 g/kg |
| **Fatty acids** | C16: o Palmetic | Not present | 7 g/kg% |
| | C18: o Stearic | Not present | 2 g/kg |
| | C18: 1ω9 Oleic | Not present | 12 g/kg |
| | C18:2ω6 (n-6 LC-PUFA or linoleic acid, LA) | 35.7 g/kg | 31 g/kg |
| | C18:3ω3 (n-3 LC-PUFA α-linolenic acid or ALA) | 4.8 g/kg | 3 g/kg |
| | Total saturated | 11 g/kg | 9 g/kg |
| | Total monounsaturated | 15.9 g/kg | 13 g/kg |
| | Total polyunsaturated | 40.4 g/kg | 34 g/kg |

interrogation given the absence of added isoflavones. The absence of isoflavone supplementation at baseline in the powdered chow diet was of interest because this study was a precursor to an intervention study investigating the benefits of maternal dietary supplementation with apigenin, an isoflavone, on brain development in offspring with Down syndrome (Guedj et al., 2018). It was therefore important to examine the effects of a diet that could be supplemented with apigenin and did not already contain other isoflavones that could confound the evaluation of apigenin's effect.

All fetuses and offspring reported here are wild-type fetuses exposed to the intrauterine environment of wild-type/C57Bl/6J dams that consumed one of two diets during pregnancy without additional isoflavone supplementation. A subsequent study compared brain development in mouse models of Down syndrome versus their wild-type littermates with and without isoflavone (apigenin) supplementation using only powdered chow (Guedj et al., 2018).

Dams were started on the study diet at the time of initial breeding with a sire, in order to isolate the impact of maternal dietary intake in pregnancy and lactation on fetal brain development and offspring behavior. The dams continued on the diet throughout pregnancy and lactation. Offspring were weaned to the same diet they were exposed to during pregnancy and lactation. With respect to the breeding strategy, females were bred with males overnight. Vaginal plugs were checked by 9 am the next day, with the presence of a vaginal plug defined as embryonic day 0.5 (E0.5). To exclude the possibility of daytime mating, males were separated from females during

the day. Male sires were consistently fed the same single diet as per their initial breeding pair. Females were weighed at embryonic days 0 (day of mating), 10, and 15.5, just prior to euthanasia. Greater than or equal to 10% weight gain at E10 was used to confirm pregnancy (Johnson et al., 2010). Animals were housed in cages with standard bedding and nestlets. Animals were given *ad libitum* access to chow and water. The colony was maintained on a standard 12:12 light-dark cycle, with lights on at 07:00. All experiments were approved by the Tufts Medical Center Institutional Animal Care and Use Committee (IACUC, protocol #B2013-20).

## Maternal Diet Composition Differences

**Table 1** demonstrates the components of the two different chow diets. They differ significantly in several respects. The powdered chow was relatively enriched for carbohydrates compared to the pelleted diet. The powdered chow also contained significantly higher concentrations of n-3 long-chain polyunsaturated fatty acids (LC-PUFAs, linolenic acid) compared to pelleted chow, with a more favorable n-6/n-3 LC-PUFA ratio (7.4 for powdered versus 10.3 for pelleted chow, with an n-6/n-3 ratio of <10 recommended for infant and adult nutrition) (Gerster, 1998; Abedi and Sahari, 2014). The remainder of differences between the two diets favored enrichment of the pelleted diet over the powdered. Compared to the powdered chow, the pelleted chow was enriched for: (1) The isoflavones daidzein and genistein (phytoestrogens known to cross the blood-brain barrier and exert antioxidant effects) (Bang et al., 2004; Zeng et al., 2004); (2) the macroelements calcium, phosphorus, sodium, potassium, chloride, and magnesium (all two- to approximately four-fold higher in pelleted chow); (3) the amino acids alanine, arginine and glycine (all approximately two-fold higher in the pelleted chow); (4) micronutrients zinc, manganese, copper, iodine, iron, and selenium (all higher in pelleted chow, ranging from approximately 2-fold to as high as 70-fold) and vitamin content, with the pelleted chow containing universally higher vitamin concentrations than the powdered chow. With respect to micronutrients, manganese, iodine, and iron have the greatest fold differences between the two diets (9. 5-, 71-, and 5.4-fold higher in pelleted chow, respectively). With respect to vitamins, the difference between the two diets was most notable for Vitamin E (more than 1,000-fold higher in the pelleted chow). Vitamins A, B1, B2, Niacin, B6, pantothenic acid, B12, Biotin, and folate were all two- to four-fold higher in the pelleted chow. In summary, the powdered chow was relatively enriched for carbohydrate content and favorable LC-PUFAs compared to pelleted chow, but was relatively deficient in macroelements, specific amino acids, and micronutrients (including all vitamins and most minerals), compared to the pelleted chow. The experimental paradigm is depicted in **Figure 1**.

## Tissue Collection

On embryonic day 15.5 (E15.5), pregnant dams were euthanized with isoflurane followed by decapitation. Embryos were rapidly dissected from the uterine horns and placed in ice-cold 1X phosphate-buffered saline (PBS) containing RNA preservative (RNALater, Qiagen). Theiler staging was performed to confirm the gestational age of E15.5 (Theiler, 1989, accessed April, 2018). On a cold platform, embryonic brains were rapidly removed from skulls, and forebrain was isolated and snap frozen in liquid nitrogen, prior to storage at −80°C. Tail snips were obtained for Ts1Cje and sex genotyping. Only mice that had the wild-type genotype were used for analysis here.

## Fetal Brain Gene Expression Studies

Total RNA was isolated from embryonic forebrains using the NucleoSpin II RNA/protein kits (Machery-Nagel, Duren, Germany) per the manufacturer's instructions. The isolation included an on-column DNase digestion step to remove genomic

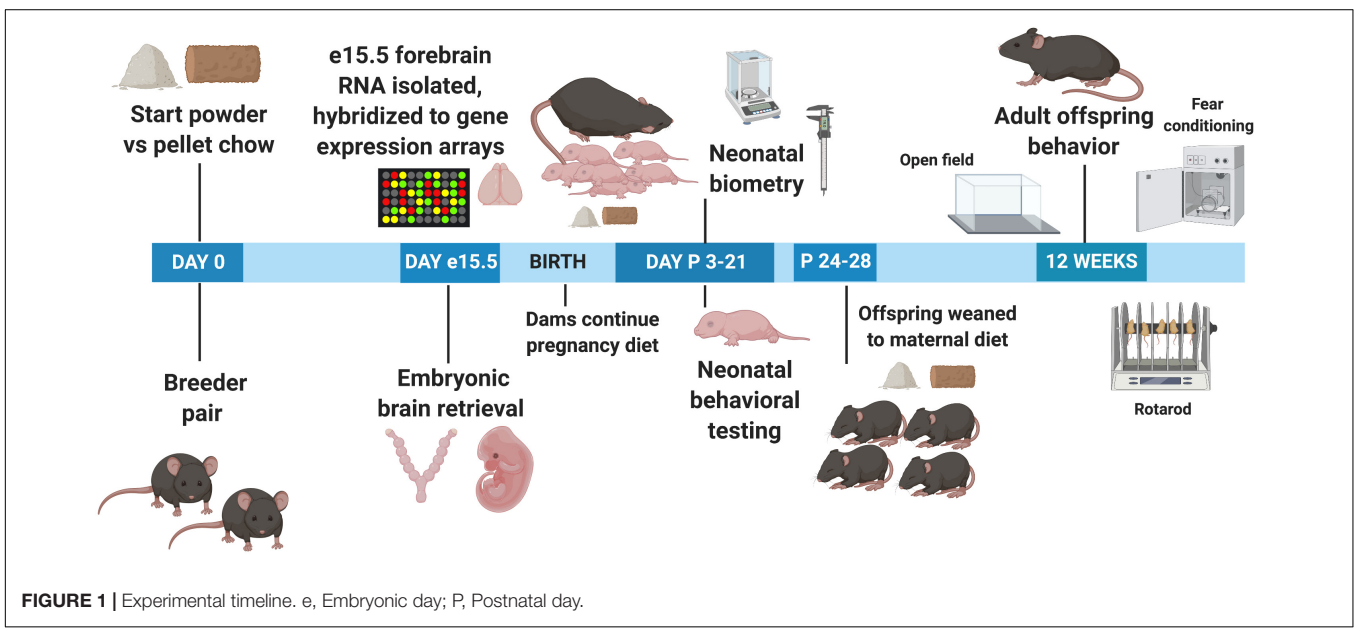


**FIGURE 1 |** Experimental timeline. e, Embryonic day; P, Postnatal day.

DNA. RNA purity, integrity and quantity were assessed using the NanoDrop ND-800 (NanoDrop, Wilmington, DE, United States) and the Bioanalyzer system (Agilent 2100; Agilent Technologies Inc., Palo Alto, CA, United States). RNA was processed and hybridized to Mouse gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, United States) as previously described (Edlow et al., 2016a; Guedj et al., 2016). Five arrays per experimental group were used, with each array corresponding to one embryonic brain. One embryo per litter from five different litters per diet group was included in microarray analyses, to minimize litter effects. Five biological replicates per group has been demonstrated to be sufficient to detect global gene expression changes in microarray studies (Allison et al., 2006; Tarca et al., 2006).

Normalization was performed using the robust multichip average algorithm (RMA) and the MBNI custom CDF[1] version #15 for the Mouse Gene 1.0 ST array. Output consisted of data for 21,225 probe sets each corresponding to unique Entrez Gene IDs. Statistical analyses were performed using R software (version 3.1.2 or later). Fetal brain gene expression data from day 15.5 embryos of dams eating pelleted chow were compared to those from day 15.5 embryos of dams eating powdered chow.

### Statistical Analyses: Gene Expression

Student's *t*-test was used to identify differentially-expressed genes (DEGs) between the two diet groups. *P*-values were jointly corrected for multiple testing by calculating the Benjamini–Hochberg false discovery rate (FDR) (Benjamini, 1995). Differentially expressed genes between groups were defined as those with a raw $p$-value of $<0.001$, an adjusted $p$-value (FDR) of $<0.01$, and an absolute fold-change value of $>1.5$. Gene expression changes were further visualized by Principal Component Analyses (PCA) using R. The pelleted chow group was selected as the referent group; upregulated genes are more highly expressed in the brains of powdered-chow exposed embryos compared to pelleted-chow exposed embryos, downregulated genes are more lowly expressed in the brains of powdered-chow exposed embryos compared to those exposed to pelleted-chow.

Functional analysis was performed on the differentially expressed genes using Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood, CA, United States). The working file for the IPA analysis may be found in **Supplementary File 1**. Statistical significance within IPA was defined as $p < 0.05$, and activation state was predicted based on $Z$-scores $\geq 2$ (activated) or $\leq -2$ (inhibited), in accordance with recommended thresholds (Kramer et al., 2014; Ingenuity Systems, 2019a,b [accessed April 12, 2019]). Only pathways including three or more differentially expressed genes were considered. We also performed whole transcriptome analysis of functional gene set regulation using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), with a developmentally focused annotation (the Developmental Functional Annotation at Tufts or DFLAT) (Wick et al., 2014; Edlow et al., 2015). Gene sets with an FDR $q < 0.05$ were considered significantly dysregulated.

[1]http://brainarray.mbni.med.umich.edu/

## Offspring Biometry and Neurobehavioral Analyses

### Neonatal Biometry and Developmental Milestone Assessment

For neonatal evaluations, 62 offspring exposed to pelleted chow *in utero* and during lactation and 31 offspring exposed to the maternal powdered chow diet were evaluated, with 1–2 offspring per sex per litter evaluated to avoid litter effects ($n = 17$ pelleted chow litters, $n = 9$ powdered chow litters). There were 15 females and 16 males in the powdered chow group, and 38 females and 24 males in the pelleted chow group. Biometry was performed on neonates from both diet groups daily from postnatal day 3 (P3) through P21 (weights), or P3-P15 (length measurements, only performed until eye opening due to limited accuracy secondary to pup movement after eye opening), to establish growth trajectories. Neonatal behavior was evaluated daily from P3 to either P15 when eye opening occurred (if this would confound the behavioral test) or P21 (weaning). Behavioral assessments started on P3 to avoid maternal stress and subsequent pup neglect or cannibalism that could impact neonatal survival and behavior. The amount of time (latency) needed to complete each test was recorded and analyzed. The neurobehavioral test protocols have been described in detail in previous publications (Hill et al., 2008; Guedj et al., 2015; Goodliffe et al., 2016). All behavioral experiments were conducted in the light phase, between 08:00 and 13:00. Test apparati were thoroughly cleaned with Sani-Cloth Plus wipes or 70% ethanol spray between mice, to minimize olfactory cues from previous trials. Mice were acclimated in the testing room in their home cages for at least 1 h prior to evaluation. For neonatal behavioral testing, pups were placed with nesting material in a bowl heated to 37°C. An investigator with extensive experience in neonatal developmental milestones performed all neonatal biometry and behavioral testing (Guedj).

A modified Fox scale (Hill et al., 2008) was used to evaluate developmental milestones in wild-type offspring exposed to powdered versus pelleted chow *in utero* and during lactation. This validated battery of behavioral tests evaluates body righting mechanisms, coordination and strength (surface righting and negative geotaxis), strength and coordination (cliff aversion and forelimb grasp), sensory system maturation (auditory startle, ear twitch, and eye opening), labyrinthine reflex (air righting), and the developmental transition from rotatory locomotion behavior to straight-line walking, reflecting the rostrocaudal development of limb coordination (open field) (Fox, 1965; Hill et al., 2008; Guedj et al., 2015, 2016; Goodliffe et al., 2016). The day of achievement of a developmental milestone was defined as the day at which the pup performed the task successfully for 2 days in a row. The time to achieve a developmental milestone (latency) and the presence or absence of a reflex was evaluated by a single investigator as stated above.

### Adult Neurobehavioral Studies

Adult behavioral testing was performed on a subset of male offspring at 3 months of age, including 16 offspring exposed to pelleted chow *in utero* and during lactation, and 14 offspring exposed to maternal powdered chow diet. The

open field test was used to evaluate locomotor activity and exploratory behavior, contextual fear conditioning was used to evaluate hippocampal learning and memory, and Rotarod test was used to evaluate motor coordination. Testing paradigms have been detailed in prior publications (Aziz et al., 2018; Guedj et al., 2018).

As previously described, open field testing utilized a 60-min trial paradigm in a 40 cm × 40 cm × 40 cm opaque plastic box, with animal tracking performed by the Ethovision 10.5 system (Noldus, Leesburg, VA, United States). The fear conditioning test was performed in a sound-attenuating cubicle with exhaust fan and stainless-steel grid floor (Med Associates, Fairfax, VT, United States), with a 5-min day one training session involving two mild foot shocks (0.5 mA for 2 s) administered at 180 and 240 s. On day two (the testing session), the mice were placed into the identical conditioning chamber for 5 min with no foot shocks, and mice were monitored for freezing (fear) behavior. Reduced freezing on day 2 was evaluated as a measure of hippocampal learning/memory deficit (failure to remember receiving a shock in the same environment on the day prior). Data were analyzed using the Freeze View software (Med Associates, Fairfax, VT, United States). Motor coordination was investigated using the rotarod test (Med Associates, Fairfax, VT, United States) using a 16 RPM, 24 RPM, and 32 RPM fixed speed protocol with two 120 s trials at each speed, separated by a 15 min inter-trial interval, as described previously (Aziz et al., 2018; Guedj et al., 2018). The latency to fall was recorded in seconds and analyzed for each mouse.

#### *Statistical analyses: behavior*

The Kolmogorov–Smirnov normality test and the Fisher variance equality test were performed on behavioral and biometric data to determine appropriate subsequent statistical tests. Significant differences between groups in biometry and in latency to developmental milestone achievement were evaluated via Mann–Whitney tests for single measures, or Wilcoxon signed-rank tests for repeated measures. Significant differences between groups were defined as $p < 0.05$ and $p$-values were corrected for multiple comparisons. Two-way ANOVA (maternal diet × offspring sex) was used to evaluate for the presence of significant interactions between maternal pregnancy diet and offspring sex on achievement of neonatal milestones.

## Genotyping

Genotype and sex of embryos and offspring were determined via multiplex PCR amplification of DNA extracted from tail snips (embryos) or ear punches (neonates). Cite-F/Cite R primers targeting the neomycin cassette (present only in Ts1Cje mice) were used to determine Ts1Cje or wild-type status. Sry-F/Sry-R primers directed against the *Sry* gene (present only in males) was used to determine fetal/neonatal sex. For each reaction, Fez-F/Fez-R primers were used as endogenous controls. DNA extraction and purification methods, PCR conditions, and primer information and amplicon sizes have been described in detail in previous publications (Guedj et al., 2015, 2018; Ferres et al., 2016). Only wild-type embryos and offspring were evaluated in these experiments.

# RESULTS

## Pelleted Versus Powdered Chow Does Not Significantly Impact Dam Weight Gain During Pregnancy Nor Embryo Size

There were no significant differences between maternal diet groups with respect to dam weight trajectories in pregnancy (**Figure 2A**). Maternal pelleted versus powdered chow diet did not have a significant impact on embryo weights or crown rump lengths at E15.5 (**Figure 2B**).

## Embryonic Day 15.5 Brain Gene Expression Profile Is Significantly Impacted by Maternal Pregnancy Diet Composition

There were 1647 differentially-expressed genes (DEGs) in the embryonic brain exposed to maternal powdered chow diet compared to maternal pelleted chow diet. Principal component analysis demonstrated strong clustering of fetal brain gene expression by maternal pregnancy diet, with PC1 (indicating maternal pregnancy diet) accounting for 63% of the variation in fetal brain gene expression (**Figure 3**). **Supplementary File 2** contains the list of significant DEGs including the Entrez Gene ID, gene name, chromosome location, fold change value, raw $p$- and BH-$p$ values for expression levels between powdered chow-exposed embryonic brain compared to pelleted chow-exposed embryonic brain. Two-way ANOVA model (maternal diet × fetal sex) found no significant interaction effects between maternal diet and fetal sex on fetal brain gene expression, and no overlap between the genes affected by diet and those affected by sex.

### Pathways Analyses

#### *Ingenuity pathways analysis*

Pathway analyses performed in IPA suggested significant potential biological impact of the dysregulated brain gene expression due to different maternal diet composition. The canonical pathway Mitotic Roles of Polo-Like Kinase was significantly upregulated in the brains of powdered-chow exposed embryos ($Z$-score 2.4, key in cell cycle regulation). Other canonical pathways that were significantly affected with an adjusted $p$-value of $<0.05$ but for which a definite up- or down-regulation could not be determined based on the pattern of gene expression included: (1) Protein Ubiquitination Pathway, (2) Oxidative Phosphorylation Pathway, (3) Ataxia Telangiectasia Mutated Protein (ATM) Pathway (a canonical pathway key in regulation of the cell cycle and response to cellular stress and injury), and (4) Sirtuin Signaling Pathway (canonical pathway key in regulating metabolism and energy homeostasis by controlling lipid and glucose metabolism, ketone body synthesis, urea cycle and insulin secretion). Significantly dysregulated Canonical Pathways and their constituent differentially expressed genes in the embryonic brain exposed to powdered versus pelleted chow *in utero* are depicted in **Table 2**.

Significant activation and inhibition of several transcriptional factors and other regulatory elements of interest was predicted

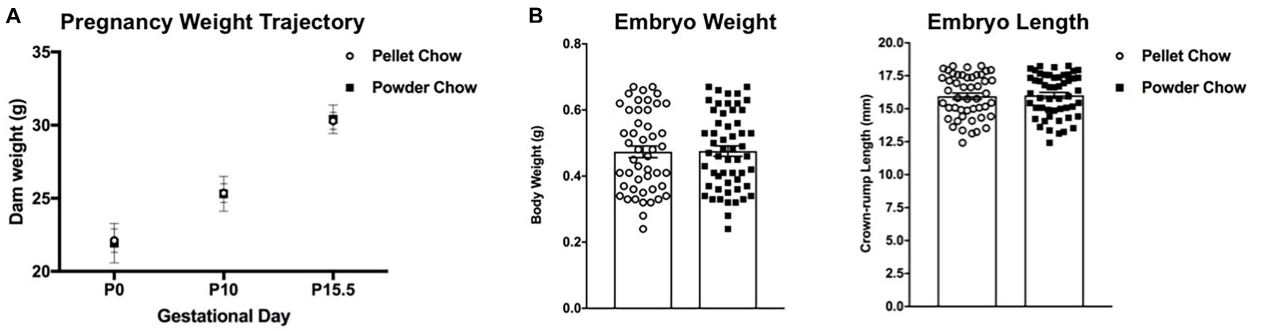


**FIGURE 2 |** Dam weight trajectories in pregnancy, embryo weights and crown-rump lengths at embryonic day 15.5 (e15.5). There are no differences between pelleted and powdered chow for **(A)**: dam weight gain in pregnancy at pregnancy day 0 (P0, day of mating), P10 and P15 or **(B)**: embryonic (e15.5) weight (g) and crown-rump length (mm). *N* = 14 powdered chow and 14 pelleted chow litters for maternal weight gain and embryo size analyses.

by the Upstream Regulator analysis within IPA (bias-corrected *Z*-score of $\geq$2.0 or $\leq$−2.0 predict significant activation or inhibition of a specific upstream regulator as described in Section "Fetal Brain Gene Expression Studies" (Ingenuity Systems, 2019a,b [accessed April 12, 2019]). Significantly activated upstream regulators in fetal brains exposed to powdered chow *in utero* included transcriptional regulators and G-protein coupled receptors implicated in negative regulation of apoptosis, regulation of the cell cycle, synaptic plasticity, brain immune and inflammatory response, sensory nervous system development, and circadian rhythm regulation. Significantly inhibited upstream regulators included transcriptional regulators, growth factors, peptidases and cytokines implicated in cognition and learning, mitochondrial apoptosis, maintenance of vascular integrity, and vasoprotection and neuroprotection in the setting of hypoxic stimuli. Significantly activated or inhibited upstream regulators and their downstream differentially expressed genes in the embryonic brain exposed to powdered versus pelleted chow *in utero* are depicted in **Table 3**.

Within the Downstream Effect Analysis, 56 Molecular, Cellular, and Physiological System Development Functions met criteria for significant dysregulation in the brains of powdered-chow exposed embryos compared to pelleted ($p$-value $< 0.05$ and involving three or more genes in the dataset). Many pathways related to cell cycle regulation were significantly dysregulated, including pathways related to the G1/S Phase, G2 Phase, G2/M transition, Mitosis, Interphase, formation of mitotic spindle, segregation of chromosomes, centrosome duplication, and cytokinesis. The function "segregation of chromosomes" within the Cell Cycle category was predicted to be significantly increased (activation *Z*-score 2.2, $p = 0.003$). Many terms related to DNA damage response were also significantly dysregulated. Other themes that emerged from the IPA Downstream Effects pathways analyses included dysregulation of pathways related to cell death (predicted to be decreased, activation *Z*-score -3.53, $p < 0.001$) and neuronal survival; to brain innate immune response (pathways related to microglia, macrophage phagocytic activity, and natural killer cell production); to synaptic transmission and plasticity (transmission in the hippocampal region was highlighted twice); to dopaminergic neuron firing and morphology; to amino acid transport (predicted to be decreased, activation *Z*-score −2.9, $p = 0.03$); and to fatty acid transport and lipid storage. All significantly dysregulated pathways and biofunctions identified by IPA in embryonic brains exposed to powdered compared to pelleted chow *in utero* are described in **Supplementary File 3**.

### *Gene set enrichment analysis with a development-specific annotation (GSEA/DFLAT)*

Two hundred sixty-one gene sets were significantly dysregulated (FDR $q < 0.05$) in the fetal brain exposed to powdered compared to pelleted chow *in utero* (215 sets upregulated and 46 downregulated). A complete list of significantly dysregulated gene sets may be found in **Supplementary File 4**. GSEA/DFLAT identified many of the same dysregulated biological processes as IPA, with cell cycle dysregulation, DNA damage response, brain immune function, amino acid transport, and neurotransmitter regulation again figuring prominently.

Cell cycle regulation was the most disrupted biofunction in powdered-chow exposed brains, with more than half of the upregulated gene sets relating to cell cycle function and regulation. Representative dysregulated gene sets related to the cell cycle include chromatin assembly and disassembly, spindle formation, mitotic spindle assembly checkpoint, centromere complex assembly, regulation of chromosome segregation (and many similar gene sets), cell cycle regulation by ubiquitin-protein ligase, regulation of the metaphase to anaphase transition, M/G1 transition, sister chromatid cohesion, G2/M transition, and many others. DNA damage response and DNA processing were the second most affected biofunctions based on the number of dysregulated gene sets (double-stranded break repair, signal transduction in response to DNA damage, DNA integrity checkpoint regulation, K63-linked polyubiquitin binding, telomere maintenance, telomere organization, recombinational repair, replication fork, double-strand break repair via homologous recombination, DNA packaging and other similar), followed by RNA processing (mRNA splicing via spliceosome/multiple spliceosome-related gene sets, RNA splicing via transesterification reactions, mRNA transport and localization, ribosomal RNA metabolism and

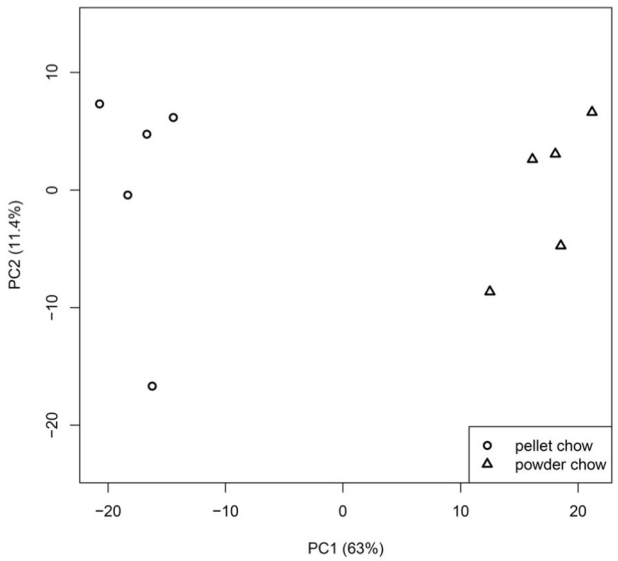


**FIGURE 3 |** Principal component analysis (PCA) of embryonic day 15.5 brain gene expression profiles. Fetal brain gene expression clusters strongly by maternal pregnancy diet, with principal component or PC 1 (maternal pregnancy diet), accounting for 63% of the variation in fetal brain gene expression.

processing, mRNA export from nucleus and other similar). Immune function (both humoral and innate) was another area in which many gene sets were upregulated, including production of molecular mediators of immune response, immunoglobulin production and diversification of immune receptors, immunoglobulin-mediated immune response, somatic recombination of immunoglobulin gene segments, B cell activation involved in immune response and other similar. Multiple gene sets related to protein synthesis and function were dysregulated (primarily downregulated) including ribosome biogenesis, translational initiation and termination, downregulation of amino acid transport/amino acid transmembrane transporter activity, glutamine amino acid metabolic process, and multiple sets related to carboxylic and organic acid transporter activity. Downregulation of gene sets related to neuronal apoptosis and cell death was also noted, as were gene sets related to neurotransmitter transport, neurotransmitter regulation, and neurotransmitter receptor activity. Regulation of cAMP biosynthesis and metabolism, and adenylate cyclase activity were also downregulated in the powdered-chow exposed brains, as were gene sets related to membrane lipid metabolism, endoplasmic reticulum function, and potassium and calcium ion transport.

## Maternal Dietary Composition Has a Significant Impact on Neonatal Biometry and Behavior

### Neonatal Biometry

Biometric analyses were performed on 31 powder-chow exposed and 62 pelleted-chow exposed neonates. These included 15 females and 16 males in the powdered chow group and 38 females and 24 males in the pelleted chow group. There was no significant difference between the two maternal diet groups with respect to offspring weight trajectory, which was true when male and female offspring were grouped and when they were examined in a sex-stratified fashion (**Figure 4A**). The powdered chow-exposed offspring were significantly longer at every postnatal day than pelleted chow-exposed counterparts, which was true both for grouped and sex-stratified analyses (**Figure 4B**).

### Body Righting, Strength and Coordination

Powdered-chow exposed neonates achieved the body righting, strength, and coordination-related milestones significantly faster than the pelleted-chow exposed neonates. The negative geotaxis task was used to evaluate body righting mechanisms, strength, and coordination. The powdered-chow neonates had a significantly shorter daily latency on the negative geotaxis task and achieved that milestone an average of 1.7 days sooner than the pelleted-chow neonates ($p$ = 0.0002, **Figure 5A**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on achievement of the negative geotaxis milestone [$F_{(1,88)}$ = 0.65 $p$ = 0.42 for interaction term, $F_{(1,88)}$ = 0.14, $p$ = 0.71 for offspring sex].

The forelimb grasp task was used to evaluate strength. Powdered chow-exposed offspring had significantly shorter daily latency on the forelimb grasp task, in addition to a faster overall acquisition of the forelimb grasp milestone (approximately 0.5 days earlier, $p$ = 0.01, **Figure 5B**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on achievement of the forelimb grasp milestone [$F_{(1,88)}$ = 0.87, $p$ = 0.35 for interaction term, $F_{(1,88)}$ = 0.04, $p$ = 0.85 for offspring sex].

Surface righting task was used to evaluate body righting mechanisms, strength and coordination. The powdered-chow neonates did not have a significantly shorter latency on the surface righting task on any specific day, but achieved the surface righting milestone significantly earlier than the pelleted chow neonates (PND 7 versus 8, $p$ = 0.007, **Figure 5C**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on achievement of the surface righting milestone [$F_{(1,88)}$ = 0.65, $p$ = 0.42 for interaction term, $F_{(1,88)}$ = 1.31, $p$ = 0.26 for offspring sex].

The cliff aversion test was used to evaluate strength and coordination. Powdered chow-exposed neonates achieved the cliff aversion milestone a mean of 0.6 days earlier than pelleted chow-exposed, but this finding did not achieve statistical significance ($p$ = 0.11, **Figure 5D**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on achievement of the cliff aversion milestone [$F_{(1,88)}$ = 0.03, $p$ = 0.87 for interaction term, $F_{(1,88)}$ = 1.18, $p$ = 0.28 for offspring sex].

**TABLE 2 |** Significantly dysregulated canonical pathways in powdered chow-exposed fetal brain.

| Pathway and constituent genes | Gene name | Entrez gene ID (mouse) | Expr log ratio | Expr false discovery rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| **Mitotic Polo-Like Kinase Pathway $p = 7.01 \times 10^{-7}$ 23 of 61 molecules in pathway dysregulated, Z-score 2.50 (upregulated)** | | | | | | |
| ANAPC4 | Anaphase promoting complex subunit 4 | 52206 | 0.603 | 1.60E–04 | Nucleus | Enzyme |
| ANAPC5 | Anaphase promoting complex subunit 5 | 59008 | 0.817 | 3.53E–06 | Nucleus | Other |
| ANAPC13 | Anaphase promoting complex subunit 13 | 69010 | 1.682 | 7.13E–06 | Nucleus | Other |
| CDC7 | Cell division cycle 7 | 12545 | 0.929 | 4.89E–05 | Nucleus | Kinase |
| CDC16 | Cell division cycle 16 | 69957 | 0.739 | 6.44E–05 | Nucleus | Other |
| CDC26 | Cell division cycle 26 | 66440 | 0.784 | 6.28E–05 | Nucleus | Other |
| CDC25C | Cell division cycle 25C | 12532 | 0.916 | 9.78E–04 | Nucleus | Phosphatase |
| FBXO5 | F-box protein 5 | 67141 | 0.911 | 1.06E–04 | Nucleus | Enzyme |
| KIF11 | Kinesin family member 11 | 16551 | 0.953 | 1.21E–03 | Nucleus | Other |
| KIF23 | Kinesin family member 23 | 71819 | 0.641 | 1.94E–03 | Cytoplasm | Other |
| PLK2 | Polo like kinase 2 | 20620 | 0.751 | 2.12E–04 | Nucleus | Kinase |
| PLK3 | Polo like kinase 3 | 12795 | −0.649 | 1.33E–03 | Nucleus | Kinase |
| PPP2R2A | Protein phosphatase 2 regulatory subunit B alpha | 71978 | 0.64 | 4.54E–05 | Cytoplasm | Phosphatase |
| PPP2R2C | Protein phosphatase 2 regulatory subunit B gamma | 269643 | −0.606 | 7.46E–05 | Nucleus | Phosphatase |
| PPP2R3A | Protein phosphatase 2 regulatory subunit B alpha | 235542 | 0.589 | 2.94E–05 | Nucleus | Phosphatase |
| PPP2R5B | Protein phosphatase 2 regulatory subunit B beta | 225849 | −0.63 | 2.40E–05 | Cytoplasm | Phosphatase |
| PPP2R5E | Protein phosphatase 2 regulatory subunit B epsilon | 26932 | 0.684 | 1.05E–04 | Cytoplasm | Phosphatase |
| PRC1 | Protein regulator of cytokinesis 1 | 233406 | 1.286 | 6.32E–04 | Nucleus | Other |
| PTTG1 | Pituitary tumor-transforming 1 | 30939 | −0.645 | 3.78E–03 | Nucleus | Transcription regulator |
| SLK | STE20 like kinase | 20874 | 0.642 | 5.30E–05 | Nucleus | Kinase |
| SMC3 | Structural maintenance of chromosomes 3 | 13006 | 1.111 | 2.99E–05 | Nucleus | Other |
| SMC1A | Structural maintenance of chromosomes 1A | 24061 | 0.905 | 5.41E–05 | Nucleus | Transporter |
| STAG2 | Stromal antigen 2 | 20843 | 0.838 | 1.03E–04 | Nucleus | Transcription regulator |
| **Protein Ubiquitination Pathway $p = 7.9 \times 10^{-4}$, 46 of 249 molecules dysregulated, Z-score NA** | | | | | | |
| ANAPC4 | Anaphase promoting complex subunit 4 | 52206 | 0.603 | 1.60E–04 | Nucleus | Enzyme |
| ANAPC5 | Anaphase promoting complex subunit 5 | 59008 | 0.817 | 3.53E–06 | Nucleus | Other |
| B2M | Beta-2-microglobulin | 12010 | −1.193 | 4.03E–05 | Plasma Membrane | Transmembrane receptor |
| BAP1 | BRCA1 associated protein 1 | 104416 | −0.799 | 3.53E–06 | Nucleus | Peptidase |
| BIRC2 | Baculoviral IAP repeat containing 2 | 11797 | 0.764 | 6.38E–04 | Cytoplasm | Enzyme |
| CUL1 | Cullin 1 | 26965 | 0.768 | 8.98E–05 | Nucleus | Enzyme |
| DNAJC1 | DnaJ heat shock protein family (Hsp40) member C1 | 13418 | 0.877 | 2.00E–05 | Cytoplasm | Transcription regulator |
| DNAJC2 | DnaJ heat shock protein family (Hsp40) member C2 | 22791 | 0.925 | 3.10E–05 | Nucleus | Transcription regulator |
| DNAJC3 | DnaJ heat shock protein family (Hsp40) member C3 | 100037258 | 0.7 | 8.26E–05 | Cytoplasm | Other |
| DNAJC7 | DnaJ heat shock protein family (Hsp40) member C7 | 56354 | 0.692 | 3.40E–05 | Cytoplasm | Other |
| DNAJC8 | DnaJ heat shock protein family (Hsp40) member C8 | 68598 | 0.903 | 1.09E–03 | Nucleus | Other |
| DNAJC9 | DnaJ heat shock protein family (Hsp40) member C9 | 108671 | 0.772 | 2.55E–04 | Nucleus | Other |

*(Continued)*

**TABLE 2 |** Continued

| Pathway and constituent genes | Gene name | Entrez gene ID (mouse) | Expr log ratio | Expr false discovery rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| DNAJC10 | DnaJ heat shock protein family (Hsp40) member C10 | 66861 | 0.81 | 4.67E–05 | Cytoplasm | Enzyme |
| DNAJC14 | DnaJ heat shock protein family (Hsp40) member C14 | 74330 | −0.965 | 1.52E–05 | Cytoplasm | Other |
| DNAJC19 | DnaJ heat shock protein family (Hsp40) member C19 | 100503724 | 0.667 | 1.26E–03 | Cytoplasm | Other |
| DNAJC21 | DnaJ heat shock protein family (Hsp40) member C21 | 78244 | 1.18 | 2.94E–05 | Other | Other |
| DNAJC30 | DnaJ heat shock protein family (Hsp40) member C30 | 66114 | −0.81 | 6.44E–04 | Cytoplasm | Other |
| ELOC | elongin C | 67923 | −0.674 | 2.48E–04 | Nucleus | Transcription regulator |
| FBXW7 | F-box and WD repeat domain containing 7 | 50754 | 0.637 | 2.65E–05 | Nucleus | Enzyme |
| HSPA4L | Heat shock protein family A (Hsp70) member 4 like | 18415 | 0.794 | 7.77E–05 | Cytoplasm | Other |
| MDM2 | MDM2 proto-oncogene | 17246 | 0.628 | 1.08E–04 | Nucleus | Transcription regulator |
| PSMA2 | Proteasome subunit alpha 2 | 19166 | 0.789 | 2.18E–05 | Cytoplasm | Peptidase |
| PSMA4 | Proteasome subunit alpha 4 | 26441 | 1.429 | 1.36E–05 | Cytoplasm | Peptidase |
| PSMA7 | Proteasome subunit alpha 7 | 26444 | 2.048 | 4.96E–06 | Cytoplasm | Peptidase |
| PSMB1 | Proteasome subunit beta 1 | 19170 | 0.828 | 2.49E–05 | Cytoplasm | Peptidase |
| PSMB2 | Proteasome subunit beta 2 | 26445 | −0.729 | 1.78E–04 | Cytoplasm | Peptidase |
| PSMB3 | Proteasome subunit beta 3 | 26446 | −1.71 | 1.15E–06 | Cytoplasm | Peptidase |
| PSMB4 | Proteasome subunit beta 4 | 19172 | −0.756 | 2.91E–03 | Cytoplasm | Peptidase |
| PSMB6 | Proteasome subunit beta 6 | 19175 | 1.201 | 5.89E–05 | Nucleus | Peptidase |
| PSMC1 | Proteasome 26S subunit, ATPase 1 | 19179 | 0.732 | 1.05E–04 | Nucleus | Peptidase |
| PSMC2 | Proteasome 26S subunit, ATPase 2 | 19181 | 0.945 | 5.53E–05 | Nucleus | Peptidase |
| PSMC5 | Proteasome 26S subunit, ATPase 5 | 19184 | 0.636 | 1.38E–03 | Nucleus | Transcription regulator |
| PSMC6 | Proteasome 26S subunit, ATPase 6 | 67089 | 0.646 | 4.40E–04 | Nucleus | Peptidase |
| PSMD7 | Proteasome 26S subunit, non-ATPase 7 | 17463 | 0.589 | 6.59E–04 | Cytoplasm | Other |
| PSMD11 | Proteasome 26S subunit, non-ATPase 11 | 69077 | 1.09 | 1.99E–05 | Cytoplasm | Other |
| PSMD12 | Proteasome 26S subunit, non-ATPase 12 | 66997 | 0.636 | 3.26E–04 | Cytoplasm | Other |
| PSME1 | Proteasome activator subunit 1 | 19186 | 0.89 | 3.19E–05 | Cytoplasm | Other |
| PSME2 | Proteasome activator subunit 2 | 19188 | −0.639 | 3.67E–04 | Cytoplasm | Peptidase |
| SUGT1 | SGT1 homolog, MIS12 kinetochore complex assembly cochaperone | 67955 | 0.662 | 6.36E–04 | Nucleus | Other |
| UBE2L3 | Ubiquitin conjugating enzyme E2 L3 | 22195 | −0.726 | 2.08E–05 | Nucleus | Enzyme |
| UBE2V1 | ubiquitin conjugating enzyme E2 V1 | 66589 | 1.141 | 4.21E–04 | Nucleus | Transcription regulator |
| UBE2V2 | Ubiquitin conjugating enzyme E2 V2 | 70620 | −0.929 | 7.41E–05 | Cytoplasm | Enzyme |
| UBE2Z | Ubiquitin conjugating enzyme E2 Z | 268470 | −0.627 | 2.56E–05 | Nucleus | Enzyme |
| UCHL5 | Ubiquitin C-terminal hydrolase L5 | 56207 | 0.752 | 1.28E–04 | Cytoplasm | Peptidase |
| USP25 | Ubiquitin specific peptidase 25 | 30940 | 0.769 | 3.59E–05 | Cytoplasm | Peptidase |
| USP47 | Ubiquitin specific peptidase 47 | 74996 | 0.82 | 3.64E–05 | Cytoplasm | Peptidase |
| **Oxidative Phosphorylation Pathway *p*-value 0.01, 21 of 92 molecules dysregulated, Z-score 0.22** | | | | | | |
| ATP5F1C | ATP synthase F1 subunit gamma | 11949 | 0.775 | 1.50E–03 | Cytoplasm | Transporter |
| ATP5F1D | ATP synthase F1 subunit delta | 66043 | −1.576 | 4.29E–05 | Cytoplasm | Transporter |
| COX11 | Cytochrome c oxidase copper chaperone COX11 | 69802 | −0.958 | 3.03E–05 | Cytoplasm | Enzyme |
| COX17 | Cytochrome c oxidase copper chaperone COX17 | 12856 | 1.965 | 2.95E–06 | Cytoplasm | Enzyme |
| COX6A1 | Cytochrome c oxidase subunit 6A1 | 12861 | 0.804 | 3.30E–05 | Cytoplasm | Enzyme |

*(Continued)*

**TABLE 2 |** Continued

| Pathway and constituent genes | Gene name | Entrez gene ID (mouse) | Expr log ratio | Expr false discovery rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| Cox6c | Cytochrome c oxidase subunit 6C | 12864 | −0.6 | 2.13E–04 | Cytoplasm | Enzyme |
| COX7A2L | Cytochrome c oxidase subunit 7A2 like | 20463 | 2.175 | 1.32E–06 | Cytoplasm | Enzyme |
| COX8A | Cytochrome c oxidase subunit 8A | 12868 | −0.819 | 5.82E–05 | Cytoplasm | Enzyme |
| CYB5A | Cytochrome b5 type A | 109672 | −0.586 | 3.74E–04 | Cytoplasm | Enzyme |
| CYC1 | Cytochrome c1 | 66445 | −1.226 | 1.10E–04 | Cytoplasm | Enzyme |
| NDUFA1 | NADH:ubiquinone oxidoreductase subunit A1 | 54405 | 1.593 | 2.91E–05 | Cytoplasm | Enzyme |
| NDUFA4 | NDUFA4, mitochondrial complex associated | 17992 | 1.402 | 8.33E–06 | Cytoplasm | Enzyme |
| NDUFA6 | NADH:ubiquinone oxidoreductase subunit A6 | 67130 | 0.774 | 1.86E–04 | Cytoplasm | Enzyme |
| NDUFB2 | NADH:ubiquinone oxidoreductase subunit B2 | 68198 | 0.751 | 5.90E–04 | Cytoplasm | Enzyme |
| NDUFB6 | NADH:ubiquinone oxidoreductase subunit B6 | 230075 | −0.899 | 3.84E–03 | Cytoplasm | Enzyme |
| NDUFB9 | NADH:ubiquinone oxidoreductase subunit B9 | 66218 | 1.305 | 3.93E–06 | Cytoplasm | Enzyme |
| NDUFB11 | NADH:ubiquinone oxidoreductase subunit B11 | 104130 | −0.749 | 2.27E–04 | Cytoplasm | Enzyme |
| NDUFS4 | NADH:ubiquinone oxidoreductase subunit S4 | 17993 | −0.801 | 8.94E–05 | Cytoplasm | Enzyme |
| NDUFS6 | NADH:ubiquinone oxidoreductase subunit S6 | 407785 | 0.609 | 1.67E–03 | Cytoplasm | Enzyme |
| NDUFS7 | NADH:ubiquinone oxidoreductase core subunit S7 | 75406 | −1.177 | 1.01E–05 | Cytoplasm | Enzyme |
| UQCRFS1 | Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 | 66694 | 0.704 | 2.81E–05 | Cytoplasm | Enzyme |
| **Ataxia Telangiectasia Mutated Protein Pathway *p*-value 0.02, 20 of 89 molecules dysregulated, *Z*-score 0.24** | | | | | | |
| CDC25C | Cell division cycle 25C | 12532 | 0.916 | 9.78E–04 | Nucleus | Phosphatase |
| CDK2 | Cyclin dependent kinase 2 | 12566 | 0.678 | 6.59E–04 | Nucleus | Kinase |
| GADD45A | Growth arrest and DNA damage inducible alpha | 13197 | 0.682 | 6.13E–05 | Nucleus | Other |
| GADD45G | Growth arrest and DNA damage inducible gamma | 23882 | 0.827 | 3.22E–04 | Nucleus | Other |
| MDM2 | MDM2 proto-oncogene | 17246 | 0.628 | 1.08E–04 | Nucleus | Transcription regulator |
| PPM1D | Protein phosphatase, Mg2 + Mn2 + dependent 1D | 53892 | −0.588 | 5.01E–05 | Cytoplasm | Phosphatase |
| PPP2R2A | Protein phosphatase 2 regulatory subunit Balpha | 71978 | 0.64 | 4.54E–05 | Cytoplasm | Phosphatase |
| PPP2R2C | Protein phosphatase 2 regulatory subunit Bgamma | 269643 | −0.606 | 7.46E–05 | Nucleus | Phosphatase |
| PPP2R3A | Protein phosphatase 2 regulatory subunit B"alpha | 235542 | 0.589 | 2.94E–05 | Nucleus | Phosphatase |
| PPP2R5B | Protein phosphatase 2 regulatory subunit B'beta | 225849 | −0.63 | 2.40E–05 | Cytoplasm | Phosphatase |
| PPP2R5E | Protein phosphatase 2 regulatory subunit B'epsilon | 26932 | 0.684 | 1.05E–04 | Cytoplasm | Phosphatase |
| RAD50 | RAD50 double strand break repair protein | 19360 | 0.626 | 6.95E–04 | Nucleus | Enzyme |
| RAD51 | RAD51 recombinase | 19361 | 0.72 | 9.83E–04 | Nucleus | Enzyme |
| RNF8 | Ring finger protein 8 | 58230 | 0.66 | 7.22E–05 | Nucleus | Enzyme |
| SMC2 | Structural maintenance of chromosomes 2 | 14211 | 0.644 | 6.72E–04 | Nucleus | Transporter |
| SMC3 | Structural maintenance of chromosomes 3 | 13006 | 1.111 | 2.99E–05 | Nucleus | Other |

*(Continued)*

**TABLE 2 |** Continued

| Pathway and constituent genes | Gene name | Entrez gene ID (mouse) | Expr log ratio | Expr false discovery rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| SMC1A | Structural maintenance of chromosomes 1A | 24061 | 0.905 | 5.41E–05 | Nucleus | Transporter |
| SUV39H1 | Suppressor of variegation 3-9 homolog 1 | 20937 | −0.598 | 2.77E–05 | Nucleus | Enzyme |
| TLK1 | Tousled like kinase 1 | 228012 | 0.803 | 2.21E–04 | Nucleus | Kinase |
| TLK2 | Tousled like kinase 2 | 24086 | 0.767 | 1.88E–04 | Cytoplasm | Kinase |
| **Sirtuin Signaling Pathway *p-value* 0.04, 40 of 251 molecules dysregulated, *Z* = −0.76** | | | | | | |
| ATG13 | Autophagy related 13 | 51897 | −0.765 | 7.34E–05 | Cytoplasm | Other |
| ATP5F1C | ATP synthase F1 subunit gamma | 11949 | 0.775 | 1.50E–03 | Cytoplasm | Transporter |
| ATP5F1D | ATP synthase F1 subunit delta | 66043 | −1.576 | 4.29E–05 | Cytoplasm | Transporter |
| BPGM | Bisphosphoglycerate mutase | 12183 | 1.425 | 2.29E–05 | Extracellular Space | Phosphatase |
| CYC1 | Cytochrome c1 | 66445 | −1.226 | 1.10E–04 | Cytoplasm | Enzyme |
| GABARAPL1 | GABA type A receptor associated protein like 1 | 57436 | −0.756 | 7.58E–05 | Cytoplasm | Other |
| GABPA | GA binding protein transcription factor subunit alpha | 14390 | 0.632 | 8.98E–04 | Nucleus | Transcription regulator |
| GADD45A | Growth arrest and DNA damage inducible alpha | 13197 | 0.682 | 6.13E–05 | Nucleus | Other |
| GADD45G | Growth arrest and DNA damage inducible gamma | 23882 | 0.827 | 3.22E–04 | Nucleus | Other |
| H1FX | H1 histone family member X | 243529 | −0.828 | 3.19E–05 | Nucleus | Other |
| HIST1H1C | Histone cluster 1 H1 family member c | 50708 | −0.686 | 1.03E–03 | Nucleus | Other |
| MAPK3 | Mitogen-activated protein kinase 3 | 26417 | −0.752 | 1.89E–04 | Cytoplasm | Kinase |
| MAPK15 | Mitogen-activated protein kinase 15 | 332110 | −0.699 | 8.98E–04 | Cytoplasm | Kinase |
| NDUFA1 | NADH:ubiquinone oxidoreductase subunit A1 | 54405 | 1.593 | 2.91E–05 | Cytoplasm | Enzyme |
| NDUFA4 | NDUFA4, mitochondrial complex associated | 17992 | 1.402 | 8.33E–06 | Cytoplasm | Enzyme |
| NDUFA6 | NADH:ubiquinone oxidoreductase subunit A6 | 67130 | 0.774 | 1.86E–04 | Cytoplasm | Enzyme |
| NDUFB2 | NADH:ubiquinone oxidoreductase subunit B2 | 68198 | 0.751 | 5.90E–04 | Cytoplasm | Enzyme |
| NDUFB6 | NADH:ubiquinone oxidoreductase subunit B6 | 230075 | −0.899 | 3.84E–03 | Cytoplasm | Enzyme |
| NDUFB9 | NADH:ubiquinone oxidoreductase subunit B9 | 66218 | 1.305 | 3.93E–06 | Cytoplasm | Enzyme |
| NDUFB11 | NADH:ubiquinone oxidoreductase subunit B11 | 104130 | −0.749 | 2.27E–04 | Cytoplasm | Enzyme |
| NDUFS4 | NADH:ubiquinone oxidoreductase subunit S4 | 17993 | −0.801 | 8.94E–05 | Cytoplasm | Enzyme |
| NDUFS6 | NADH:ubiquinone oxidoreductase subunit S6 | 407785 | 0.609 | 1.67E–03 | Cytoplasm | Enzyme |
| NDUFS7 | NADH:ubiquinone oxidoreductase core subunit S7 | 75406 | −1.177 | 1.01E–05 | Cytoplasm | Enzyme |
| POLR1B | RNA polymerase I subunit B | 20017 | −0.696 | 2.49E–04 | Nucleus | Enzyme |
| POLR1D | RNA polymerase I and III subunit D | 20018 | 0.738 | 1.55E–04 | Nucleus | Enzyme |
| POLR2F | RNA polymerase II subunit F | 69833 | −1.462 | 7.63E–06 | Nucleus | Enzyme |
| PPIF | Peptidylprolyl isomerase F | 105675 | −0.614 | 2.65E–04 | Cytoplasm | Enzyme |
| SIRT3 | Sirtuin 3 | 64384 | −0.596 | 1.30E–04 | Cytoplasm | Enzyme |
| SIRT6 | Sirtuin 6 | 50721 | −1.22 | 8.33E–06 | Nucleus | Enzyme |
| SLC25A4 | Solute carrier family 25 member 4 | 11739 | 0.819 | 3.35E–05 | Cytoplasm | Transporter |
| SMARCA5 | SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5 | 93762 | 1.405 | 1.36E–04 | Nucleus | Transcription regulator |

*(Continued)*

**TABLE 2** | Continued

| Pathway and constituent genes | Gene name | Entrez gene ID (mouse) | Expr log ratio | Expr false discovery rate (q-value) | Location | Type(s) |
|---|---|---|---|---|---|---|
| SOD2 | Superoxide dismutase 2 | 20656 | −0.601 | 6.71E–05 | Cytoplasm | Enzyme |
| SUV39H1 | Suppressor of variegation 3-9 homolog 1 | 20937 | −0.598 | 2.77E–05 | Nucleus | Enzyme |
| TIMM44 | Translocase of inner mitochondrial membrane 44 | 21856 | 0.69 | 2.08E–05 | Cytoplasm | Transporter |
| TIMM17A | Translocase of inner mitochondrial membrane 17A | 21854 | 0.604 | 8.33E–06 | Cytoplasm | Transporter |
| TOMM6 | Translocase of outer mitochondrial membrane 6 | 66119 | −0.93 | 2.99E–05 | Cytoplasm | Other |
| TOMM70 | Translocase of outer mitochondrial membrane 70 | 28185 | 0.896 | 4.80E–05 | Cytoplasm | Transporter |
| TUBA4A | Tubulin alpha 4a | 22145 | −0.708 | 2.79E–03 | Cytoplasm | Other |
| UQCRFS1 | Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 | 66694 | 0.704 | 2.81E–05 | Cytoplasm | Enzyme |
| ZIC2 | Zic family member 2 | 22772 | −0.645 | 1.52E–04 | Nucleus | Transcription regulator |

### Transition From Rotatory/Pivoting Locomotion Behavior to Straight-Line Walking

There were no significant differences between powdered-chow and pelleted-chow neonates with respect to daily time spent ambulating in a rotatory fashion and latency to achieve the extinction of rotatory behavior on open field testing (∼day 13 for both groups, **Figure 6A**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on extinction of rotatory behavior [$F_{(1,88)} = 0.43$, $p = 0.51$ for interaction term, $F_{(1,88)} = 0.15$, $p = 0.69$ for offspring sex].

### Sensory Maturation

Unlike the body strength and coordination tasks, powdered chow-exposed neonates were significantly delayed in achievement of the sensory maturation reflexes compared to their pelleted chow counterparts. Powdered chow exposed neonates were delayed by 1.5 days in achieving the air righting reflex (evaluates the labyrinthine reflex initiated by the vestibular system, in addition to body righting and coordination) compared to pelleted chow-exposed neonates ($p < 0.0001$, **Figure 6B**). Two-way ANOVA demonstrated no significant interaction terms between maternal diet and offspring sex, and no significant main effects of offspring sex on achievement of the air righting milestone [$F_{(1,88)} = 1.45$, $p = 0.23$ for interaction term, $F_{(1,88)} = 0.71$, $p = 0.40$ for offspring sex]. Powdered chow offspring were 2 days delayed in achieving the ear twitch (tactile reflex, $p < 0.0001$), 3 days delayed in auditory startle (auditory reflex, $p < 0.0001$), and 0.4 days delayed in eye opening ($p = 0.06$), compared to their pelleted chow-exposed counterparts. Similar to the other neonatal milestones, two-way ANOVA demonstrated no significant interactions between maternal diet and offspring sex and no significant main effects of offspring sex on attainment of any sensory maturation reflexes [ear twitch $F_{(1,88)} = 0.21$, $p = 0.65$ for interaction term, $F_{(1,88)} = 1.78$, $p = 0.19$ for offspring sex; auditory startle $F_{(1,88)} = 1.86$, $p = 0.18$ for interaction term, $F_{(1,88)} = 0.02$, $p = 0.88$ for offspring sex; eye opening $F_{(1,88)} = 0.35$, $p = 0.55$ for interaction term, $F_{(1,88)} = 1.48$, $p = 0.23$ for offspring sex]. **Figures 6C–E** depicts the day of milestone achievement for powdered versus pelleted chow-exposed neonates for the aforementioned sensory maturation reflexes.

## Maternal Dietary Composition in Pregnancy and Lactation Has a Significant Impact on Adult Locomotor Activity and Hippocampal Learning

Powdered chow-exposed adult offspring traveled a significantly greater distance in the open field arena compared to pellet chow-exposed adults, consistent with hyperactivity (23,296 ± 1019 cm versus 18,853 ± 779.1 cm, $p = 0.001$, **Figure 7A**). Powdered chow-exposed adults demonstrated significantly reduced freezing on day 2 of fear conditioning at 60, 120, and 180 s, consistent with a hippocampal learning/memory deficit ($p = 0.01$, $p = 0.017$, and $p = 0.039$, respectively, **Figure 7B**). There were no significant differences between powdered chow-exposed and pellet chow-exposed adults on the rotarod test at any speed (**Figure 7C**).

# DISCUSSION

In this study, we demonstrated that maternal diet in pregnancy has a significant impact on fetal brain gene expression signatures and neonatal behavior, even in the absence of overt maternal over- or undernutrition. Specifically, we found that a maternal diet relatively enriched for carbohydrates and n-3 long-chain polyunsaturated fatty acids, and relatively deficient in micronutrients, antioxidants, macroelements, and the amino acids alanine, alargine, and glycine, was associated with differential expression of over 1600 fetal brain genes, significantly increased neonatal length, earlier achievement of strength

**TABLE 3 |** Significantly dysregulated upstream regulators and constituent downstream genes in powdered chow-exposed fetal brain.

| Upstream regulator | Entrez gene name | Putative function | Molecule type | Predicted activation state | Bias-corrected Z-score | Target molecules in dataset |
|---|---|---|---|---|---|---|
| *EP400* | E1A binding protein p400 | Cell cycle regulation; chromatin organization; histone H2A acetylation; histone H4 acetylation | other | Activated | 2.063 | *CDCA3, CENPF, FBXO5, INCENP, MCM3, SUV39H2* (direction of regulation predicts *EP400* activation in 6/6) |
| *ISL1* | ISL LIM homeobox 1 | Encodes a member of the LIM/homeodomain family of transcription factors. The encoded protein may play an important role in regulating insulin gene expression. | Transcription regulator | Activated | 2.009 | *CRABP1, NPY, NXPH4, OLIG1* (direction of expression predicts *ISL1* activation in 4/4) |
| *PTGER2* | Prostaglandin E receptor 2 | Encodes a receptor for prostaglandin E2, a metabolite of arachidonic acid. Within the brain, PGE2 receptors are involved in the regulation of synaptic activity and plasticity, in brain maturation, and are key mediators of the brain's response to inflammation. This gene has been implicated in negative regulation of the apoptotic process | G-protein coupled receptor | Activated | 3.623 | *ARFGEF1, CENPE, CENPF, CEP55, CKAP2L, ECT2, KIF11, KIF15, KIF20A, KIF2C, MELK, MKI67, NUF2, PRC1, STIL, TPX2, TTK* (direction of expression predicts *PTGER2* activation in 17/17) |
| *POU4F1* | POU class 4 homeobox 1 | Encodes a member of the POU-IV class of neural transcription factors. Implicated in sensory nervous system development, axonogenesis, and negative regulation of central nervous system apoptosis | Transcription regulator | Activated | 2.013 | *CHRNA3, CRABP1, NECAB2, NPY, NXPH4, OLIG1, RTN4RL2, SSTR4* (direction of expression predicts activation of *POU4F1* in 4/8) |
| *CLOCK* | Clock circadian regulator | Encodes a protein that is a key regulator of circadian rhythms. Polymorphisms in this gene are associated with obesity and metabolic syndrome in certain populations. | Transcription regulator | Activated | 2.248 | *CADM2, CLIP1, CTGF, GCC2, GTF2E1, ITM2C, LYPD6, OPN3, OSTM1, PCGF5, PIP4K2C, QSER1, THUMPD1, TIMP4* (direction of expression predicts *CLOCK* activation in 11/14 genes) |
| *BDNF* | Brain derived neurotrophic factor | Encodes a member of the nerve growth factor family of proteins. Inhibition of expression is associated with cognitive deficits and neurogenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease. | Growth factor | Inhibited | −2.781 | *ALDH7A1, ANXA5, Cdkn1c, DNAJC21, DRD2, EGR1, HSD17B4, HSPA4L mir-10, mir-154, NCDN, NPY, PCDH8, RGS4, SCCPDH, SFR1, TMED2, Tmsb4x, VAMP2, VGF* (direction of expression predicts *BDNF* inhibition in 12/20) |
| *IL33* | Interleukin 33 | Encodes a cytokine that binds to the IL1RL1/ST2 receptor. Encoded protein is involved in the maturation of Th2 cells and the activation of mast cells, basophils, eosinophils and natural killer cells. Gene has been implicated in microglial activation and the brain's innate immune response. | cytokine | Inhibited | −3.102 | *ACAT1, ARRB1, CCL3L3, ENO2, HACD3, NFKBIB, PRPF4B, RAI14, RAMP3, RASGRP1* (direction of expression predicts *IL33* inhibition in 8/12) |
| *BNIP3L* | BCL2 interacting protein 3 like | Encodes a protein that belongs to the pro-apoptotic subfamily within the Bcl-2 family of proteins. The encoded protein directly targets mitochondria and causes apoptotic changes, including loss of membrane potential and the release of cytochrome c. | Other | Inhibited | −2.94 | CCND3, CENPE, CENPF, CKAP2, CST3, GADD45A, GPSM2, KIF11, NT5C3A, NUF2, PRIM1, RAD54L, TOP2A (direction of expression predicts inhibition in 12/13) |
| *KLF3* | Kruppel like factor 3 | Transcription factor that is a key regulator of adipogenesis and B cell development. KLF3 serves as a key regulator of neuronal development, and dysregulation of regulators in the KLF famiuly has been linked to has been linked to various neurological disorders. KLFs may play a key role in brain vasoprotection and neuroprotection in response to ischemic or hypoxic stimuli. | Transcription regulator | Inhibited | −2.241 | AGGF1, ANXA5, CEP63, CHCHD10, CSNK1G3, EEF1AKMT1, EPRS, HNRNPH1, HYPK, IGF2BP3 (direction of expression predicts inhibition in 25/35) |

*(Continued)*

**TABLE 3 |** Continued

| Upstream regulator | Entrez gene name | Putative function | Molecule type | Predicted activation state | Bias-corrected Z-score | Target molecules in dataset |
|---|---|---|---|---|---|---|
| *F2* | Coagulation Factor II; Thrombin | Coagulation factor II is proteolytically cleaved to form thrombin in the first step of the coagulation cascade which ultimately results in the stemming of blood loss. F2 also plays a role in maintaining vascular integrity during development and postnatal life. | Peptidase | Inhibited | −2.821 | B4GALT1, BIRC2, CTGF, DOK5, EGR1, F3, PPIF, RAC1, RHOJ, SLC2A6 (direction of expression predicts inhibition in 10/12) |
| *C5* | Complement 5 | Encodes a component of the complement system, part of the innate immune system that plays an important role in inflammation, host homeostasis, and host defense against pathogens | Cytokine | Inhibited | −2.101 | CCL3L3, EFNB2, EGR1, F3, PLK3 (direction of expression predicts inhibition in 4/5) |

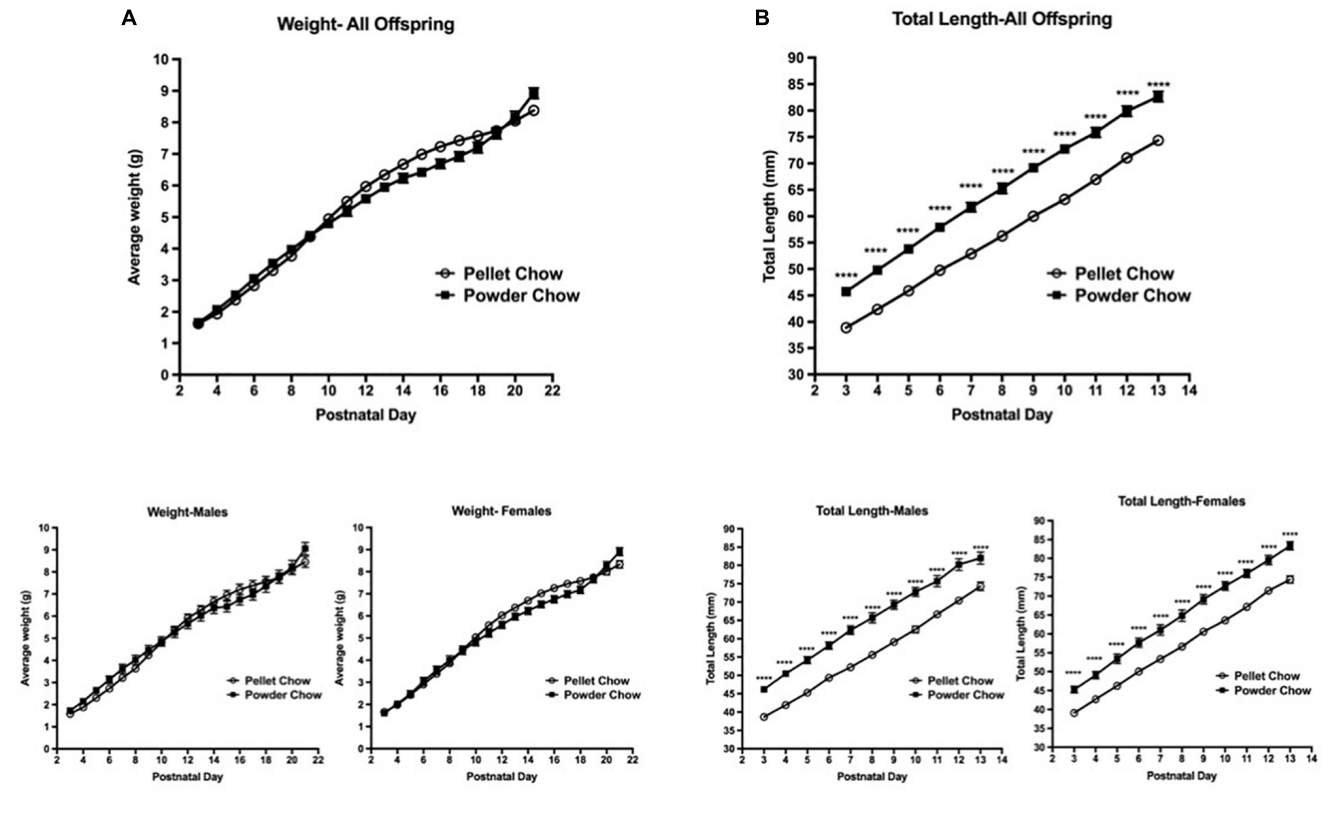


**FIGURE 4 |** Neonatal offspring weight and length trajectory. There were no significant differences in offspring weight **(A)** between maternal diet groups, both when sexes were grouped (above) and in sex-stratified analyses (below). Powdered chow-exposed offspring were significantly longer at every postnatal day than pelleted chow-exposed counterparts **(B)**, both when sexes were grouped (above) and in sex-stratified analyses (below). $N = 31$ powder and 62 pelleted chow-exposed neonates. $^{****}p < 0.0001$.

and coordination-associated milestones, and delayed neonatal sensory maturation. Importantly, these changes occurred in the absence of any differences in maternal weight gain in pregnancy or fetal weight/length changes at embryonic day 15.5, and in the absence of any neonatal weight differences, suggesting that the differences in fetal brain gene expression and neonatal behavior were not attributable to overt nutrient excess or deficiency. We also found neurobehavioral changes persisting into adult life in the offspring, with maternal powdered chow diet in pregnancy and lactation associated with hyperactivity of on open field testing, and hippocampal learning deficits on contextual fear conditioning. Both diets were commercially available "chow" diets, highlighting the critical importance of selection of appropriate control diets for those examining the impact of maternal dietary or other environmental manipulations on fetal brain development and offspring behavior.

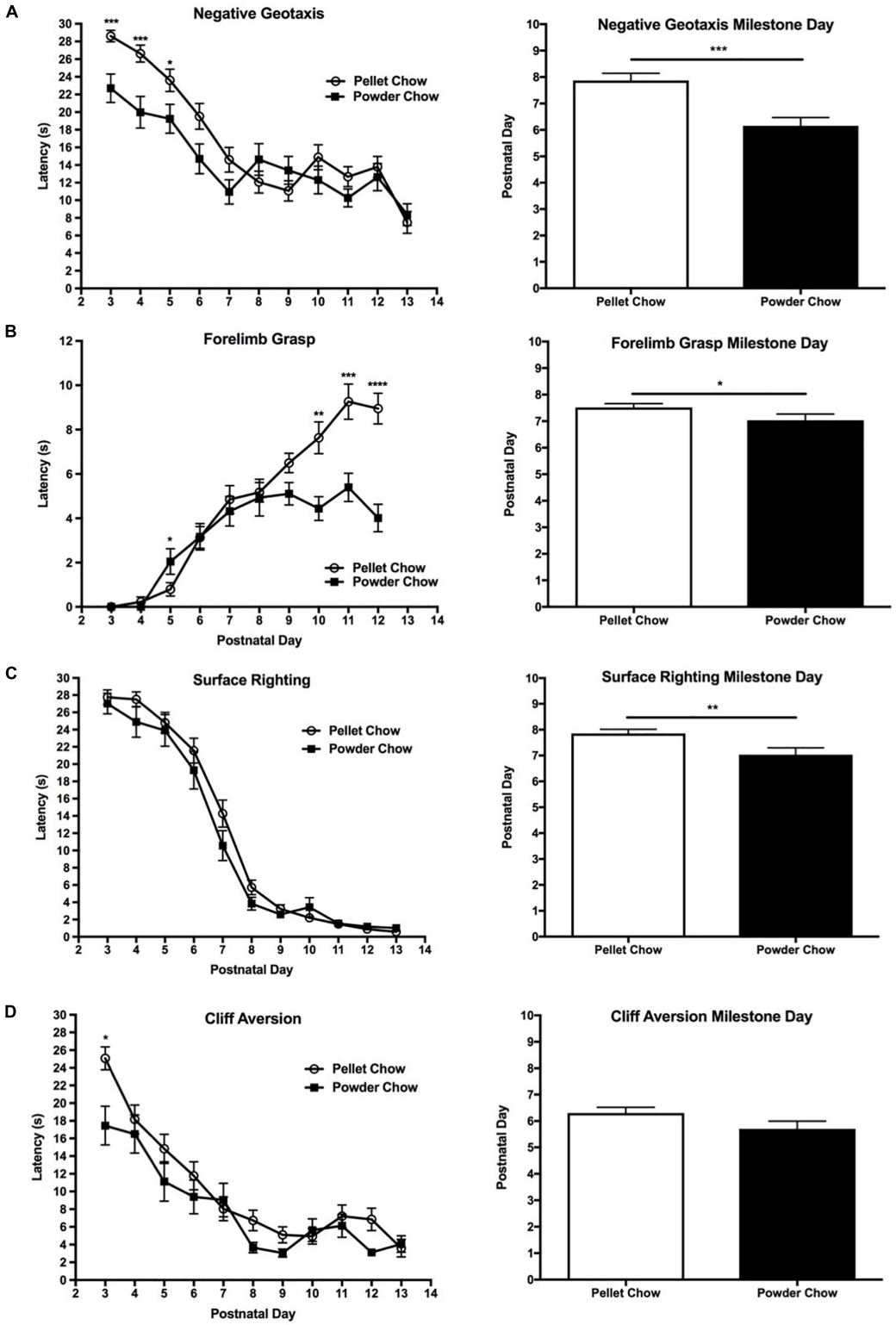


**FIGURE 5 |** Neonatal performance on tests of coordination, strength and body righting. Powdered chow-exposed neonates achieved coordination, strength, and body-righting milestones significantly earlier than pelleted-chow exposed neonates. Graphs on the left depict neonatal latency (mean ± SEM) to complete the behavioral task by postnatal day. Graphs on the right depict differences between neonates from the two maternal diet groups in the day of developmental milestone achievement (defined as the day at which the pup performed the task successfully for 2 days in a row). **(A)** Negative geotaxis test; **(B)** Forelimb grasp test; **(C)** Surface righting test; **(D)** Cliff aversion test. $N = 31$ powder and $N = 62$ pellet-chow exposed neonates. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

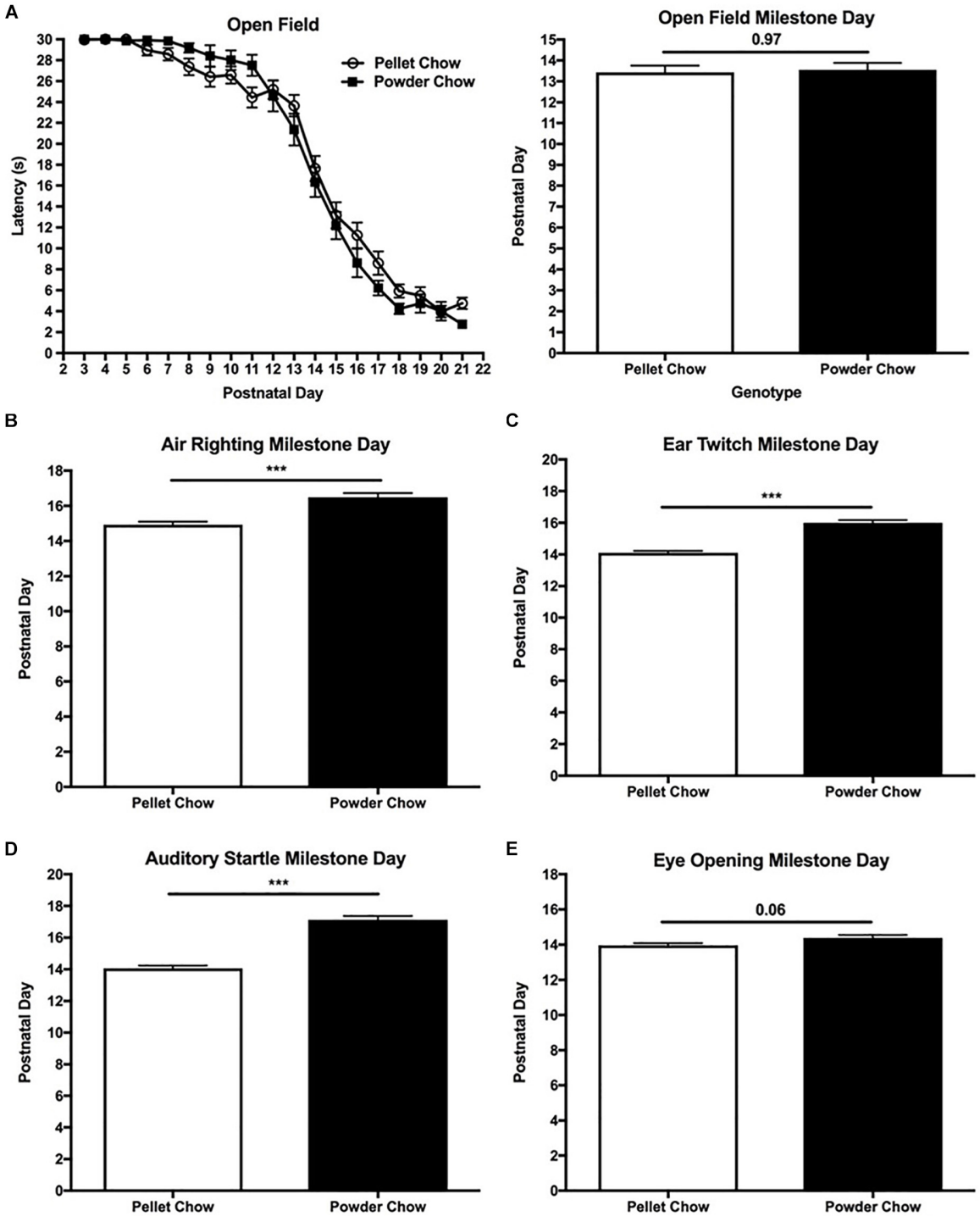


**FIGURE 6 |** Neonatal extinction of rotatory behavior and acquisition of sensory maturation reflexes. Powdered chow-exposed neonates were significantly delayed in their achievement of sensory maturation reflexes including air righting **(B)**, ear twitch **(C)**, and auditory startle **(D)**, compared to pelleted chow-exposed neonates. Eye opening **(E)** also trended toward delay in the powdered chow-exposed neonates. There were no significant differences in neonatal extinction of rotatory behavior between maternal diet groups (evaluated by open field testing, **A**). $N = 31$ powder and $N = 62$ pellet-chow exposed neonates. $^{***}p < 0.001$.

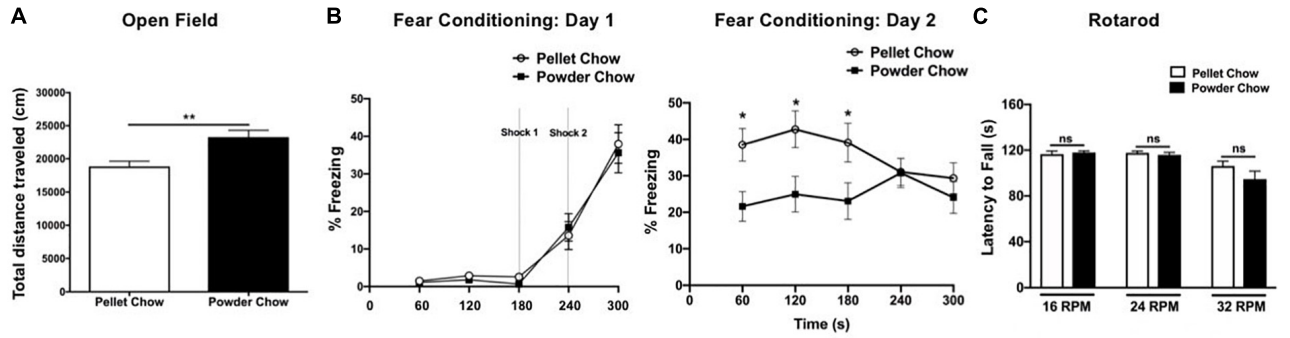


**FIGURE 7 |** Adult offspring locomotor behavior, hippocampal learning, and motor coordination. Powdered chow-exposed adults demonstrated hyperactivity (significantly greater distance traveled in the open field test, **A**) and hippocampal learning/memory deficits (significantly reduced freezing on day 2 of contextual fear conditioning, **B**). There were no differences between groups in adult motor coordination (Rotarod testing, **C**). $N = 14$ powder and $N = 16$ pellet-chow exposed adults, except for open field where $N = 13$ powder and 16 pellet-chow exposed adults. $^{*}p < 0.05$; $^{**}p < 0.01$.

## Impact of Dietary Micro- and Macronutrients on Brain and Organismal Development

The powdered-chow diet was relatively deficient in manganese, iodine, iron, zinc, and copper, which were 9.5-fold, 71-, 5. 4-, 2-, and 2.5-fold higher in pelleted chow, respectively. These micronutrients have all been shown to influence neurodevelopment in both human and animal model studies (Beard and Connor, 2003; Armony-Sivan et al., 2004; Hirzel et al., 2006; Corniola et al., 2008; Gao et al., 2009; Adamo and Oteiza, 2010; Gogia and Sachdev, 2012; Chung et al., 2015; Claus Henn et al., 2017). Maternal supplementation of these micronutrients has been demonstrated to improve neonatal and pregnancy outcomes, including incidence of congenital anomalies, low birth weight, low IQ or developmental delay, preterm birth, preeclampsia, and preterm premature rupture of membranes (Keats et al., 2019; Mousa et al., 2019).

It is important to note the limitations of available data on the impact of particular micronutrients on fetal and offspring brain development, particularly data from human cohorts. Key limitations include: (1) Many of the associations between maternal micronutrient deficiency and offspring neurodevelopmental outcomes are based on small observational studies that utilize maternal dietary report/recall (therefore are subject to recall bias); (2) Deficiency of a particular micronutrient is likely to co-occur with other nutrient deficiencies and potentially other conditions which may also impact fetal and offspring brain development, such as low socioeconomic status/food insecurity, poor maternal health in general, and prematurity (raising the potential for confounding). Thus, data linking any single micronutrient deficiency to adverse neurodevelopmental outcomes, particularly in observational human cohorts, should be interpreted with caution.

Maternal manganese deficiency and manganese excess have both been associated with worse performance on psychomotor and mental development indexes in human cohorts (Chung et al., 2015; Claus Henn et al., 2017). Zinc deficiency during brain development is associated with adverse neurodevelopmental outcomes in humans and in animal models, with deficits including motor delay and impairments, impaired task attention, engagement, and social behavior (Hirzel et al., 2006; Gogia and Sachdev, 2012). In rodent models, zinc deficiency is associated with cell cycle arrest, mediated in part through dysregulation of the ERK1/2, p53, and NF-kappa B pathways, and zinc deficiency during development is associated with both impaired neuronal precursor cell proliferation and induction of apoptosis (Corniola et al., 2008; Gao et al., 2009; Adamo and Oteiza, 2010). Iodine was the most discrepant micronutrient among the two experimental diets, and has been demonstrated to have a significant impact on neurodevelopment, with effects likely mediated by thyroid hormone deficiency (Skeaff, 2011). Iodine is required for neuronal growth, synapse formation, and myelination during brain development (Prado and Dewey, 2014). Iron was also significantly different between the two diets (relatively deficient in powdered chow), and has been shown to be critical to early life brain development. Iron deficiency has been associated with deficits in learning and memory in children, as well as alterations in neuron energy metabolism, dopamine signaling, and dendrite complexity in animal models (Beard and Connor, 2003; Georgieff, 2007; Prado and Dewey, 2014; Bastian et al., 2016). Particularly relevant to the deficits observed on neonatal behavioral testing in the powdered chow-exposed neonates, iron deficiency has been associated with abnormal neonatal reflexes and impaired auditory processing and auditory cortex development in human cohorts (Armony-Sivan et al., 2004; Siddappa et al., 2004).

The powdered chow was also relatively deficient in all vitamins, with vitamins A, B1, B2, Niacin, B6, pantothenic acid, B12, biotin, and folate all two- to four-fold higher in pelleted chow and Vitamin E 1000-fold higher in pelleted chow. Vitamins A, E, B6, B12 and folic acid have all been demonstrated to impact brain development, with deficiency during key developmental windows associated in human cohorts with adverse neurodevelopmental outcomes including cognitive and motor deficits, as well as autism spectrum disorder (Dias et al., 2013; Wachs et al., 2014; Altamimi, 2018;

Mousa et al., 2019). Vitamin E, which was 1000-fold lower in the powdered chow diet, is known to serve as an antioxidant, with fetal neuroprotective effects demonstrated primarily inrat and hamster models in the setting of maternal toxic exposures and/or oxidative stress (Erdemli et al., 2016; Sampayo-Reyes et al., 2017; Sakamoto et al., 2018; Zhang Y. et al., 2018). Supraphysiologic/supra-nutritional maternal vitamin E consumption has been demonstrated in rodent models to induce enduring changes in hippocampal synaptic plasticity of offspring, with increased synaptic density/reduced synaptic pruning observed in the adult offspring hippocampus, and associated cognitive/learning deficits (Betti et al., 2011; Salucci et al., 2014). Thus, both Vitamin E deficiency and excess appear to have deleterious impact on the developing fetal brain.

In addition to being deficient in the antioxidants Vitamin A and E, the powdered chow lacked the isoflavones daidzein and genistein. Studies in rodent models have demonstrated an important effect of dietary soy-derived phytoestrogens on learning and memory, and in mediating anti-inflammatory and neuroprotective effects on the brain (Wang et al., 2013). Both genistein and daidzein have been demonstrated to cross the blood-brain barrier, and exert antioxidant and neuroprotective effects by several mechanisms including mediation of programed cell death and reduction of RAGE-related NF-kB activation and neurovascular production of pro-inflammatory cytokines (Zeng et al., 2004; Xi et al., 2013; Zhang Q. et al., 2018). Both isoflavones have also been demonstrated to improve cognitive function. One putative mechanism for this is upregulation of brain-derived neurotrophic factors (Pan et al., 1999; Lund et al., 2001). Daidzein has been demonstrated in rodent models to exert neuroprotective and cell-proliferative effects in the context of stroke, and in the setting of high-fat diet-induced apoptosis and gliosis (Subedi et al., 2017).

While the powdered chow was deficient for micronutrients and antioxidants, it was relatively enriched for omega-3 long-chain polyunsaturated fatty acids (in particular the n-3 LC-PUFA linolenic acid), which have been demonstrated to influence neurodevelopment. The powdered chow contained 1.6-fold higher n-3 LC-PUFAs than pelleted chow, and had a more favorable n-6/n-3 long-chain PUFA ratio than the pelleted chow (Abedi and Sahari, 2014). LC-PUFAs are fatty acids with at least 18–20 carbons, whose omega-6 (n-6) or omega-3 (n-3) designations depend on the position of the first double bond relative to the methyl end group of the fatty acid (Venegas-Caleron et al., 2010). LC-PUFAs are important in neurotransmitter synthesis and release, immune system regulation, the clotting cascade, phospholipid membrane structure in the brain and retina, and cholesterol metabolism (Li and Hu, 2009; Abedi and Sahari, 2014). N-3 LC-PUFAs are highly concentrated in the mammalian retina and brain, and play a key role in normal visual and brain function due to their involvement in neurotransmitter biosynthesis, signal transduction, and monoamine neurotransmitter receptor binding and activity (Li and Hu, 2009). The powdered chow also had a higher content of carbohydrates when compared with the pelleted chow. There is a dearth of information in the literature about the impact of dietary carbohydrates on neurodevelopment, although overall maternal dietary quality, one measure of which includes total caloric consumption, has been demonstrated to be associated with neurodevelopmental outcomes (Malin et al., 2018). The slight increase in calories in the powdered chow (3.7 kcal/g versus 3.1 kcal/g in the pelleted chow), which is primarily attributable to the increased carbohydrate content, could impact overall organismal development, including of the developing brain.

## Pathways Analysis of Fetal Brain Transcriptome Data in Context

Key themes in the functional analyses of differentially expressed fetal brain genes included dysregulation of the cell cycle, of DNA damage response, and of apoptosis. Significant upregulation of the canonical pathway Mitotic Roles of Polo-Like Kinase (key in cell cycle regulation), and significant dysregulation of multiple downstream pathways implicated in cell cycle regulation, was noted in the powdered chow-exposed fetal brain. The polo-like kinases are a highly conserved family of proteins identified in yeast, *Xenopus*, *C. elegans* and mammals, playing a key regulatory role for entry into and exit from mitosis, centrosome separation and maturation, and promoting the onset of cytokinesis, among other functions (Glover et al., 1998; Donaldson et al., 2001). Polo-like kinases have been implicated in neurogenesis and synaptic plasticity (Kauselmann et al., 1999; Sakai et al., 2012; Genin et al., 2014), suggesting that activation of polo-like kinases might reflect increased demand for new neural progenitor cells or new neuronal connections. Whether the significant upregulation of this canonical pathway means that there is increased flux through the cell cycle in the brains of powdered chow-exposed embryos, and if there is increased flux, whether this reflects increased neurogenesis, increased cell death, or both, is beyond the scope of these experiments to determine. These results point to the need for additional experiments investigating the impact of micronutrient deficiency on cellular proliferation and death in the developing brain, specifically as these processes relate to increased flux through the cell cycle mediated by polo-like kinases.

DNA damage response was another key dysregulated biological process in the functional analyses, and might be related to the significant dysregulation of cell-cycle related biological functions and canonical pathways. There are numerous examples of micronutrient deficiencies associated with DNA damage (Ames, 1999). In particular, iron is an essential cofactor for proteins which regulate DNA replication, repair, and cell cycle progression (Zhang, 2014; Jung et al., 2019). One such protein for which iron is an essential cofactor is the enzyme GADD45, which was noted to be significantly dysregulated in the powdered chow-exposed embryonic brains, and is involved in cell cycle arrest after DNA damage (Gao et al., 1999; Steegmann-Olmedillas, 2011). Dietary zinc supplementation has also been linked with reduced DNA strand breaks in human subjects; proteins involved in DNA repair, antioxidant, and immune functions were restored after dietary zinc was increased (Song et al., 2009; Zyba et al., 2017).

Negative regulation of apoptosis in the powdered chow-exposed fetal brain was another key theme in the functional

analyses, including activation of both upstream regulators that downregulate apoptosis, and downstream pathways that negatively regulate apoptosis. This finding is consistent with gene expression patterns suggesting reduced apoptosis in the human fetal brain exposed to maternal obesity, which may itself be a state of relative micronutrient deficiency (Edlow et al., 2014). Multiple micronutrients that are deficient in the powdered chow diet have been linked to increased apoptosis in animal models, including iodine, manganese, zinc, and copper (Beard and Connor, 2003; Armony-Sivan et al., 2004; Vanlandingham et al., 2005; Georgieff, 2007; Tamm et al., 2008; Gao et al., 2009; Cui et al., 2018). Zinc deficiency, in particular, is strongly associated with apoptosis and abnormal neural progenitor regulation in the developing brain (Corniola et al., 2008; Gao et al., 2009; Adamo and Oteiza, 2010). Thus, downregulation of apoptosis-related pathways in the powdered chow-exposed fetal brain could be compensatory. Determining whether apoptosis is up- or down-regulated in the powdered chow-exposed/relatively micronutrient-deficient brain, and whether the downregulation of apoptosis seen on pathways analysis is compensatory or causative are important directions for future investigation.

Other key themes in both the IPA and GSEA/DFLAT analyses included decreased amino acid transport in the powdered chow-exposed fetal brain; dysregulated synaptic transmission and plasticity; brain innate immune response (including pathways related to microglia/macrophage phagocytic activity, as well as killer cell activity); and dopaminergic signaling. The changes in amino acid transport pathways could reflect the powdered chow's relative deficiency of multiple amino acids, especially glycine, alanine and arginine. Several components deficient in the powdered chow diet have been demonstrated to play an important role in synaptic plasticity, in particular zinc and iron (Beard and Connor, 2003; Jorgenson et al., 2005; Georgieff, 2007). LC-PUFAs, which are relatively enriched in the powdered chow diet, have also been demonstrated to play an important role in synaptogenesis, learning and memory, and synaptic plasticity (Georgieff and Innis, 2005; Georgieff, 2007; Crupi et al., 2013). Deficiency of iron, zinc, and copper, and reduced antioxidant capacity conferred by relative deficiency of antioxidants (including the specific isoflavones daidzein and genistein), have been associated with altered microglial function and brain innate immune response (Cunningham-Rundles et al., 2009; Ibrahim et al., 2010; Chinta et al., 2013). Iron and copper deficiency (both relatively deficient in the powdered-chow exposed diet) have been demonstrated to impact dopamine synthesis, metabolism and release in the striatum (Prohaska and Brokate, 2001; Beard and Connor, 2003; Georgieff, 2007).

## Contextualizing Neonatal Biometric Parameters and Behavioral Testing

The multiple differences between the two diets preclude attribution of neonatal growth changes or performance on any particular behavioral test to relative excess or deficiency of a particular dietary component. One possible interpretation of the neonatal length and behavioral differences between diet groups, however, is that the increased length and improved attainment of strength-and coordination-related tasks in the powdered chow-exposed pups may be attributable to aspects of the diet that were relatively enriched in powdered chow, while the delay in sensory milestone achievement might be attributable to relative deficiencies in the powdered chow diet. Thus, the relative enrichment of powdered chow for the n-3 LC-PUFA linolenic acid and carbohydrates could be a driver for increased length and improved strength and coordination in the pups, while the relative deficiency in macroelements, micronutrients and antioxidants could be a driver of the delays in sensory maturation noted.

Although our study design does not permit the explicit testing of these hypotheses, the existing literature suggests there is biologic plausibility for both omega-3 LC-PUFAs and carbohydrate enrichment increasing body length, and for omega-3 LC-PUFA enrichment improving strength and coordination. Prior studies have found an association between omega-3:omega-6 maternal dietary ratios and neonatal length in mice (Santillan et al., 2010). Maternal diet correlates with neonatal length in humans, as well (Rodriguez-Bernal et al., 2010; Hjertholm et al., 2018). Associations have also been noted between maternal dietary carbohydrate content and offspring body size, although weight and/or fat mass is more frequently reported to be significantly increased in the setting of increased carbohydrate consumption, compared to length (Renault et al., 2015; Crume et al., 2016).

LC-PUFAs are highly transferred between mother and neonate in breast milk and play a key role in synaptogenesis, synaptic plasticity, and myelination (Georgieff, 2007; Crupi et al., 2013). The majority of animal studies have demonstrated omega-3 fatty acid supplementation is associated with improvement in cognitive tasks, visual acuity, neurogenesis, and other brain-development-related endpoints (Lauritzen et al., 2016). Studies evaluating the benefit of maternal omega-3 fatty acid supplementation on cognition in humans, however, have been mixed. Inconsistent with our results demonstrating sensory maturation deficits in powdered chow-exposed offspring, a study in Turkish infants demonstrated that DHA-enriched formula was associated with more rapid acquisition of brainstem auditory-evoked potentials (Unay et al., 2004). Consistent with our results demonstrating improved coordination in powdered-chow exposed offspring, a small randomized trial found that omega-3 fatty acid supplementation was associated with improved hand-eye coordination in 2.5-year-old children (Dunstan et al., 2008). However, two comprehensive reviews, including one of nine randomized controlled studies, found no consistent sustained benefit of omega-3 supplementation for infant cognition or visual development (Lo et al., 2012; Prado and Dewey, 2014). Overall, studies evaluating the benefit of maternal omega-3 fatty acid supplementation on offspring developmental outcomes have demonstrated the most benefit in preterm infants (SanGiovanni et al., 2000; O'Connor et al., 2001; Makrides et al., 2010).

With respect to strength, omega-3 LC-PUFAs have been demonstrated to improve muscle contractility in rats (Patten et al., 2002), and in humans when omega-3 fatty acid

supplementation was combined with an exercise and resistance-training regimen (Rodacki et al., 2012). This may be mediated by enhanced sensitivity of the muscle to acetylcholine, a neurotransmitter that stimulates muscle contraction (Jeromson et al., 2015). Finally, it is possible that the increased body length itself had a favorable impact on strength and coordination-related neonatal behavioral tasks; this association has not been previously reported, and may be an interesting direction for future study.

There is also biologic plausibility for micronutrient and antioxidant deficiency impacting sensory maturation. In a guinea pig model, mild maternal iron deficiency during pregnancy and lactation was associated with abnormal auditory function in postnatal day 24 offspring (Jougleux et al., 2011). Maternal iodine deficiency, even if mild, has also been associated with disorders of auditory processing in human offspring (Azizi et al., 1995; Hynes et al., 2013), and in rodents, early postnatal iodine deficiency and hypothyroidism resulted in decreased dendritic branching in the visual and auditory cortex (Dussault and Ruel, 1987). Maternal and neonatal zinc deficiency has also been associated in multiple studies with abnormal development of brain regions that play critical roles in processing sensory information (Hagmeyer et al., 2014), and with neurosensory disorders in children (Prasad, 1996). Although maternal and perinatal zinc deficiency has more classically been associated with deficits in offspring learning and memory and social behavior (Golub et al., 1995; Hagmeyer et al., 2014), the brain areas rich in zinc-containing glutamatergic neurons (cerebral cortex and limbic structures) also play a key role in sensory processing (Hagmeyer et al., 2014). With respect to ways in which relative antioxidant deficiency may contribute to deficits in sensory maturiation, early life oxidative stress has been demonstrated to impair the development of parvalbumin-expressing fast-spiking interneurons, leading to deficits in sensory processing (Cabungcal et al., 2013).

## Contextualizing Adult Offspring Neurobehavioral Testing

With respect to the finding of hippocampal learning deficits in powdered-chow exposed adult offspring, multiple human studies have demonstrated relative micronutrient deficiency negatively impacts offspring cognition, with micronutrient supplementation of pregnant women and their infants/children associated with improved child cognition (standardized reading and math scores and information processing measures) (Prado and Dewey, 2014). However, these data are limited by concomitant increased caloric intake and/or improved protein intake during the period of supplementation for most studies, and lack of standardization of micronutrient supplementation content. While nearly all the micronutrients and antioxidants relatively deficient in the powdered-chow diet have been linked to hippocampal development and learning/memory, the strongest data in human and animal model studies are for iron and iodine deficiency in early development resulting in abnormal hippocampal development and cognitive and learning deficits that endure into adult life (Dussault and Ruel, 1987; Jorgenson et al., 2003, 2005; Lozoff et al., 2006; Beard, 2007; de Escobar et al., 2007; Walker et al., 2007; Fretham et al., 2011; Prado and Dewey, 2014).

With respect to the finding of hyperactivity/increased locomotion in powdered-chow exposed offspring, human studies evaluating the impact of vitamin and micronutrient deficiencies on offspring attention deficit hyperactivity disorder (ADHD) risk have reported mixed results (Li et al., 2019). For example, maternal multivitamin and folate intake were associated with a lower risk of ADHD diagnosis and medication use in the Danish National Birth Cohort, but not in a New Zealand birth cohort (Virk et al., 2018; D'Souza et al., 2019). Deficiencies in B vitamins and Vitamin D have also been linked to increased ADHD risk in children (Morales et al., 2015; Altun et al., 2018; Fasihpour et al., 2019; Kotsi et al., 2019), although some studies have failed to find these associations (Gustafsson et al., 2015). Inconsistent with our finding of hyperactivity in the powder-chow exposed offspring, recent human and rodent studies have reported that increased cord blood n-6/n-3 fatty acid ratio or relative maternal deficiency of n-3 fatty acids is associated with increased risk for ADHD in offspring (Fedorova and Salem, 2006; Sakayori et al., 2016; Lopez-Vicente et al., 2019), A recent systematic meta-analysis concluded that maternal omega-3 fatty acid intake has not been consistently associated with offspring ADHD risk, however (Li et al., 2019). Poor maternal dietary quality, particularly diets high in sugar, fat and processed foods (which may in some cases be a marker for micronutrient deficiency), has been more consistently linked to ADHD risk in offspring (Rijlaarsdam et al., 2017; Galera et al., 2018). Animal model studies have demonstrated more consistent associations between micronutrient deficiency during development and hyperactivity of offspring, with relative deficiency of B vitamins and iron most strongly implicated (Lalonde et al., 2008; LeBlanc et al., 2009; Fiset et al., 2015).

## Strengths, Limitations and Future Directions

As one of the first studies to directly examine the impact of maternal micronutrient and antioxidant deficiency on fetal brain development and offspring behavior in the absence of overt maternal over- or undernutrition, this study begins to address a significant knowledge gap. Pathway analysis of differentially expressed fetal brain genes provided unique insights into biological processes that could be impacted by the relative excess and deficient dietary components in the powdered chow. We hope that the gene expression changes and dysregulated pathways highlighted here can act as a starting point for future studies designed to examine the impact of specific combinations of maternal dietary components on fetal neurodevelopment and offspring behavior. The use of multiple pathways analysis tools, including a tool with annotation specific to fetal development (DFLAT), provides the most comprehensive picture of dysregulated biological processes (Edlow et al., 2015). The use of a comprehensive and validated battery of neonatal behavioral assessments performed by a single experienced investigator is also a strength. The multiple dietary components that differ between the study diets is a limitation,

as it does not permit us to attribute specific gene expression changes or putative biological functional changes to particular macro- or micronutrient components, only to demonstrate that "chow" diet composition has a profound impact on the developing fetal brain and enduring consequences for offspring behavior. However, as a recent publication notes, studies that examine the impact of deficiency or enrichment for a single micro- or macronutrient have limited "real world" applicability, and to maximize translatability, studies should focus on the neurodevelopmental impact of combinations of nutrients, given that nutrients are actually ingested in combination (Malin et al., 2018). Because offspring were weaned to the same diet they were exposed to *in utero* and during lactation, we cannot determine whether the adult behavioral differences were attributable solely to the intrauterine and lactational environment, the postnatal diet exposure, or a combination of the two. Although the study was not designed to interrogate the contribution of paternal diet, a strength of the study design is that male sires were exposed to the same diet as the dams, and male sires were kept on a powdered or pelleted chow diet throughout the duration of the study, with no crossover of male sires between maternal diet groups.

The relatively small number of microarrays per maternal diet group could be viewed as a potential limitation, but five biological replicates per group is sufficient in microarray studies to detect differences in global gene expression (Allison et al., 2006; Tarca et al., 2006). While this number of microarrays is sufficient to interrogate the impact of maternal diet on fetal brain gene expression, the study was not specifically designed or powered to evaluate sex differences in fetal brain gene expression in the setting of this maternal exposure. Therefore, the lack of a significant sex-diet interaction on two-way ANOVA modeling of the differentially expressed brain genes should be interpreted with caution. One piece of evidence in favor of no actual fetal sex effect is the additional lack of a significant effect of offspring sex in the neonatal behavioral analyses. The large number of offspring per sex in the neonatal behavioral experiments (15–37/sex/maternal diet group) suggests that in the context of this particular maternal dietary exposure, fetal and offspring sex truly may not be a significant modifier. Additional studies designed and powered specifically to look at the impact of fetal sex in the setting of these maternal dietary exposures are necessary to definitively exclude fetal sex as an effect modifier. Other future directions include specific enrichment or depletion of maternal diet for only one or a small combination of micro- or macronutrients to explicitly test some of the hypotheses put forward here regarding the impact of LC-PUFA and carbohydrate enrichment on neonatal length, strength and coordination, and the impact of relative micronutrient and antioxidant deficiency on neonatal sensory maturation. In addition, specific evaluation of the mother's milk for particular dietary components would be a useful adjunct to future studies.

## SUMMARY

In this study, we demonstrate the important role of maternal diet composition in fetal brain development and offspring behavior, even in the absence of maternal over- or undernutrition. We show that commercially available chow diets are not interchangeable, and the content of maternal chow diet significantly impacts embryonic brain gene expression, neonatal behavior, and adult behavior in mice. These findings underscore the importance of selecting a maternal chow diet matched for macro- and micronutrients when investigating fetal and offspring brain development and behavior.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO, accession number GSE133525.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Tufts Medical Center IACUC.

## AUTHOR CONTRIBUTIONS

AE, FG, and DB contributed to conceiving and designing the study. AE, FG, and JP performed the statistical analysis of behavioral and microarray data. FG performed the neonatal behavioral and microarray experiments. AE performed *in silico* microarray experiments and wrote the first draft of the manuscript. DS wrote sections of the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version.

## FUNDING

This study was funded by the National Institutes of Child Health and Human Development (NICHD R01HD058880 to DB; 2K12HD000849 to AE via the Reproductive Scientist Development Program; and NICHD R01HD100022-01 to AE), and a sponsored research grant from Verinata Health, Inc., an Illumina company (to DB). This work was also supported in part by the National Human Genome Research Institute's Intramural Research Program, National Institutes of Health, Bethesda, MD, United States through grant Z1A HG200399-04 (Investigator: Diana Bianchi). The funders had no role in the study design, interpretation of results, or writing of the manuscript. Funding for open access publication fees is provided by the Massachusetts General Hospital Vincent Memorial Fund.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.01335/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 31 January 2020
doi: 10.3389/fnins.2019.01454

# Increased Hypothalamic Projections to the Lateral Hypothalamus and Responses to Leptin in Rat Neonates From High Fat Fed Mothers

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OPEN ACCESS

**Edited by:**
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**Specialty section:**
This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience

**Received:** 07 November 2019
**Accepted:** 30 December 2019
**Published:** 31 January 2020

**Citation:**
Kelley L, Verlezza S, Long H, Loka M and Walker C-D (2020) Increased Hypothalamic Projections to the Lateral Hypothalamus and Responses to Leptin in Rat Neonates From High Fat Fed Mothers. Front. Neurosci. 13:1454. doi: 10.3389/fnins.2019.01454

The lateral hypothalamus (LHA) is a central hub in the regulation of food intake and metabolism, as it integrates homeostatic and hedonic circuits. During early development, maturing input to and output from the LHA might be particularly sensitive to environmental dietary changes. We examined the effects of a maternal high fat diet (HFD, 60% Kcal in fat) on the density of hypothalamic projections to the orexin (ORX-A) field of the LHA in 10 day-old (PND10) rat pups using retrograde labeling with fluorescent microspheres. We also compared responsiveness of phenotypically identified LHA neurons to leptin administration (3 mg/kg, bw) between pups from control (CD) or high fat (HFD) fed mothers on PND10 and 15-16, at the onset of independent feeding. HFD pups exhibited a higher density of LHA projections ($p$ = 0.05) from the ventromedial hypothalamus (VMH) compared to CD pups and these originated from both SF-1 and BDNF-positive neurons in the VMH. Increased circulating leptin levels in HFD pups, particularly on PND15-16 was consistent with enhanced pSTAT3 responses to leptin in the orexin (ORX-A) field of the LHA, with some of the activated neurons expressing a GABA, but not CART phenotype. ORX-A neurons colocalizing with pERK were significantly higher in PND15-16 HFD pups compared to CD pups, and leptin-induced increase in pERK signaling was only observed in CD pups. There was no significant effect of leptin on pERK in HFD pups. These results suggest that perinatal maternal high fat feeding increases hypothalamic projections to the ORX-A field of the LHA, increases basal activation of ORX-A neurons and direct responsiveness of LHA neurons to leptin. Since these various LHA neuronal populations project quite heavily to Dopamine (DA) neurons in the ventral tegmental area, they might participate in the early dietary programming of mesocorticolimbic reward circuits and food intake.

**Keywords: maternal programming, high fat diet, neonatal, leptin, lateral hypothalamus, orexin**

## INTRODUCTION

The lateral hypothalamus (LHA) is central to the regulation of food intake as it integrates incoming metabolic signals from several hypothalamic nuclei with the hedonic regulation of food intake, involving the mesolimbic circuit and in particular, dopamine (DA) activity in the ventral tegmental area (VTA). Homeostatic hypothalamic nuclei such as the arcuate nucleus (ARC), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and hypothalamic paraventricular nucleus (PVN) project either directly or indirectly to the LHA (Hahn and Swanson, 2015) and together with multiple cortical, subcortical and brain stem inputs, regulate LHA activity (Bonnavion et al., 2016). In the LHA, multiple cell types are known to project to the VTA where they control dopamine and GABA interneuron firing activity and among these, several harbor functional leptin receptors (Lept$R_b$), including Neurotensin (Nts), Cocaine and Amphetamine Regulated Transcript (CART) neurons as well as different populations of GABA-containing neurons projecting to VTA GABA neurons (Nieh et al., 2016; Stuber and Wise, 2016; Godfrey and Borgland, 2019). Orexin-A (ORX-A) containing neurons in the LHA directly project to VTA dopamine neurons and stimulation of these cells has been shown to increase VTA DA firing activity and increase food intake (Tyree and de Lecea, 2017). While ORX-A neurons projecting to the VTA do not contain leptin receptors (Leinninger et al., 2009; Leinninger, 2011) they are indirectly inhibited by leptin in an energy-dependent state, whereby high fat feeding dampens the inhibitory effect of leptin on these neurons in adult mice (Liu et al., 2017), resulting in increased food intake.

In rodents, connections between "homeostatic" hypothalamic nuclei and the LHA are not fully established at birth and mature over the first weeks postnatally, making them susceptible to changes in the perinatal nutritional environment (Muscatelli and Bouret, 2018). Projections from the ARC to the DMH mature by postnatal day (PND) 6, whereas those reaching the PVN and the LHA are observed between PND8-10 and PND12, respectively (Bouret et al., 2004a). In neonatal mice, projections from the DMH to the PVN and LHA are fully established by PND6, followed by projections from the VMH to the LHA, which develop by PND10, prior to those from the ARC (Bouret et al., 2004b). While the development of hypothalamic homeostatic connections has been well studied, much less is known on the development of efferent projections from the LHA to the VTA. Using retrograde tracing, we previously showed that functional connections already exist between these two nuclei by PND6 and that some of these originated from ORX-A neurons (Gjerde et al., 2016). Thus, by PND10, the LHA is already connected to both homeostatic and hedonic centers even though independent food intake does not start until PND17 in rat pups from control mothers or PND16 in pups from high fat diet (HFD)-fed mothers (Doerflinger and Swithers, 2004). Similarly to the earlier onset of independent feeding, central food intake mechanisms, food seeking behavior, and food preferences are altered by changes in the perinatal nutritional environment, increasing vulnerability to adult metabolic diseases (Bouret, 2012; Ribaroff et al., 2017; Romaní-Pérez et al., 2017). The metabolic hormone leptin inhibits food intake and increases energy expenditure in adult rodents. Yet, during perinatal life, it may be critical to developmental programming because of its important role in neurite outgrowth and synapse formation (Bouret et al., 2004b; Bouret, 2010). Circulating leptin concentrations in the offspring are also significantly increased when mothers are fed a HFD (Trottier et al., 1998).

During hypothalamic circuit maturation, leptin's role on the development of neuronal projections might supersede its primary role to suppress food intake. The production of leptin-induced intracellular signaling molecules such as phospho-STAT3 (pSTAT3) and phospho-ERK (pERK) (Kwon et al., 2016) might not only indicate the presence of functional Lept$R_b$ receptors, but also provide an index of tissue responsiveness and/or resistance to increased circulating leptin levels. We previously documented a gradual and site-specific emergence of tissue responsiveness to leptin in naïve neonates (Naef et al., 2014; Gjerde et al., 2016) with significant pSTAT3 production in the ARC already by PND10 and by PND16 in the VTA. In the LHA, increases in pERK were documented on PND16. However, it is currently unknown whether exposure to a HFD in early neonatal life modifies LHA responsiveness to leptin and induces early resistance to leptin as observed in adult rats (Matheny et al., 2011). Given the ability of maternal HFD feeding to increase leptin levels in offspring during the maturation of food intake circuits and the central role of the LHA in coordinating homeostatic and hedonic regulation of food ingestion, the aim of our studies were to determine whether perinatal maternal HFD feeding influences the density of hypothalamic projections to the LHA and modifies responsiveness to leptin in phenotypically identified LHA neurons. Our results show that VMH projections to the LHA are significantly increased in PND10 neonates nursed by HFD mothers and that contrary to the adult condition, leptin still elicits a robust intracellular response in LHA neurons from HFD preweaning offspring at the time of the onset of independent feeding.

## MATERIALS AND METHODS

### Animals

Pregnant Sprague–Dawley females (Charles River Laboratories, Inc., St. Constant, QC, Canada) were obtained on gestation day (GD) 13–14 and housed under controlled conditions of light (12 h light-dark cycle), temperature, and 70–80% relative humidity. All rats were provided food and water *ad libitum*. On the day of arrival, half of the mothers were placed either on a chow diet (CD; Teklad Global diet, 6% kilocalories (Kcal) from fat; Harlan Laboratories) or a HFD [Teklad diet (TD) 06414, 60% Kcal from fat; Harlan Laboratories, Indianapolis, IN, United States] supplemented with peanut butter (approximately 30 g per day; 72% Kcal from fat, 6.7 Kcal/gram) to ensure higher caloric consumption between gestation day (GD)13–14 and postpartum day 15–16 (**Table 1**). We chose to expose mothers to the HFD during the last week of gestation and throughout lactation in order to (1) target the period of hypothalamic nuclei

**TABLE 1 |** Nutritional information for the control (CD) and High fat (HFD) diets.

| Nutritional information | CD: Teklad 18% protein diet | HFD: Teklad 60% fat diet |
|---|---|---|
| % Kcal from fat | 6.0% | 60.3% (37% saturated, 47% monounsaturated, 16% polyunsaturated) |
| % Kcal from protein | 18.9% | 18.3% |
| % Kcal from carbohydrates | 75.1% | 21.4% |
| Kcal per gram | 3.3 | 5.1 |

birth and development (between E13-19 in rats) and circuit maturation in the fetus/neonate and (2) avoid the potential confounding effects of maternal obesity that develops when dams are fed a HFD during the entire pregnancy. The day of parturition was considered PND 0 and on PND1, litters with both male and female pups were culled to either 8 (for retrograde tracing experiments) or 10 pups per mother (for immunohistochemistry experiments). Animals were maintained on their diet until pups were sacrificed at either PND9-10 or PND15-16. Body weight and food intake was recorded on various gestational and postpartum days. All procedures were reviewed and approved by the Animal Care Committee at McGill University and followed ethical guidelines from the Canadian Council on Animal Care (CCAC).

## Retrograde Tracer Injections in the LHA ORX-A Cell Field of Neonates

On PND5-6, pups from both diet groups (CD: $n$ = 8, average 16.2 g; HFD: $n$ = 10, average 17.8 g at time of injection) were anesthetized with isoflurane and placed in a stereotaxic frame with neonatal rat ear bars and anesthesia adaptor (Kopf Instruments, Tujunga, CA, United States). Once anesthetized, the skull of the pup was aligned using bregma and lambda as reference points for the coordinates. Injections were made to target the medial and lateral LHA ORX-A field between the adult coordinates of −2.28 and −3.36 mm anterior/posterior (A/P) from the bregma (Paxinos and Watson, 2005). These adult coordinates were adapted by a factor of 0.663 (and further adjusted based on trial and error in preliminary surgeries) to make injections to neonatal pups (Gjerde et al., 2016). The resulting coordinates used for targeting the medial and lateral portions of the LH ORX cell field were the following: A/P −1.99 to −2.6 mm, lateral: 0.8–1.2 mm, and ventral: −5.95 to −6.08 mm. Red fluorescent fluorospheres (20 nl, #F-8793; Invitrogen, Life technologies Inc., Burlington, ON, United States) were injected using a 0.5-µl Hamilton neurosyringe and a Harvard nanopump mounted on the stereotaxic arm (Harvard Instruments, St-Laurent, QC, Canada). Fluorospheres were injected at a rate of 10 nl/min, and the syringe was left in place for another 5 min before it was slowly removed from the injection site over 1–2 min. The skin was sutured using surgical silk, and pups were returned to their mother only after reaching full consciousness. Four days after microinjection, pups were anesthetized with ketamine/xylazine (subcutaneous injection 0.1 ml/kg body weight) and perfused with ice-cold 0.9% saline (50 ml) followed by 200 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7. Before perfusion, a blood sample was collected on EDTA via cardiac puncture to determine plasma leptin levels. Blood samples were centrifuged (13,000 rpm, 5 min, 4°C), and the plasma was collected and stored at −20°C until leptin assays were performed. Brains were post-fixed for 2 h in 4% PFA-PBS and transferred to 20% sucrose in 0.1 M phosphate buffer (PB) overnight before being frozen on dry ice and stored at −80°C until slicing. Brain tissue was sliced on a cryostat at 50 µm in two series to include at least the LHA (injection placement), VMH, and DMH regions. All slides were coverslipped with DAPI mounting medium (Vector Labs, Burlingame, CA, United States) and kept in the dark to preserve fluorosphere fluorescence until imaging. Each brain was examined for appropriate injection placement to the LHA ORX-A neuronal field, and was only taken for further image analysis if the bottom of the injection fell between the appropriate coordinates as previously indicated (see **Figure 1** for injection placement). Images were acquired for both the injection site and the projection neurons.

## Identification of VMH Neurons Projecting to LHA in Neonates by RNAscope

Brain sections adjacent to those used for estimating projection density were used to phenotype the VMH neurons that send LHA projections (colocalizing with fluorospheres). RNAscope *in situ* hybridization (ACDBio, Newark, NJ, United States) for SF-1 and BDNF was performed on tissue sections from the neonatal VMH containing fluorospheres from correct LHA injection sites. Procedures were adapted from the manufacturer instructions. Briefly, VMH sections (50 µm) were transferred to negatively charged slides, incubated for 1 h at 60°C and post-fixed in 4% PFA for 1 h at 4°C. Sections were dehydrated by successive ethanol rinses and incubated for 10 min with 1% $H_2O_2$. After washing (manufacturer wash solution), sections were incubated with RNAscope Protease III for 10 min at 40°C followed by probe hybridization at 40°C for 2 h. After washing, sections were kept overnight at room temperature in 5x SSC buffer. On the next day RNAscope amplification steps were performed prior to HRP developing (15 min) of the signal using specific fluorophores such as Opal520 (BDNF) and Opal 690 (SF-1) exposure for 30 min. Sections were washed and coverslipped with DAPI containing mounting medium (Thermo Fisher Scientific, Canada). Images were acquired with an Olympus FluoView FV-1200 confocal microscope.

## Leptin Treatment and Tissue Collection

On PND10 or 15-16, male and female pups from either the CD or the HFD mothers were randomly assigned to receive an intraperitoneal (0.1 ml) injection of either vehicle (0.9% saline) or leptin (3 mg leptin/kg BW; PeproTech, Rocky Hill, NJ, United States). Pups were returned to their mothers after injection and 1 h later, they were perfused transcardially with 50 ml of ice-cold 0.9% saline, followed by either 10% formalin (for pSTAT3 detection) or 4% PFA (for pERK detection) in 0.1 M PBS as described above. This time point is optimal to determine activation of second messenger signaling molecules

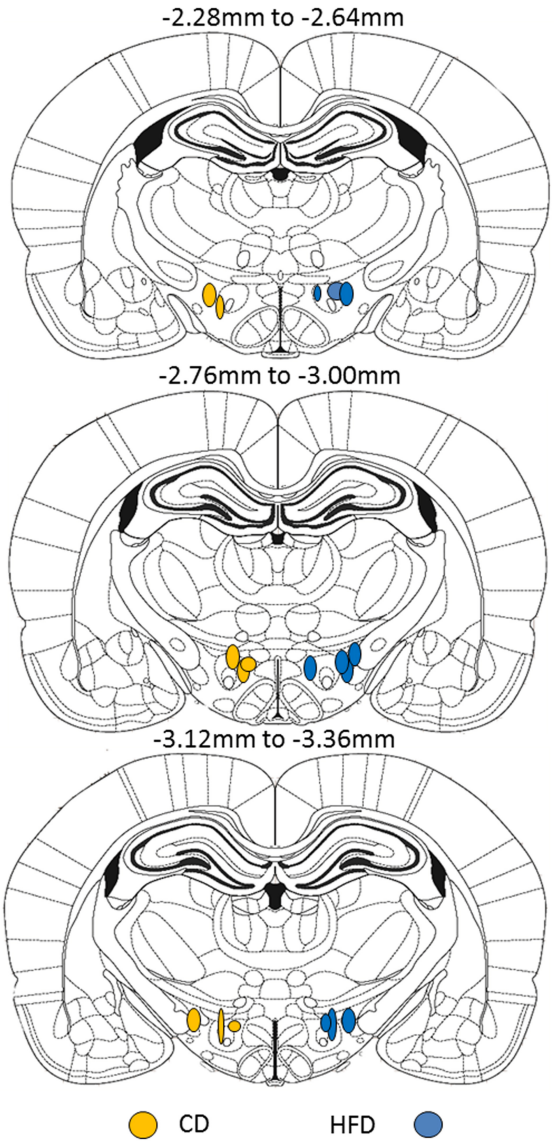


**FIGURE 1 |** Schematic representation of the site of fluorescent microsphere injections in PND5-6 rat pups from either CD (left side, $n = 8$) or HFD (right side, $n = 10$) litters. Each circle represents one animal and the antero-posterior (A/P) level of injection is indicated according to the atlas of the rat brain from Paxinos and Watson (2005). Within a diet group, injections were randomized between left and right LHA and were only considered accurate within the A/P coordinates shown. For ease of illustration and comparison between diet groups, all injections in CD pups are displayed on the left and injection sites in HFD pups are displayed on the right. Because of the relatively small number of animals per diet group, we did not distinguish between medial and lateral injection sites.

such as pSTAT3 and pERK in neonates (Walker et al., 2007). After blood collection, perfusion, and post-fixation, brains were transferred to 20% sucrose (in 0.1 M PB) overnight. The following day, brains were frozen on dry ice and stored at −80°C until processed for immunohistochemistry. Brains were collected as 50 μm floating sections in cryoprotectant (30% ethylene glycol, 20% glycerol, 37.5% diethylpyrocarbonate $H_2O$, 8% NaCl, 0.02% KCl, 1.4% $Na_2HPO_4$, and 0.24% $KH_2PO_4$ in $dH_2O$, pH 7.4) and stored at −20°C until staining. All primary and secondary antibodies, fixation conditions, and blocking solutions for each IHC are summarized in **Table 2**.

## Single or Double Immunohistochemistry (IHC) of Neonatal LHA After Leptin Treatment

In the first experimental series, we examined leptin-induced activation of pSTAT3 in LHA neurons (single pSTAT3 IHC). Sections were brought to room temperature (RT), washed 3 × 5 min in 0.1 M Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated in 0.5% $H_2O_2$ and 0.5% NaOH in TBS-T for 20 min at RT. Sections were washed again (4 × 5 min in TBS-T) and placed in 0.3% glycine in TBS-T for 10 min at RT, followed by a wash (3 × 5 min in TBS-T) and another incubation with 0.03% SDS in TBS-T for 10 min at RT. After washing, sections were placed in a blocking solution (**Table 2**) for 1 h at RT, treated for 15 min with avidin-biotin (Vectastain, Burlingame, CA, United States) at RT, and washed before incubation with the primary rabbit anti-pSTAT3 antibody (Cell Signaling 9131S, 1:500 dilution in blocking solution; New England Biolabs, Ipswich, MA, United States) for 1 h at RT and then overnight at 4°C. The following day, sections were washed and placed in biotinylated goat-anti-rabbit secondary antibody (Vector Cat. No: BA-1000, 1:500 in blocking solution; Vector Labs, Burlingame, CA, United States) for 2 h at RT. Next, sections were washed and placed in streptavidin conjugated to Cy3 (Jackson ImunoResearch 016-160-084, 1:1000 in blocking solution; Jackson ImmunoResearch, West Grove, PA, United States) for 2 h at RT. Sections were washed (3 × 5 min in TBS-T), coverslipped using mounting medium with DAPI (Vector Labs, Burlingame, CA, United States), dried and stored at 4°C prior to imaging.

In the second experimental series, we performed double IHC to identify the phenotype of leptin-activated neurons in the LHA using pSTAT3 IHC with either CART or GAD67 as a marker of GABAergic neurons. The protocol remained the same through the avidin and biotin treatment and thereafter, tissue sections were placed in the appropriate blocking solution (**Table 2**) for 1 h at RT. Sections were washed and incubated in either mouse-anti-GAD67 (Millipore MAB5406, 1:500 in blocking solution, MilliporeSigma, Burlington, MA, United States) or goat-anti-CART (R&D Systems AF163, 1:100 in blocking solution; R&D Systems, Minneapolis, MN, United States) primary antibody for 2 h at RT, and overnight at 4°C. The following day, sections were washed and placed in the appropriate secondary antibody: goat-anti-mouse Alexa568 for GAD67 (A11031 Invitrogen, 1:500 in blocking solution; Life Technologies, Carlsbad, CA, United States) or donkey-anti-goat Alexa488 for CART (Life Technologies A11055, 1:200 in blocking solution; Thermo Fisher Scientific Corporation, Carlsbad, CA, United States). Sections were stained for pSTAT3 detection as described above, coverslipped, and stored at 4°C prior to analysis.

**TABLE 2 |** List of antibodies and immunohistochemistry procedures.

| Primary AB | Fixative | Blocking solution | Primary AB dilution | Secondary AB |
|---|---|---|---|---|
| Rabbit anti-pSTAT3 (Cell Signaling #9131S) | 10% formalin | 4% Normal goat serum 0.4% Triton-X100 1% BSA (in TBS-T) | 1:500 | Biotinylated goat-anti-rabbit 1:500 (Vector Labs Cat# BA-1000) |
| Goat anti-CART (R&D Systems #AF163) | 10% formalin | 4% Normal horse serum 0.4% Triton-X100 1% BSA (in TBS-T) | 1:100 | Donkey-anti-goat Alexa488 1:200 (Life Technologies #A11055) |
| Mouse anti-GAD (Millipore MAB#5406) | 10% formalin | 2% Normal goat serum 0.04% Triton-X100 1% BSA (in TBS-T) | 1:500 | Goat-anti-mouse Alexa568 1:500 (Invitrogen #A11031) |
| Rabbit anti-pERK (Cell Signaling #4370S) | 4% PFA | 3% Normal horse serum 0.3% Triton-X100 1% BSA (in TBS-T) | 1:200 | Donkey-anti-rabbit Cy3 (Jackson ImmunoResearch #711-165-152) 1:500 |
| Goat anti-ORX-A (Santa Cruz SC#8070) | 4% PFA | 3% Normal horse serum 0.3% Triton-X100 1% BSA (in TBS-T) | 1:200 | Donkey anti-goat Alexa 488 (Life Technologies #A11055) 1:200 |

*AB, antibody; BSA, bovine serum albumin; ORX-A, orexin A; PFA, paraformaldehyde; TBS-T, 0.1 M Tris buffered saline containing 0.1% Tween20.*

In the third experimental series, we performed double IHC for detection of pERK and ORX-A. Sections were brought to room temperature (RT), washed 3 × 5 min in 0.1 M TBS-T and treated with 1% NaOH + 1% $H_2O_2$ in TBS-T for 30 min at RT, followed by TBS-T washes (3 × 5 min). Sections were treated with 0.3% glycine in TBS-T for 10 min at RT, washed and treated with 0.03% SDS in $dH_2O$, washed again (TBS-T, 3 × 5 min), and treated with 100% methanol at −20°C. Following washes, the sections were incubated in blocking solution (**Table 2**) for 1 h at RT. Thereafter, sections were incubated in rabbit anti-pERK primary antibody (Cell Signaling 4370S, 1:200 dilution in blocking solution; Cell Signaling Technology, Danvers, MA, United States) for 1 h at RT and overnight at 4°C. The following day, sections were washed and incubated for 2 h in Cy3 donkey-anti-rabbit secondary antibody (Jackson ImmunoResearch 711-165-152, 1:500 dilution in blocking solution; Jackson ImmunoResearch, West Grove, PA, United States) at RT. Sections were washed and treated with a goat anti-ORX-A primary antibody (Santa Cruz SC8070, 1:200 dilution in blocking solution; Santa Cruz Biotechnology, Dallas, TX, United States) overnight at 4°C. On the final day, sections were washed, treated with Alexa 488 donkey anti-goat secondary antibody (Life Technologies A11055, 1:200 dilution in blocking solution; Thermo Fisher Scientific Corporation, Carlsbad, CA, United States) for 2 h at RT, washed as previously described, and coverslipped using mounting medium with DAPI (Vector Labs, Burlingame, CA, United States), and stored in the dark at 4°C until imaged.

## Image Acquisition and Signal Quantification

For fluorosphere-injected brains with proper placement in the LHA ORX-A cell field, serial z-stack tiling images of the DMH and VMH were taken at 20x using an Olympus BX63 microscope with a step size of 0.35 mm to visualize retrogradely transported beads. Images were reconstructed using CellSens Software (version 1.12 to 1.15; Olympus Canada Inc., Toronto, ON, Canada) and the VMH and DMH regions were contoured using nuclear DAPI staining. Projections originating from the DMH and VMH were quantified using the optical fractionator probe for unbiased stereology (Microbright Field Bioscience, MBF, Williston, VT, United States). Within these regions, clusters of beads clearly showing a neuronal shape (either contained in the axon or clustered in the cell body) were counted as one projection. In the VMH region, a counting frame of 100 μm X 100 μm was chosen with a 200 μm X 200 μm sampling grid and a dissector height of 30 μm. Counted projections in the VMH began at −1.72 mm and extended to −3.36 mm from the bregma. For the DMH region, the entire region was counted with a 150 μm X 150 μm counting frame and a 20 μm dissector height. The DMH region began at −3.00 mm and extended to −3.48 mm from the bregma (Paxinos and Watson, 2005). The Gundersen coefficient of error ($m$ = 1) (Gundersen et al., 1999) was 0.09 ± 0.008 and 0.14 ± 0.009 for the VMH and DMH, respectively. Projection density was calculated using the estimated population of cells (# of cells containing fluorospheres) and the volume of the counted regions (MBF). The average volume for the VMH was 5.82 ± 0.238 × $10^8$ $\mu m^3$ (CD) and 6.26 ± 0.223 × $10^8$ $\mu m^3$ (HFD). The average volume for the DMH was 0.503 ± 0.037 × $10^8$ $\mu m^3$ (CD) and 0.535 ± 0.051 × $10^8$ $\mu m^3$ (HFD). Additionally, 4X serial images were taken of the injection site in the LHA and used to normalize the density of projections counted to the maximum volume of beads injected. This was determined for each brain using the LHA section with highly fluorescent beads at the most ventral point, and was considered the reference point of the bead injection. In this particular section, we calculated the volume of bead injection by multiplying the surface area (obtained using ImageJ Software; ImageJ 1.50i; National Institutes of Health, United States) by the thickness of the section (measured with stereology, MBF). This value was used to normalize the density of projections per animal by dividing the density of projections counted from the DMH and VMH (cells/$mm^3$) by the volume of beads ($mm^3$) injected. To obtain a higher-resolution visualization of both immunostaining and DMH/VMH projections, selected sections were imaged using an Olympus FV1200 confocal microscope with a motorized stage (Olympus Canada Inc., Toronto, ON, Canada). Images were acquired using 2X line averaging, and were 2048 pixels X 2048 pixels. Both the 405 nm and 543 nm lasers were used, with emission barrier filters BA430-470 and BA550-660, respectively.

Images of immunostained tissue sections (for pSTAT3, pSTAT3/CART, pSTAT3/GAD67, or pERK/ORX-A) were taken

with an Olympus BX63 microscope at either 20X or 40X using an Olympus DP80 camera and motorized stage (Olympus Canada Inc., Toronto, ON, Canada). For immunostained tissue, multichannel tiling images were taken at 20X. All tiled images were reconstructed using CellSens Software. For the detection and quantification of pERK and ORX-A double IHC, colocalized and single stained cells were counted using ImageJ. Both ORX-A and pERK cell density (number of cells/mm$^2$ area) were calculated and the percent of ORX-A cells co-localizing with pERK was determined. Nuclear pSTAT3 signal in single IHC experiments was quantified using the QuPath software (Bankhead et al., 2017). We evaluated the precision and reliability of the QuPath quantification by performing parallel manual counting of all sections for pSTAT3 analysis on PND10 pups. The correlation between both modes of counting was significant ($p < 0.001$) with an $r^2 = 0.3899$ ($n = 141$ determinations).

## Plasma Leptin Concentrations

Plasma leptin levels were measured using a specific rat leptin ELISA kit (Sigma Aldrich, St. Louis, MI, United States) with a detection range of 30–8,000 pg/ml and following the manufacturer's indications. Plasma samples (50 µl) of neonates were assayed for all cohorts in duplicates and within the same assay plate if possible.

## Statistical Analysis

For projection analysis, a one-way ANOVA was used to compare the normalized density of projections between diet (CD vs. HFD) groups. For the quantification of immunofluorescent signal in the LHA after leptin treatment, we performed a two-way ANOVA using diet (CD vs. HFD) and treatment (saline vs. leptin injection) as between factors. Analysis of variance was followed by *post hoc* Tukey HSD tests as appropriate. Values are represented as mean ± SEM and statistical differences with $p < 0.05$ are considered significant.

# RESULTS

## Maternal High Fat Diet Effects on Caloric Intake, Pup Body Weight and Leptin Concentrations

Maternal Kcal intake (**Figure 2**, top panel) varied significantly as a function of diet ($F(1,19) = 7.84$, $p = 0.011$) and time ($F(1,19) = 125.3$, $p < 0.001$) and *post hoc* Tukey HSD test showed that Kcal intake was significantly ($p = 0.021$) increased in HFD compared to CD mothers during gestation, but not when measured postpartum ($p = 0.061$). Pup weight during the first 10 days of life did not change significantly with the maternal diet (**Figure 2**, middle panel) in line with the lack of significant diet effect on leptin levels on PND9-10 pups (**Figure 2**, bottom panel). In contrast, plasma leptin levels on PND15-16 were significantly increased in HFD compared to CD-fed pups ($p = 0.002$, *t*-test).

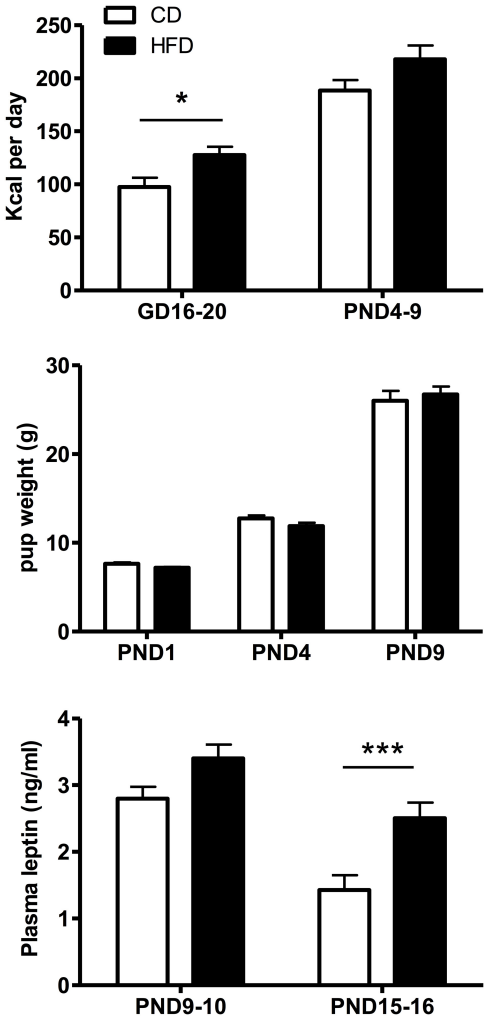


**FIGURE 2 |** Top: Time-related changes in maternal caloric intake (Kcal/day) pre- and postpartum in mothers fed a control (CD, $n = 11$) or high fat (HFD, $n = 10$) diet. Caloric intake was calculated over an interval of 4–5 days during gestation (GD16-20) or postpartum (PND4-9) for four experimental series. Middle: Age-related changes in CD or HFD pup weight between PND1 and 9. Bottom: Changes in plasma leptin levels as a function of age and diet exposure in PND9-10 ($n = 23$–$24$) or PND15-16 ($n = 20$) pups. Values represent the mean ±SEM. *$p < 0.05$; ***$p < 0.001$ compared to the CD group (*post hoc* Tukey HSD test).

## Density of Hypothalamic (DMH and VMH) Projections to the LHA ORX-A Neuronal Field

We determined whether maternal HFD modified the density of hypothalamic projections to the ORX-A field of the LHA in the offspring using injections with fluorescent retrograde microbeads to the LHA ORX-A cell field at PND5-6 (**Figure 1**). Retrograde labeling in the DMH and VMH was measured 4 days later (PND9-10). These nuclei were chosen because of their important role in metabolic regulation and because the maturation of their projections to the LHA is relatively early compared to afferents

from the ARC for instance. By PND9-10, projections to the LHA were observed from both the DMH and VMH in animals of both diet groups, thus confirming that axons from these regions have reached the LHA ORX-A cells at least by this age. As illustrated in **Figure 3**, projection density originating from either the VMH (**Figures 3A,C**) or the DMH (**Figures 3B,D**) was higher in pups from HFD mothers. When the normalized projection density was quantified (**Figure 4**), we found a significant effect of diet on the projection density originating from and observed in the VMH ($F(1,10) = 4.79$, $p = 0.05$), with HFD pups exhibiting a higher density compared to CD pups. The projection density originating from the DMH also tended to be increased with exposure to the HFD, although the values did not reach statistical significance ($F(1,10) = 3.24$, $p = 0.10$). Other regions such as the mPFC also displayed modest labeling, that was not quantified. Labeling of the ARC nucleus was very sparse with occasional cells displaying beads, consistent with the later onset of ARC to LHA projections around PND10-12 (Bouret et al., 2004b).

We next wanted to determine whether the origin of early projections to the LHA ORX-A field in the VMH was cell population specific and we performed VMH RNAscope analysis for SF-1 and BDNF neuronal populations on sections exhibiting retrograde fluorospheres. As illustrated in **Figure 3F**, projections to the LHA in PND9-10 pups originated from either neurons expressing BDNF alone or from those co-localizing SF-1 with BDNF.

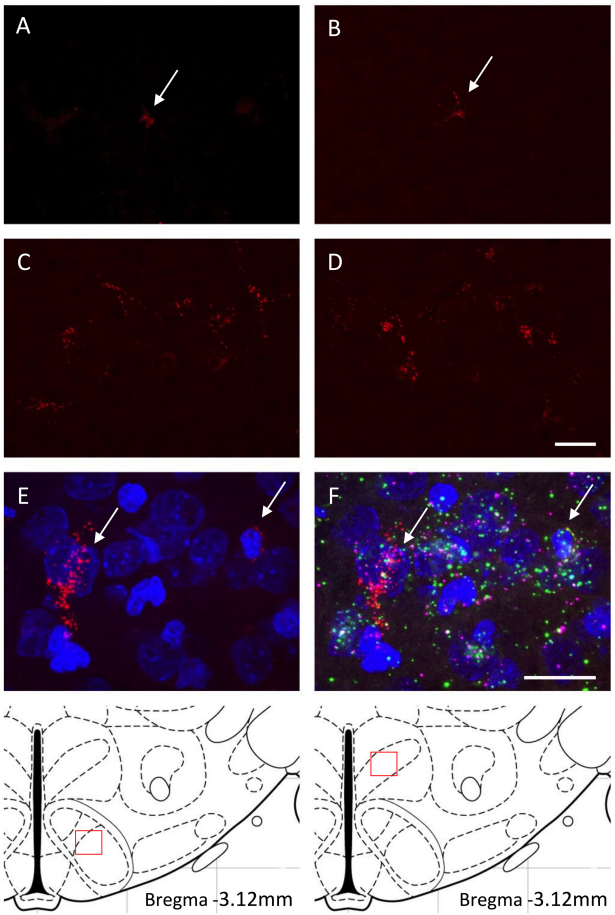


**FIGURE 3 | (A–D)** Retrogradely labeled neurons of the VMH **(A,C)** and DMH **(B,D)** with fluorescent microspheres in PND9-10 rat pups from either the control diet **(A,B)** or the high fat diet **(C,D)** groups. Fluorescent microspheres were injected on PND5-6 in the ORX-A neuronal cell field of the lateral hypothalamic area (LHA). White arrows on the top photographs indicate few projection neurons that contain microspheres in the CD pups. Higher density of labeled cells is observed in the HFD pups. A schematic of the area analyzed is provided on the bottom of the figure. All projection photographs **(A–D)** were taken at 40X magnification and the scale bar represents 20 μm. Phenotypic identification of VMH neurons projecting to the ORX-A region of the LHA in PND9-10 rat pups by RNAscope **(E,F)**. Subpopulations of VMH neurons displaying retrograde microspheres **(E)** were identified by expression of either BDNF (green) or SF-1 (pink) mRNA molecules **(F)**. Photographs for the RNAscope analysis were taken on an Olympus FluoView FV1200 confocal microscope at 60X magnification. Scale bar for **(E,F)** panels represents 20 μm.

## Leptin-Induced pSTAT3 Activation in PND10 and PND16 Neonates

Given the increase in projections to LH ORX-A cells in HFD pups, we next examined the ability of peripheral leptin injection (3 mg/kg BW i.p.) to modify responsiveness to leptin in the LHA-ORX-A field on PND10 and later, at the onset of independent feeding on PND16. **Figure 5** illustrates the changes in pSTAT3 immunostaining in the LHA of PND16 pups following either vehicle or leptin administration in CD and HFD pups. On PND10, there was no significant treatment or diet effect and no treatment X diet interaction on the expression of pSTAT3 immunostaining in the LHA ORX-A field as shown in an earlier

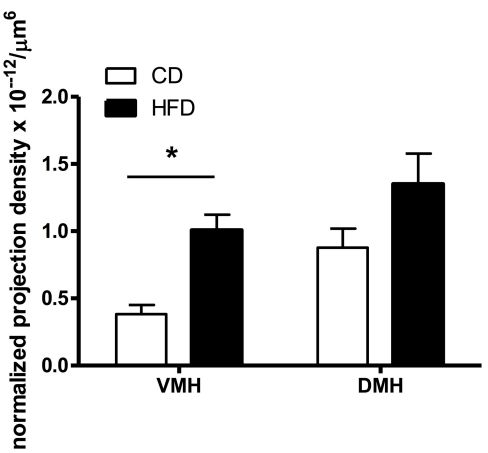


**FIGURE 4 |** Quantification of the density of VMH and DMH neurons expressing retrogradely transported microspheres from the ORX-A field of the LHA in CD or HFD PND9-10 pups. Projection density was calculated using the estimated population of cells (# of cells containing fluorospheres) and the volume of the counted regions (#cells/$\mu m^3$), further normalized by the volume of beads injected ($\mu m^3$) in the LHA region for each injected animal. The resulting normalized density of projections (cells/$\mu m^6$) is compared between CD ($n = 8$) and HFD ($n = 10$) pups (see "Materials and Methods" for detailed description). Values represent the Mean ±SEM. *$p < 0.05$ compared to CD group (one way ANOVA).

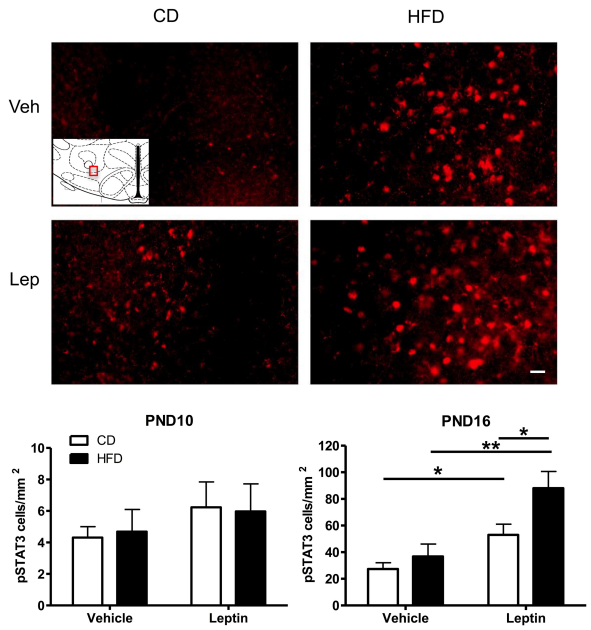


**FIGURE 5 |** Changes in Phospho-STAT3 (pSTAT3) immunoreactivity in the ORX-A field of the LHA in PND16 rat pups receiving either vehicle or leptin (3 mg/kg bw, ip) injection 60 min earlier. Expression of pSTAT3 staining after either treatment is compared between CD (left) or HFD (right) pups. The insert indicates the LHA region that is used for illustration (Bregma level = –3.12 mm). Photographs were taken at a 20X magnification and the scale bar represents 20 μm. Bottom panels: Quantification of immunoreactive pSTAT3 cell density (#cells/mm$^2$) in the LHA of PND10 ($n$ = 5/treatment group) and PND16 ($n$ = 4–5/treatment group) pups from either CD or HFD litters. Values are Mean ±SEM. *$p < 0.05$; **$p < 0.01$ *post hoc* Tukey HSD test.

study (Gjerde et al., 2016). In contrast, on PND16, HFD pups exhibited significantly higher pSTAT3 activation in response to leptin compared to CD pups. ANOVA for pSTAT3 positive cell density showed a significant effect of diet ($F(1,14) = 6.53$, $p = 0.023$) and treatment ($F(1,14) = 19.60$, $p < 0.001$), but no significant diet X treatment interaction. Tukey HSD *post hoc* tests showed that the effect of diet was significant for the leptin-treated group ($p = 0.013$), but not the vehicle-treated group. Increase in pSTAT3 immunostaining after leptin injection was significant both for the CD ($p = 0.044$) and the HFD ($p = 0.0015$) groups, showing that the onset of functional LHA cellular responses to leptin on PND16 can be modulated by maternal diet exposure.

In order to phenotype leptin activated neurons in the LHA of PND16 pups, we performed double immunocytochemistry for pSTAT3 and CART or GAD67 (GABAergic neurons). As indicated in **Figures 6A–C**, there was no pSTAT3 co-localization with CART in either CD or HFD groups, as these were distinct cell populations. However, as a control, we found quite extensive co-localization between pSTAT3 and CART in the arcuate region of the hypothalamus on PND16 (**Figure 6D**). In contrast to CART, we observed a small percentage of pSTAT3 neurons that co-localized with GAD67 on PND16 (**Figures 6E–G**), but only in leptin treated pups (Percentage of colocalization: CD-Lept = 1.59 ± 1.17, $n = 4$; HFD-Lept: 1.81 ± 0.65, $n = 5$). Immunostaining for GAD67 was generally lower in the LHA compared to the hippocampus (**Figure 6H**) on PND16 and the number of GAD67-positive cells was not modified by either diet or leptin treatment (number of GAD cells LHA: CD-Veh: 67.3 ± 9.47, $n = 4$; CD-Lept: 69.3 ± 11.3, $n = 4$; HFD-Veh: 60.8 ± 12.9, $n = 5$; HFD-Lept: 77.8 ± 13.9, $n = 5$).

## Indirect Activation of LHA ORX-A Neurons in the Neonatal LHA

Although LHA ORX-A neurons do not harbor leptin receptors, they might still be activated indirectly by intra-hypothalamic or extra-hypothalamic leptin-sensitive projections, some of these being potentially increased by the maternal HFD. Thus, we examined leptin-induced indirect ORX-A activation by evaluating the co-localization of ORX-A with pERK (a marker of neuronal activation) in pups (**Figure 7**). At PND10, there was no significant effect of diet or treatment on the number of pERK-positive and ORX-A cells, or the proportion of ORX-A cells co-localizing with pERK (data not shown). On PND16, there was a significant effect of treatment ($F(1,18) = 6.54$, $p = 0.0198$) on pERK density and a close to significant diet X treatment interaction ($p = 0.058$). Leptin treatment increased pERK-positive cells in the LHA of CD ($p = 0.0062$), but not HFD pups. Neither diet nor treatment modified the number or ORX-A cells at this age. However, early diet had a significant effect to increase pERK and ORX-A co-localization (ANOVA: diet effect $F(1,18) = 12.22$, $p = 0.0025$), in particular in vehicle-treated pups ($p = 0.0016$). Leptin's effect to increase pERK/ORX-A colocalization was close to significant in CD pups ($p = 0.065$), but was not modified in the HFD pups.

# DISCUSSION

The aims of the present study were to determine whether perinatal maternal feeding with a HFD could modify the establishment of hypothalamic projections to the LHA in neonatal pups and alter the sensitivity of LHA neurons to the metabolic hormone leptin, which is increased by maternal high fat feeding and exerts neurodevelopmental actions in neonatal rodents (Bouret et al., 2004a). Our results demonstrate that maternal high fat feeding increases the number of VMH projections to the LHA in pups already by PND10 and increases direct responsiveness of LHA neurons to exogenous leptin administration at the time of onset of independent feeding around PND15-16. Interestingly, leptin-activated neurons that exhibit pSTAT3 immunoreactivity in neonates are not co-expressing CART and might constitute only a small subset of GABAergic LHA neurons. These results suggest that maternal diet has a significant effect on the hypothalamic control and responsiveness of the LHA during a developmental time period that is critical for the establishment of feeding circuits.

The LHA is a large heterogeneous structure and a critical hub between homeostatic and hedonic feeding centers. We focused more specifically on afferents to the ORX-A field of

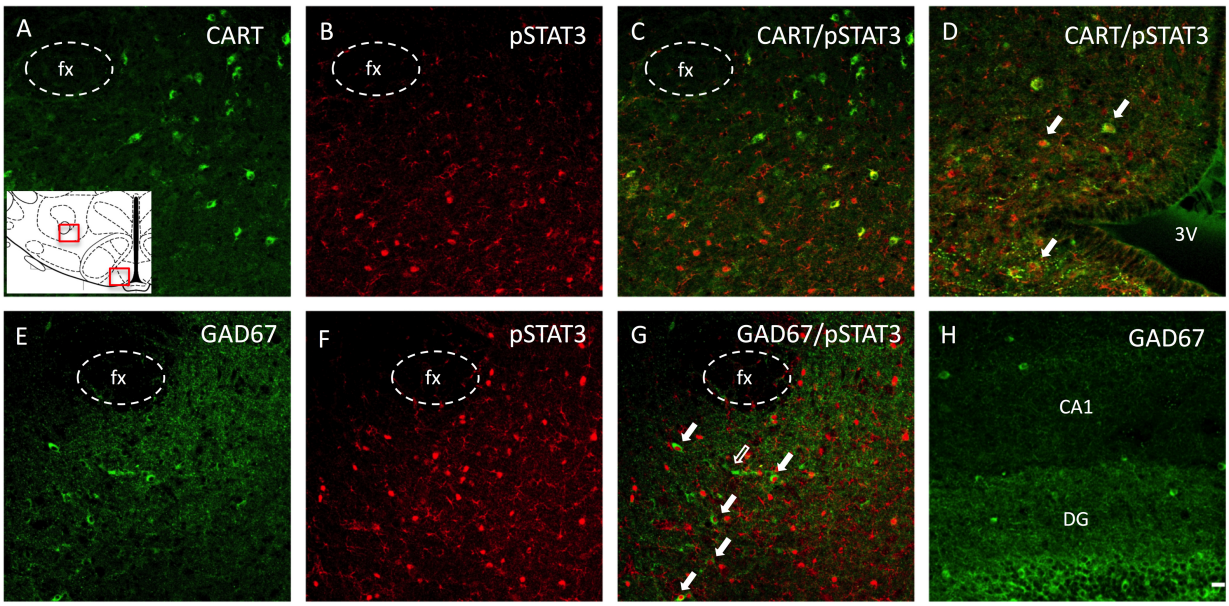


**FIGURE 6 |** Phenotypic identification of neurons expressing pSTAT3 in the ORX-A field of the LHA in a leptin-treated PND16 rat pup by double immunohistochemistry. **(A)** CART immunoreactive neurons are found lateral to the fornix (fx), **(B)** pSTAT3 positive neurons, **(C)** lack of colocalization between CART and pSTAT3 in the LHA. **(D)** In contrast to the LHA, pSTAT3 highly colocalizes with CART neurons in the arcuate nucleus. Another set of sections were examined for GABA neurons (GAD67), **(E)**, pSTAT3 neurons **(F)**, and neurons colocalizing both GAD67 and pSTAT3 **(G)** in the LHA. Few cells in the neonatal LHA express GAD67 **(E)**, but some of these co-localize with pSTAT3 expression [**(G)**, full arrows]. **(H)** The dentate gyrus (DG) of the hippocampus expresses a higher density of GAD67 neurons compared to the LHA **(E)** at this age. All photographs were taken at bregma level = –3.12 mm, at 20X magnification and the scale bar represents 20 μm. The insert represents the areas that are depicted in this figure for the LHA and the arcuate nucleus. 3V, third ventricle; fx, fornix; DG, dentate gyrus; CA1, pyramidal CA1 region of the hippocampus.

the LHA because this neuronal population directly regulates dopamine (DA) neurons in the VTA (Moorman and Aston-Jones, 2010; Leinninger et al., 2011; Muschamp et al., 2014; Godfrey and Borgland, 2019). Although these cells do not express the LepRb, they are regulated by leptin-sensitive afferent regions (Louis et al., 2010; Goforth et al., 2014) and accordingly modulate DA reward. To evaluate the density of projections to this area, we stereotaxically injected fluorescent retrograde tracer beads to the ORX-A field in CD and HFD PND5-6 pups and examined hypothalamic VMH and DMH labeling on PND9-10. We chose this experimental time course because this window during hypothalamic maturation potentially captured the development of afferents and provided enough time for substantial labeling in afferent nuclei. Though labeling was observed in various regions, we focused on afferents from the DMH and VMH because these nuclei express leptin receptors early in life (Bouret, 2010) and regulate food intake in the adult (Choi et al., 2013; Stuber, 2016; Jeong et al., 2017). Furthermore, ARC connections reach the DMH by PND6 (Bouret et al., 2004a) and the VMH at an unknown stage during development. While the ARC is highly sensitive to leptin by the first week of postnatal development (Bouret, 2010; Naef et al., 2014), the direct ARC-LHA fibers do not mature until PND12 (Bouret et al., 2004a). Thus, the DMH and VMH are likely to relay leptin-sensitive input from the ARC to the LHA neurons before PND12, although it is currently unclear when projections from the DMH and VMH reach the LHA. Here, we examined if these projections are sensitive to maternal diet and mature earlier than the ARC-LHA connections.

Both HFD and CD pups showed retrograde labeling in the DMH and VMH by PND10, which confirmed that at least some of the projections between these regions and LHA ORX-A neurons are already present at this age. When compared to CD pups, HFD pups displayed significantly more retrograde labeling in the VMH and tended to have more labeling in the DMH. Our results suggest that a maternal HFD either accelerates the onset of projection development or increases the number of fibers projecting to the LHA. In contrast to VMH and DMH, we observed little to no labeling in the ARC at PND10, confirming the absence of direct ARC-LHA projections at this age. In the adult, the VMH preferentially innervates the medial portion of LH ORX-A cells, while the DMH innervates both medial and lateral areas of the ORX-A neuronal field (Yoshida et al., 2006). Our data does not indicate any difference in projection density in these regions between medially- vs. laterally-injected pups, however we have a small sample size for each group which prevents a full examination of labeling as a function of LHA injection site. By combining retrograde labeling and RNAscope techniques, we showed that VMH neurons projecting to LHA in neonates did not constitute a unique cell population, but that both Steroidogenic factor 1 (SF-1) and brain-derived neurotrophic factor (BDNF) expressing VMH neurons projected to the LHA. Signature SF-1-expressing VMH neurons harbor leptin receptors and are known to increase

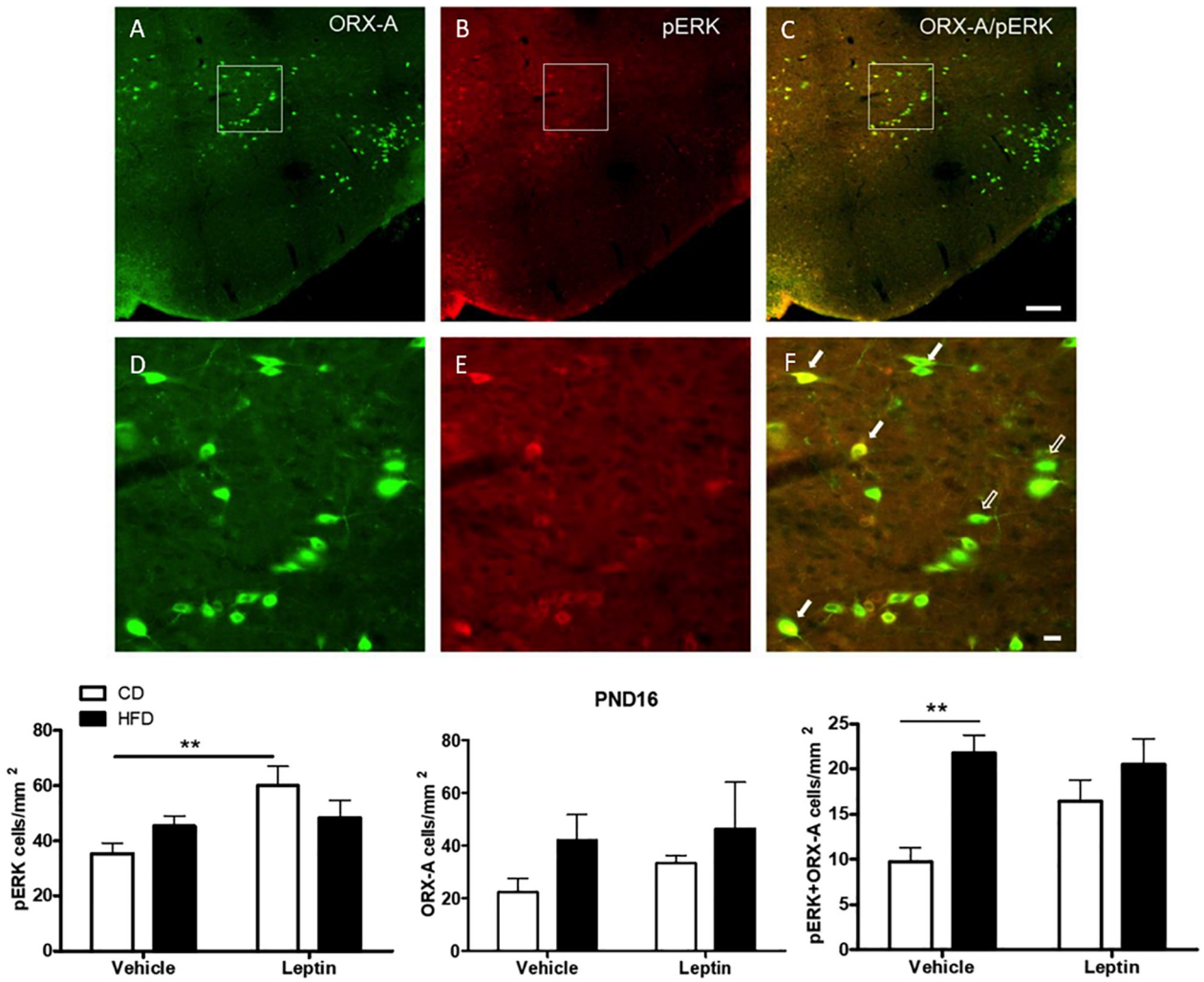


**FIGURE 7 |** Immunohistochemical detection of ORX-A **(A)**, pERK **(B)** cells in the LHA of a leptin-treated PND16 pup. Phospho-ERK expression is observed in some ORX-A neurons [**(C)**, full arrows], but not in others [**(C)**, empty arrows] at this age. Details of individual and co-localized immunoreactive neurons are provided in panels **(D–F)**. Photographs were taken at a 20X magnification and the scale bar represents 200 μm **(A–C)** or 20 μm **(D–F)**. Quantification of immunostained neurons in the LHA. $^{**}p < 0.01$ Posthoc Tukey HSD test.

thermogenesis via activation of sympathetic output rather than regulating food intake (Dhillon et al., 2006; Kim et al., 2011). Also present in the VMH are unidentified neuronal populations that regulate food intake rather than energy expenditure, which may be sensitive to leptin and project to the LHA (Choi et al., 2013). Early increases in circulating leptin concentrations in pups from the HFD mothers might be important to activate these neurons and promote the development and maintenance of their projections to other hypothalamic areas. Although not significant, maternal HFD also tended to increase projection density from the DMH in PND10 pups.

Interestingly, we observed an increased fiber density in the VMH in PND10 HFD pups despite the fact that at this age, circulating leptin levels were not significantly increased compared to CD pups. While this does not eliminate an important effect of leptin on the development of projections, it suggests that other factors present in or triggered by the HFD exposure, such as BDNF (Genzer et al., 2016) might promote neurite outgrowth in neonatal life. It is also possible that specific fatty acids (FA) present in the maternal HFD and transmitted to the offspring through the milk may be responsible for increased neuronal proliferation and/or the increased density of projections that we observed in HFD neonates. In adult mice, supplementation with dietary unsaturated omega-3 fatty acids was shown to induce hypothalamic cell proliferation specifically in the ARC nucleus (Nascimento et al., 2016) and other *in vitro* studies found that docosahexaenoic acid (DHA) induces neurite outgrowth and promotes neuronal differentiation in isolated hippocampal neurons (Calderon and Kim, 2004). Given that our diet was relatively rich is unsaturated fatty acids (63%) this might explain why, by PND10, HFD pups displayed increased hypothalamic projections despite no

differences in weight gain or leptin concentrations compared to CD pups. Alternatively, changes in dietary fat could also modify the number of hypothalamic VMH or DMH neurons by altering their survival. Although hypothalamic neurogenesis (mostly ARC) ends by the second week of gestation in rodents, proliferation extends until the late fetal and perinatal periods, precisely when mothers were exposed to the HFD (Bouret, 2017). One limitation to our study is that in our experiments, retrograde fluorescent microbeads were injected into the region of the LHA containing ORX-A cells, but they may have been taken up by synapses formed with other cell types as well. For instance, we have previously shown that ORX-A neurons are intermingled with Neurotensin neurons in neonates (Gjerde et al., 2016) and thus, microbeads may have been transported by afferent fibers innervating these or other cells in this area.

Given the diet-induced increase in afferents from the VMH to the ORX-A cell field of the neonatal LHA, we next examined whether leptin-induced neuronal activation of LHA neurons was enhanced by the maternal diet. In contrast to ORX-A neurons, many cell types in the LHA harbor LepRb, including CART, GABAergic cells co-localizing with Neurotensin for instance (Berthoud and Münzberg, 2011; Leinninger, 2011; Goforth et al., 2014) and other phenotypically uncharacterized cells. As observed earlier (Gjerde et al., 2016), leptin-induced pSTAT3 expression was substantially higher on PND15-16 compared to PND10, when neither diet nor treatment effect was significant. On PND15-16, leptin treatment induced a robust pSTAT3 response in both CD and HFD pups and a larger response in HFD pups, suggesting that the LHA has become more responsive to leptin signaling as a result of either higher circulating levels of leptin and/or increased density of projections to the LHA. This result might be unique to the developmental period and/or represents early re-programming of hypothalamic pathways since Fos responses to central leptin administration in the LHA of adult male rats was reduced by adult exposure to a HFD (Alsuhaymi et al., 2017). Robust leptin-induced pSTAT3 immunostaining on PND15-16 was also observed in the ARC, VMH, and DMH, possibly reflecting heightened leptin receptor expression in these areas in the HFD pups and/or increased sensitivity of intracellular pathways at this age. Leptin-induced pSTAT3-positive neurons in the LHA co-localized modestly with GAD67, a marker of GABA neurons, but not with CART. This was surprising as adult CART neurons of the LHA express LepRb mRNA (Berthoud and Münzberg, 2011; Farzi et al., 2018) and we observed robust CART immunostaining and co-localization with pSTAT3 in the ARC in young neonates. However, this could also represent an early adaptation to favor an anabolic state during development because, in contrast to LHA CART, stimulation of ARC CART reduces energy expenditure in adult mice (Farzi et al., 2018). The lack of leptin-induced activation of CART in the neonatal LHA indicates that the orexigenic and motivational actions of CART (Somalwar et al., 2017) in this structure might not be fully developed by the second week of life.

In older pups (PND15-16), leptin-induced pSTAT3 activation was observed in a subset of LHA GABAergic neurons despite the fact that expression of GAD67 immunoreactivity was sparse in the LHA of either CD or HFD pups when compared to other regions such as the hippocampus. It is possible that leptin-responsive GABAergic cells in the LHA mature later than GABAergic cells in other regions and that GABAergic tone in the LHA is low during neonatal life. The mature LHA contains many subsets of GABAergic cells and some co-express LepRb and various neuropeptides (Leinninger et al., 2009; Berthoud and Münzberg, 2011; Leinninger, 2011). In adult mice, GABAergic LHA fibers innervate both VTA GABA and Dopamine neurons although preferential innervation of VTA GABA neurons results in disinhibition of DA neurons (Stuber and Wise, 2016). The low percentage of leptin-induced pSTAT3/GAD67 cells in both CD and HFD groups compared to the robust pSTAT3 activation observed in the same groups (**Figure 5**) suggests that other non-GABAergic cell populations are activated by leptin in neonates. Further experiments should better characterize these phenotypically unknown activated neurons and determine if they project to the VTA to regulate DA function.

Orexin-A cells of the LHA directly regulate VTA DA neurons, but they do not express LepRb (Goforth et al., 2014). These ORX-A cells are already functionally connected to the VTA by the end of the first week of age (Gjerde et al., 2016) and thus might be important to regulate the development of DA function (Naef et al., 2014). To examine the potential effect of maternal HFD on differential ORX-A activation, we used pERK as a marker of general neuronal activation in response to leptin-sensitive afferent projections to these neurons. Production of pERK is stimulated by leptin (Frühbeck, 2006; Kwon et al., 2016) and other ligands and is especially important during neonatal development due to its role in cell proliferation and survival (Zhang and Liu, 2002). On PND10 there was no effect of diet or leptin challenge on the number of pERK cells or the proportion of ORX-A cells co-localizing with pERK in the LHA (not shown). On PND15-16, Significant pERK responsiveness to leptin was observed in CD pups, but not in HFD pups. This was unexpected given the increased pSTAT3 responsiveness observed in this region after leptin administration in HFD pups (**Figure 5**). This results suggests that in HFD pups, pERK might not be an accurate indicator of leptin responsiveness. The maternal HFD dramatically increased pERK/ORX-A co-localization in vehicle-treated pups, suggesting a larger activation of ORX-A neurons after the stress of vehicle injection. However, it is unclear why we did not observe a significant increase in pERK/ORX-A colocalization after leptin injection, in particular in the HFD pups. It is possible that pERK activation reached a ceiling effect in HFD pups because of the high levels observed even after vehicle injection. Interestingly, the number of ORX-A neurons was not significantly altered by the maternal diet and this is consistent with other reports indicating that a maternal HFD during pregnancy (Poon et al., 2012) or neonatal leptin administration (Proulx et al., 2002) alters the expression of other appetite-regulated peptides but not ORX-A.

In summary, our experiments demonstrate that a maternal HFD provided during the last week of gestation and during lactation increases the density of VMH afferents to the LHA in neonatal PND10 offspring, and more specifically in the region where ORX-A cells are located. This is associated with increased

responsiveness of LHA neurons to elevated leptin levels and could lead to potential dysregulation of VTA DA neurons since these are primarily regulated by LHA afferents. What remains to be determined is whether other metabolic signals also regulate LHA function during this critical developmental period and participate in the programming of the feeding circuitry. Because our study does not allow distinguishing between the prenatal and/or postnatal effects of the maternal dietary environment on the offspring, future experiments should establish more precise critical windows for maturation of these circuits. Further research in this area will greatly contribute to our understanding of the developmental origins of dysregulated feeding behavior and metabolic disease in adulthood.

## 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 Douglas Institute Animal Care Committee, McGill University and followed ethical guidelines from the Canadian Council on Animal Care (CCAC).

## AUTHOR CONTRIBUTIONS

LK and C-DW designed the study, analyzed the data, and wrote the manuscript. LK performed the experiments and collected all data together with HL and SV. ML helped in the analysis of the ORX-A/pERK data.

## FUNDING

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR) to C-DW, grant MOP# 298195.

## ACKNOWLEDGMENTS

We thank Ms. Angela Guadagno for her help with the figures. The present study used the services of the Molecular and Cellular Microscopy Platform at the DHRC.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 04 February 2020
doi: 10.3389/fnins.2020.00013

# Neurodegenerative Susceptibility During Maternal Nutritional Programing: Are Central and Peripheral Innate Immune Training Relevant?

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Maternal overnutrition modulates body weight, development of metabolic failure and, potentially, neurodegenerative susceptibility in the offspring. Overnutrition sets a chronic pro-inflammatory profile that integrates peripheral and central immune activation nodes, damaging neuronal physiology and survival. Innate immune cells exposed to hypercaloric diets might experience trained immunity. Here, we address the role of maternal overnutrition as a trigger for central and peripheral immune training and its contribution to neurodegeneration and the molecular nodes implicated in the Nod-like receptor protein 3 (NLRP3) inflammasome pathway leading to immune training. We propose that maternal overnutrition leads to peripheral or central immune training that favor neurodegenerative susceptibility in the offspring.

**Keywords: maternal programing, neurodegeneration, inflammation, immune training, cytokines**

OPEN ACCESS

***Edited by:***
*Kathleen C. Page, Bucknell University, United States*

***Reviewed by:***
*Juan C. Saez, Pontifical Catholic University of Chile, Chile*
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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *18 November 2019*
***Accepted:*** *08 January 2020*
***Published:*** *04 February 2020*

***Citation:***
*Cárdenas-Tueme M, Montalvo-Martínez L, Maldonado-Ruiz R, Camacho-Morales A and Reséndez-Pérez D (2020) Neurodegenerative Susceptibility During Maternal Nutritional Programing: Are Central and Peripheral Innate Immune Training Relevant? Front. Neurosci. 14:13. doi: 10.3389/fnins.2020.00013*

## INTRODUCTION

According to the World Health Organization, nearly 39% of adults were overweight in 2016, and 13% were obese. Epidemiological data from human catastrophes such as the Dutch famine (1944), the siege of Leningrad (1942–1944), the great Chinese famine (1958–1961), and the Överkalix study (1890-present) propose that changes in nutrient intake such as fasting or overnutrition during pregnancy and lactation are associated with metabolic and behavioral disorders in the offspring (Vaiserman, 2017). Maternal nutritional programing can influence offspring body weight, adiposity, and development of metabolic syndrome in diverse ways (Friedman, 2018; Patro Golab et al., 2018).

For instance, nutritional programing adversely impacts both maternal and offspring health, increasing susceptibility to show metabolic abnormalities later in life such as obesity, dyslipidemia, type 2 diabetes (T2D) mellitus and hypertension, as well as behavioral disorders related to schizophrenia, autism, and compulsive eating (Shapiro et al., 2017; Friedman, 2018; Patro Golab et al., 2018; Derks et al., 2019).

Recent experimental evidence shows that exposure to hypercaloric diets programs the immune nodes that modulate metabolism and neuronal survival during embryogenesis. For instance, the immune response is involved in the balance and maintenance of an adequate metabolic state; however, conditions of altered metabolic demand promote a pro-inflammatory state (Christ et al., 2018). On this context, overnutrition might trigger an epigenetic process called "trained immunity" in innate immune cells, such as microglia in the central nervous system (CNS) and macrophages or natural killer cells in the periphery, which results in enhanced immune responses following infections or immune stimulation. The epigenetic mechanisms that mediate trained immunity are better understood in macrophages and monocytes, and a few have been described in microglia. Wendeln et al. (2018) demonstrated that microglia, just like peripheral macrophages, can be trained to confer long-lasting memory. In microglia, "trained immunity" is dependent on chromatin remodeling and activation of inflammatory signaling pathways (NF-κB, JNK, and ERK1/2). Also, by knocking down Hdac1/2 or Tak1 in long-lived CX3CR1 + cells (most of them microglia) cytokine levels were reduced, suggesting that epigenetic reprograming of inflammatory responses is key to accomplish immune training (Wendeln et al., 2018).

In this review, we address the role of maternal nutritional programing on central and peripheral immune training and crosstalk during positive metabolic scenarios, as well as their potential relevance in neurodegenerative susceptibility in the offspring.

## CENTRAL AND PERIPHERAL IMMUNE SYSTEM COMMUNICATION

The innate immune system is the first line of defense against pathogens and tissue damage; it includes physical barriers such as the skin, and specific cell types that modulate the inflammatory response such as macrophages, microglia, and complement proteins. The inflammatory response consists of an innate cellular system and humoral responses that occur during injury to restore homeostasis (Ciaccia, 2010). In this scenario, microglia, play the role of the innate immune system in the brain, infiltrating during early embryogenesis, coordinating neurogenesis, synaptic pruning, connectome establishment, and acting as major antigen-presenting cells (Colonna and Butovsky, 2017; Maldonado-Ruiz et al., 2017). In healthy brains, microglia remain in a ramified stated, and when activated, they enlarge their cell body, change to a phagocytic state, release pro-inflammatory cytokines, and increase antigen presentation and ROS production, leading to neuroinflammation (Colonna and Butovsky, 2017). As shown in **Figure 1**, under an altered physiological scenario, peripheral immune cells infiltrate the brain, become pro-inflammatory and secrete cytokines. This causes an exacerbated immune response by M1-activated microglia which leads to neuroinflammation, favoring protein aggregation and brain damage consistent with neurodegenerative pathologies (Colonna and Butovsky, 2017).

In the past years, the concept of the CNS as an immune-privileged site has changed, and interactions between the peripheral and central immune system have been demonstrated. Furthermore, these interactions are necessary to maintain homeostasis in the CNS, which was once thought to be impossible. For instance, the myeloid cells such as the macrophages and dendritic cells (DCs) in the meninges (dura, arachnoid, and pia mater), choroid plexus, and perivascular spaces in the CNS parenchyma can mount a robust protective and restorative response when necessary.

In addition, there is a strong relationship between inflammation and metabolic disorders in the immune response against infection. It is known that the immune response is involved in the balance and maintenance of an adequate metabolic state; however, it can have adverse effects under conditions of altered metabolic demand (Agrawal, 2014). In this way, positive energy balance during overnutrition promotes an inflammatory state in the CNS. For instance, saturated fatty acids from the diet are considered powerful candidates to trigger immune response in the peripheral system and in the CNS. However, how central immunity communicates with the peripheral immune response during overnutrition remains to be clarified (Maldonado-Ruiz et al., 2017). Based on this complex and delicate regulation, we next address how much the cells of the immune system can be influenced by selective diets.

## MATERNAL PROGRAMING BY EXPOSURE TO HIGH-FAT-HIGH-SUGAR DIETS ACTIVATES IMMUNITY AND REGULATES NEURODEGENERATIVE SUSCEPTIBILITY

Experimental data in animal models and humans have contributed to understand the effect of caloric overnutrition during pregnancy on immunity and neurodegenerative susceptibility later in life. The nervous system develops through a sophisticated and precise process from embryonic stages through puberty and adulthood. Proliferation, migration and differentiation of major brain cell types followed by rapid synaptogenesis integrate and establish a selective and efficient brain connectome. The immune system shares a time-dependent development and maturation with the nervous system during embryogenesis and post-natal periods (Estes and McAllister, 2016), which renders these systems extremely responsive to environmental factors, potentially modulating central and peripheral cross-talk and neurodegenerative susceptibility.

Initial experimental evidence in animal models and humans showing defects in glucose homeostasis such as T2D, have

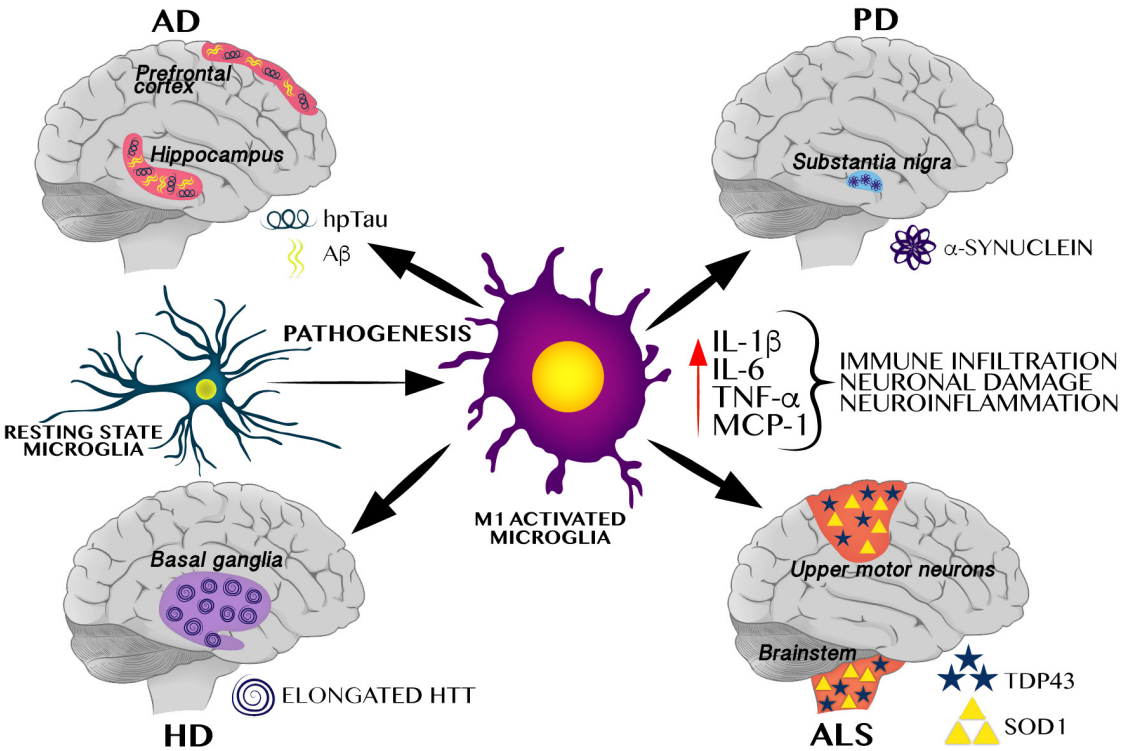


**FIGURE 1 |** M1 Microglial activation induces a pro-inflammatory cytokines favoring neuroinflammation and protein aggregation-related neurodegenerative pathologies. M1 activated microglia induces a pro-inflammatory profile, including increase of IL-1β, TNF-α, MCP-1, and IL-6, all of these cytokines have been identified in different neurodegenerative pathologies including AD, PD, HD, and ALS. These neurodegenerative pathologies are characterized by selective protein aggregation, including amyloid beta (Aβ) and hyperphosphorylated Tau (hpTau) in AD, α-synuclein in PD, elongated huntingtin (HTT) in HD, and TAR DNA binding protein 43 (TDP43) and superoxide dismutase 1 (SOD1) in ALS.

reported to contribute to neurodegeneration. For instance, rats fed a high-fat/glucose die develop insulin resistance and exhibit impaired spatial learning ability, reduced hippocampal dendritic spine density, and reduced long-term potentiation in the CA1 region due to glucotoxicity or altered insulin signaling (Stranahan et al., 2008). Moreover, Alzheimer's disease (AD) transgenic mice models fed with a high-fat diet showed T2D-like peripheral insulin resistance but also more severe cognitive deficits compared to normoglycemic AD mice (Procaccini et al., 2016), demonstrating the possible interplay between defects in glucose metabolism and AD development. Of note, patients with T2D show two-fold higher risk to develop AD than normal subjects (Ott et al., 1999).

Experimental and clinical data support the hypothesis that inflammation itself might be a risk factor for neurodegeneration during AD (Ott et al., 1999; Bertram et al., 2007; Takahashi et al., 2007; Tartaglione et al., 2016). For instance, Ling Z. et al. (2004) and Ling Z. D. et al. (2004) exposed rats to LPS prenatally and then administered with a subtoxic dose of the dopaminergic (DA) neurotoxin rotenone (1.25 mg/kg per day for 14 days) when 16 months old. Of note, prenatal LPS and postnatal rotenone exposure exacerbate DA cell loss compared with the effects of single LPS or rotenone exposure. Also, initial genomic analysis of AD patients identified several risk genes that encode proteins involved in neural repair and remyelination and in the modulation of microglial responses, including phagocytosis (Bertram et al., 2007; Takahashi et al., 2007).

Obesity or maternal programing by exposure to high-fat-high-sugar diets in animal models and humans is in fact associated with overactivation of the immune system, triggering a process of chronic inflammation named "meta-inflammation" or "metabolic inflammation," which ultimately links central and peripheral immune activation (O'Brien et al., 2017). For instance, maternal overnutrition of high-fat-high-sugar diets during pregnancy in murine models programs metabolic and hormonal settings that modulate neuronal development, axonal pruning, synaptic plasticity and connectome establishment during embryogenesis (Bilbo and Tsang, 2010; Morin et al., 2017; Mucellini et al., 2019). Also, mice born from obese dams have shown significant metabolic alterations including higher body fat, altered serum leptin levels, pancreatic islet hypertrophy, and lower expression of metabolism-related genes (Bilbo and Tsang, 2010; Gomes et al., 2018; Mucellini et al., 2019). In addition, human obesity is associated with the low-grade peripheral immune activation characteristic of an acute phase reaction, including C-reactive protein and serum amyloid A (SAA), an increase of cytokines, such as tumor necrosis factor (TNF-α), IL-1β, and IL-6 in serum, and an increase

in white cell counts (Esser et al., 2014). In fact, systemic immune activation in psoriasis, a skin disorder, efficiently favors neuroinflammation (O'Brien et al., 2017). There are also other evidences in humans verifying that obesity is closely associated with neurodegenerative susceptibility including Alzheimer's, Parkinson's, and Huntington's diseases and nervous system sclerosis (Procaccini et al., 2016), and an increase in the Body Mass Index in healthy mothers is negatively associated with white matter development in their offspring (Ou et al., 2015; Procaccini et al., 2016; Widen et al., 2018). These results suggest that positive energy balance such as during obesity or maternal overnutrition leads to neurodegenerative susceptibility, which potentially involves an immune activation pathway.

Initial reports of maternal programing by overnutrition using murine models identified sex-specific differences in fetal size and gene expression signatures in fetal-brains, showing that male offspring are the most affected. Importantly, the offspring displays a pro-inflammatory gene signature as the top regulated gene pathway (Edlow et al., 2016a). Later on, these results were confirmed in humans in a pilot study that analyzed the amniotic fluid from obese and lean pregnant women on the second trimester; in this study, 205 genes were found differentially regulated, where the lipid regulator, Apolipoprotein D, was the most upregulated gene (9-fold). Also, genes involved in apoptotic cell death were significantly downregulated particularly within pathways involving the cerebral cortex, such as the Serine/threonine kinase 24 ATPase (*STK24*). These biomarkers also correlated with the activation of the transcriptional regulators estrogen receptor, FOS, and STAT3, suggesting a pro-estrogenic, pro-inflammatory milieu (Edlow et al., 2014). In addition, this molecular signature was then confirmed in a prospective case-control study were 701 differentially regulated genes were identified, integrating a neurodegeneration gene signature, including Occludin (*OCLN*), Kinesin Family Member 14 (*KIF14*), and Nuclear receptor subfamily 2, group E, member 1 (*NR2E1*) (Edlow et al., 2016b). Upregulation of *OCLN* and *KIF14* genes have been previously associated to AD and vascular dementia in post-mortem human brain tissue (Romanitan et al., 2007), and in negative regulation of neuron apoptosis and regulation of myelination (Fujikura et al., 2013), respectively. Also, *NR2E1* is implicated in neurogenesis and neuronal differentiation modulating aggressive behavior and fear response (Young et al., 2002). Notably, all of these gene alterations also correlated with a pro-inflammatory signature of upregulated genes: chemokine (C-C motif) receptor 6 (CCR6), O-linked N-acetylglucosamine (GlcNAc) transferase (OGT), chemokine (C-C motif) receptor 2 (CCR2), caspase 4, apoptosis-related cysteine peptidase (CASP4), toll-like receptor 1 (TLR1), nucleoporin 107 kDa (NUP107), decapping enzyme, scavenger (D), among others (Edlow et al., 2016b). Together, maternal nutritional programing by overnutrition activates the central and peripheral immune systems that intimately communicate with each other to modulate neuroinflammation, thus increasing neurodegenerative susceptibility in offspring.

We will now discuss the experimental data addressing the potential role of maternal nutritional programing on the Nod-like receptor protein 3 (NLRP3) inflammasome pathway activation and its effects on neurodegeneration.

## POTENTIAL ROLE OF THE NOD-LIKE RECEPTOR PROTEIN 3 INFLAMMASOME PATHWAY ON NEURODEGENERATIVE SUSCEPTIBILITY BY NUTRIENT OVER SUPPLY

The NLRP3- inflammasome pathway is linked to a danger-associated molecular pattern released from damaged or dying neurons that bind and activate the Toll-like receptor (TLR) –dependent myeloid differentiation primary response protein MyD88 (MYD88)–nuclear factor-κB (NF-κB) pathway. At first, the TLR-MYD88-NF-κB pathway positively produces pro-IL-1β and NLRP3 synthesis, activating a positive feedback loop. Then, negative stimuli, including changes in potassium efflux or reactive oxygen species, trigger the inflammasome assembly and processing of pro-IL-1β into IL-1β by caspase 1 activation (Heneka et al., 2018). Finally, the NF-κB transcription factor also regulates a variety of different processes, including stress response and a pro-inflammatory profile activation.

Initial reports by Christ et al. (2018) identified that murine models exposed to high-fat-high-sugar diets set an epigenetic program that primes B lymphocytes into an exacerbated pro-inflammatory phenotype. These, become much more responsive under physiologic stimuli which depend on the NLRP3-inflammasome pathway (Christ et al., 2018). Selective lipid species, such as palmitate and stearate, as well as, carbohydrates have been identified to activate the NLRP3-inflammasome pathway (Wen et al., 2011; Ann et al., 2018). For instance, saturated lipids from diet intake such as palmitic and stearic acids promote IL-1β release from bone marrow-derived macrophages of rodents and humans, respectively (Wen et al., 2011; L'homme et al., 2013), an effect replicated in murine macrophages (Ann et al., 2018). Of note, immune activation by palmitic and stearic acids precisely depends on the NLRP3-inflammasome pathway (Wen et al., 2011; L'homme et al., 2013). Conversely, unsaturated fatty acids, including oleate and linoleate, prevent IL-1β release and are unable to activate the NLRP3-inflammasome pathway in human monocytes/macrophages (L'homme et al., 2013; Sui et al., 2016). Also, a high-fructose diet in mice positively activates the NLRP3-inflammasome pathway and IL-1β release in human macrophage and liver cell lines, which correlates with neutrophil infiltration (Mastrocola et al., 2016; Nigro et al., 2017; Choe and Kim, 2017). Finally, the role of metabolic species regulating the NLRP3-inflammasome and neurodegeneration was recently evidenced by showing that the 25-hydroxycholesterol also activates the NLRP3-inflammasome pathway, promoting progressive neurodegeneration in X-linked adrenoleukodystrophy, a nervous disease with cerebral inflammatory demyelination (Jang et al., 2016). Moreover, the NLRP3 inflammasome pathway has been identified to contribute to PD (Fan et al., 2017;

Gordon et al., 2018; Lee et al., 2018), AD and ALS (Heneka et al., 2013; Johann et al., 2015), HD (Glinsky, 2008), as well as to behavioral alterations in mice at later stages, such as anhedonia (Zhu et al., 2017), anxiety (Lei et al., 2017) and depression-like behavior (Pan et al., 2014; Su et al., 2017).

Altogether, the evidence suggests that overnutrition during pregnancy might promote microglia activation, which correlates with peripheral pro-inflammatory profiles and brain abnormalities in the offspring that are related with neurodegenerative susceptibility. We next discuss the role of diet-induced central and peripheral immune training on neurodegeneration.

## DOES CENTRAL AND PERIPHERAL INNATE IMMUNE TRAINING BY NLRP3 INFLAMMASOME CONTRIBUTES TO NEURODEGENERATION?

Trained immunity is a selective innate immune memory that induces an enhanced response to an inflammatory stimulus in innate immune cells (for instance, microglia), with a much stronger effect than that observed in basal conditions. Trained immunity depends of signal imprinting on transcription factors and epigenetic reprograming (**Figure 2A**). At this state, microglia in the CNS, or macrophages, leukocytes, natural killer cells, etc., in the peripheral system undergo apparent changes in their morphology, elevated secretion of cytokines and other inflammatory mediators, as well as substantial epigenetic program settings (Netea et al., 2016). Recent reports in different models and also in humans have confirmed robust innate immune training of myeloid cells following a pro-inflammatory challenge (Arts et al., 2018; Bekkering et al., 2018; Christ et al., 2018; Kaufmann et al., 2018; Mitroulis et al., 2018). For instance, exposure to a brief high-fat diet for 3 days promoted the activation of the NLRP3 inflammasome pathway, leading to central immune training, which potentially associates to deficits in long-term memory formation in mice (Sobesky et al., 2016). Also, peripherally applied inflammatory stimuli induce acute immune training in microglia, which correlates with differential epigenetic reprograming that modulates the microglial Tak1 or Hdac1/2 genes. Notably, microglia immune training persists for at least 6 months and increases neuronal death in an animal model of AD and ischemia (Wendeln et al., 2018). These results support the importance of central immune training as a positive regulator of neurodegenerative susceptibility that, during nutritional programing, depends on the NLRP3 inflammasome pathway, as we mentioned before (Christ et al., 2018).

Major advances demonstrating the role of the NLRP3 inflammasome pathway on neurodegenerative susceptibility have been identified in animal models. In fact, it is believed that the NLRP3 inflammasome pathway goes beyond IL-1β synthesis, activating the function of the NF-κB factor and modulating neurodegenerative susceptibility. At first, experimental studies using murine models propose that NF-κB seems to have a role in the development of synaptic plasticity and neuronal survival (Monje et al., 2003), however, over activation of the IKKβ/NF-kB binomial might set the scenario toward a new pro-inflammatory state involving TNF-α through the paracrine actions modulating neuronal survival (**Figure 2B**). For instance, microglia IKKβ/NF-κB ablation in the hypothalamus reverts cognitive decline in rodents (Zhang et al., 2013), whereas IKKβ/NF-κB activation under long-time exposure to a hypercaloric diet depletes and impairs neuronal differentiation of adult hypothalamic neural stem cells (Li et al., 2012).

In the context of overnutrition, the IKKβ/NF-kB pathway (**Figure 2C**) seems to activate metabolic-related neurodegenerative markers including the suppressor of cytokine signaling 3 (SOCS3), the tyrosine-protein phosphatase non-receptor type 1 (PTP1B), and potentially, the T-cell protein tyrosine phosphatase (TCPTP) (Milanski et al., 2009). SOCS3 is implicated in neuronal damage during aging (Yadav et al., 2005; DiNapoli et al., 2010), and is also activated during Wallerian degeneration in injured peripheral nerves (Girolami et al., 2010; McNamee et al., 2010). The PTP1B marker leads to neuronal damage in the developing brain (Liu et al., 2019) and is actively implicated in apoptotic neuronal death in a high-glucose *in vitro* model (Arroba et al., 2016). Of note, PTP1B inhibition significantly inactivates GSK-3β-suppressing, amyloid β (Aβ)-induced tau phosphorylation and ameliorates spatial learning and memory in an animal model of AD (Kanno et al., 2016). On the other hand, the TCPTP marker was initially identified as a contributor to leptin resistance in the hypothalamus during obesity in rodents (Loh et al., 2011) and as a modulator of the homeostasis of body glucose (Dodd et al., 2015, 2018). Recently, TCPTP was reported to modulate cytokine release by the expression of connexin 30 (CX30) and connexin 43 (CX43) from astrocytes in a reciprocal interplay with PTP1B following LPS administration (Debarba et al., 2018). In fact, connexin43 or pannexin1 in astrocytes has been demonstrated to promote activation of the inflammasome in glial cells (Yi et al., 2016) and, its blockade with boldine, inhibited hemichannel activity in astrocytes and microglia without affecting gap junctional communication in culture and acute hippocampal slices. Also, when tested in animal models, AD mice with long-term oral administration of boldine prevented the increase in glial hemichannel activity, astrocytic $Ca^{2+}$ signal, ATP and glutamate release which in turn, alleviated hippocampal neuronal suffering (Yi et al., 2017). These evidences allow us to propose that the IKKβ/NF-kB pathway activation during overnutrition integrates the PTP1B – TCPTP cross-talk which might be potentially implicated in neuronal survival. Together, given that overnutrition is associated to immune training by regulating the NF-κB activation linked to the NLRP3 inflammasome pathway (Christ et al., 2018), we propose that maternal nutritional programing by exposure to hypercaloric diets during pregnancy might set microglia immune training by activation of the IKKβ/NF-kB pathway, amplifying its effects on the SOCS3, the PTP1B, and the TCPTP (**Figure 2C**). Altogether, these proteins might increase susceptibility to neurodegenerative diseases in the offspring at later stages.

Potential molecular mechanisms that regulate peripheral immune training linked to NLRP3 inflammasome and

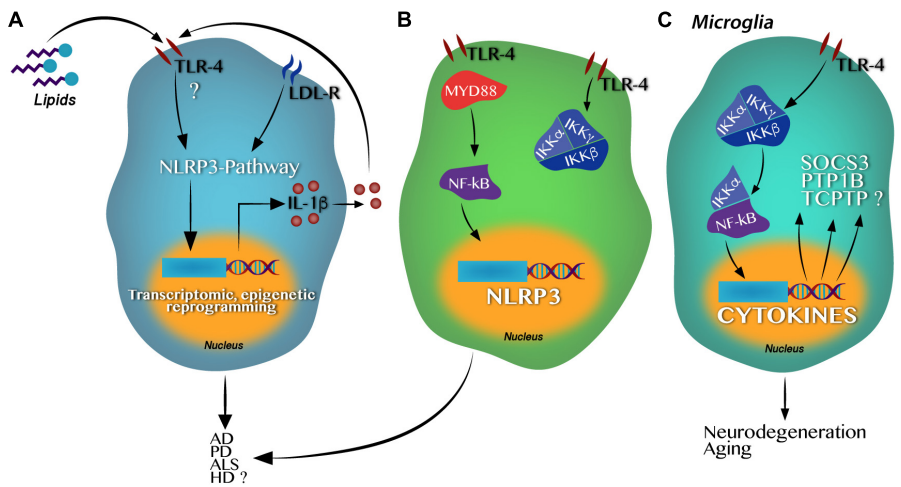


**FIGURE 2 |** Toll-like receptor 4 activation by hypercaloric diet regulates inflammasome activation in innate cells leading to neurodegeneration. **(A)** In granulocyte monocyte precursor cells lipids activate Toll-like receptor 4 (TLR-4) leading to the inflammasome-pathway (NLRP3) activation, epigenetic, and transcriptomic changes in nucleus and IL-1β synthesis and release. IL-1β itself might stimulate an autocrine loop activating the TLR-4. **(B)** Classic activation of the IKK kinase complex (IKKα and IKKβ) by lipids in granulocyte monocyte precursor cells over supply leads to the NF-κB transcription factor activation and translocation into the nucleus positively regulating the NLRP3 synthesis. Dual activation of the **(A)** and **(B)** pathways result in neuronal death found in AD, PD, HD, and potentially in ALS. **(C)** Activation of the TLR-4 in microglia stimulates the IKK kinase complex following by the NF-κB activation and translocation into the nucleus leading to cytokines, suppressor of cytokine signaling 3 (SOCS3), protein-tyrosine phosphatase 1B (PTP1B) and probably, T-cell protein tyrosine phosphatase (TCPTP) synthesis, which altogether promote neurodegeneration and aging.

neurodegeneration are being elucidated. Qiao et al. (2018) identified that hepatic NLRP3 inflammasome inhibition by *in vivo* siRNA administration decreases the TNF-α, IL-β, IL-12, and IL-18 serum levels. Of note, inhibition of the hepatic NLRP3 inflammasome partially prevents dopaminergic neuronal death in the substantia nigra of a murine Parkinson model by decreasing the pro-inflammatory profile in plasma (Qiao et al., 2018). It seems that immune activation, at least for viral infections, depends on CXCR6, a chemokine receptor of hepatic NK cells involved in the persistence of cellular memory (Netea et al., 2011). This suggests a potential direct link between peripheral immune training in the liver and neuroinflammation that leads to neurodegeneration in the CNS (**Figure 3A**).

Moreover, initial central immune training and its cross-talk with peripheral immunity also seems to be involved in neurodegeneration. For instance, recently, a substantial accumulation of peripheral FOXP3 + regulatory T cells in the mouse brain following ischemic stroke (Ito et al., 2019) was described. Of note, central FOXP3 + regulatory T cells accumulation inhibits neurotoxic astrogliosis by releasing amphiregulin, a low-affinity ligand of the epidermal growth factor receptor (Ito et al., 2019). Central FOXP3 + regulatory T cell accumulation appears to have a binary role on neuronal survival (**Figure 3A**). Transient depletion of FOXP3 + regulatory T cells decrease amyloid-β plaque accumulation, reverts the neuroinflammatory response and improves cognitive performance in the 5XFAD AD mouse model, therefore is beneficial under acute central inflammation (Baruch et al., 2015). Conversely, peripheral immune training in the liver might also be regulated by central immune inflammation (**Figure 3B**). Initial reports show that extracellular vesicles released from astrocytes and microglia during central immune training might be implicated in the peripheral-central immune cross-talk (Wang et al., 2017; Yang et al., 2018). For instance, in murine models, intracerebral injection of IL-1β promotes the release of extracellular vesicles from astrocytes, leading to leukocyte migration into the brain and regulation of the liver cytokines profile by inhibition of the peroxisome proliferator-activated receptor α (Dickens et al., 2017). It is important to mention that extracellular vesicles released by central immune activation depend on glutamine modulation by glutaminase enzyme activation (Dickens et al., 2017; Wang et al., 2017; Wu et al., 2018). In fact, substantial programing of metabolic related pathways coordinates innate immunity. For instance, M1 pro-inflammatory state in macrophages depends largely on glycolytic metabolism, impairment of oxidative phosphorylation and disruption of the Krebs cycle; glycolysis activation during immune training was recently reported to be dependent on the mevalonate pathway (Bekkering et al., 2018). Conversely, macrophages M2 phenotype, which are involved in tissue repair and homeostasis, are dependent on the Krebs cycle (Netea et al., 2011). These results suggest that a shift from oxidative phosphorylation toward glycolysis seems to be a potential metabolic node to regulated immune training. In line with this, the Akt/mTOR/HIF-1α–dependent pathway and the brain neutral sphingomyelin hydrolase 2 have been reported to be

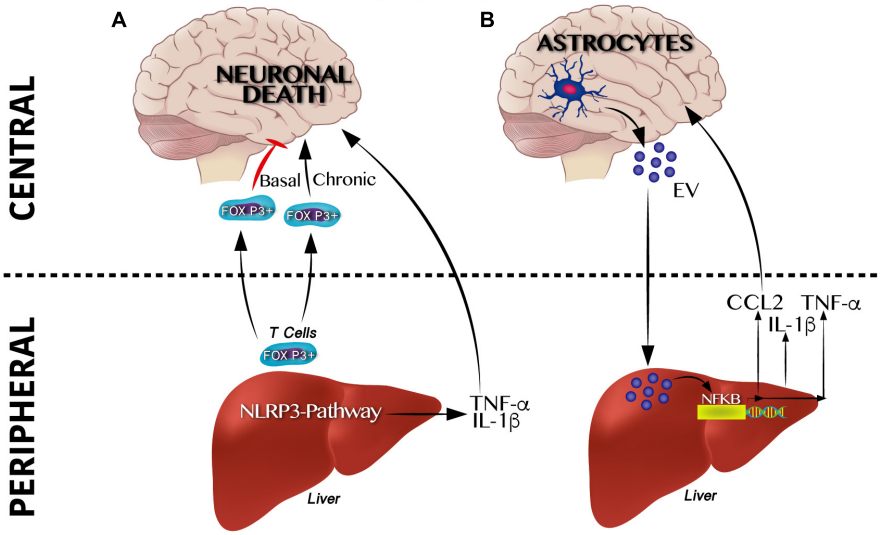


**FIGURE 3 |** Cross-talk between peripheral and central immune system on neurodegenerative susceptibility. **(A)** Peripheral immune system regulates neurodegeneration. Under a physiological scenario, Foxp3 + T cells are released from the liver to circulation reaching the central nervous system (CNS) positively modulating neuronal survival, whereas soon after a chronic activation Foxp3 + T cells become overactive promoting neuronal death. Likewise, activation of the inflammasome pathway in the liver produces IL-1β and TNF-α which exacerbate neurodegeneration. **(B)** Central immune system regulates neurodegeneration. Astrocytes secrete extracellular vesicles (EV) to the circulation reaching the liver and positively modulating the NF-κB transcriptional activation program, which includes IL-1β, TNF-α and CCL2 synthesis and release. This pro-inflammatory profile has been reported to contribute centrally to neuronal damage.

essential for trained immunity (Cheng et al., 2014; Saeed et al., 2014; Dickens et al., 2017).

A pro-inflammatory profile has been demonstrated to contribute to four major neurodegenerative pathologies and either suppressing or ameliorating this inflammatory process can have a good outcome for these diseases. According to this, we next discuss four of the major neurodegenerative pathologies in which inflammation has been proven to play a key role.

## CENTRAL AND PERIPHERAL INFLAMMATORY NODES IN NEURODEGENERATIVE DISEASES

### Active Immunity in Alzheimer Disease (AD)

Alzheimer disease is the most common type of senile dementia. Worldwide, approximately 50 million people are living with dementia and this number will triple in the next 40 years (Venigalla et al., 2015). AD is characterized by Aβ aggregation and intracellular bundles of hyperphosphorylated Tau protein (Colonna and Butovsky, 2017), which contribute to cognitive decline (Heneka et al., 2015).

High levels of TNF-α have also been identified in murine models of AD that resemble the classical behavioral abnormalities of the disease in humans (Gabbita et al., 2012; Kinney et al., 2018). Blocking the TNF-α pathway decreases central immune infiltration and prevents AD neurochemical and behavioral phenotypes (Tweedie et al., 2012; Prasad Gabbita et al., 2015), suggesting that central–peripheral cross-talk may be implicated in the disease. Notably, experimental studies using transgenic AD models have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) can reduce AD pathology (Kinney et al., 2018). In addition, not only vertebrates seem to have dysregulation of the immune system during AD: In a *D. melanogaster* AD-like model, there is an upregulation of all five antimicrobial peptides (AMPs) related to inflammatory genes, suggesting the activation of innate immunity in AD-like flies. To counteract this neuroinflammation, they fed the flies with *Gardenia jasminoides* extracts (iridoid glycosides and aglycones, such as gardenoside, crocin, geniposide, and genipin) whose components can rescue cognitive deficits in a memory impairment mouse model (Nam and Lee, 2013) and show neuroprotective effect in cell culture (Sun et al., 2014). This experiment demonstrated that even though these extracts did not alter the Aβ concentration, they regulated the expression of immune-related genes (Drosomycin, Diptericine, Attacin, Cecropin, Mtk) in the brain of the flies and ameliorated memory deficits (Wei-Wei et al., 2017). The amelioration of memory loss and the counteraction of inflammation occurs through the modulation of the activity of acetylcholine esterase and the regulation the genes in apoptosis for neuroprotection, which have been recently accounted for in glycosides and aglycones present in *Gardenia jasminoides* extracts (Sun et al., 2014). Moreover, the robust effect of central-peripheral immune activation on AD-like neuropathology is also simulated using prenatal immune activation by the bacterial endotoxin lipopolysaccharide (LPS) or polyinosinic:polycytidylic

acid (PolyI:C) (Chen et al., 2011; Krstic et al., 2012; Giovanoli et al., 2015, 2016) these findings suggest that prenatal immune activation may be an important environmental risk factor that can cause hippocampus-related cognitive and synaptic deficits in the absence of chronic inflammation across aging.

Initial reports in humans have identified a proinflammatory profile of cytokines such as TNF-α, IL-6, and IL-1β in the prefrontal cortex, hippocampus and serum of AD subjects (Cacabelos et al., 1994; Mrak and Griffin, 2005; Calsolaro and Edison, 2016). In fact, the risk for progression from mild cognitive impairment to AD and cognitive decline is higher in subjects with elevated IL-6 and TNF-α levels in the cerebral spinal fluid (Heneka et al., 2015). In the 1980s, when neuroinflammation was described in AD and immune-related cells where described to be close to Aβ plaques, several large epidemiological and observational studies were published concluding that long-term use of NSAIDs showed protective qualities against developing AD. However, recent studies have identified variable outcomes and no convincing evidence regarding the positive effect of NSAIDs in AD (Kinney et al., 2018). Despite these experimental evidences, it is still unknown whether elevated levels of IL-1β, IL-6 and TNF-α lead directly to neurodegeneration in humans (**Figure 1**).

## Active Immunity in Parkinson's Disease (PD)

Parkinson's disease is a the second most common neurodegenerative condition, affecting about 1.6% of people over 60 years old; it acts by promoting dopaminergic neuronal death in the substantia nigra pars compacta (Bassani et al., 2015). PD shows intraneuronal aggregates (called Lewy bodies) formed by α-synuclein that contribute to dopaminergic neuronal death in the basal ganglia, leading to the classical parkinsonian motor symptoms (De Virgilio et al., 2016). Neurodegeneration in PD is associated with the activation of microglia (Heneka et al., 2015), increased TNF-α, IL-1β, and IL-6 levels in the midbrain, serum and cerebral spinal fluid (Hofmann et al., 2009; Montgomery and Bowers, 2012) and activation of astrocytes (Wang et al., 2015).

Potential systemic triggers of immune activation in PD have identified that maternal immune activation in murine models reproduces the neurochemical and neuropathological symptoms of the disease (Wang et al., 2009; Vuillermot et al., 2012). Also, TNF-α and IL-6 levels correlate with dopaminergic neuronal death in the PD brain – it seems that both cytokines show a dual role on neurodegeneration and neurophysiology (**Figure 1**). Another *in vivo* example in which neuroinflammation is involved in neurodegeneration is the *D. melanogaster* Rotenone PD-like model. Lakkappa et al. (2018) used a new compound, PTUPB [(4-(5-phenyl-3-3-3-(4-trifluoromethyl-phenyl)-ureido-propyl-pyrazol-1-yl)-benzenesulfonamide)], that inhibited neuroinflammation and in turn prevented the loss of dopaminergic neurons, slightly ameliorating the PD-like pathology.

Similar to the animal models, post-mortem PD patients show infiltration of T-lymphocytes into the substantia nigra (Montgomery and Bowers, 2012). The immune hypothesis as a tentative cause of PD is still active given that, similar to AD genetic risk factors, PD involves the LRRK2, NR4A2, PARK7, and CD74 genes, which are capable of regulating immune function and microglial activation (Pandey et al., 2009). In summary, the involvement of immune responses in PD is not fully understood, but they influence the inflammation response and seem to affect disease progression.

## Active Immunity in Huntington's Disease (HD)

Huntington's disease is an autosomal-dominant neurodegenerative disease caused by a CAG trinucleotide repeat expansion that gives place to a polyglutamine region in the huntingtin protein (HTT). HD patients show a positive pro-inflammatory profile (**Figure 1**) correlated with the disease's progression, including increases of IL-1β, IL-6, and TNF-α in the striatum, cerebral spinal fluid and plasma that were also confirmed in mouse models (Rocha et al., 2016). Central immune activation in HD subjects was confirmed using positron emission tomography (PET) and in pre-symptomatic HD-gene carriers (Rocha et al., 2016). Even though pharmacologic immune modulation using the XPro1595 TNF-α inhibitor has shown neuroprotection against the cytokine-induced neurotoxicity in primary R6/2 neurons and human neurons derived from iPSCs of HD patients (Hsiao et al., 2014), the information available is contradictory based on one report describing that neuroinflammation seems a consequence rather than a cause of neurodegeneration in late HD (Vinther-Jensen et al., 2016).

## Active Immunity in Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis is the most prevalent kind of motor neuron disease in adults, affecting 4–6 per 100,000 people, and is considered a fatal neurodegenerative disease (Filippi and Agosta, 2016). ALS is characterized by the degeneration of motor spinal neurons, brain stem and primary motor cortex mainly associated to a neuroinflammatory response by central (microglial) and peripheral (T-cells) immune activation and infiltration into the affected brain regions (Evans et al., 2013; Filippi and Agosta, 2016). Central immune activation also occurs in patients with either sporadic or familiar ALS, as well as in transgenic models of the disease (Geloso et al., 2017). Immune activation has also been reported in a model of ALS in *D. melanogaster*, in which the flies were supplemented with potent anti-inflammatory natural extracts of *Withania somnifera* (Wse) and *Mucuna pruriens* (Mpe), which significantly rescued climbing impairment (De Rose et al., 2017). Also, ALS patients and mouse models of the disease have elevated levels of TNFα, IL-6, and IL-1β in blood and cerebrospinal fluid. In addition to TNF-α upregulation in ALS, TNFR1, and TNFR2 transcripts have also been found to be overexpressed in ALS patients compared with non-neurological controls (Han et al., 2015; Tortarolo et al., 2017). Similar to Alzheimer, Parkinson and Huntington, modulation of inflammatory cytokine–dependent pathways prevent neuronal death in ALS models. As we commented previously, the contribution of TNFα or IL-6

(**Figure 1**) as etiological causes of ALS pathophysiology remains controversial because of their physiological functions or dual role on the central and peripheral immune activation or as a neurotrophic factors (Tortarolo et al., 2017).

Altogether, these results propose a very new and not-yet-understood pathway that integrates metabolism, central and peripheral immune training and cross-talk which might actively modulate neurodegeneration.

## CONCLUSION

Maternal nutritional programing and immune training in microglia or peripheral innate cells are complex and require a profound integration of glycolytic and oxidative metabolism as well as the reprograming of molecular signatures that modulate susceptibility to neuronal damage at earlier stages of development in the offspring. Peripheral or central innate immune training during maternal nutritional programing might integrate a significant node that regulates neurodegenerative susceptibility in the offspring. Potential integration of the NLRP3 inflammasome and the IKKβ/NF-kB pathways in microglia at early stages, and peripheral or central immune cross-talk at later stages, including the FOXP3 + regulatory T cells or the extracellular vesicles, might actively regulate neuroinflammation and neuronal death/survival. This information allows us to propose that maternal nutritional programing leads to peripheral or central immune training, favoring neurodegenerative susceptibility.

## AUTHOR CONTRIBUTIONS

MC-T, LM-M, RM-R, AC-M, and DR-P: conceptualization and writing – review and editing. AC-M and DR-P: supervision and visualization.

## FUNDING

This work was funded by the National Council of Science and Technology in Mexico (CONACYT), 650620 CONACYT for MC-T, 582196 CONACYT for LM-M, and 573686 CONACYT for RM-R.

## ACKNOWLEDGMENTS

We thank Alejandra Arreola-Triana for her support on editing this manuscript and Arlette Yaressi Vinaja-Romero for her figure artworks.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 03 April 2020
doi: 10.3389/fnins.2020.00318

# Prenatal Exposure to Gossypol Impairs Corticogenesis of Mouse

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Gossypol is a yellow polyphenolic compounds extracted from roots, stems and seeds of cotton plants. Excessive intake of gossypol induces severe pathological signs of toxicity in livestock and wildlife. Currently, gossypol has received widespread attention for its toxic effects on the reproductive system. However, reports of the effects of gossypol during corticogenesis and the development of the mouse cerebral cortex are unavailable. In the present study, gossypol was orally administrated at a dose of 0, 20, and 50 mg/kg body weight/day to pregnant mice from embryonic day 6.5 to the time of sample collection. We used *in utero* electroporation and immunofluorescence to demonstrate that gossypol impaired cortical neuronal migration. Furthermore, labeling with 5-bromo-2-deoxyuridine and western blot analysis revealed that gossypol disturbed the balance between proliferation and differentiation of neural progenitors, inhibited neural progenitor cell proliferation, neuronal differentiation, and maturation. Additionally, cortical progenitor apoptotic cell death increased in the developing gossypol-treated cortex, which was associated with NF-κB and MAPK pathways. In conclusion, our findings indicate that gossypol exposure disrupted neurogenesis in the developing neocortex, suggesting the potentially harmful impact of gossypol on the cerebral cortex development of humans and livestock.

**Keywords: gossypol, corticogenesis, neural progenitor cells, proliferation, differentiation**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *01 December 2019*
***Accepted:*** *18 March 2020*
***Published:*** *03 April 2020*

***Citation:***
*Zhu X, Wu Y, Li C, Yan W, Pan J, Wang S and Zhao S (2020) Prenatal Exposure to Gossypol Impairs Corticogenesis of Mouse. Front. Neurosci. 14:318. doi: 10.3389/fnins.2020.00318*

## INTRODUCTION

Cottonseed oil and cottonseed meal flour are by-products of cottonseeds and cotton plants commonly consumed by humans and food-producing animals as they are rich in oil and proteins (Huang et al., 2006; Camara et al., 2016). However, high concentrations of gossypol and its potential toxicity limit the efficient utilization of cottonseed and cotton plants (Chen et al., 2019). Gossypol, a yellow polyphenol compound, was initially investigated as a male contraceptive in China (Coutinho, 2002; Lopez et al., 2005). Previous studies have focused on the toxic effects of gossypol on the reproductive system, and immune function, while reducing resistance to infections, and impairing the efficiency of vaccines (Gadelha et al., 2014b). Cumulative evidence suggests that gossypol exhibits various pharmacological properties such as anti-inflammatory, anti-fungal, anti-fertility, and anti-cancer properties (Moon et al., 2011; Keshmiri-Neghab and Goliaei, 2014; Zeng et al., 2019). Molecular research shows that gossypol causes mitochondrial dysfunction by inhibiting cell respiration. Furthermore, it induces oxidative stress viewed as an imbalance between antioxidants and pro-oxidants, which results in the accumulation of reactive oxygen species (Kovacic, 2003; Keshmiri-Neghab and Goliaei, 2014; Santana et al., 2015). It has been reported that gossypol inhibits fast axonal transport and accumulates in the nerve, raising concerns about

its possible neurotoxicity (Kanje et al., 1986). Therefore, the risk of gossypol toxicity has aroused worldwide attention due to the consumption of agricultural by-products such as cottonseed oil, milk, and meat from the affected animals.

The mammalian cerebral neocortex is a well-organized, six-layered structure composed of neurons. During cortical development, newborn neurons derive from the ventricular zone (VZ)/subventricular zone (SVZ) transition from multipolar to bipolar state and undergo radial glial-dependent migration to their final destination to form the cortical plate (CP) (Kriegstein and Noctor, 2004; Cooper, 2008; Frotscher, 2010). The development of the mammalian neocortex is indispensable to coordinate the proliferation, migration, and differentiation of neural progenitor cells (NPCs) (Florio and Huttner, 2014). It is widely believed that these processes are closely related to complex brain functions such as learning, memory, and cognition. The dysplasia of neocortex may lead to neurological disorders such as epilepsy, cognitive impairment, microcephalies, or hemimegalencephaly (Stouffer et al., 2016; Wang et al., 2017). However, the process of neurogenesis is regulated by intrinsic molecules, extrinsic signals and various environmental stimuli (Shimojo et al., 2008; Gotz et al., 2016). It has been reported that gossypol can cause hippocampal hemorrhage and changes in membrane permeability. It interferes with microtubule assembly, which may lead to the formation of apparent neurofibrillary tangles in rats (Sharma et al., 1966; Semon, 2012). Previous reports suggest that neurogenesis is regulated by a variety of pathological conditions that are commonly associated with microglial activation and inflammation in the brain, such as chronic stress and prenatal stimulation (Sierra et al., 2014). These findings may bring new ideas in investigating the neurotoxicity of gossypol in the central nervous system, especially during the development of the cerebral cortex.

The present study was undertaken to investigate the effect of gossypol exposure during embryonic neurogenesis in the developing neocortex. Here, we reported that gossypol exposure inhibited neuronal proliferation, differentiation, and maturation, as well as increased progenitor apoptotic cell death. Our results demonstrated the neurotoxicity of gossypol during corticogenesis and may help to understand the mechanism of gossypol neurotoxicity.

# MATERIALS AND METHODS

## Reagents and Chemicals

Gossypol was purchased from Sigma-Aldrich (St. Louis, MO, United States). It was initially dissolved in a small volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, United States), further diluted in 0.9% saline, and stored at −20°C. Working solutions of gossypol (0, 2.0, and 5.0 mg/ml) were freshly prepared every day. 5-Bromo-2′-deoxyuridine (BrdU) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, United States). 4′, 6′-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Carlsbad, CA, United States). All other reagents and instruments used in this experiment are indicated below.

## Ethics Statement and Animals

All experimental procedures concerning animal care and handling were conducted according to the guidelines for Care and Experimental Use of Laboratory Animals of Northwest A&F University. All animal experiments were approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University (certificate NO.: SCXK [SHAAN] 2017-003), in accordance with ARRIVE guidelines[1].

Three months old male and female ICR mice were purchased from the Experimental Center of Xi'an Jiaotong University and adapted to the laboratory environment for 1 week. Mice were housed in a temperature-controlled room (22–26°C) on a light cycle (12 h light/12 h dark; light on from 8 a.m to 8 p.m), with *ad libitum* access to food and water. The time of vaginal plug appearance was designated as embryonic day (E) 0.5. Each dam was housed individually during the experiment.

## Drug Administration

Pregnant mice were randomly and assigned to three groups of five dams, and the investigators were blinded to study conditions. Pregnant mice received gossypol orally at a dose of 0, 20, and 50 mg/kg body weight/day from E6.5 to the time of sample collection. These doses were determined based on previous studies (Hahn et al., 1981; Li et al., 1989) and a preliminary study. A high dose was determined as the point when gossypol significantly altered neuronal migration of fetal cerebral cortex, but did not affect the maintenance of pregnancy and delivery of dams. Only one pregnant mouse was placed in each cage to prevent overcrowding. The number of pups per little was culled to 10 at postpartum day (P) 0. In every experiment, for one dosage group, newborn pups were selected randomly from five dams with five offspring in each group.

## Open Field Test (OFT)

Open field test (OFT) measures the exploratory activity and anxiety behavior of mice in a novel environment (Hei et al., 2019). The mice at P21 were placed individually in the center of an open field box (50 × 50 × 30 cm) with inner and external areas. The box was divided into 25 equal squares including 9 cells in the center and the remaining 16 cells in the outer area (**Figure 1E**). Each session lasted 15 min, and dejection amounts, distance, times, proportion of the time traveled in the inner area, and jumping frequency were recorded immediately by using a video tracking/computer-digitizing system (ANY-maze) after the mice were put in the box. The box was wiped with 75% ethanol every time after each trail to avoid the influence of the residual odor on the experiment.

## *In Utero* Electroporation (IUE)

The plasmid expressing enhanced green fluorescence protein (*pCAG-GFP*) was purified using the E.Z.N.A.TM Plasmid Maxi Kit (Omega, United States) according to the manufacturer's protocol. The *in utero* electroporation (IUE) procedure was performed as previously described (Li et al., 2018). The pregnant

[1]https://www.nc3rs.org.uk/arrive-guidelines

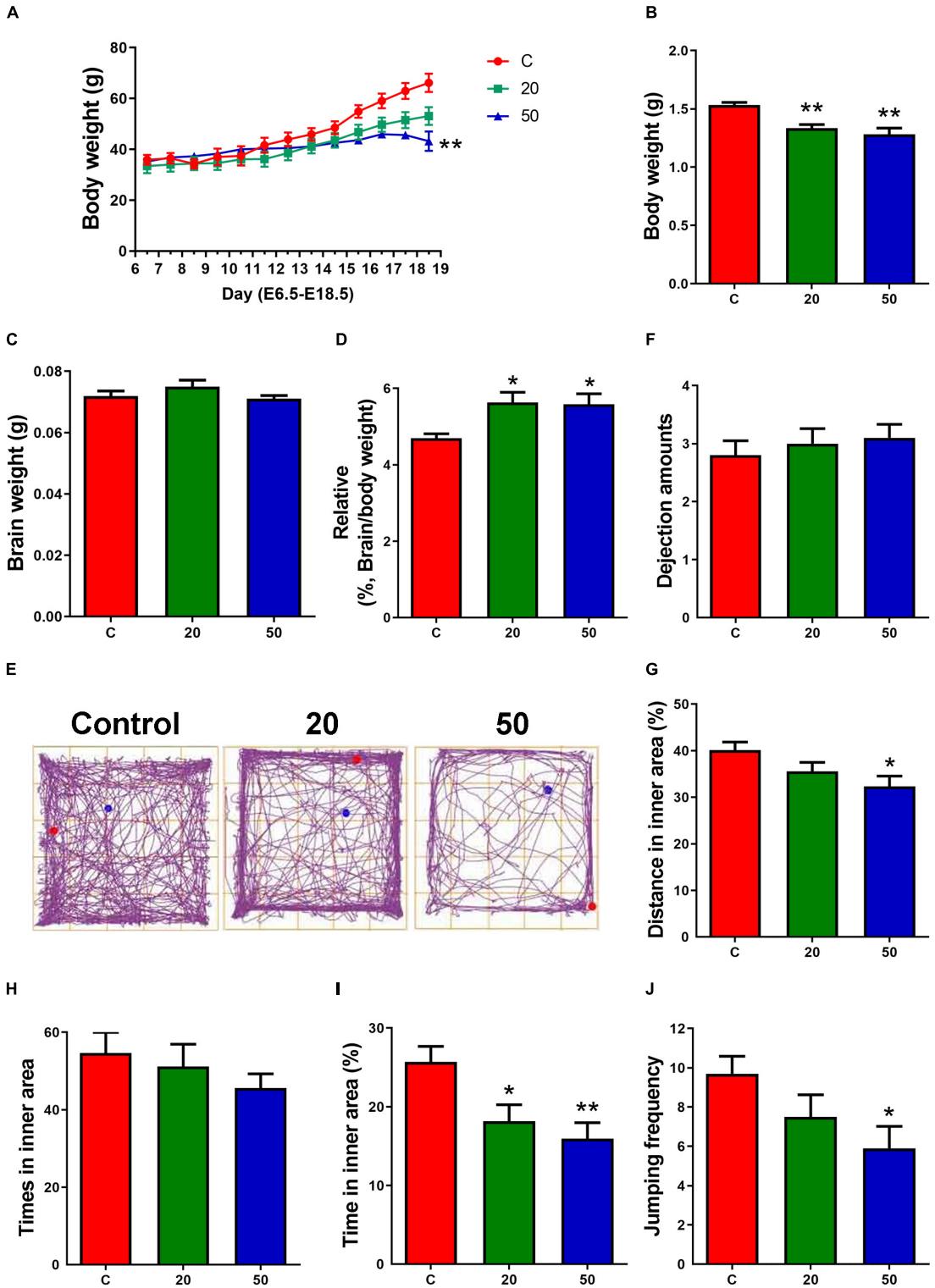


**FIGURE 1 |** Exposure to gossypol suppressed the body weight of dams, body weights and the ration of brain weight to body weight of offspring. **(A)** Body weight of dams during E6.5 to E18.5. **(B)** Body weights of offspring at E18.5. **(C)** Brain weight of offspring at E18.5. **(D)** The ratio of brain weight to body weight of offspring at E18.5. **(A–D)** $n = 5$ per group. **(E)** Trace chart of open field test at P21 of offspring. The blue point represents the initial position, and the red point represents the end position. **(F–J)** Quantitative analysis of dejection amounts, distance, times, time traveled in the inner area, and jumping frequency. $n = 10$ per group. Difference was found between the control group and gossypol-treated groups. Results are presented as the mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group. E, embryonic day.

mice with embryos at E15.5 were anesthetized with amobarbital sodium (Sigma), followed by exposure of the uterine horns. Approximately 1.5 μl of a 3.0 μg/μl plasmid containing Fast Green was injected into the lateral ventricle of the embryonic brain with a fine pre-pulled glass micropipette. Electroporation was performed with a BTX electroporator (30 V, 50 ms, five times, 950 ms interval). Subsequently, the uterine horns were repositioned into the abdominal cavity. Following the recovery period, pups were collected and post-fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C.

## BrdU Labeling

BrdU, a marker of neurogenesis, is incorporated into the DNA of dividing cells during the S-phase of the mitotic process (Kee et al., 2002). BrdU was dissolved freshly in 0.9% saline before use. To assess the cell proliferation, pregnant mice were received a single intraperitoneal injection of BrdU (50 mg/kg body weight) 2 h before sacrificed at E15.5. To examine the proliferation, differentiation and maturation of cells, pregnant mice were given intraperitoneal injection of BrdU at E15.5 and sacrificed at E16.5, E17.5, or E18.5. Brains were dissected at the different stages indicated and post-fixed with 4% PFA in 0.1 M PB (pH 7.4) at 4°C.

## Immunofluorescence Analysis

Brain sections were sliced into 60-μm thick coronal sections using a vibratome (VT 1000S, Leica, Germany). The sections were rinsed three times in 0.1 M PB (pH 7.4) and incubated overnight at 4°C with primary antibodies diluted in blocking solution (4% BSA, 1% normal goat serum and 0.3% Triton X-100 in PB). Sections were rinsed in 0.1 M PB and incubated for 3 h at room temperature with adequate fluorescent secondary antibodies. After three consecutive washes in 0.1 M PB, the sections were counterstained for 15 min in 0.1 μg/ml DAPI or PI. The slices were washed in 0.1 M PB, dried, and coverslipped using Fluoromount G (Southern Biotechnology). Negative controls were processed using 1% normal goat serum instead of primary antibody to show the specificity of the immunostaining. Immunofluorescence labeling was visualized using a structured illumination microscope (Zeiss observer Z1) or a confocal laser scanning microscope (TCS SP8, Leica, Germany).

Immunofluorescent detection of BrdU was performed as described previously (Li et al., 2017; Xu et al., 2018). Brain sections were denatured in 2 M HCl at 37°C for 30 min, rinsed with 0.1 M PB, neutralized in 0.1 M borate buffer (pH 8.5) for 30 min, and rinsed with 0.1 M PB, before incubation with primary antibodies.

The following primary antibodies were used in this study: rabbit anti-GFP (1:1000, Invitrogen, Carlsbad, CA, United States); rat anti-BrdU (1:500, Millipore, Temecula, CA, United States); rabbit anti-Ki67 (1:500, Abcam, Cambridge, United Kingdom); rabbit anti-p-histone H3 (1:500, Millipore, Temecula, CA, United States); mouse anti-Nestin (1:500, Invitrogen, Carlsbad, CA, United States); mouse anti-Tuj1 (1:500, Chemicon, Nuremberg, Germany); goat anti-Brn2 (1:300, Santa Cruz Biotechnology, United States). The following secondary antibodies were used: Alexa Fluor 647 goat anti-rat IgG (1:500, Chemicon, Nuremberg, Germany); Alexa Fluor 488 donkey anti-rabbit IgG (1:500, Invitrogen, Carlsbad, CA, United States); Alexa Fluor 568 donkey anti-goat IgG (1:500, Cell Signaling Technology, Boston, MA, United States); Alexa Fluor 555 goat anti-mouse IgG (1:500, Abcam, Cambridge, United Kingdom).

## Western Blot Analysis

Western blot analysis was performed as described previously (Li et al., 2018; Hei et al., 2019). In brief, the E16.5 and E18.5 neocortices of pups were isolated and lysed in ice-cold RIPA buffer (Solarbio, Beijing, China) containing 1 mM PMSF (Solarbio, Beijing, China). 10% acrylamide gels (SDS-PAGE) with an equal amount of 20 μg protein load in each lane were electrophoresed, and the proteins were transferred onto a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, United States). After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with specific primary antibodies against p-histone H3 (1:1000, Millipore, Temecula, CA, United States), Nestin (1:1000, Invitrogen, Carlsbad, CA, United States), Tuj1 (1:1000, Sigma-Aldrich, St. Louis, MO, United States), Brn2 (1:1000, Santa Cruz Biotechnology, United States), nuclear transcription factor-kappa B (NF-κB), caspase-3, p44/42 MAP kinase (ERK1/2), phospho-p44/42 MAP Kinase (p-ERK1/2), p38, phospho-p38 (p-p38), and β-actin (1:1000, Cell signaling Technology, Danvers, MA, United States). After rinsing three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody, donkey anti-goat IgG antibody, or goat anti-mouse IgG HRP linked antibody (1:5000, Cell signaling Technology, Danvers, MA, United States). The protein bands were detected by ECL plus (GE Healthcare, Buckinghamshire, United Kingdom) using the GelDoc XR Gel Documentation System (Bio-Rad). The band intensities were analyzed using ImageJ analysis software.

## Statistical Analysis

All statistical analyses were performed with GraphPad Prism 7 (GraphPad Software Inc., United States). Data were expressed as mean ± standard error of the mean (SEM), and n referred to the number of animals per experimental group. Statistical tests included one-way ANOVA followed by Tukey's multiple comparisons test between groups. Statistical significance was set at $p < 0.05$.

# RESULTS

## Reduced Body Weight and Induced Depression-Like Behaviors in Gossypol-Treated Offspring

The dams treated with gossypol and the control group mice had indistinguishable weights from E6.5 to E14.5 of the gestational period. Later, the weight of the gossypol-treated dams displayed

a slow-growth trend compared to the controls, and a significant difference in body weight was observed at E18.5 (**Figure 1A**). The body weight of mice offspring in the gossypol-treated group (20 and 50 mg/kg) at E18.5 was significantly lower than that in the control group (**Figure 1B**). In contrast, the brain weights of offspring were not significantly different between gossypol-exposed and control groups (**Figure 1C**), while the ratio of brain weight of the offspring mice showed a significant difference between the control group and the group treated with gossypol (**Figure 1D**). Moreover, we investigated the behavior of offspring regarding brain function at P21 through open field test (**Figure 1E**). The gossypol-treated offspring showed an obvious reduction in the distance (**Figure 1G**) and time (**Figure 1I**) in the inner area, and jumping frequency (**Figure 1J**) in the box, and no differences were observed in the dejection amounts (**Figure 1F**) and times in inner area (**Figure 1H**). The results indicated anxiety- and depression-like behaviors of brain in the gossypol-treated offspring.

## Gossypol Inhibited Neuronal Migration

To investigate the role of gossypol in the developing brain specifically during the period of prominent neurogenesis, we orally administered different concentrations of gossypol to time-pregnant mice from E6.5, injected *pCAG-GFP* at E15.5 using IUE, and performed histological analysis 3 days after electroporation (E18.5) to determine the neuronal migration (**Figures 2A,B,B',B"**). We found that about 39.8% of GFP-positive cells had migrated into the intermediate zone (IZ). In contrast, numerous (43.0 and 57.1%) GFP-positive cells were found in the IZ of gossypol groups, at respective doses of 20 and 50 mg/kg. Small percentage (9.5%) of neurons labeled with GFP migrated into the CP in the group dosed with gossypol at 50 mg/kg, while the percentage of neurons was significantly higher in the control group (30.2%) (**Figure 2C**). These results suggested that gossypol treatment inhibited neuronal migration in the mouse cerebral cortex.

## Gossypol Impaired the Balance Between Proliferation and Differentiation of Neural Progenitor Cells

The balance between NPCs proliferation and differentiation is of critical importance in the development of cortical cortex. To investigate the effect of gossypol on NPCs, we injected BrdU at E15.5, and performed immunofluorescent staining at E17.5 and E18.5, respectively (**Figure 3A**). The brain slices were immunostained with antibodies against BrdU and proliferative marker Ki67, a nuclear protein expressed in all proliferating cells (**Figures 3B,B',B",C,C',C**"). Statistical analysis showed that 14.92% of the cells in the gossypol 50 mg/kg group were $BrdU^+$ $Ki67^+$ double-labeled cells at E17.5, which was a significant reduction when compared to controls with almost 19.76% of co-labeled cells in the VZ/SVZ (**Figure 3D**). Similarly, this difference remained significant at E18.5. In the control group, 23.08% of $BrdU^+$ $Ki67^+$ double-labeled cells were present in the VZ/SVZ. In contrast, the co-expression neurons significantly reduced to 14.61 and 13.86% in the gossypol groups, at respective doses of 20 and 50 mg/kg (**Figure 3E**). This result suggested that gossypol may influence proliferating cells or their progeny by promoting cell exit from the germ zone of neocortex.

## Reduction in the Proliferation of Neural Progenitor Cells of Gossypol-Treated Mice

As the VZ and SVZ are the proliferation zones of the cerebral cortex, we analyzed the number of proliferating NPCs to examine the inhibitory effect of gossypol on neuronal proliferation. E15.5 embryos were incorporated with BrdU 2 h before harvesting (**Figures 4A,B,B',B**"). Statistical analysis showed a significant difference in the number of $BrdU^+$ cells between the gossypol-treated and control groups (**Figure 4D**). To confirm the loss of progenitors in the gossypol treated groups, we performed pHH3, Nestin and Ki67 immunostaining in the VZ/SVZ. pHH3 is a marker for mitotic cells. We found that the number of $pHH3^+$ cells had a decreasing dose-dependent trend following exposure to gossypol, which was significantly lower in the 50 mg/kg group than in the control group (**Figure 4E**). Nestin, a characteristic marker for NPCs, is widely expressed in the cerebral cortex and can mark the fibrous protuberance of NPCs. The results showed no significant differences in the area, sparse distribution, and arrangement of Nestin positive cells between gossypol-exposure and control groups (**Figures 4C,C',C**"). Consistent with these results, we also found that the protein level of pHH3 was decreased following gossypol-treated group at a dose of 50 mg/kg compared to the control (**Figures 4F,G**), and no difference was observed in the expression of Nestin between gossypol-treated and control cortices (**Figures 4F,H**). To understand the link between the loss of proliferating progenitors in gossypol-treated mice and changes in the mode of cell differentiation, we injected BrdU into the abdominal cavity of pregnant mice on E15.5 and embryos were harvested 24 h after operation (E16.5) (**Figure 4A**). Cells that were co-labeled with $BrdU^+$ and $Ki67^+$ reentered the cell cycle. Consistent with the decreased numbers of $BrdU^+$ and $pHH3^+$ cells, the analysis revealed a significant decrease in the ratio of $BrdU^+$ $Ki67^+$ double-labeled cells over total $BrdU^+$ cells in the gossypol-treated group (**Figures 4I,I',I",J**). Therefore, we concluded that the reduced number of proliferating NPCs in the VZ/SVZ of gossypol-treated mice was a result of decreased production of mitotic cells and a reduced cell-cycle reentry.

## Inhibited Neuronal Differentiation and Maturation in Gossypol-Treated Cortex

To further confirm if gossypol could influence neuronal differentiation, we injected BrdU at E15.5 and cortical slices were stained with Tuj1, a marker for young neurons (**Figures 5A,B,B',B"**). The number of $BrdU^+$ positive cells in Tuj1 positive cells located in the IZ was significantly lower in the gossypol-exposed groups at doses of 20 and 50 mg/kg than in the control group (**Figure 5D**). However, we studied the expression level of Tuj1 in cortex (**Figure 5F**) and found no significant difference between gossypol-exposed and control groups (**Figure 5G**). We performed $Brn2^+$ immunostaining to examine the effect of gossypol on the maturation of neurons

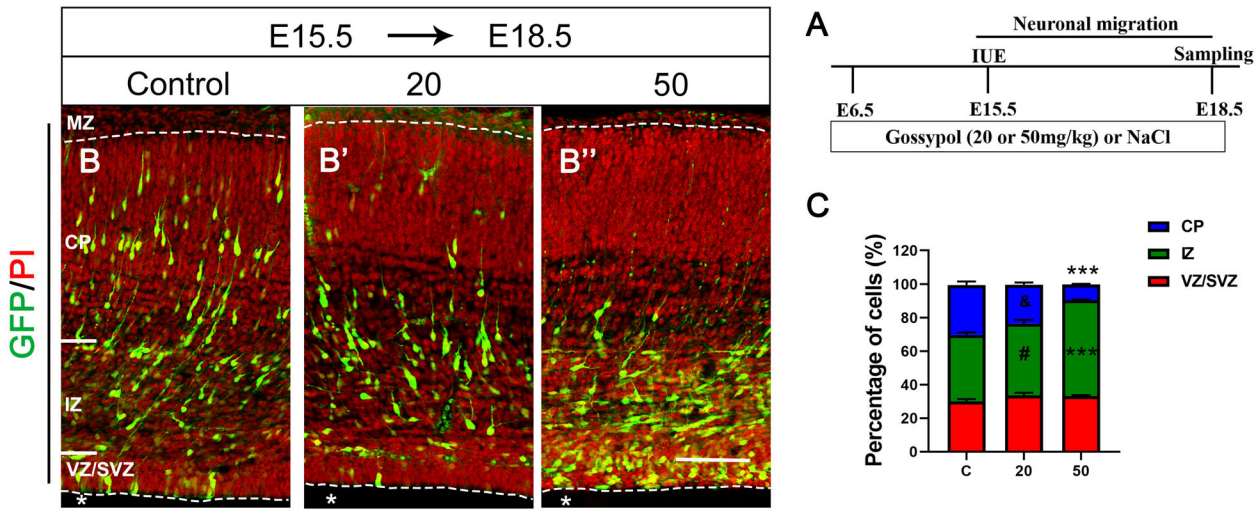


**FIGURE 2 |** Exposure to gossypol inhibited neuronal migration in the developing cerebral cortex. Brain were electroporated with GFP (green) at E15.5 and fixed at E18.5 after IUE. Sections were counterstained with PI (red). **(A)** Protocol of IUE for testing the neuronal migration of cerebral cortex. **(B)** Representative immunofluorescence images of the $GFP^+$ migrating neurons in the developmental cerebral cortex. **(C)** Distribution of $GFP^+$ cells in each layer of cerebral cortex. Quantification of the number of neurons showed that, in control slices, one third neurons invaded the CP, whereas a large number of neurons accumulated in the IZ in the exposure gossypol groups. Results are presented as the mean ± SEM ($n$ = 5 per group). Scale bars 100 μm. ***$p < 0.001$ compared with the control group; & and # showed no significant difference but has the trends compared with the control group. IUE, *in utero* electroporation; E, embryo; GFP, green fluorescent protein; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone. VZ/SVZ, ventricular zone/subventricular zone. Asterisks indicate the ventricular lumen. Dashed lines indicate cortex boundaries.

(**Figures 5A,C,C',C"**). The analysis revealed a decreasing dose-dependent trend following gossypol exposure in the percentage of $BrdU^+$ $Brn2^+$ positive cells in total $BrdU^+$ cells, which was significantly lower at a dose of 50 mg/kg than in the control group (**Figure 5E**). Moreover, significant decreases of Brn2 protein levels were observed when we performed western blot analysis for E18.5 gossypol-treated cortices (**Figures 5F,H**). Collectively, these results indicated that maternal gossypol exposure inhibited neuronal progenitor cells toward a neuronal fate and affected neuronal differentiation and maturation, which impeded neuronal development.

## Gossypol Induced Apoptotic Cell Death Associated With NF-κB and MAPK Pathway in Gossypol-Treated Cortex

Multiple signals control the proliferation and differentiation of neural progenitor cells during corticogenesis. To understand the molecular mechanisms for gossypol regulated cortical neurogenesis, specifically the decreased neuronal maturation, we investigated the possibility of gossypol-induced apoptotic cell death in the neonatal cortex during the gestational period, using western blot analysis (**Figure 6A**). The protein levels of caspase-3, a marker of apoptosis, were examined. The results showed that the levels of caspase-3 significantly increased in gossypol-treated mice than in controls (**Figure 6B**). This suggested that the apoptotic cell death is likely a significant factor contributing to the reduced neuronal number in the gossypol-treated mice. Given the evidence that the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways play a crucial role in cell fate, the effects of gossypol on the activation of associated proteins were investigated (**Figure 6A**). The results showed that the protein levels of NF-κB significantly increased following gossypol exposure (**Figure 6C**). However, gossypol significantly decreased phosphorylation of ERK and p38 (**Figures 6D,E**). The results demonstrated that gossypol induced cortical progenitor apoptotic cell death and was possibly related to NF-κB and MAPK signaling.

# DISCUSSION

Gossypol is slowly eliminated and tends to accumulate in the body (Sharma et al., 1966). It is known to reach the brain and bind randomly to vital cellular structures (Semon, 2012). Long-term feeding of cotton seed without gossypol detoxification or incomplete detoxification may result in slow growth, reduced reproductive efficiency, and death of livestock and poultry. The accumulation of gossypol in the human body may result in bleeding, loss of appetite, weight loss, infertility, hypokalemia, gastroenteritis, and neurological disorders. As the blood-brain barrier is not fully developed in the embryonic period, it provides limited protection against harmful substances entering the central nervous system. In the present study, we elucidated that exposure to gossypol during pregnancy disrupts neuronal migration, proliferation, and differentiation of offspring, which may be associated with progenitor apoptotic cell death. To our knowledge, this is the first report that demonstrates the direct effects of gossypol in mouse cerebral corticogenesis.

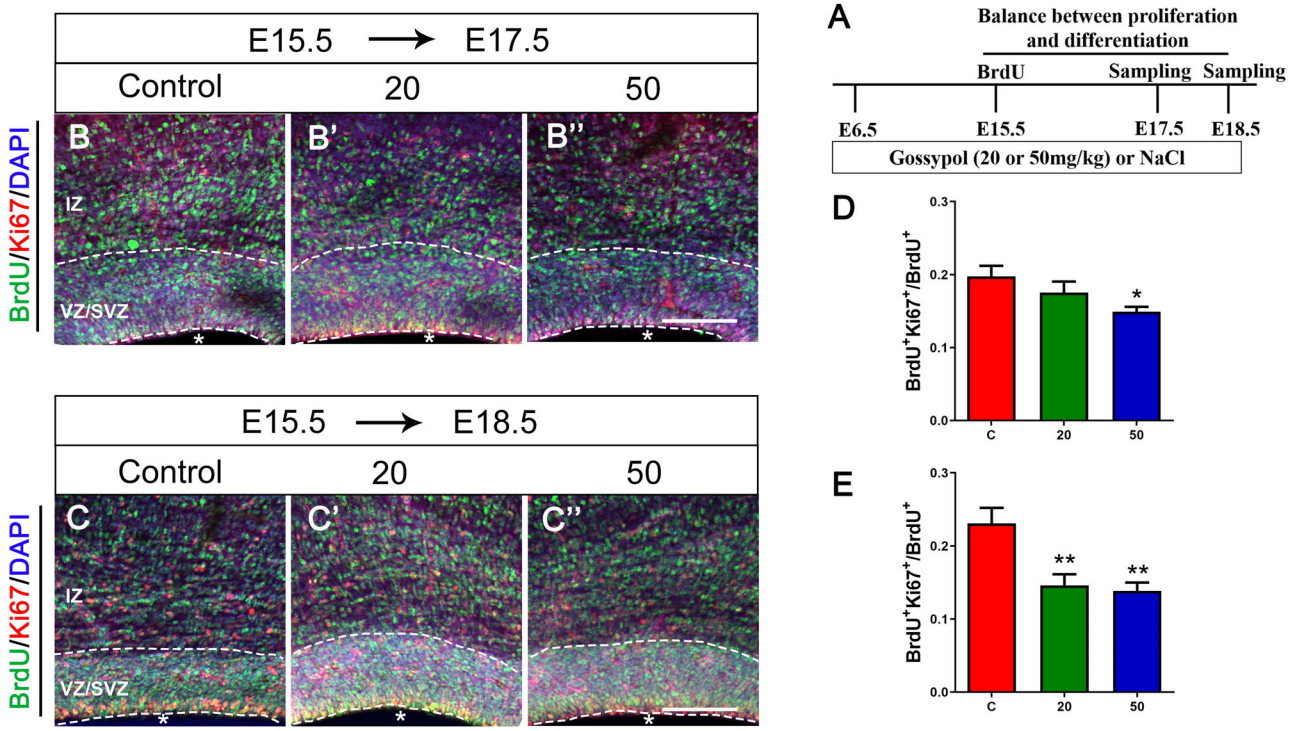


**FIGURE 3 |** Exposure to gossypol break the balance between proliferation and differentiation of neural progenitor cells. A single pulse of BrdU was injected at E15.5 and fixed at E17.5 and E18.5. The brain slices were immunostained with antibody against BrdU (green), Ki67 (red), and counterstained with DAPI (blue). **(A)** Protocol of BrdU injections for testing the balance between proliferation and differentiation of neural progenitor cells. **(B)** Representative immunofluorescence images of the $BrdU^+$ and $Ki67^+$ cells at E17.5 in the developmental cerebral cortex. **(C)** Representative immunofluorescence images of the $BrdU^+$ and $Ki67^+$ cells at E18.5 in the developmental cerebral cortex. **(D)** Ratio of BrdU and Ki67 co-labeled neurons at E17.5 in the VZ/SVZ. **(E)** Ratio of BrdU and Ki67 co-labeled neurons at E18.5 in the VZ/SVZ. Exposure to gossypol caused a decrease in Ki67 incorporation in BrdU cells. Results are presented as the mean ± SEM ($n = 5$ per group). Scale bars 100 μm. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group. E, embryo; IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Asterisks indicate the ventricular lumen. Dashed lines indicate cortex boundaries.

In the present study, decreased weight gain was observed in dams and offspring treated with gossypol. This finding is in agreement with Henry et al. (2001) indicating that the addition of gossypol to feed led to significant reductions in body weight and feed intake in chickens. Recent studies have also reported that reduced weight gain is a common sign of gossypol toxicity (Blevins et al., 2010; Zeng et al., 2014). Given that growth hormone plays a crucial role in growth and development, we suspect that these alterations may be related to the influence of gossypol on the regulation of the somatotropic axis in mice, and the decreased levels of growth hormone. However, the detailed mechanisms remain to be elucidated.

As the fetal thyroid gland produces inadequate amounts of thyroid hormone, the maternal thyroid supplies thyroxine in embryonic development. Gadelha et al. (2014a) reported that gossypol administered to rats was responsible for a significant reduction in serum thyroxine. Other studies showed the maternal thyroxine deficiency before the onset of fetal thyroid function (E17.5) affects reelin and its downstream signaling cascade, thereby response to aberrant neocortical neuronal migration and affecting neocortex development (Pathak et al., 2011; Chai et al., 2016). Our study showed that gossypol treatment inhibited neuronal migration in the cerebral cortex of embryonic mice. Although gossypol exposure may not influence the mature nervous system of dams, it may affect the development and function of the embryonic neocortex of the offspring by suppressing the maternal thyroid function. This observation explains, to some extent, that the nervous system of mammalian embryos is more sensitive than that of adults. It may be easily affected by neurotoxic exposure and lead to developmental disorders. This could be explained by the investigation of OFT in mice at P21. OFT is a classical method to evaluate anxiety-like and depression-like state in rodents. Mice treated with gossypol travelled less distance and spent less time in inner area, demonstrating the decline of curiosity and exploratory response in face of unfamiliar environment and increase of anxiety-like and depression-like behaviors.

The development of the mouse neocortex is a complex process initiated by the proliferation of neuronal precursors in the VZ, followed by neuronal differentiation and migration (Sidman et al., 1959). Recently, there have been considerable evidence to demonstrate that developing neocortex can be influenced by adverse effects, including prenatal stress and physical or chemical factor (s) and so on. Some researchers found that maternal exposure to bisphenol A affected neurogenesis in the developing neocortex and disrupted normal neocortical

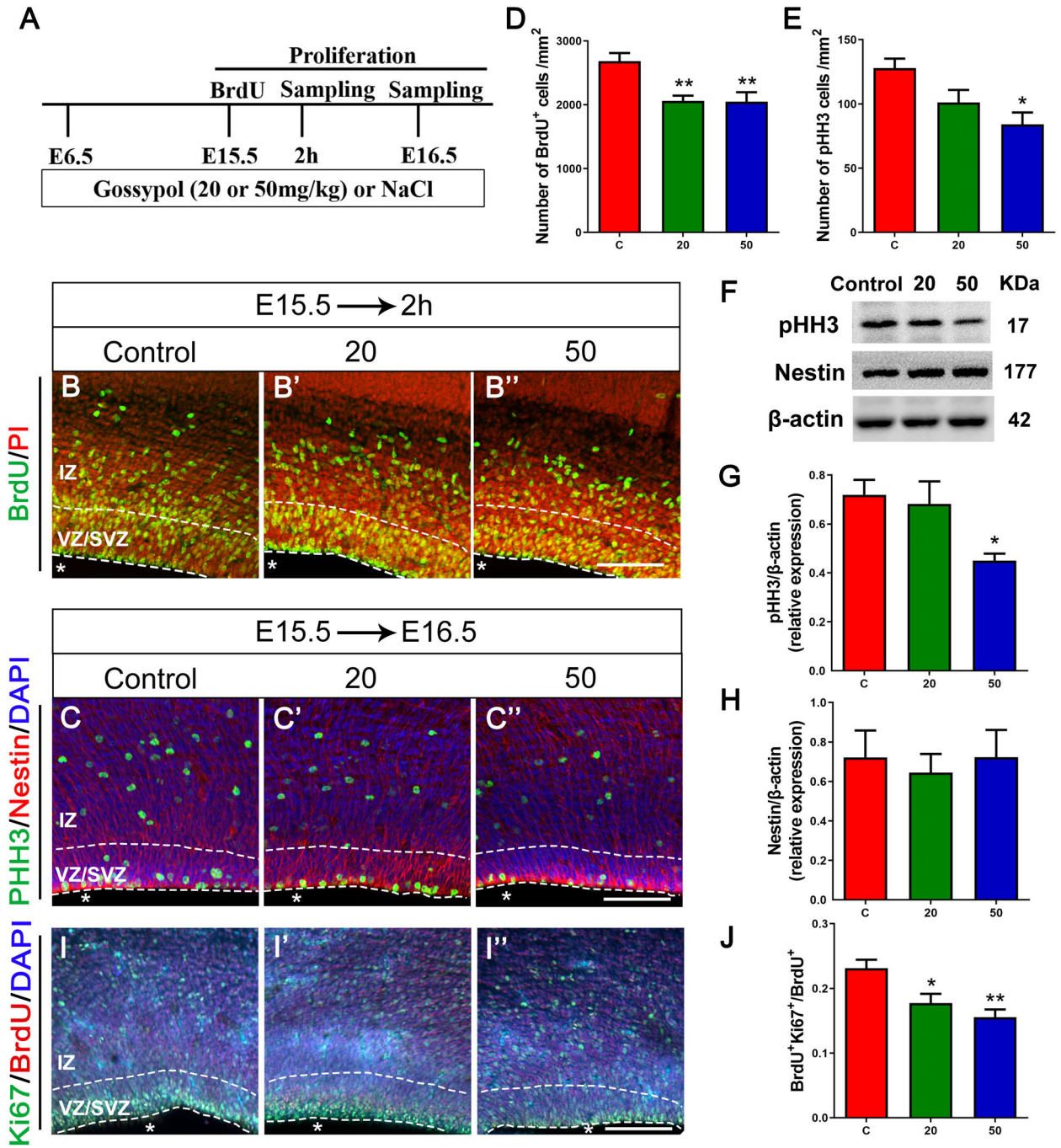


**FIGURE 4 |** Exposure to gossypol inhibited proliferation of neural progenitor cells. **(A)** Protocol of BrdU injections for testing the proliferation of neural progenitor cells. **(B)** Representative immunofluorescence images of the $BrdU^+$ cells at E15.5 after 2 h BrdU injection in the developmental cerebral cortex. The brain slices were immunostained with antibody against BrdU (green) and counterstained with PI (red). **(C)** Representative immunofluorescence images of the $pHH3^+$ cells at E16.5 in the developmental cerebral cortex. The brain slices were immunostained with antibody against pHH3 (green) and Nestin (red), and counterstained with DAPI (blue). **(D)** Quantification of BrdU labeling cells. **(E)** Quantification of pHH3-positive cells. **(F)** Representative Western blots of pHH3, Nestin and β-actin are shown and densitometry was used to quantify the protein levels in the developmental cerebral cortex. **(G,H)** Relative protein levels of pHH3 **(G)** and Nestin **(H)** at E16.5, the expression of pHH3 was decreased after the treatment of gossypol. **(I)** Representative immunofluorescence images of the $Ki67^+$ cells at E16.5 in the developmental cerebral cortex. The brain slices were immunostained with antibody against Ki67 (green), BrdU (red), and counterstained with DAPI (blue). **(J)** Quantification of Ki67-positive cells. Quantification of the number of neurons showed that, the number of $BrdU^+$ cells, $pHH3^+$ cells and the ratio of BrdU and Ki67 co-labeled neurons were significantly decreased in the slices of exposure to gossypol when compared with the control group. Results are presented as the mean ± SEM ($n = 5$ per group). Scale bars 100 μm. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group. E, embryo; IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Asterisks indicate the ventricular lumen. Dashed lines indicate cortex boundaries.

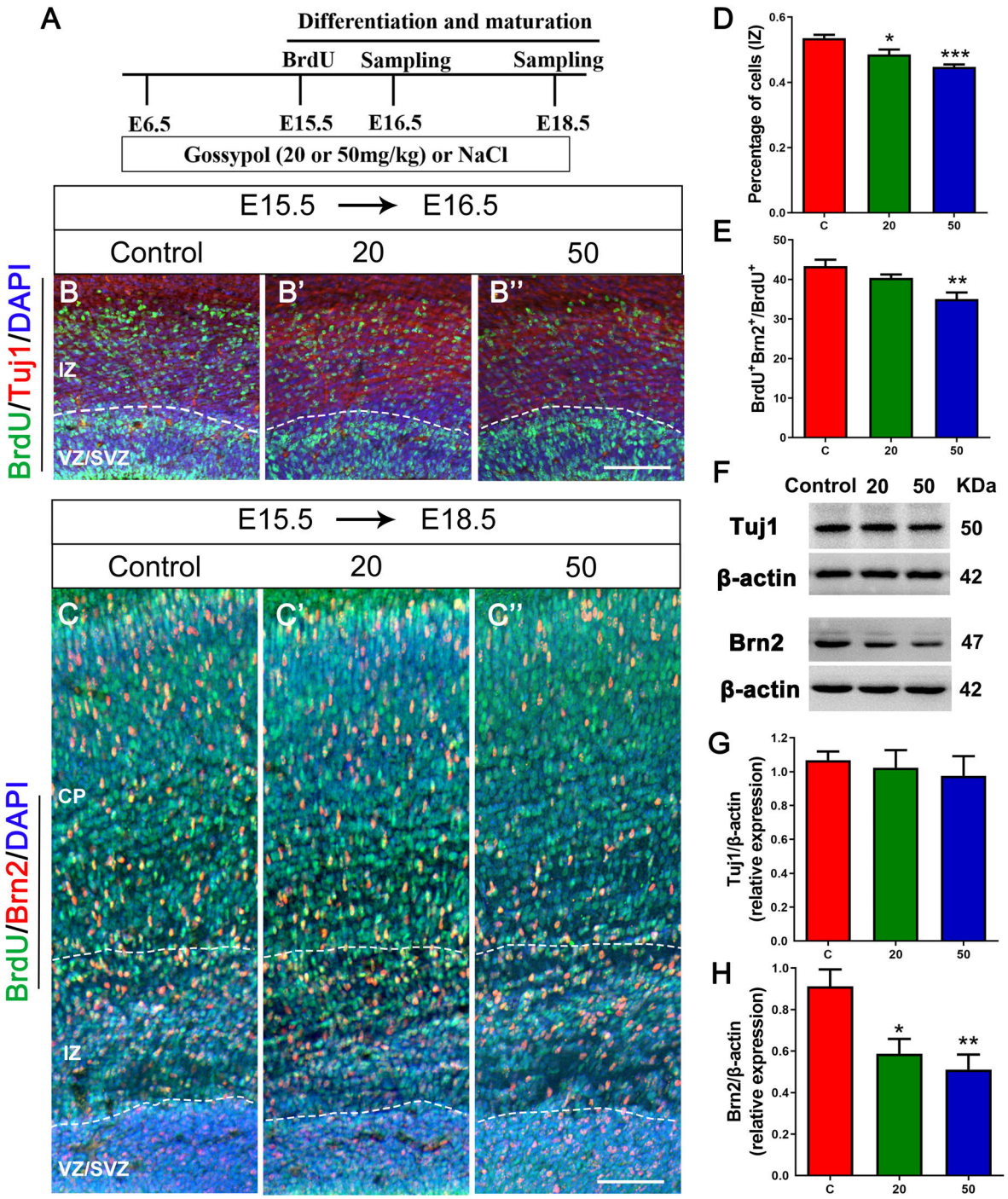


**FIGURE 5 |** Exposure to gossypol inhibited neuronal differentiation and maturation. **(A)** Protocol of BrdU injections for testing the neuronal differentiation and maturation. **(B)** Representative immunofluorescence images of immunostaining for BrdU at E16.5. A single pulse of BrdU was injected at E15.5 and fixed at E16.5. The brain slices were immunostained with antibody against BrdU (green) and counterstained with DAPI (blue). Tuj1 (red) was used to label the IZ of the cerebral cortex. **(B)** Representative immunofluorescence images of the $Brn2^+$ cells at E18.5. **(C)** A single pulse of BrdU was injected at E15.5 and fixed at E18.5. The brain slices were immunostained with antibody against BrdU (green) and Brn2 (red), and counterstained with DAPI (blue). **(D)** Quantification of BrdU labeling cells located in IZ. Less neurons entered the IZ in the gossypol exposure groups. **(E)** Quantification of $BrdU^+$ $Brn2^+$ positive cells in the total number of $BrdU^+$ cells. Less cells differentiated into neurons at E18.5 in gossypol exposure groups. **(F)** Representative Western blots of Tuj1, Brn2 and β-actin are shown and densitometry was used to quantify the protein levels in the developmental cerebral cortex. **(G,H)** Relative protein levels of Tuj1 **(G)** at E16.5 and Brn2 **(H)** at E18.5, the expression of Brn2 was decreased after the treatment of gossypol. Results are presented as the mean ± SEM ($n = 5$ per group). Scale bars 100 μm. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group. E, embryo; CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular zone/subventricular zone. Dashed lines indicate cortex boundaries.

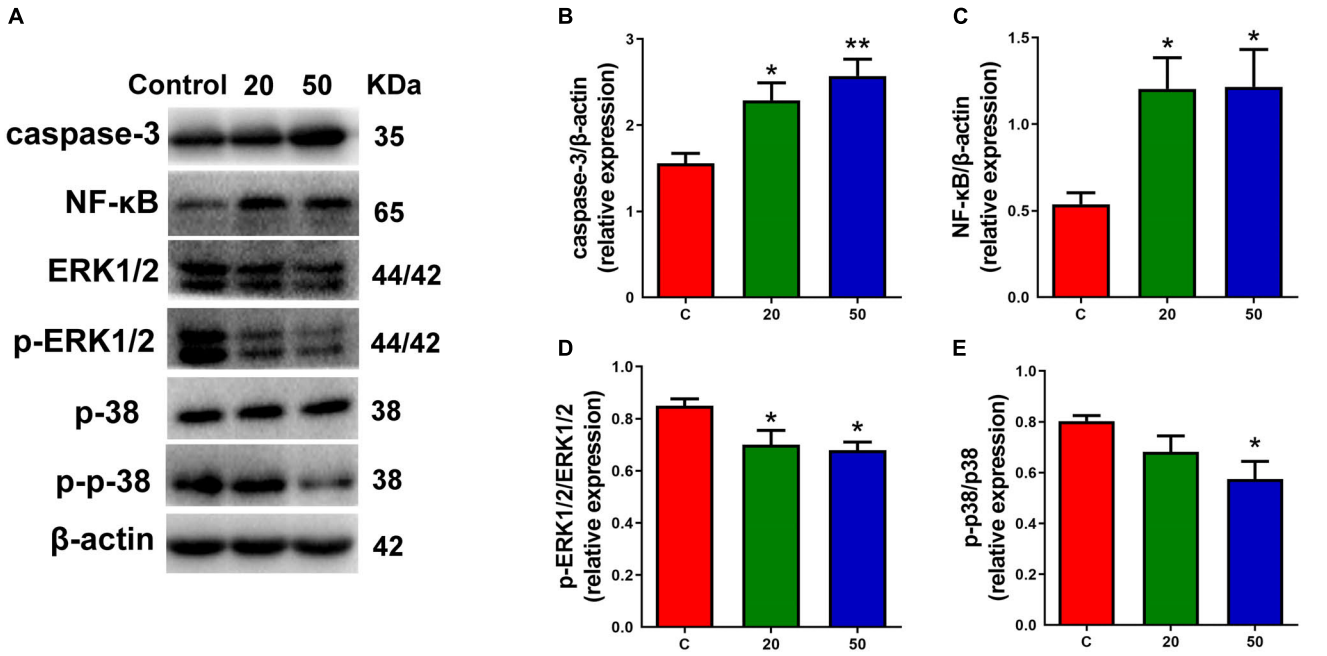


**FIGURE 6 |** Exposure to gossypol induced progenitor apoptotic cell death. **(A)** Representative Western blots of caspase-3, NF-κB, ERK, p-ERK, p38, p-p38 and β-actin are shown and densitometry was used to quantify the protein levels in the developmental cerebral cortex. **(B,C)** Relative protein levels of caspase-3 and NF-κB, the expressions of caspase-3 and NF-κB were increased after the treatment of gossypol. **(D,E)** Ratio of phosphorylation of ERK and ERK, and ratio of phosphorylation of p38 and p38. Exposure to gossypol decreased the phosphorylation of ERK and p38. Results are presented as the mean ± SEM ($n$ = 5 per group). *$p < 0.05$ and **$p < 0.01$ compared with the control group. p-ERK: phospho-ERK; p-p38: phospho-p38.

development by accelerating neuronal differentiation/migration (Nakamura et al., 2006; Komada et al., 2012). Other experiments revealed the impaired dendritic structure, cortical dysgenesis, and behavioral abnormalities following perinatal dioxin exposure (Haijima et al., 2010; Kakeyama et al., 2014; Kimura et al., 2015). In the present study, we found that gossypol disturbed the balance between proliferation and differentiation of NPCs. Although few $BrdU^+$ cells were detected, the findings could not confirm that gossypol exposure inhibited the proliferation of NPCs. To provide additional support, we performed pHH3 immunofluorescent staining and western blot, found decreased numbers of $pHH3^+$ cells and its expression in the gossypol group dosed at 50 mg/kg, indicating that gossypol exposure inhibited the mitotically active cells. Moreover, the cell cycle is a sequence of events by which a cell duplicates its genome, grows, and divides (Poon, 2016). In neural cells, proliferation and growth arrest are regulated by a balance of intrinsic and extrinsic factors that direct entry and exit from the cell cycle (Cunningham and Roussel, 2001; Dehay and Kennedy, 2007). Ki67 labels cells in the active phases of the cell cycle. Its expression within cells labeled with BrdU indicates that the cells are remaining in the active cell cycle (Wang et al., 2011). Previous study found significant increase of cell cycle exit in the bisphenol A treated mice (Komada et al., 2012). Interestingly, fewer cells remained in the active cell cycle in the gossypol-administrated group, suggesting that the inhibition of gossypol on NPCs cell cycle results not from remaining cells in the cell cycle, and rather from promoting cells to exit the cell cycle. The implication is that gossypol may shorten the duration of the cell cycle by inducing cell cycle exit. Thus, gossypol exposure inhibited neuronal proliferation, depending on the mitotic activity and the cell cycle of NPCs.

In addition to its inhibitory effect on NPCs proliferation, we demonstrated that gossypol could determine the fate of cortical neurons produced. The results showed that gossypol decreased the number of $BrdU^+$ positive cells in Tuj1 positive cells located in the IZ. Thus, gossypol reduced NPCs by inhibiting their proliferation and neuronal differentiation. This phenotype may be caused by developmental delay following exposure to gossypol. Furthermore, Munekazu Komada et al. (2012) reported that drug exposure affected estrogen signaling, resulting in defective neuronal maturation and migration (Komada et al., 2012). Gossypol has been shown to affect estrogen production by specifically interacting with a nucleophilic site on rat alpha-fetoprotein that influences estrogen binding (Baker, 1984; Basini et al., 2009). Estrogens have neurotrophic and differentiation-promoting effects on neurons, which are critical during the period of brain development (Toran-Allerand et al., 2002). By co-labeling BrdU and Brn2, we observed a decrease of $BrdU^+Brn2^+$ mature cells following maternal exposure to gossypol at a dose of 50 mg/kg. These results presumably reflect that gossypol may influence estrogenic activity through down-regulation of estrogenic signaling target gene expression in the central nervous system. Therefore, future studies should examine the signaling pathways for a better understanding of the underlying mechanism of gossypol neurotoxicity.

We rationalized that gossypol induced a significant increase in the number of cortical progenitors exiting the cell cycle, thereby implying that the number of cells increased as they underwent differentiation. However, the mature neurons were significantly decreased on E18.5. Notably, gossypol increased caspase-3 levels, which revealed that gossypol-induced apoptotic cell death led to a decrease in mature neurons. It has been reported that NF-κB pathway activation stimulates cellular production of proinflammatory cytokines and apoptotic factors, resulting in neurotoxicity to the brain (Yepes et al., 2005). Previous study found that treatment with 10 μM gossypol induced apoptosis, and suppressed NF-κB activity and NF-κB-related gene expression in human leukemia U937 cells, which suggested that NF-κB is an essential target for the apoptotic effects of gossypol (Moon et al., 2008). However, in the present study, the influence of gossypol on NF-κB levels is of interest in further research as this transcription factor is activated following exposure to gossypol and contributes to neuronal apoptotic cell death. These disparate results can be shown that the role of NF-κB under gossypol treatment is diverse in different models. Furthermore, it should be noted that ERK and p38 have been associated with many cellular responses, including cell survival, proliferation, differentiation and apoptosis (Raman et al., 2007; Burmistrova et al., 2011). Gossypol decreased the phosphorylation of p38 and biphasic phosphorylation of ERK, suggesting that ERK and p38 may be involved in survival signals following gossypol exposure. Although the mechanisms by which gossypol decreased the phosphorylation of ERK and p38 are unknown, a possible explanation could be that the production of inflammatory cytokines and apoptotic factors downregulated ERK and p38 activities. Thus, apoptotic cell death and neurogenesis in the presence of maternal stimulation is still poorly understood and requires in-depth research.

## CONCLUSION

Using *in utero* electroporation and immunofluorescence analysis, the present study, for the first time, provided the evidence for the effects of maternal oral exposure to gossypol on neurogenesis in the developing neocortex, apart from its role in steroidogenesis. Maternal exposure to gossypol was associated with the disruption of neuronal migration. Notably, gossypol inhibited NPCs proliferation and neuronal differentiation. It disrupted the cell cycle, which consequently impeded neuronal development. Gossypol may be associated with increased progenitor apoptotic cell death related to NF-κB and MAPK pathways. These findings indicate that gossypol, as an agricultural by-product, is potentially hazardous to fetal development. This study may help to address a previously less-touched perspective of gossypol. Future studies on brain development following *in utero* exposure to gossypol are needed to evaluate the pathological changes in the cerebral cortex and elucidate the potential mechanism of gossypol neurotoxicity.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

All experimental procedures concerning animal care and handing were conducted according to the guide lines for Care and Experimental of Laboratory Animals of Northwest A&F University. All animal experiments were reviewed and approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University (certificate NO.: SCXK [SHAAN] 2017-003) in accordance with ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

## AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. XZ and SZ conceived the project idea, performed the data analysis, and finalization of the manuscript. XZ, YW, and CL performed majority of the experiments, participated in the discussion and analysis of data. WY, JP, and SW conducted parts of the experiments, and participated in discussion and analysis of the data. SZ supervised the project. XZ is the corresponding author and SZ is the co-corresponding author of this manuscript.

## FUNDING

This work was financially supported by the National Natural Science Foundation of China (No. 31802154), the China Postdoctoral Science Foundation (No. 2018M631210), the Doctoral Scientific Research Foundation of Northwest A&F University (No. 2452017210), and the Key Research and Development Project of Shaanxi Province (No. 2018ZDXM2).

## ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 19 May 2020
doi: 10.3389/fnins.2020.00504

# Concurrent and Delayed Behavioral and Monoamine Alterations by Excessive Sucrose Intake in Juvenile Mice

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Our daily diet in the modern society has substantially changed from that in the ancient past. Consequently, new disorders associated with such dietary changes have emerged. For instance, excessive intake of compounds, such as sucrose (SUC), has recently been reported to induce pathological neuronal changes in adults, such as food addiction. It is still largely unclear whether and how excessive intake of such nutrients affects neurodevelopment. We investigated changes in behavior and monoamine signaling caused by excessive, semi-chronic intake of SUC and the non-caloric sweetener saccharin (SAC) in juvenile mice, using a battery of behavioral tests and high-performance liquid chromatography. Both SUC and SAC intake induced behavioral alterations such as altered amphetamine responses, sucrose preference, stress response, and anxiety, but did not affect social behavior and cognitive function such as attention in juvenile and adult mice. Moreover, SUC and SAC also altered dopamine and serotonin transmission in mesocorticolimbic regions. Some of these behavioral and neural alterations were triggered by SAC and SUC but others were distinct between the treatments. Moreover, alterations induced in juvenile mice were also different from those observed in adult mice. These results suggest that excessive SUC and SAC intake during the juvenile period may cause concurrent and delayed behavioral and monoamine signaling alterations in juvenile and adult mice, respectively.

**Keywords: neurodevelopment, monoamine, reward, stress, anxiety, sucrose**

OPEN ACCESS

***Edited by:***
*Kathleen C. Page, Bucknell University, United States*

***Reviewed by:***
*Gina L. Forster, University of Otago, New Zealand*
*Emmanuel N. Pothos, Tufts University School of Medicine, United States*

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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *29 January 2020*
***Accepted:*** *22 April 2020*
***Published:*** *19 May 2020*

***Citation:***
*Choe W-H, Lee K-A, Goto Y and Lee Y-A (2020) Concurrent and Delayed Behavioral and Monoamine Alterations by Excessive Sucrose Intake in Juvenile Mice. Front. Neurosci. 14:504. doi: 10.3389/fnins.2020.00504*

## INTRODUCTION

Several major components of our diets, such as sugar, fat, and caffeine, have been reported to induce addictive behavior. In modern society, food addiction is becoming a serious social problem, and although it has not yet been classified, food addiction is currently under debate for inclusion into the category of substance use disorder in further diagnostic manuals (Meule and Gearhardt, 2014). The prevalence of food addiction has increased exponentially since the 2000s (Randolph, 1956; Hebebrand et al., 2014; Meule, 2015). Moreover, food addiction could be more prevalent in the presence of other psychiatric disorders (Murphy et al., 2014; Cathelain et al., 2016).

Dopamine (DA) plays a central role in the brain's reward system. In particular, sucrose (SUC) consumption evokes DA release in the nucleus accumbens (NAcc) in normal mice

(Hajnal et al., 2004), whereas dopamine transporter (DAT) knockout mice exhibit decreased SUC preference (Cinque et al., 2018), suggesting that SUC is a robust modulator of the DA system. Moreover, excessive intake of nutrients such as SUC has been demonstrated to cause dysfunction of the DA reward system (Avena et al., 2008; Volkow et al., 2011). Serotonin (5-HT) is monoamine and is also involved in reward mechanisms, which may or may not be mediated through interaction with the DA system (Smolders et al., 2001; Müller and Homberg, 2015).

Accumulating evidence suggests that food addiction involves neural mechanisms that may substantially overlap with those causing drug addiction (Lenoir et al., 2007). Nevertheless, the mechanisms of food addiction have largely remained unclear. Cocaine and nicotine addiction induce structural alterations of neurons in the prefrontal cortex (PFC) and the NAcc (Robinson and Kolb, 2004), which could be associated with impairments of cognitive and affective function (Quello et al., 2005; Gould, 2010). However, whether food addiction also involves similar neuronal and behavioral alterations is mostly unexplored. In addition, since drug addiction is primarily a problem of adolescent and adult subjects, neurodevelopmental aspects have rarely been considered. Given that addictive diets such as SUC are consumed from a very early developmental stage, the impact of exposing humans to such addictive diets at early neurodevelopment may play a critical role.

Excessive intake of both caloric sweeteners, such as SUC, and non-caloric artificial sweeteners, such as saccharin (SAC) and aspartame, has been reported to induce addiction (Murray et al., 2016). Excessive aspartame consumption has been reported to cause irritable mood, depression, and to impair spatial memory (Lindseth et al., 2014). Long-term consumption of artificial sweeteners also disrupts passive avoidance learning memory (Erbaş et al., 2018). These studies raise the question of whether behavioral and neural changes associated with food addiction are induced by excessive intake of caloric nutrients or just by an excessive rewarding (sweet taste) experience.

In this study, we investigated the effects of excessive intake of SUC and SAC during the early neurodevelopmental period on cognitive, affective, and social functions and on DA/5-HT signaling in mesocorticolibmic brain regions of mice. We hypothesized that excessive intake of both SAC and SUC causes persistent behavioral and monoamine signaling alterations until adulthood and that such alterations may partly differ between SAC and SUC.

# MATERIALS AND METHODS

## Animals

All animal experiments were conducted in accordance with the Research Ethics Policy of the Korean Association of Laboratory Animal Science and were approved by the Institutional Animal Care and Use Committee of Daegu Catholic University. Pregnant female ICR mice at gestational day 14 were individually housed. Male pups were weaned from dams at postnatal day (PD) 21 and housed in cages of four to five mice per cage for subsequent experiments. To avoid (to minimize) litter effects, a number of mice born from each dam was limited to 3 offspring. Moreover, these littermates were separated at birth and raised by different dams.

## SUC and SAC Treatments in Juvenile Mice

Starting at PD21, animals were assigned into three groups. The first group of animals with control (CTR) treatment received only tap water. Animals in the second and third groups received 20% SUC or 0.2% SAC solution, respectively. These solutions were available *ad libitum* until PD35. All animals received tap water after PD36 (**Figure 1A**). The concentrations of SAC and SUC solutions were based on previous studies (Feijó et al., 2013; Bissonnette et al., 2017). SUC and SAC treatments during the juvenile period resulted in no changes in body weight gain compared to CTR treatment (**Figure 1B**).

## Behavioral Tests

All behavioral tests were conducted during the juvenile period (PD28–35; **Figure 1A**) and adulthood (PD56 or older; **Figure 1A**).

### Locomotion Test With Amphetamine Treatment

The DA response was examined with locomotion tests after amphetamine (AMP) administration (Salahpour et al., 2008). Animals were placed in an open-field chamber and the locomotor distance that animals traveled during 20 min was measured, either after AMP or saline administration. AMP at doses of 1, 2, and 3 mg/kg dissolved in 0.1 mL saline and the equivalent volume

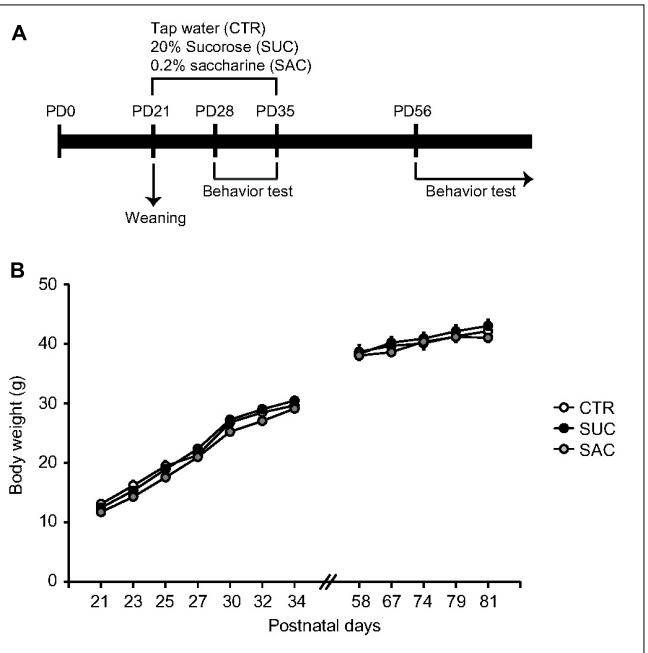


**FIGURE 1 |** Experimental schedule and body weight gain. **(A)** Schematic diagram illustrating the timeframe of experiments. **(B)** Graph showing body weight gain of animals with vehicle (CTR), 20% sucrose (SUC), and 0.2% saccharine (SAC) treatments. Error bars indicate SEM.

of saline was given intraperitoneal repeatedly to the same animals once per day over 4 days. These injections were administered 10 min before the animal was subjected to the locomotion test.

### SUC Preference Test

A SUC preference test was conducted to examine whether SUC and SAC treatments affected reward sensitivity (Hajnal et al., 2004). During this test, animals were individually housed in cages with two water bottles: one with tap water and the other with SUC solution. Different concentrations of SUC (0, 0.25, 0.5, 1.0, 2.0, and 5.0%) were offered to the animals on each day. The amount of SUC solution and tap water intake was measured. SUC solution and tap water were offered for 16 h (17:00–09:00) at each SUC concentration condition. The preference to SUC over tap water was expressed as the preference ratio, which was the amount of SUC solution consumption compared to tap water consumption.

### Forced Swimming Test

A forced swimming test was conducted to examine responsiveness to stress (Yankelevitch-Yahav et al., 2015). We utilized the forced swimming test over other test such as Morris water maze test, as the aim of conducting this study was not focused on spatial learning and memory, but sorely stress response. A glass cylinder (50 cm height × 11 cm diameter) was filled with tap water at 23 ± 1°C (35 cm from the bottom). Animals were placed into the glass-cylinder for 10 min and the total duration of immobility in the water was measured.

### Elevated Plus Maze Test

An elevated plus maze test was conducted to examine anxiety of animals (Walf and Frye, 2007). The elevated plus maze consisted of two diagonal arms without side walls (open arms) and two arms with walls perpendicular to the open arms (enclosed arms). Animals were placed in the center area of the maze and allowed to freely enter the arms for 10 min. Duration and number of entries in the open and enclosed arms were measured.

### Object Exploration Test

An object exploration test that we have developed previously (Kim et al., 2018) was conducted to examine attention. In this test, four objects with different features were placed in an open-field chamber. Animals were then placed in the chamber and allowed to explore these objects freely for 10 min. Their exploratory behaviors (touching, sniffing, and riding) over the objects were considered as animals paid attention to the objects, allowing us to measure the time spent to explore these objects.

### Social Interaction Test

A social interaction test was conducted to examine sociality of animals (Innos et al., 2011). In this test, an animal was placed in the open-field chamber with another, age-matched, unfamiliar animal. Duration and frequency of demonstrated social behaviors such as sniffing and chasing were measured for 10 min. In cases where one of the animals showed aggressive attacks, such as biting and jumping, the experiment was stopped, and a new experiment was conducted on another day.

## High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was conducted to investigate tissue concentrations of DA and 5-HT and their metabolites in mesocorticolimbic brain regions. Animals were euthanized by administration of an overdose of sodium pentobarbital [100 mg/kg, intraperitoneal (i.p.)] and zoletil (60 mg/kg, i.p.), followed by decapitation. The brains were removed and cut into coronal sections according to the stereotaxic coordinates of the atlas (Franklin and Paxinos, 2008). Then tissue samples from the medial PFC, the dorsal striatum (dSTR), the NAcc, the basolateral and lateral nuclei of the amygdala (AMY), the dorsal part of the hippocampus (HPC), and the ventral tegmental area (VTA) were obtained using disposable biopsy punch (diameter 1.0 mm, Kai Industries, Co., Ltd., Japan). The tissue samples were processed as described previously (Lee et al., 2018; Jeon et al., 2019). Quantification of DA, 5-HT, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) was conducted according to the formula $(ISO_{std}/TA_{std})\ x\ (TA_{spl}/ISO_{spl})\ x\ A\ x\ (1/B)$, where $ISO_{std}$ and $TA_{std}$ are areas under the curves of the peaks in chromatograms for isoproterenol (ISO) added in standard and sample solutions as the internal standard marker) and a target substance in the standard solution, respectively. $ISO_{spl}$ and $TA_{spl}$ are areas under the curves of the peaks for ISO and target substances in the sample solution, respectively. *'A'* is the amount of ISO added to sample solution. *'B'* is the amount of tissue proteins in sample solution.

## Immunostaining

Fluorescent immunostaining was conducted to examine DAT and serotonin transporter (SERT) expression in mesocorticolimbic regions. An overdose of sodium pentobarbital (100 mg/kg, i.p.) and zoletil (60 mg/kg, i.p.) was administered to animals and once no response to tail pinches was confirmed, the abdomen was surgically opened. Then, transcardiac perfusion of phosphate buffer saline (PBS) and 4% paraformaldehyde was conducted to remove the blood and fix the brain. After perfusion, the brains were removed and immersed in 4% paraformaldehyde, followed by 30% SUC solution in PBS for anti-freezing protection. After these procedures, the brains were cut into slices of 30 μm thickness using a microtome and placed on gelatin-coated slide glasses. The slides were washed with 0.3% Triton X in PBS and incubated with DAT (Catalog #: sc14002, Rabbit polyclonal, dilution at 1:300, Santa Cruz Biotechnology) and SERT (Catalog #: sc33724, Mouse monoclonal, dilution at 1:300, Santa Cruz Biotechnology) antibodies with 10% normal goat serum diluted in 0.3% Triton X in PBS at 4°C for 24 h. Afterward, the slides were incubated with Alexa Fluor 488 goat anti-mouse IgG (H+L) (Catalog #: A-11001, dilution at 1:300, Thermo Fisher Scientific) and Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Catalog #: A-11012, dilution at 1:300, Thermo Fisher Scientific) secondary antibodies for DAT and SERT, respectively, diluted in 0.3% Triton X in PBS at room temperature for 2 h. The slides were covered with cover glasses with mounting medium. Fluorescence intensity was captured with the fluorescence

microscope (DM2500, Leica), and images were analyzed using ImageJ (version 1.51j8). The images were converted into 8-bit grayscale, and then intensity was quantified under the threshold set equally applied to all images. As a control for these specific expressions, shadow effects of primary antibodies were also examined, and illustrated in **Supplementary Figure S1**.

## Data Analysis

All data are expressed as means ± SEM $p < 0.05$ was considered statistically significant different. Statistical analysis for comparisons among CTR, SUC, and SAC groups was conducted using analysis of variance (ANOVA), with *post hoc* Turkey test for pair-wise comparisons.

# RESULTS

## Alterations of the AMP Response

Whether juvenile SUC and SAC intake affects the monoamine system was examined via AMP modulation of locomotion in the open field chamber (**Figure 2A**). When tested in juvenile mice (PD28–35), CTR, SUC, and SAC animals exhibited no difference in locomotor distance with SAL and 1 and 2 mg/kg AMP administration. However, locomotor distance in SAC animals with 3 mg/kg AMP administration was lower than in CTR animals (two-way ANOVA with repeated measures; $F_{2,180} = 8.905$, $p < 0.001$ for groups; $F_{3,180} = 106.423$, $p < 0.001$ for AMP; $F_{6,180} = 5.353$, $p < 0.001$ for interaction; *post hoc* Tukey test; $p = 0.021$, CTR vs. SAC). In adulthood (PD56 or older), shifts of the dose-response curves were observed, for example of the AMP response in SUC animals toward higher AMP sensitivity along with a significantly higher locomotor distance than in CTR animals with 1 mg/kg AMP ($F_{2,140} = 39.132$, $p < 0.001$ for groups; $F_{3,140} = 42.540$, $p < 0.001$ for AMP; $F_{6,140} = 2.897$, $p = 0.011$ for interaction; $p = 0.010$, CTR vs. SUC). In contrast, the AMP response in SAC animals was shifted toward more blunted sensitivity along with a significantly lower locomotor distance than in CTR animals with 3 mg/kg AMP ($p = 0.021$, SAC vs. CTR). These results suggest that both SAC and SUC treatments may cause alterations in AMP responses that persist up until adulthood. However, these alterations differed between SAC and SUC.

## Alterations of SUC Preference

Juvenile CTR animals exhibited higher SUC preference over increasing SUC concentrations (**Figure 2B**). Two-way ANOVA with repeated measures revealed significant differences in the interaction between groups and SUC concentrations ($F_{2,148} = 11.484$, $p < 0.001$ for groups; $F_{4,148} = 18.526$, $p < 0.001$ for SUC concentrations; $F_{8,148} = 3.578$, $p < 0.001$ for interaction). *Post hoc* analysis revealed that SUC preference in CTR animals was higher at 2% ($p = 0.018$) and 5% ($p < 0.001$) SUC than at 0.25%. This increase was not observed in SUC and SAC mice, resulting in significantly lower SUC preference than in CTR mice at 5% SUC ($p = 0.006$, CTR vs. SUC; $p = 0.025$, CTR vs. SAC). In contrast, no difference in SUC preference was observed between adult CTR, SUC, and SAC animals. Some of the mice were already sucrose-exposed prior to the sucrose test, such that a novelty aspect of the sucrose might be unequal among the groups. However, as shown in **Figure 2B**, our results indicate that differences were prominent at higher doses of sucrose solution, which were the conditions examined after animals had already experienced lower doses. Thus, such difference suggest that a novelty might be less likely involved, although the possibility that a novelty to the sucrose was still influencing its preference cannot be excluded. These results suggest that both SUC and SAC treatments may cause a similar, transient SUC preference change in juvenile mice.

## Alterations of Stress Response

The forced swimming test was conducted to evaluate stress responses (**Figure 2C**). In juvenile mice, SUC animals exhibited longer immobility than CTR and SAC animals (one-way ANOVA; $F_{2,25} = 7.825$, $p = 0.002$; $p = 0.017$, CTR vs. SUC; $p = 0.004$, SUC vs. SAC). In contrast, no difference was observed in adult CTR, SUC, and SAC animals. These results suggest that SUC, but not SAC, intake may cause a transient alteration of stress response in juvenile mice.

## Alterations of Anxiety

The elevated plus maze test was conducted to evaluate anxiety (**Figure 2D**). Juvenile SAC animals stayed in the open arms longer than CTR and SUC animals ($F_{2,39} = 9.604$, $p < 0.001$; $p = 0.004$, CTR vs. SAC; $p = 0.001$, SUC vs. SAC). Moreover, juvenile SAC animals entered into the enclosed more often than and SUC ($F_{2,39} = 5.189$, $p = 0.010$; $p = 0.007$, SUC vs. SAC). When tested in adulthood, SUC animals spent less time in the open arms than SAC animals ($F_{2,35} = 4.410$, $p = 0.020$; $p = 0.030$, SUC vs. SAC; $p = 0.051$, CTR vs. SUC). Moreover, SAC animals entered into the enclosed arm more often than SUC and CTR animals ($F_{2,35} = 4.515$, $p = 0.018$; $p = 0.037$, CTR vs. SAC; $p = 0.034$, SUC vs. SAC). These results suggest that both SAC and SUC treatments may cause alterations of anxiety but in a distinct manner across ages.

## No Alteration of Attention and Social Behavior

In the object exploration test to examine attention (**Figure 2E**) and the social interaction test (**Figure 2F**), no difference in time spent on object exploration per visit and time spent on social interaction was observed in juvenile and adult CTR, SUC, and SAC animals.

## Alterations of DA and 5-HT

To understand the neural mechanisms that may be associated with the observed behavioral alterations, changes in DA and 5-HT levels in the PFC, dSTR, NAcc, HPC, AMY, and VTA of juvenile and adult animals were examined.

In juvenile mice, DA levels were lower in the dSTR of SUC and SAC animals than of CTR animals ($F_{2,15} = 73.415$, $p < 0.001$; $p < 0.001$, CTR vs. SUC; $p < 0.001$, CTR vs. SAC; $p < 0.001$, SUC vs. SAC; **Figure 3A**). In the NAcc, the DA concentration was also lower in SAC animals than in CTR and SUC animals

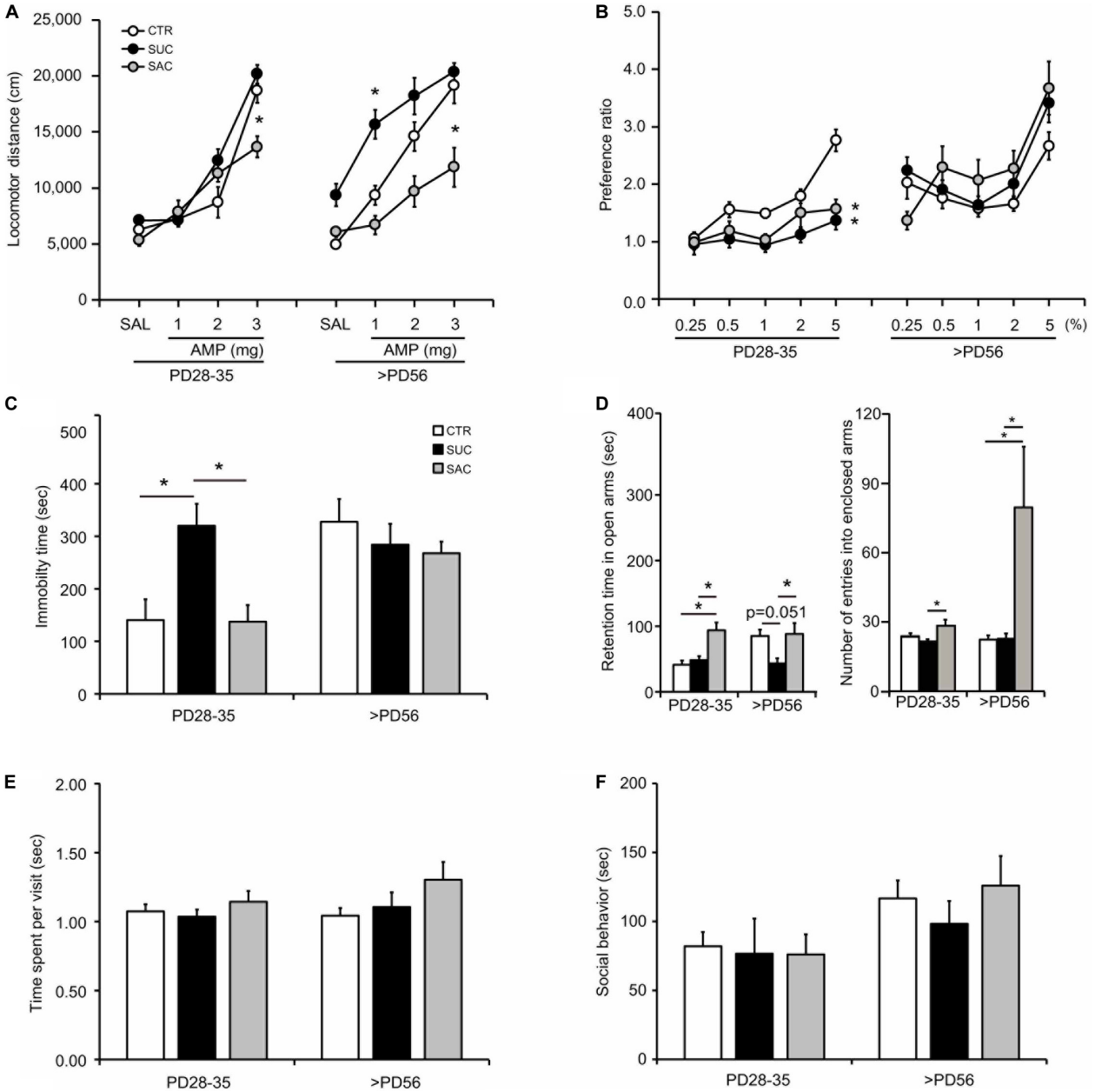


**FIGURE 2 |** Behavioral alterations in animals subjected to sucrose and saccharine treatments. **(A)** Graph showing amphetamine (AMP) modulation of locomotion in juvenile (PD28–35) and adult (>PD56) animals. **(B)** Graph showing sucrose preference expressed as the ratio of sucrose solution intake over tap water intake in the sucrose preference test. **(C)** Graph showing the duration of immobility in the forced swimming test. **(D)** Graph showing the time spent in the open arms (left) and the number of entries into the enclosed arms (right) in the elevated plus maze. **(E)** Graph showing the time spent for object exploration per visit in the object exploration test. **(F)** Graph showing the time spent on social interaction with a mate in the social interaction test. *$p < 0.05$.

($F_{2,15} = 23.573$, $p < 0.001$; $p < 0.001$, CTR vs. SAC; $p < 0.001$, SUC vs. SAC; **Figure 3A**). In addition, the 5-HT concentration in the dSTR of SUC and SAC animals was lower than in CTR animals ($F_{2,15} = 32.473$, $p < 0.001$ for groups; $p = 0.003$, CTR vs. SUC; $p < 0.001$, CTR vs. SAC; $p = 0.003$, SUC vs. SAC; **Figure 3C**). The 5-HT concentration in the NAcc was also lower in SAC animals than in CTR animals ($F_{2,15} = 5.750$, $p = 0.014$ for groups; $p = 0.013$, CTR vs. SAC, **Figure 3C**).

In adulthood, DA was higher in the NAcc of SUC animals than in CTR ($F_{2,15} = 6.130$, $p = 0.011$; $p = 0.022$) and SAC ($p = 0.021$) animals (**Figure 3B**). In the HPC, DA in SAC animals was higher than in CTR ($F_{2,15} = 9.430$, $p = 0.002$; $p = 0.034$) and SUC ($p = 0.002$) animals (**Figure 3B**). In the AMY, SUC and SAC animals exhibited lower DA than CTR animals ($F_{2,15} = 5.726$, $p = 0.014$; $p = 0.025$, CTR vs. SUC; $p = 0.028$, CTR vs. SAC; **Figure 3B**), whereas this was opposite in the VTA. DA in SUC

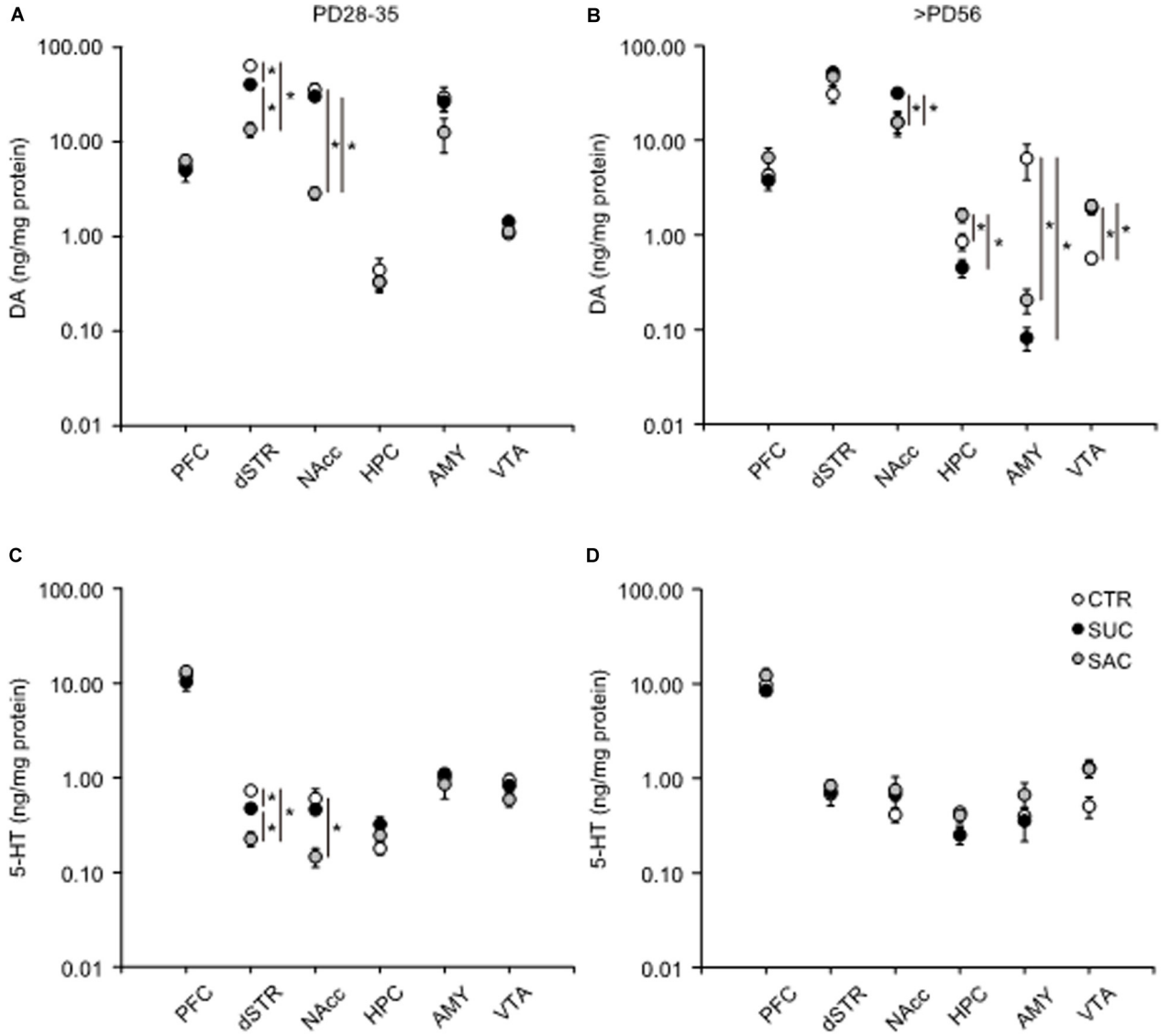


**FIGURE 3 |** Alterations of tissue DA and 5-HT concentrations in mesocorticolimbic regions of animals subjected to sucrose and saccharine treatments. Graphs showing tissue DA concentrations in the PFC, dSTR, NAcc, HPC, AMY, and VTA of juvenile **(A)** and adult **(B)** animals. Graphs similar to a and b but showing tissue 5-HT concentrations in juvenile **(C)** and adult **(D)** animals. *$p < 0.05$.

and SAC animals was higher than in CTR animals ($F_{2,15} = 11.616$, $p < 0.001$; $p = 0.003$, CTR vs. SUC; $p = 0.002$, CTR vs. SAC, **Figure 3B**). In contrast, 5-HT did not differ between groups, except the VTA where an overall group difference, but no pair-wise difference, was observed ($F_{2,15} = 3.802$, $p = 0.046$ in the VTA; **Figure 3D**).

DA and 5-HT metabolisms were further analyzed and the ratio of DOPAC/DA, HVA/DA and HIAA/5-HT, which are thought to reflect synaptic release of DA and 5-HT (Best et al., 2010; Meiser et al., 2013), were examined. In juvenile mice, the DOAPC/DA ratio was higher in the dSTR ($F_{2,15} = 6.924$, $p = 0.007$; $p = 0.009$, CTR vs. SAC; $p = 0.030$, SUC vs. SAC) and the NAcc ($F_{2,15} = 43.310$, $p < 0.001$; $p < 0.001$, CTR vs. SAC; $p < 0.001$, SUC vs. SAC) of SAC animals than in CTR and SUC animals (**Figure 4A**). Consistent with such alterations, the HVA/DA ratio was also higher in the dSTR ($F_{2,15} = 8.944$, $p = 0.003$; $p = 0.004$, CTR vs. SAC; $p = 0.010$, SUC vs. SAC) and the NAcc ($F_{2,15} = 36.042$, $p < 0.001$; $p < 0.001$, CTR vs. SAC, $p < 0.001$, SUC vs. SAC) of SAC animals than in CTR and SUC animals (**Figure 4C**). Similarly, the 5-HIAA/5-HT ratio was higher in the dSTR ($F_{2,15} = 30.347$, $p < 0.001$; $p < 0.001$ for CTR vs. SAC, $p < 0.001$ for SUC vs. SAC) and the NAcc ($F_{2,15} = 28.199$, $p < 0.001$; $p < 0.001$ for CTR vs. SAC, $p < 0.001$ for SUC vs. SAC) of SAC animals than in CTR and SUC animals (**Figure 4E**).

In adulthood, an overall group difference, but no pair-wise difference, of the DOPAC/DA ratio was found in the PFC ($F_{2,15} = 3.785$, $p = 0.047$; **Figure 4B**). In the HPC, the DOPAC/DA

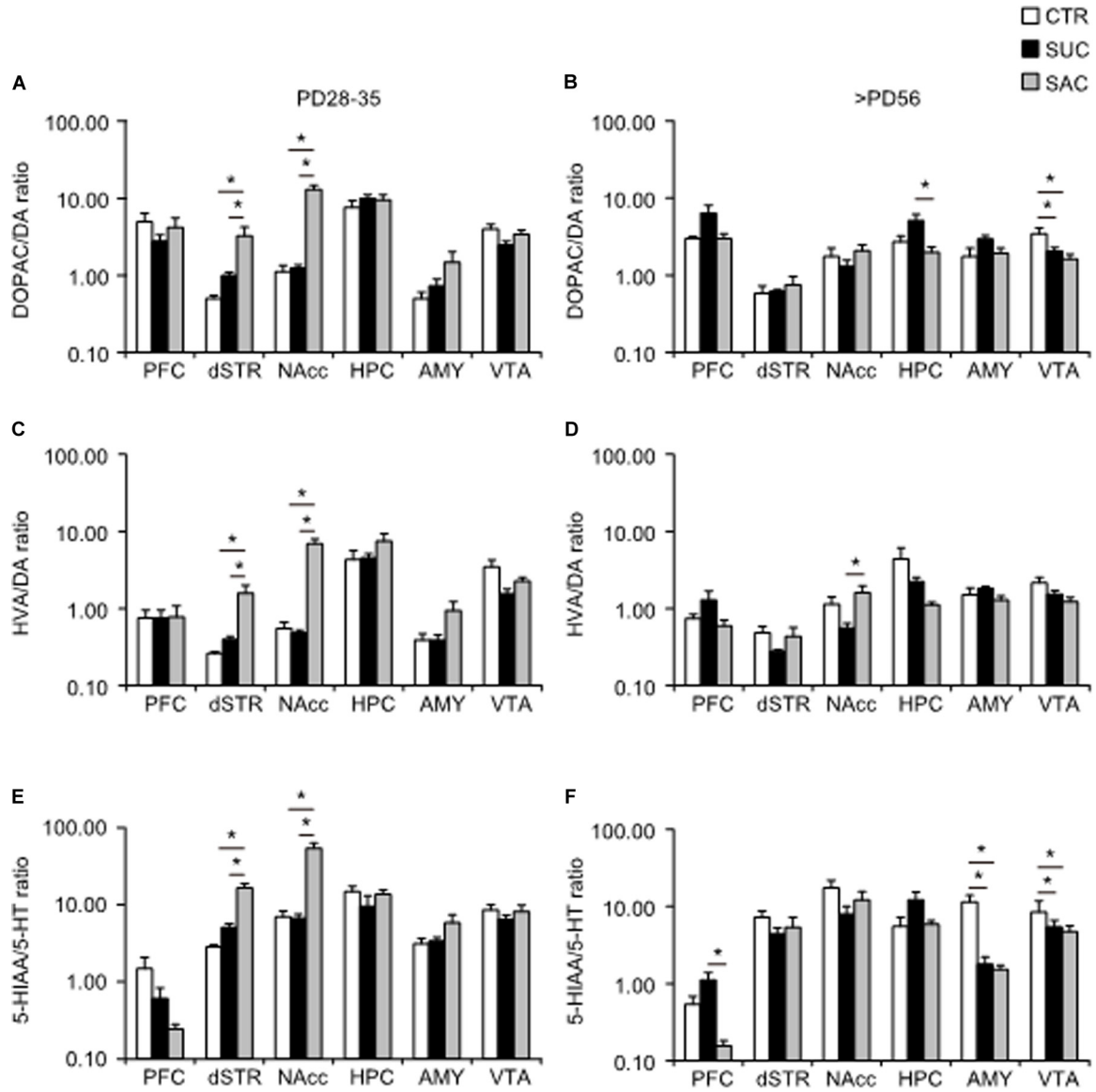


**FIGURE 4 |** Alterations of ratios between DA/5-HT and their metabolites in mesocorticolimbic regions of animals subjected to sucrose and saccharine treatments. Graphs showing the DOPAC/DA ratio in the PFC, dSTR, vSTR, HPC, AMY, and VTA of juvenile **(A)** and adult **(B)** animals. Graphs similar to a and b but showing HVA/DA ratio in juvenile **(C)** and adult **(D)** animals. Graphs similar to a and b but showing 5-HIAA/5-HT ratio in juvenile **(E)** and adult **(F)** animals. *$p < 0.05$.

ratio was higher in SUC animals than in SAC but not CTR animals ($F_{2,15} = 5.916$, $p = 0.013$; $p = 0.013$, SUC vs. SAC; **Figure 4B**). The DOPAC/DA ratio in the VTA of SUC and SAC animals was significantly lower than in CTR animals ($F_{2,15} = 29.069$, $p < 0.001$; $p < 0.001$, CTR vs. SUC; $p < 0.001$, CTR vs. SAC; **Figure 4B**). The HVA/DA ratio in the NAcc of SUC animals was also lower than in SAC but not CTR animals ($p = 0.048$; **Figure 4D**). The 5-HIAA/5-HT ratio in the AMY ($F_{2,15} = 16.237$, $p < 0.001$; $p = 0.001$, CTR vs. SUC; $p = 0.001$, CTR vs. SAC) and the VTA ($F_{2,15} = 5.280$, $p = 0.018$; $p = 0.041$, CTR vs. SUC; $p = 0.027$, CTR vs. SAC) of both SUC and SAC animals was lower than in CTR animals (**Figure 4F**), whereas the 5-HIAA/5-HT ratio in the PFC of SAC animals was lower than in SUC but not CTR animals ($F_{2,15} = 6.721$, $p = 0.008$; $p = 0.007$; **Figure 4F**).

These results suggest that excessive juvenile SUC and SAC intake may induce distinct patterns of immediate and persistent alterations in mesocorticolimbic DA and 5-HT transmission.

## Alterations of DAT and SERT Expression

Alterations of tissue DA and 5-HT levels and their metabolites suggested that synaptic DA and 5-HT release mechanisms may be affected by excessive SUC and SAC intake in juvenile mice. Thus, we also investigated DAT and SERT expression levels in mesocorticolimbic regions of juvenile and adult animals.

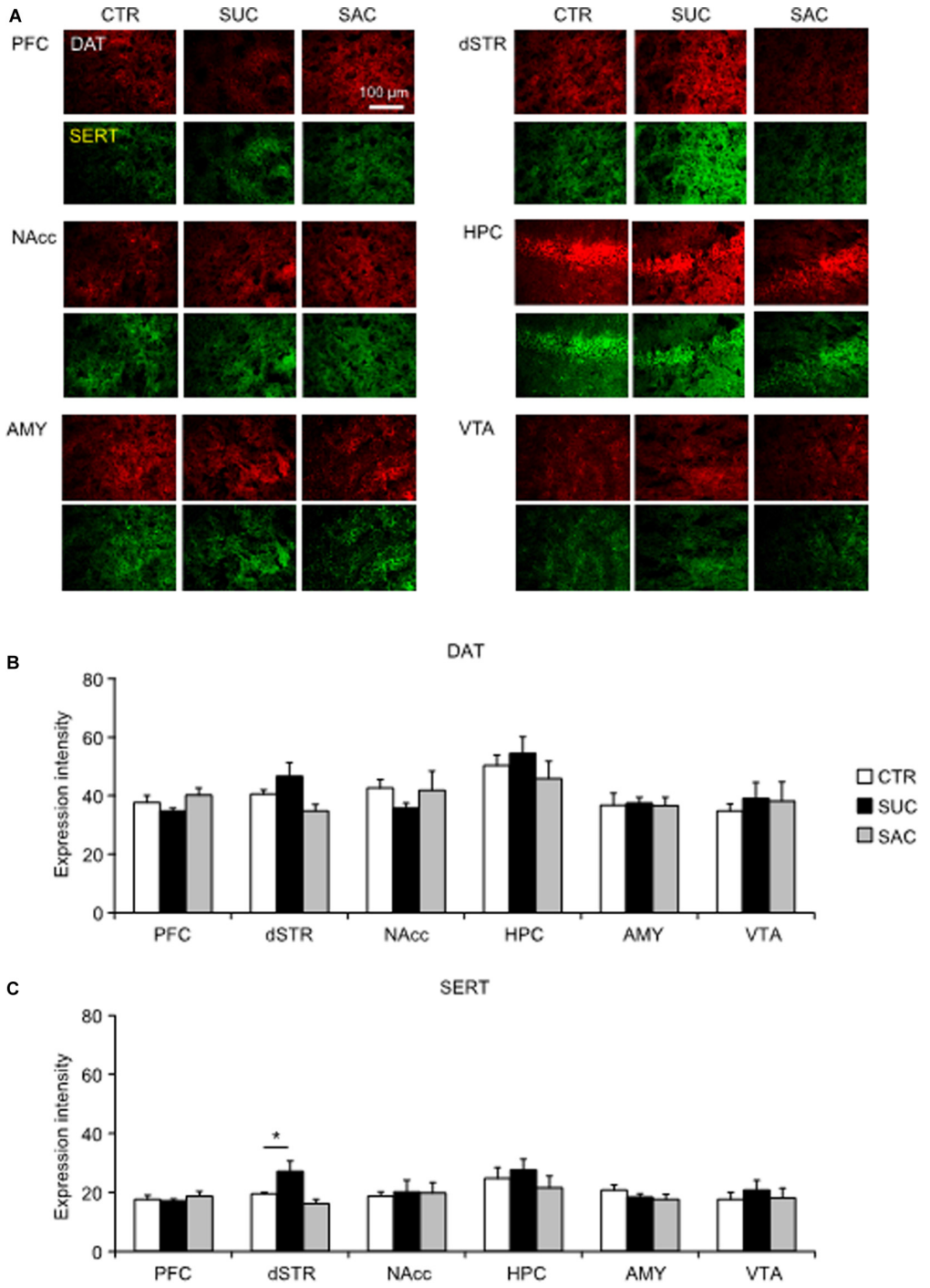


**FIGURE 5 |** Alterations of dopamine and serotonin transporter expression levels in mesocorticolimbic regions of juvenile animals subjected to sucrose and saccharine treatments. **(A)** Representative images of DAT (red) and SERT (green) expression levels in mesocorticolimbic regions of juvenile animals. Graph showing fluorescence intensities of DAT **(B)** and SERT **(C)** expression in juvenile animals. *$p < 0.05$.

In juvenile mice, DAT and SERT expression of SUC and SAC animals did not differ from those in CTR animals (**Figures 5A–C**). The only difference was a higher SERT expression in the dSTR of SUC animals compared to CTR animals ($F_{2,13} = 5.410$, $p = 0.020$; $p = 0.019$; **Figure 5C**).

In adulthood, significant DAT and SERT expression changes were observed (**Figures 6A–C**). In the PFC and the dSTR, both SUC and SAC animals exhibited significantly higher DAT expression levels than CTR animals ($F_{2,15} = 12.211$, $p < 0.001$ for the PFC; $p = 0.002$ for CTR vs. SUC, $p = 0.002$ for CTR vs. SAC;

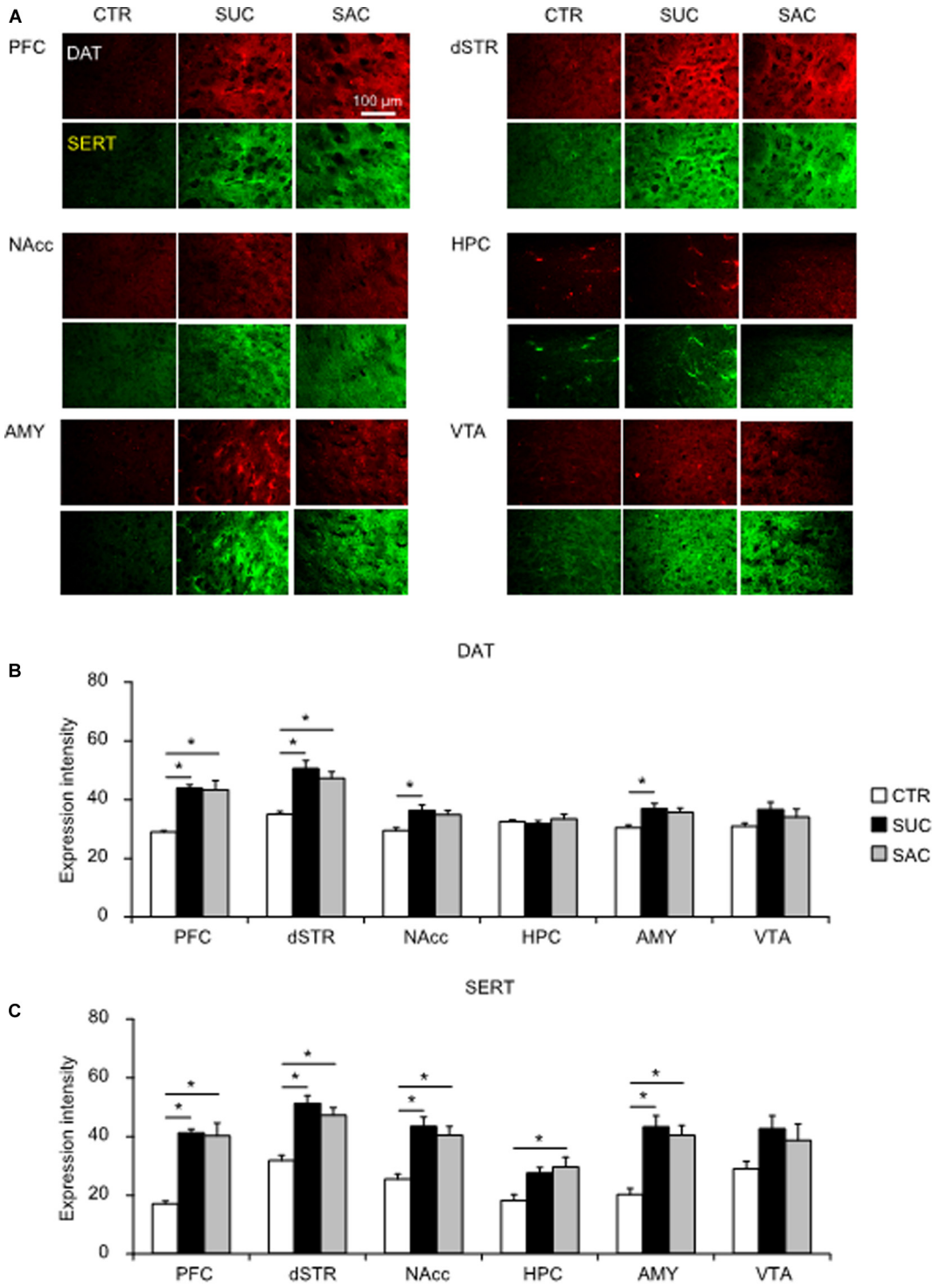


**FIGURE 6 |** Alterations of dopamine and serotonin transporter expression levels in mesocorticolimbic regions of adult animals subjected to sucrose and saccharine treatments. **(A)** Representative images of DAT (red) and SERT (green) expression in mesocorticolimbic regions of adult animals. Graph showing fluorescence intensities of DAT **(B)** and SERT **(C)** expression in adult animals. *$p < 0.05$.

$F_{2,15} = 11.682$, $p = 0.001$ for the dSTR; $p = 0.001$ for CTR vs. SUC, $p = 0.005$ for CTR vs. SAC; **Figure 6B**). In the NAcc ($F_{2,15} = 5.303$, $p = 0.018$; $p = 0.018$ for SUC vs. CTR) and the AMY ($F_{2,14} = 5.000$, $p = 0.023$; $p = 0.023$ for SUC vs. CTR), DAT expression levels were higher in SUC animals than in CTR animals (**Figure 6B**). Group differences of SERT expression levels were observed in all but

the VTA ($F_{2,15} = 16.893$, $p < 0.001$ for the PFC; $F_{2,15} = 15.570$, $p < 0.001$ for the dSTR; $F_{2,15} = 10.323$, $p = 0.002$ for the NAcc; $F_{2,14} = 5.367$, $p = 0.019$ for the HPC; $F_{2,14} = 13.475$, $p = 0.001$ for the AMY; **Figure 6C**). In these areas, SERT expression was higher in SUC and SAC animals than in CTR animals ($p < 0.001$ for CTR vs. SUC, $p < 0.001$ for CTR vs. SAC in the PFC; $p < 0.001$ for CTR vs. SUC, $p = 0.002$ for CTR vs. SAC in the dSTR; $p = 0.002$ for CTR vs. SUC, $p = 0.006$ for CTR vs. SAC in the NAcc; $p = 0.001$ for CTR vs. SUC, $p = 0.002$ for CTR vs. SAC in the AMY; **Figure 6C**), although in the HPC, SERT expression was only higher in SAC animals compared to CTR animals ($p = 0.020$ for CTR vs. SAC in the HPC; **Figure 6C**).

These results suggest that excessive SUC and SAC intake may induce distinct and delayed alterations of DAT and SERT expression levels in mesocorticolimbic regions in adulthood.

## DISCUSSION

In this study, we found that excessive SUC and SAC intake during the juvenile period in rodents induced behavioral and neural alterations, some of which were already apparent in juvenile mice while others became apparent when the animals reached adulthood. The immediate alterations in juvenile mice and delayed alterations in adult mice were different. Moreover, some of the alterations induced by SUC and SAC treatments were common while others differed between SUC and SAC.

This study had two aims. First, we examined how excessive SUC and SAC intake during the juvenile period affected behavioral and neural alterations during development and whether excessive SUC and SAC intake affected behaviors and neural mechanisms not only in juvenile but also in adult animals. Previous studies in adult animals have shown that excessive SUC intake abnormally increases DA release in the NAcc, which is more prominent in the core than the shell regions. Such alteration is similar to that caused by excessive ethanol intake (Kranz et al., 2010; Bassareo et al., 2015). Excessive SUC intake also promotes abnormal 5-HT release in the HPC (Smolders et al., 2001). However, consistent with studies in adult animals, we found that excessive SUC and SAC intake in juvenile mice also altered DA and 5-HT transmission and associated behaviors not only in juvenile but also in adult animals (**Supplementary Tables S1–S3**). On the other hand, SUC preference was decreased both in SUC and SAC animals during the juvenile period, but not adulthood. Moreover, juvenile but not adult SUC animals exhibited greater vulnerability to stress, suggesting that some behavioral alterations were induced immediately and transiently. In contrast, alterations in AMP modulation of locomotion were observed both in juvenile and adult mice. Importantly, such alterations observed both in juvenile and adult mice were not identical, suggesting that the alterations observed in adulthood are not merely a consequence of excessive SUC and SAC intake during the juvenile period, but become apparent by interaction between juvenile alterations and maturation during adolescence. Consistent with these behavioral alterations, DAT and SERT expression levels in mesocorticolimbic regions were not changed in juvenile SUC and SAC animals, whereas these molecules were significantly up-regulated in several mesocorticolimbic regions of adult animals. In this regard, it is interesting to note that DAT and SERT expression levels were not modulated by SAC and SUC in juvenile animals. Moreover, in adult animals, DAT and SERT expression levels were altered mostly in the regions where tissue DA and 5-HT concentrations and their metabolites were not altered. In contrast, in other regions where DAT and SERT expression levels were not altered, alterations of tissue DA and 5-HT concentrations and their metabolites were observed. These results suggest that up-regulation of DAT and SERT expression in adult animals that had excessive SUC and SAC intake during the juvenile period could be a compensatory action of altered DA and 5-HT transmission.

Several studies have established the role of the intrauterine environment and immediate postnatal period in inducing central DA deficiencies in obesity-prone animals (Geiger et al., 2008). Indeed, the effects of SUC and SAC exposure during an early developmental phase like gestation and weaning would yield substantial impacts on the monoamine systems. In contrast, alterations of monoamine systems observed with the effects of SUC and SAC exposure during the juvenile period were less robust. However, we investigated the effects during the juvenile period, since this study was particularly motivated to investigate the effects of SUC and SAC intakes during the juvenile period, in order to find an insight for the effects of excessive intakes in kids at school ages as human cases, in which excessive intakes of SUC and SAC have often been reported problematic and deteriorating.

Accumulating evidence also suggests that obesity induces specific patterns of DA changes in the STR of both rodents and humans. In rats, obesity has been demonstrated to be associated with decrease of extracellular DA level, concurrent with decreased DAT and D2 receptor expression in the NAcc (Geiger et al., 2008, 2009). Consistent with it, a human study has shown decreased D2 receptor availability in the STR of obese people (Wang et al., 2001). Since we did not examine extracellular DA level or D2 receptor expression, it remains largely unclear how the results are similar or different with those previous findings; however, it appears that the effects of juvenile SAC and SUC intakes could be quite distinct, as tissue DA concentration was found higher in the NAcc, along with higher DAT expression in adult mice exposed to juvenile SUC intake. One possible explanation for such discrepancy is that the effects of SUC and SAC intake are not sorely determined by high caloric intake.

The second aim of our study was to compare the effects of SUC and SAC, for which we directly compared behavioral and neural alterations caused by excessive SUC and SAC intake during the juvenile period with the identical administration procedure. A previous study has shown activation of the striatum with caloric drink but not with non-caloric drink consumption (Smeets et al., 2011). In another study, both SUC and non-caloric artificial sucralose have been reported to similarly activate the primary gustatory cortex. However, SUC, but not sucralose, activates several additional brain regions, such as the anterior insula, the frontal operculum, the striatum, and the anterior cingulate (Burke et al., 2018). In this study, we found that some behavioral and neural alterations were common but we

also found differences between the treatments (**Supplementary Tables S1–S3**). In particular, adult SUC animals exhibited higher sensitivity to AMP, whereas the AMP response was blunted in adult SAC animals. Moreover, juvenile, SAC, but not SUC, animals were less anxious. In adulthood, SUC, but not SAC, animals were more anxious. Juvenile SUC, but not SAC, animals also exhibited greater vulnerability to stress. Consistent with these behavioral alterations, some, but not all, DA and 5-HT alterations were also distinct between SUC and SAC intake. The ratio between DA/5-HT and their metabolites has been defined as the rates of DA and 5-HT turnovers (Nissbrandt and Carlsson, 1987). Studies have shown that cocaine self-administration increases DA turnover in the dSTR and NAcc (Kimmel et al., 2003; Smith et al., 2003). Lower tissue DA and 5-HT concentrations and higher turnovers of them were observed in the dSTR of both juvenile SUC and SAC animals. In addition, lower tissue DA and 5-HT concentrations and higher turnovers of them in the NAcc were also found in juvenile SAC, but not SUC, animals. Such differences in the NAcc may contribute to behavioral alterations observed in juvenile SAC, but not SUC, animals, such as the lower AMP response at the high dose, and the lower anxiety in SAC animals. In adulthood, distinct alterations between SUC and SAC animals were also observed in the NAcc, which may contribute to behavioral alterations observed in adult SUC and SAC animals, such as the higher anxiety in SUC animals and the augmented and blunted AMP responses in SUC and SAC animals, respectively. SAC is a non-caloric, whereas SUC is caloric, sweeteners. Thus, if DA and 5-HT alterations were induced by only repeated exposure to the sweet taste, alterations of DA and 5-HT concentrations and turnovers would not be different between SUC and SAC conditions. However, SUC animals exhibited some distinct behavioral and neural alterations from those in SAC animals, suggesting that alterations induced by excessive SUC and SAC intake during the juvenile period may involve both repeated strong pleasure (sweet taste) experience and the amount of caloric consumption.

In this study, we investigated the effects of SUC and SAC exposure in male animals. Although there may be some profits of using male mice, including controlling of subjects to minimize influence of various factors such as menstrual cycles, and enabling to compare with other studies investigating relevant issues in male animals, substance-related addiction has been reported more vulnerable to female than male subjects (Becker et al., 2017). Indeed, neurotransmitter mechanisms involved in addiction have been shown under tight relationships with sex hormones, such as estrogen and progesterone (Lightfoot, 2008). Thus, investigation with female animals remains a very important issue that was not addressed in the current study. Repeating all experiments conducted in the current study with female mice could double the amount of the data, and thereby, beyond the scope of the current study. However, we will investigate the effects of juvenile SUC and SAC exposure in female mice in our next study, with which we can compare the results with the current study conducted in male mice to elucidate if there are any gender differences.

Excessive consumption of SUC has been suggested as one of the major dietary factors associated with various chronic diseases, including obesity, diabetes, and cardiovascular disease (Howard and Wylie-Rosett, 2002; Tsilas et al., 2017; Burke et al., 2018; Togo et al., 2019). Thus, we expected to observe such augmented weight gain with repeated sucrose administration, but actually did not. As one of possible reasons, we suspect that these mice with sucrose administration compensated for their caloric intakes by eating less food pellets, so that they maintained body weight gain comparable to that of control mice. In addition, although chronic SUC consumption in adulthood has been shown to induce obesity with increasing body weight, the effects depend on the life-cycles, duration and amount of consumption, and genetic backgrounds of animals (Glendinning et al., 2010), such that excessive SUC intake only for 2 weeks in the juvenile period at the concentration of 20% solution might not be enough to induce apparent obesity. Recent studies suggested that excessive SUC consumption also induces brain dysfunctions, such as learning impairments and addiction-like behavior (Reichelt et al., 2015; Abbott et al., 2016; Wiss et al., 2018). Excessive SUC intake during pregnancy in rodents has also been reported to induce ADHD-like behavioral alterations with increased striatal DAT expression in offspring (Choi et al., 2015). Our study suggests that excessive SUC and SAC consumptions during the juvenile period induce an assortment of behavioral and neural alterations, some of which could further interact with maturation processes during adolescence, consequently causing both concurrent juvenile and adult expressions of alterations. Thus, excessive SUC consumption in early development may be a predisposition that increases the vulnerability of developing psychiatric disorders.

## CONCLUSION

Excessive intake of SUC in the juvenile period induced behavioral alterations caused by dysregulations of dopaminergic and serotonergic neuronal pathways both in juvenile and adult animals.

## DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Daegu Catholic University.

## AUTHOR CONTRIBUTIONS

Y-AL conceived the idea and designed the research. W-HC, K-AL, and YG performed the experiments. W-HC and K-AL contributed to data analyzation and sample preparation. Y-AL

and YG wrote the manuscript. All authors contributed to the interpretation of the results, and provided critical feedback and helped shape the research, analysis and manuscript.

## FUNDING

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by Korean Ministry of Education (NRF-2017R1D1A1B03028486).

## ACKNOWLEDGMENTS

We thank Ye-Jin Kim and Jae-Won Jung for technical assistance.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00504/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 11 September 2020
doi: 10.3389/fnins.2020.527258

# Dietary Exposure to Excess Saturated Fat During Early Life Alters Hippocampal Gene Expression and Increases Risk for Behavioral Disorders in Adulthood

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**Purpose:** Maternal and postnatal diets result in long-term changes in offspring brain and behavior; however, the key mediators of these developmental changes are not well-defined. In this study, we investigated the impact of maternal and post-weaning high-fat diets on gene expression of key components mediating hippocampal synaptic efficacy. In addition, we evaluated the risk for impaired stress-coping and anxiety-like behaviors in adult offspring exposed to obesogenic diets during early life.

**Methods:** Dams were fed a control (C) or high-fat (HF) diet prior to mating, pregnancy, and lactation. Male offspring from control chow and high-fat fed dams were weaned to control chow or HF diets. The forced swim test (FST) and the elevated-plus maze (EPM) were used to detect stress-coping and anxiety-like behavior, respectively. Real-time RT-PCR and ELISA were used to analyze hippocampal expression of genes mediating synaptic function.

**Results:** Animals fed a HF diet post-weaning spent more time immobile in the FST. Swimming time was reduced in response to both maternal and post-weaning HF diets. Both maternal and post-weaning HF diets contributed to anxiety-like behavior in animals exposed to the EPM. Maternal and post-weaning HF diets were associated with a significant decrease in mRNA and protein expression for hippocampal GDNF, MAP2, SNAP25, and synaptophysin. Hippocampal mRNA expression of key serotonergic and glutamatergic receptors also exhibited differential responses to maternal and post-weaning HF diets. Hippocampal serotonergic receptor 5HT1A mRNA was reduced in response to both the maternal and post-weaning diet, whereas, 5HT2A receptor mRNA expression was increased in response to the maternal HF diet. The glutamate AMPA receptor subunit, GluA1, mRNA expression was significantly reduced in response to both diets, whereas no change was detected in GluA2 subunit mRNA expression.

**Conclusion:** These data demonstrate that the expression of genes mediating synaptic function are differentially affected by maternal and post-weaning high-fat diets. The post-weaning high-fat diet clearly disturbs both behavior and gene expression. In addition,

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***Edited by:***
*Julie A. Chowen, Niño Jesús University Children's Hospital, Spain*

***Reviewed by:***
*Christine Jasoni, University of Otago, New Zealand*
*Ewan McNay, University at Albany, United States*

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***Specialty section:***
*This article was submitted to Neuroenergetics, Nutrition and Brain Health, a section of the journal Frontiers in Neuroscience*

***Received:*** *15 January 2020*
***Accepted:*** *21 August 2020*
***Published:*** *11 September 2020*

***Citation:***
*Page KC and Anday EK (2020) Dietary Exposure to Excess Saturated Fat During Early Life Alters Hippocampal Gene Expression and Increases Risk for Behavioral Disorders in Adulthood. Front. Neurosci. 14:527258. doi: 10.3389/fnins.2020.527258*

although the transition to control diet at weaning partially compensates for the adverse effects of the maternal HF diet, the negative consequence of the maternal HF diet is exacerbated by continuing the high-fat diet post-weaning. We present evidence to support the claim that these dietary influences increase the risk for anxiety and impaired stress-coping abilities in adulthood.

**Keywords: maternal diet, high-fat, post-weaning diet, hippocampus, anxiety, depression**

## INTRODUCTION

The abundance of saturated fats and carbohydrate-dense foods in the Westernized world has contributed to the prevalence of obesity. In fact, epidemiological studies show that in the last two to three decades, more than 50% of women of child-bearing age in the United States are overweight or obese. If the trend continues, more than 80% of adults will be obese by 2030 (Wang et al., 2008). The etiology of obesity is multifactorial and involves complex interactions between genes and the environment. In addition to numerous lifestyle factors which contribute to the development of obesity, a growing body of evidence suggests that genetic programming occurs while still in the womb (Barker et al., 2002).

Obesity in pregnancy represents a significant problem. Not only because of adverse effects on maternal health and pregnancy outcomes such as hypertensive disorders, gestational diabetes, and increased risk for Cesarean section, but also because it poses a risk for the developing fetus (Srinivasan et al., 2006). Childhood obesity has been associated with an increased risk of psychological and behavioral disorders which include depression and anxiety (Rofey et al., 2009), autism spectrum disorder (ASD) (Angelidou et al., 2012), learning deficits and attention deficit disorder (Cserjesi et al., 2007). Moreover, obese adults are 20% more likely to have depressive disorders compared to non-obese subjects (Simon et al., 2006; Waring and Lapane, 2008).

Animal studies have demonstrated that prolonged consumption of a diet high in saturated fat results in increased body weight, hyperinsulinemia, and insulin resistance (Woods et al., 2003). A number of studies have also demonstrated that inflammation, oxidative stress and dysregulation of hormones impair the formation of critical neural circuits in the developing brain (Sullivan et al., 2015). In fact, brain leptin resistance is a well-studied aspect of the obese state. Previous reports indicate that obesity and disturbances in leptin signaling markedly affect the expression of neuronal and glial proteins and that leptin binding to its receptor, which is expressed at a remarkably high level in the hippocampus (Caron et al., 2010), is required for neurogenesis and efficient synaptic transmission (Grillo et al., 2011; McGregor and Harvey, 2018).

It has also been shown that excess dietary fat consumption may contribute to a physiological background of chronic stress since elevations in the level of adrenocorticotropic hormone and glucocorticoid are associated with high-fat feeding (Tannenbaum et al., 1997). Stress results in loss of neurons and glia in the hippocampus (Duman and Duman, 2015), and the adverse effects of glucocorticoids on hippocampal spine synapses and neurogenesis are well-documented (McEwen et al., 2016). In addition, the hippocampus is an integral part of neural circuits regulating the HPA axis response to stress. Disruption of HPA axis-controlled glucocorticoid release is a common feature of depression (Duman, 2014a) and a heightened HPA-axis response to stress accompanies high-fat feeding (Dutheil et al., 2016). It is becoming clear that HPA axis hyperactivity and excess circulating glucocorticoid in response to a HF diet may lead to altered stress responsiveness and behavioral alterations such as mood disorders.

The claim that a HF diet disrupts HPA axis regulation (Winther et al., 2018) is further supported by studies from de Kloet's laboratory. Their results demonstrate that the capacity for an individual to cope with stress is coordinated via glucocorticoid action on the mineralcorticoid receptor (MR) network, particularly in the hippocampus. In fact, subsequent management of stress adaptation via the low affinity glucocorticoid receptor (GR) is dependent on the coordinated regulation of the MR:GR ratio where MR is the critical mediator of an animal's resilience to stress (de Kloet et al., 2016). Previous results from our lab showed that a reduction in hippocampal MR but not GR expression was associated with early life exposure to excess glucocorticoids, and that this change in the MR:GR ratio was associated with an increase in hippocampal drive and HPA axis hyperactivity (Shoener et al., 2006).

We have now turned our attention to the impact of maternal and post-weaning HF diets on the expression of genes mediating hippocampal neurogenesis and synaptic efficacy, functions that impact an individual's emotional balance. Neural plasticity and experience-dependent plasticity are particularly vulnerable to gene–environment interactions during developmental periods such as early life, and adverse conditions during these times could induce changes that increase the risk of psychopathologies such as anxiety and depression in adulthood. Although perturbations in each individual gene susceptible to gene–environment interactions contribute a small fraction to the total risk for behavioral disorders, it is likely that overlapping sets of susceptibility genes are at play. This makes the timing and dose-response outcome difficult to discern (Lopizzo et al., 2015). In order to explore the timing and extent of HF diet exposure, we chose to investigate the effects of a diet high in saturated fat during two distinct developmental times; (1) the period between gestation and the end of lactation which reflects the maternal diet and (2) the period from weaning to adulthood which constitutes the post-weaning diet. Each diet period includes environmental variables that may impact genetic programming differentially, particularly in the developing brain. We hypothesized that maternal and post-weaning diets high in saturated fat exert deleterious effects on the expression of

hippocampal genes mediating synaptic plasticity and increase the propensity for behavioral disorders in adulthood. To investigate this possibility, we employed the forced swim test (FST) and the elevated-plus maze (EPM) to assess alterations in stress-coping and anxiety-like behavior, respectively.

We also examined expression data for proteins mediating hippocampal plasticity, synaptic function, and associated behavioral manifestations. We hypothesized that expression of these key genes is perturbed in response to the consumption of a high-fat diet. We analyzed the mRNA and protein expression for GDNF, a neurotrophin required for survival and efficacy of neuronal contacts, and for MAP2, a protein which reflects the capacity for receiving synaptic inputs along dendritic outgrowths. In addition, the expression of synaptosomal-associated protein 25 (SNAP25) and synaptophysin was measured since these two proteins are critical for presynaptic vesicle transport and fusion. The mRNA levels for the key post-synaptic serotonin receptors, 5HT1A and 5HT2A, and critical subunits of the glutamate responsive AMPA receptor, GluA1 and GluA2, were also evaluated. The choice of molecular markers was based on literature focused on synaptic dysfunctions that underpin an individual's risk for emotional imbalance, particularly anxiety and depression (Duman and Duman, 2015; McEwen et al., 2016; Tsybko et al., 2017). To date, studies designed to explore the effects of high-fat diets on anxiety and stress-coping strategies are not conclusive; however, these studies indicate that disturbances in the integrity and/or functionality of the hippocampus are involved in the etiology of mood disorders.

# MATERIALS AND METHODS

## Animals

Thirty-two Sprague-Dawley dams (Hilltop Laboratories) were split into two nutritional groups as described previously (35). Sixteen of the dams were placed on standard rat chow providing 3.85 kcal/g dry wt, 20 kcal% protein, 70 kcal% carbohydrate, and 10 kcal% saturated fat (D12450H; Research Diets, New Brunswick, NJ, United States). The remaining 16 dams were fed a high-fat diet providing 4.73 kcal/g dry wt, 20 kcal% protein, 35 kcal% carbohydrate, and 45 kcal% saturated fat, mostly in the form of lard (D12451; Research Diets), for 1 month before mating. Standard and high-fat diets were sucrose matched. Pregnant dams were housed singly and continued on their specified diets. At birth, litters were culled to 10 offspring per dam to standardize lactation demands. At weaning, four male offspring were chosen at random from each of the 16 control dams and split into two nutritional groups: two male offspring from each of the 16 control dams (C) were weaned to standard chow (C) diet (CC, $n = 16$), and the other two offspring from each control dam (C) were weaned to a 45 kcal% saturated high-fat (HF) diet (CHF, $n = 16$). Four male offspring from each dam fed a HF diet were also split into two nutritional groups at weaning: two of the four offspring from each of the 16 HF fed dams (HF) were weaned to standard chow (C) diet (HFC, $n = 16$), and the remaining two male offspring from each HF fed dam (HF) were weaned to the high-fat (F) diet (HFF; $n = 16$). Results obtained from each pair of male offspring taken from a given dam were averaged and represented as $n = 1$. The final sample size was $n = 16$ for each group.

All offspring were continued on their respective diets into adulthood. At 126 to 130 days of age, adult males from each experimental treatment were separated by litter ($n = 16$) and tested for anxiety-like behavior using the EPM. Since it has been shown that anxiogenic effects remain even after a 1-week interval between behavioral tests (Andreatini and Bacellar, 1999), our animals were allowed to acclimate and return to baseline for 10 days prior to testing the animals at 136 to 140 days for depressive-like behavior using the FST.

## Behavioral Testing: Forced Swim Test and Elevated-Plus Maze

The FST was used to assess the response to acute inescapable stress which reflects the activity of neuronal networks that may be impaired in depression, ASD, and other disorders (Commons et al., 2017). More specifically, the increased immobility time during forced swim was used in our study as a measure for stress-coping behavior in rodents. In this study we used a repeated-trial procedure similar to the original FST protocol (Porsolt et al., 1977). Since rats remain immobile for a higher proportion of time in the second trial, this approach provides a more suitable baseline for measuring a decrease or increase in immobility duration. An interval of 24 h between the pre-test and the test trial was used. On day one, rats were individually immersed in a water-filled (23–25°C) plastic cylinder (46 cm high × 20 cm diameter) filled with tap water to 30 cm water depth which prevents them from touching the bottom. The animals were exposed to the forced swim for 15 min. After 24 h, the test trial was conducted similarly, however, in this trial animals were subjected to the forced swim for only 5 min and time spent immobile during each one min interval over the 5-min period was recorded along with swimming activity. All sessions were videotaped and recorded for analysis using ANY-MAZE video-tracking system and time spent immobile, swimming, or climbing for the four treatment groups was analyzed using a two-way ANOVA [maternal diet X post-weaning diet].

General exploratory and anxiety-like behaviors were evaluated using EPM apparatus consisting of a black acrylic cross with a shared central square (12 cm × 12 cm) with two opposite open arms and two opposite closed arms (50 cm × 12 cm). The closed arms were enclosed by 50-cm high walls. The test rat was placed on the central square facing a corner which allowed equal choice of entering an open or closed arm. The animal explored the apparatus during a 5 min session. The behavior was recorded using ANY-MAZE tracking system. Time spent and distance traveled in the open and closed arms were quantified. At the end of each session, the number of fecal boli were counted and the apparatus was thoroughly cleaned.

Upon completion of behavior testing the animals were maintained in their specific groups for 10–14 days prior to sacrifice to ensure that all animals had returned to a stable physiological baseline after behavioral testing. At 150 days of age, individual animals from each group were decapitated in a

staggered fashion (CC, CHF, HFC, HFF, repeat). Immediately upon death, trunk blood was drained into serum collection tubes containing gel activator, whole hippocampi were rapidly dissected from each side of the brain and rapidly frozen using liquid nitrogen. Body weight was recorded and visceral fat pads, both retroperitoneal and gonadal, were excised and weighed.

## Collection of Hippocampi

Hippocampi from each animal were rapidly dissected and frozen in liquid nitrogen. This was carried out during the morning phase of the circadian cycle (between 800 and 1000 h) when each animal was 150 days of age. These studies were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all animal procedures have been reviewed and approved by the Animal Care and Use Committee of Bucknell University.

## RNA Isolation and RT-PCR

Total RNA was isolated from each hippocampus using TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Purified total RNA was reverse transcribed using RETROscript (Ambion, Austin, TX, United States) in accordance with the manufacturer's recommended procedure. Real-time PCR was performed on an iCycler iQ Real Time PCR Detection System (Bio-Rad, Hercules, CA, United States) using SYBR Green Supermix (Bio-Rad, Hercules, CA, United States). Target genes were amplified using the thermocycling program and controls previously described (Page et al., 2014). Primers were constructed using the NCBI online database[1] and sequence specificity of each primer pair (**Table 1**) was confirmed using BLAST[2]. All primers were ordered from MWG Oligo Synthesis (High Point, NC, United States). Efficiency of the primers was calculated, and gel electrophoresis was conducted on primers to exclude those with DNA contamination. In each sample, the gene of interest was co-amplified with a standard housekeeping gene 18S ribosomal RNA. Before the experimental plates were analyzed, real-time PCR control plates were run for each primer pair: double-distilled $H_2O$ was substituted for cDNA to verify that exogenous DNA was not present and 2 μg of isolated total RNA was substituted for cDNA in the PCR reaction to rule out genomic DNA contaminants. Both negative controls showed no amplification even after 35 cycles. The following genes were amplified from hippocampal-derived cDNA: glial-derived neurotrophic factor (GDNF), microtubule-associated protein 2 (MAP2), synaptosomal-associated protein (SNAP25), synaptophysin, the ionotropic glutamate AMPA receptor subunits, GluA1 and GluA2, and the G-protein coupled serotonin receptors, 5HT1A and 5HT2A. In each sample, the gene of interest was co-amplified with the standard housekeeping gene, 18S ribosomal RNA, to control for potential differences in pipette load volume.

[1]http://www.ncbi.nlm.nih.gov

[2]https://blast.ncbi.nlm.nih.gov/Blast.cgi

## Data Analysis for Quantitative qPCR

The cycle numbers at which amplified DNA samples exceeded a computer-generated fluorescence threshold level ($C_T$) were normalized and compared to determine relative gene expression. There were no statistical differences in 18S $C_T$ values across different groups (data not shown). Higher cycle number values indicate lower initial concentrations of cDNA, and thus lower levels of mRNA expression. Each sample was run in triplicate, and averaged triplicates were used to assign cycle threshold ($C_T$) values. $dC_T$ values were generated by subtracting experimental $C_T$ values from the $C_T$ values for 18S targets co-amplified with each sample. The mean $dC_T$ value per amplified gene target for the CC (control) group was subtracted from itself to set the difference ($ddC_T$) to 0 and the $2^{ddCT}$ to 1. The mean $dC_T$ values for each of the other three groups were then subtracted from the $dC_T$ for the CC group such that the difference in values ($ddC_T$) would be set relative to the CC group. The $ddC_T$ values were then calculated as powers of 2 ($2^{ddCT}$) to account for the exponential doubling of the polymerase chain reaction and the CC group with its $ddC_T$ set to 0 would always have its $2^{ddCT}$ equal to one arbitrary unit.

## Protein Levels Determined for Hippocampal GDNF, MAP2, SNAP25, and Synaptophysin Using ELISA

Rapidly dissected whole hippocampi were immediately frozen using liquid nitrogen and kept at 80°C until use. Tissue

**TABLE 1 |** Primer sequences *(GenBank/NCBI)* used in real-time PCR.

| Primer ID | Primer sequence 5′–3′ | Accession no. | Length, nt |
|---|---|---|---|
| 18S | Fwd: AAACGGCTACCACATCCAAG Rev: GGCCTCGAAAGAGTCCTGTA | M11188 | 76 |
| GDNF | Fwd: GGAGACCGGATCCGAGGTG Rev: GCGCTTCGAGAAGCCTCTTA | NM_019139 | 136 |
| GluA1 | Fwd: CGACCCTCCCCCGGAACAGT Rev: GGCTGACCACCCGGCCATTC | NM_031608 | 75 |
| GluA2 | Fwd: TGTGGAGCCAAGGACTCGGGA Rev: TTGCCAAACCAAGGCCCCCG | NM_017261 | 103 |
| MAP2 | Fwd: CCCTCCTCGCAGGGGCGTAT Rev: TCTGACCTGGTGGTCCGTCGT | NM_2013066 | 93 |
| SNAP-25 | Fwd: CCTTCCCTCCCTACCCGGCG Rev: CGGGGCCAGCAAGTCAGTGG | NM_030991 | 115 |
| SYP | Fwd: GAATCAGCTGGTGGCTGGGGG Rev: CACGCTCAGCCGAAGCTCCC | NM_012664 | 136 |
| 5HT1A | Fwd: TGTTGCTCATGCTGGTTCTC Rev: CCGACGAAGTTCCTAAGCTG | NM_000524 | 88 |
| 5HT2A | Fwd: CTGCTGGGTTTCCTTGTCAT Rev: ATCCAGATCGCACAGAGCTT | NM_017254 | 106 |

*Final concentration of each primer was 1 μM. Nucelotides is abbreviated nt. GDNF, glial cell-derived neurotrophic factor; GluA1 and GluA2, AMPA receptor subunits; MAP2, microtutule-associated protein2; SNAP-25, synaptosomal nerve-associated protein 25; Syp, synaptophysin; 5HT1A and 5HT2A, serotonin receptors.*

lysates for ELISA analysis were prepared according to the manufacturer's protocol (My Biosource, San Diego, CA, United States). Ice-cold homogenate was centrifuged (15 min at 10,000 *g*, 4°C), and supernatant was removed. Protein concentrations of the supernatant were quantified using the Bradford method. Supernatant protein concentration for the gene products of interest, GDNF, SNAP25, MAP2 or Synaptophysin, were analyzed using target specific ELISA kits according to manufacturer's instructions (My Biosource). Target protein concentration (pg/ml) was normalized using total protein concentration of the supernatant (pg target/mg protein). Protein determinations were made using the Bradford assay.

## Statistical Analysis

Gene expression and data from the FST and EPM were analyzed using two-way ANOVA of maternal diet X post-weaning diet (maternal diet effect CC vs. HFC; CHF vs. HFF and post-weaning diet effect CC vs. CHF; HFC vs. HFF). Multiple comparisons were made using Bonferroni *post hoc* analysis (SPSS, Statistical Analysis Software, Chicago, IL, United States). Statistical significance was confirmed if $P \leq 0.05$.

# RESULTS

## Effects of Excess Saturated Fat Consumption on Behavior

We have established a metabolic profile for rats consuming high fat diets and reported in a previous study that animals exposed to excess saturated fat during early development exhibit significant increases in body weight, energy intake, adipose deposition, and higher circulating levels of leptin, insulin, and glucocorticoid (Page et al., 2009). Our current study was designed to investigate whether exposure to excess saturated fat during development would influence adult behaviors such as stress-coping or anxiety. Using the FST, we measured the behavioral response of an animal to an acute inescapable stressor. Results from this stress-coping test can be used to detect disturbances in neural networks that underpin the development of anxiety, depression, and ASD (Commons et al., 2017).

Using the FST, we found that excess saturated fat content in the post-weaning diet markedly increased the amount of time an animal spent immobile [post-weaning diet effect; $F_{(3,61)} = 6.58$, $P = 0.01$; CC vs. CHF, HFC vs. HFF] (**Figure 1A**). Moreover, the amount of time spent immobile following exposure to the maternal HF diet increased but did not reach significance [maternal diet effect; $F_{(3,61)} = 4.15$, $P = 0.058$; CC vs. HFC, CHF vs. HFF] (**Figure 1A**). In contrast, time spent swimming was significantly decreased in response to both the maternal diet ($F_{(3,61)} = 4.36$, $P = 0.038$) and post-weaning diet ($F_{(3,61)} = 5.61$, $P = 0.024$) (**Figure 1B**).

Behavioral changes were also observed during EPM testing. Time spent in the open arms was significantly decreased in response to both the maternal diet ($F_{(3,61)} = 5.17$, $P = 0.034$) and post-weaning diet ($F_{(3,61)} = 4.36$, $P = 0.049$) (**Figure 2A**), whereas, time spent in the closed arms was significantly increased in response to the maternal ($F_{(3,61)} = 6.48$, $P = 0.016$) and post-weaning HF diet ($F_{(3,61)} = 5.52$, $P = 0.026$) (**Figure 2B**). Interestingly, a reduction in the number of open arm entries ($F_{(3,61)} = 4.19$, $P = 0.042$) (**Figure 2C**) and an increase in the distance traveled in the closed arms ($F_{(3,61)} = 5.34$, $P = 0.027$) (**Figure 2D**) was significant only in response to excess saturated fat in the maternal diet.

## Effects of Excess Saturated Fat on Hippocampal GDNF and MAP2 mRNA and Protein Expressions

To evaluate the effects of HF diets on brain development, more specifically hippocampal neurogenesis, we measured the expression of GDNF, a key neurotrophic factor, as well as MAP2, a major dendritic marker. Two-way ANOVA (maternal diet X post-weaning diet) revealed a significant reduction of hippocampal GDNF mRNA (**Figure 3A**) in response to both the maternal ($F_{(3,57)} = 3.92$, $P = 0.049$) and post-weaning high-fat diet ($F_{(3,57)} = 4.87$, $P = 0.034$), although the maternal diet effect is mainly due to the significant difference between CC and HFC animals. Moreover, both the maternal ($F_{(3,57)} = 8.65$, $P = 0.004$) and post-weaning diet ($F_{(3,57)} = 11.64$, $P = 0.001$) contributed to the reduction in GDNF protein expression in an independent fashion since no interactions were detected (**Figure 3B**).

A similar expression pattern was detected for MAP2 (**Figure 4**). Again, MAP2 mRNA level was significantly reduced in response to both the maternal ($F_{(3,57)} = 3.28$, $P = 0.05$) and post-weaning diet ($F_{(3,57)} = 6.76$, $P = 0.013$). Moreover, both maternal ($F_{(3,57)} = 9.28$, $P = 0.004$) and post-weaning ($F_{(3,57)} = 5.67$, $P = 0.023$) high-fat diets contributed to the reduction in MAP2 protein expression. No significant interactions were detected using two-way ANOVA.

## Effects of Excess Saturated Fat Consumption on mRNA and Protein Expression of Hippocampal Genes Mediating Synaptic Vesicle Release

Neuronal function is exquisitely dependent on synaptic vesicle release. To determine the effects of HF diets on synaptic function, we measured the expression of two key genes mediating synaptic vesicle release, SNAP25 and synaptophysin. Expression of SNAP25 mRNA was reduced in response to both the maternal ($F_{(3,57)} = 5.11$, $P = 0.038$) and post-weaning high-fat diets ($F_{(3,57)} = 12.71$, $P = 0.001$) (**Figure 5A**). This decrease was also translated into a significant reduction in SNAP25 protein levels (pg/mg) in response to both maternal ($F_{(3,57)} = 8.36$, $P = 0.007$) and post-weaning ($F_{(3,57)} = 10.53$, $P = 0.003$) high-fat diets (**Figure 5B**). This pattern of reduced expression was once again detected in synaptophysin mRNA [maternal diet effect, $F_{(3,57)} = 4.14$, $P = 0.038$; post-weaning diet effect; $F_{(3,57)} = 7.47$, $P = 0.014$] (**Figure 6A**) and protein levels [maternal diet effect, $F_{(3,57)} = 6.65$, $P = 0.032$; post-weaning diet effect; $F_{(3,57)} = 8.64$, $P = 0.004$] (**Figure 6B**).

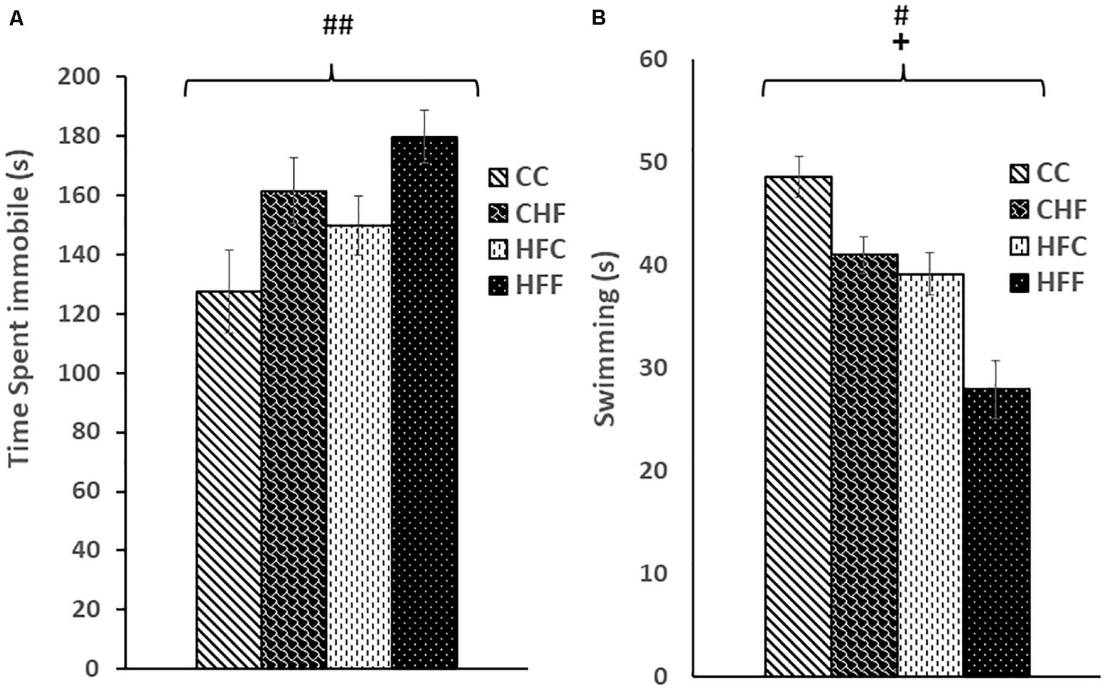


**FIGURE 1 |** Forced Swim Test performance was measured in adult male rat offspring from chow-fed dams or high-fat fed dams fed either chow or high-fat food post-weaning: control offspring fed chow (CC), control offspring fed high-fat food (CHF), high-fat offspring fed chow (HFC), and high-fat offspring fed high-fat food (HFF). **(A)** Total immobility increased significantly in response to the post-weaning high-fat diet (post-weaning diet effect: $F_{(3,61)} = 6.58$, $P = 0.01$). Increased immobility in response to a maternal high-fat diet did not reach significance (maternal diet effect: $F_{(3,61)} = 4.15$, $P = 0.058$). **(B)** Swim time was reduced by both the maternal (maternal diet effect: $F_{(3,61)} = 4.36$, $P = 0.038$) and post-weaning high-fat diet (post-weaning diet effect: $F_{(3,61)} = 5.61$, $P = 0.024$). No significant interactions were detected using two-way ANOVA. Values are means ± SEM where $n = 16$ for each group. $^{+}P \leq 0.05$ (significant maternal diet effect); $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ (significant post-weaning diet effect).

## Effects of Excess Saturated Fat Consumption on the mRNA Expression of Genes Mediating Serotonergic and Glutamatergic Function in the Hippocampus

Post-synaptic receptors are also key to synaptic efficacy and plasticity. To assess the status of key post-synaptic receptors following nutritional manipulation, we examined the hippocampal mRNA expression of important serotonin receptor subtypes, 5HT1A and 5HT2A, as well as two key subunits of the glutamate AMPA receptor, GluA1 and GluA2. Serotonin receptor 1A (5HT1A) mRNA was significantly decreased in response to both the maternal ($F_{(3,57)} = 7.13$; $P \leq 0.01$) and post-weaning diet ($F_{(3,57)} = 6.49$; $P \leq 0.01$) (**Figure 7**), whereas the 5HT2A receptor mRNA expression was significantly increased only in offspring from dams fed a diet high in saturated fat [maternal effect: ($F_{(3,57)} = 7.28$, $P \leq 0.05$)] (**Figure 7**). The mRNA expression of key subunits composing the glutamate-gated AMPA receptor was also differentially affected by nutritional manipulation. GluA1 mRNA level was significantly reduced by both the maternal ($F_{(3,57)} = 4.06$; $P \leq 0.05$) and post-weaning diet ($F_{(3,57)} = 8.04$, $P \leq 0.01$) (**Figure 8A**), whereas, the expression of the GluA2 subunit was not significantly changed (**Figure 8B**).

## DISCUSSION

Results from this study demonstrate that exposure to excess dietary fat during development adversely affects the expression of genes mediating hippocampal neurogenesis and plasticity and leads to emotional imbalance. Previous studies have shown that obesity is associated with both anxiety- (Mizunoya et al., 2013; Ogrodnik et al., 2019) and depressive-like behavior (Hryhorczuk et al., 2013), and that the negative impact on hippocampal neurogenesis in depression model rats can be alleviated by the antidepressant, clomipramine (Liu et al., 2008). In our study, the expression of genes critical to neurogenesis and plasticity were, on average, significantly reduced in response to HF diet exposure, and behaviors reflecting the animals emotional state were disturbed.

We previously investigated the impact of HF diets on key metabolic factors which showed that maternal and post-weaning HF diets are associated with significantly higher body weight and visceral fat mass as well as markedly increased serum levels of leptin, insulin, and glucocorticoid (Page et al., 2009). These characteristics are indicative of the obese phenotype that is often accompanied by neuroinflammation and oxidative stress (Edlow, 2017), two stressors implicated in the development of anxiety and depression (Patki et al., 2013).

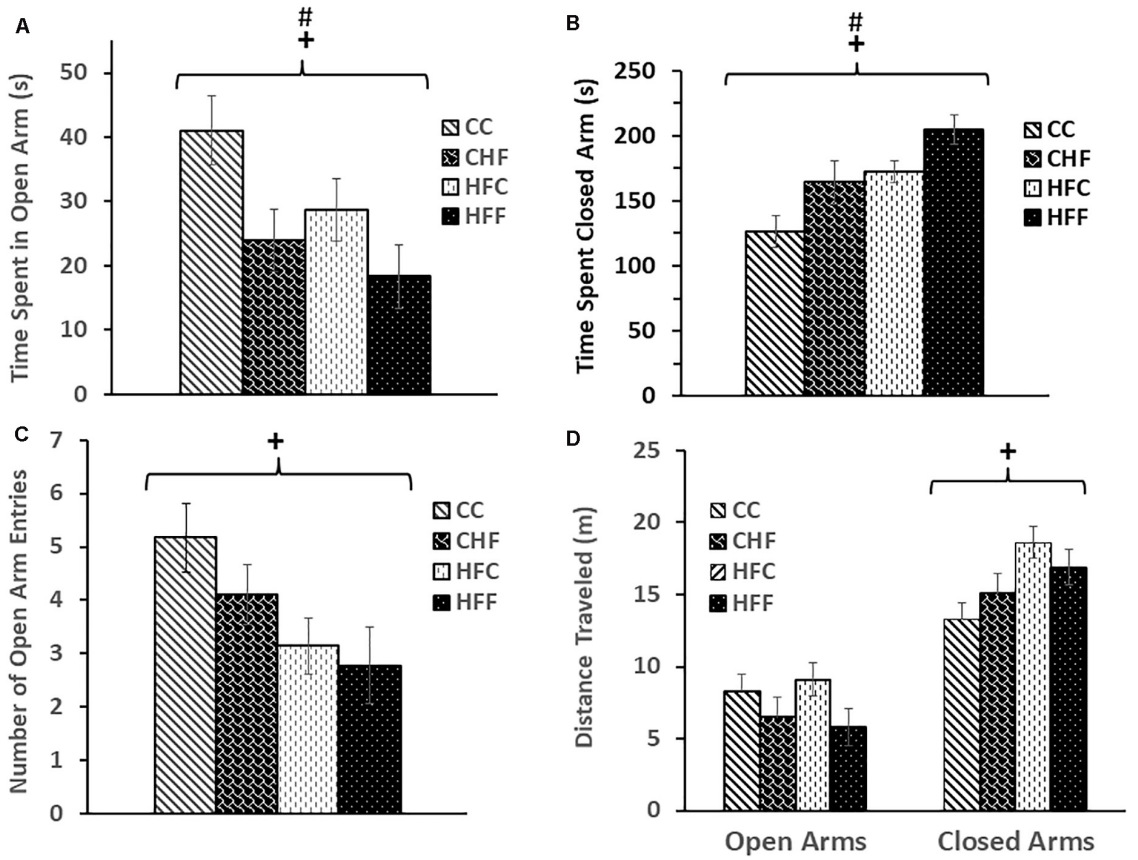


**FIGURE 2 |** Elevated-plus maze was used to evaluate anxiety-like behavior in adult male rat offspring: CC, CHF, HFC, or HFF. **(A)** Time spent in the open arms was significantly reduced in response to both the maternal (maternal diet effect: $F_{(3,61)} = 5.17$, $P = 0.034$) and post-weaning high-fat diet (post-weaning diet effect: $F_{(3,32)} = 4.36$, $P = 0.049$); whereas, **(B)** time spent in the closed arms was increased in response to both the maternal (maternal diet effect: $F_{(3,61)} = 6.48$, $P = 0.016$) and post-weaning high-fat diet (post-weaning diet effect: $F_{(3,61)} = 5.52$, $P = 0.026$). **(C)** The number of open-arm entries was significantly reduced in response to the maternal high-fat diet (maternal diet effect: $F_{(3,61)} = 4.19$, $P = 0.042$) and **(D)** the maternal diet was associated with an increase in distance traveled by adult male offspring in the closed arms (maternal diet effect: $F_{(3,61)} = 5.34$, $P = 0.027$). No significant interactions were detected using two-way ANOVA. Values are means ± SEM ($n = 16$ for each group). $^{+}P \leq 0.05$ (significant maternal diet effect); $^{\#}P \leq 0.05$ (significant post-weaning diet effect).

In our current study, we evaluated the effect of a HF diet on an animal's stress coping capacity or anxiety-like response using the FST and EPM, respectively. Results from the FST indicated that adult male offspring consuming excess saturated fat post-weaning spent significantly more time immobile. The increase in time spent immobile in animals exposed to a maternal HF diet was also detected, but this change did not reach significance. The CHF and HFF adult offspring were most susceptible to disruption in their stress coping strategies. Although depressive tendencies are not directly measured using the FST, this behavioral test does provide information with regard to an animal's ability to cope with an inescapable stressor (Molendijk and de Kloet, 2019), and changes in stress responsiveness are associated with an animal's vulnerability to depression (de Kloet et al., 2016). In fact, the neural networks that control coping strategies in response to acute stress overlap heavily with those affected by depression (Commons et al., 2017). For example, responses to acute stress are under the control of the glucocorticoid-regulated HPA axis, and dysregulation of this neuroendocrine feedback loop often accompanies depressive illness (Gold et al., 2015).

In our previous study, we found a significant increase in circulating glucocorticoid in animals exposed to excess saturated fat *in utero* and/or during the post-weaning period. This suggests that HPA axis feedback regulation has been disturbed. In fact, using the synthetic glucocorticoid, dexamethasone, we demonstrated that excess glucocorticoid exposure during the prenatal period increases hippocampal drive on the HPA axis in adulthood (Shoener et al., 2006). It is likely that the resulting HPA hyperactivity predisposes the animal to persistent physiological changes such as disturbances in the stress response and susceptibility to stress-induced anxiety and depression. In fact, rats or mice exposed to chronic HF diets develop various impairments, including inflammation, high corticosterone (CORT) levels, neuronal atrophy, decreased neurogenesis, and decreased neurotrophic factor expression in regions such as the hippocampus where glucocorticoid receptors are particularly abundant (Dutheil et al., 2016). These

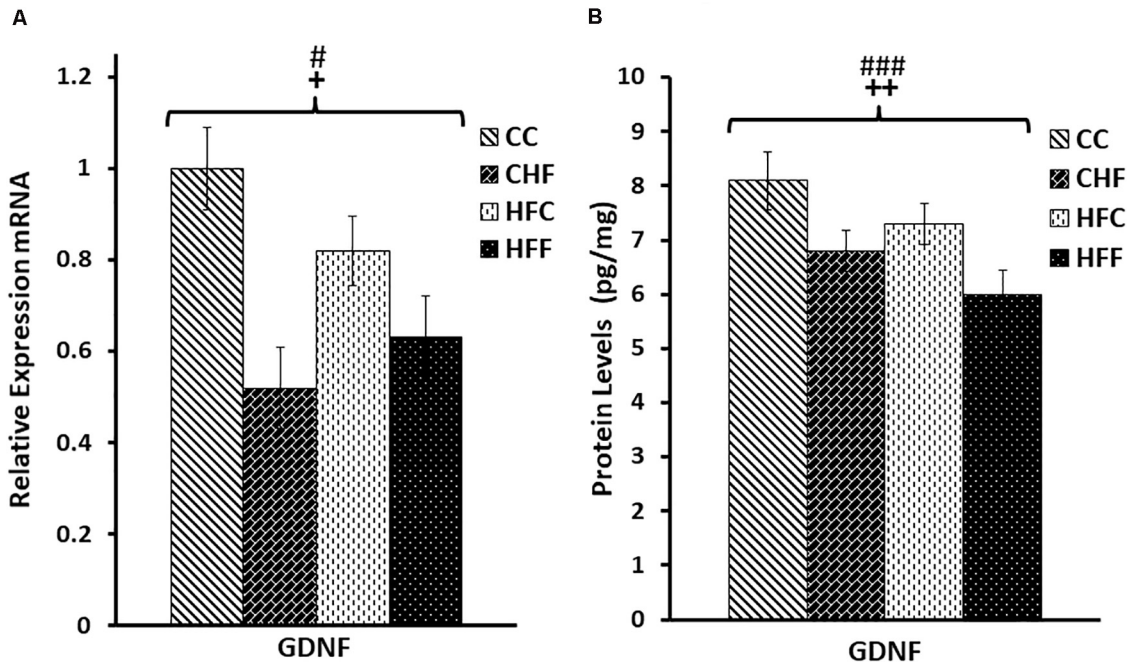


**FIGURE 3 |** Relative levels of GDNF mRNA and protein expression in the hippocampus. **(A)** Using quantitative real-time PCR (qRT-PCR), glial cell-derived neurotrophic factor (GDNF) mRNA expression was determined to be significantly reduced in response to both the maternal ($F_{(3,57)} = 3.92$, $P = 0.049$) and post-weaning HF diet ($F_{(3,57)} = 4.87$, $P = 0.034$). Values are expressed in relative units where the CC group is set to 1. **(B)** Significant reductions in GDNF protein levels (pg/mg) were also associated with both the maternal ($F_{(3,57)} = 8.65$, $P = 0.004$) and the post-weaning HF diet ($F_{(3,57)} = 11.64$, $P = 0.001$) using ELISA. No significant interactions were detected using two-way ANOVA. Values are means ± SEM ($n = 16$ for each group). $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ (significant maternal diet effect); $^{\#}P \leq 0.05$, $^{\#\#\#}P \leq 0.001$ (significant post-weaning diet effect).

disturbances most likely contribute to the impaired stress coping measured in our HF fed animals.

In addition to the diminished stress coping strategy detected in our experimental animals using the FST, results from our EPM testing demonstrate that maternal and post-weaning HF diets are also associated with a significant increase in anxiety-like behavior. In our previous study, we found that transitioning offspring from HF fed dams to a control diet at weaning lessens, but does not ameliorate, metabolic disturbances (Page et al., 2009). These results are consistent with other studies showing that transitioning pups from HF fed dams to control chow at weaning does not fully compensate for maternal diet effects associated with the significant increase in leptin and anxiogenic outcomes in the adult offspring (Bilbo and Tsang, 2010). In our current study, the excessive traveling recorded for our animals in the closed arms of the EPM and the significant reduction in open-arm time indicate an exacerbation of anxiety-like behavior in response to both the maternal and post-weaning HF diet. Our findings also agree with results from studies which demonstrate that non-human primates exposed to a maternal or post-weaning HF diet exhibit a persistent increase in anxiety and cortisol levels (Thompson et al., 2017); and that maternal hyperleptinemia and leptin resistance resulting from diet-induced obesity during pregnancy have been linked to anxiety-like behaviors in non-human primate offspring (Sullivan et al., 2010) and mice (Peleg-Raibstein et al., 2012). A recent study in rats also shows that maternal obesity and hyperleptinemia are associated with an impairment in stress adaptation and anxiety-like behavior in male offspring (Balsevich et al., 2016).

The dramatic increase in circulating leptin following exposure to a maternal and/or post-weaning HF diet suggests that leptin resistance develops in animals consuming a HF diet (Page et al., 2009; Vasselli et al., 2013). Studies also indicate that obesity and disturbances in leptin signaling markedly affect the expression of neuronal and glial proteins (Ahima et al., 1999). Disturbances in leptin signaling are particularly important in the hippocampus. The high level of hippocampal leptin receptors (Caron et al., 2010) promotes neurogenesis and efficient synaptic transmission following leptin activation (Grillo et al., 2011; McGregor and Harvey, 2018), and leptin binding stimulates rapid changes in dendritic morphology (O'Malley et al., 2007). In fact, Ahima et al. (1999) showed that rats with either a leptin deficiency or insensitivity had reduced brain weights and protein content and that the levels of several synaptic proteins were significantly lower compared to control animals, in particular, syntaxin, synaptobrevin, and SNAP25.

Our results support the claim that excessive fat consumption is associated with a disturbance in synaptic efficacy. Following exposure to maternal and post-weaning HF diets, we detected a significant reduction in both mRNA and protein levels for SNAP25 and synaptophysin, two proteins critical for the proper functioning of presynaptic vesicle release, as well as for

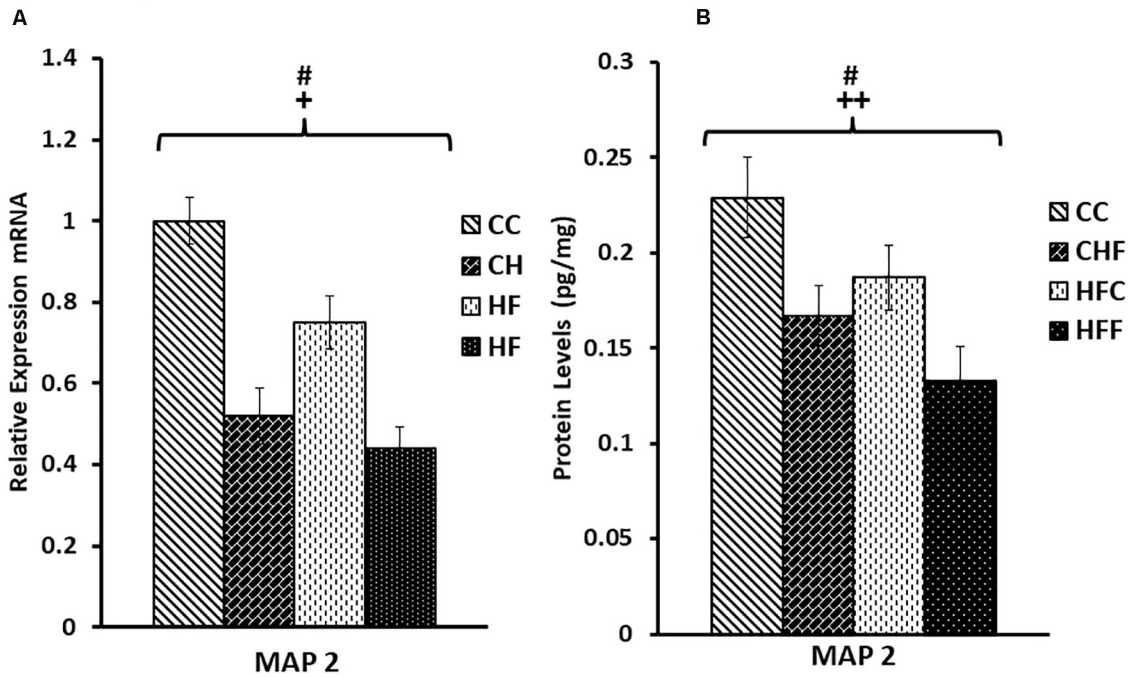


**FIGURE 4 |** Relative levels of MAP2 mRNA and protein expression in the hippocampus. **(A)** Using qRT-PCR, hippocampal microtubule-associated protein 2 (MAP2) mRNA expression was significantly reduced in response to the maternal diet ($F_{(3,57)} = 3.28$, $P = 0.05$) and post-weaning high-fat diets ($F_{(3,57)} = 6.76$, $P = 0.013$). Values are expressed in relative units where the CC group is set to 1. **(B)** Significant reductions in MAP2 protein levels (pg/mg) were also detected in response to both the maternal ($F_{(3,57)} = 9.28$, $P = 0.004$) and post-weaning HF diets ($F_{(3,57)} = 5.67$, $P = 0.023$) using ELISA. No significant interactions were detected using two-way ANOVA. Values are means ± SEM ($n = 16$ for each group). $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ (significant maternal diet effect); $^{\#}P \leq 0.05$ (significant post-weaning diet effect).

MAP2, a key dendritic marker. Our data are also consistent with studies claiming that a reduction in synaptic spines and dendritic remodeling are prominent characteristics in animal models of depression (Duman and Duman, 2015). A significant reduction in SNAP-25 expression is also associated with impaired dendritic spine formation and function (Tomasoni et al., 2013) as well as neuronal circuit disruption and altered plasticity (Antonucci et al., 2013). We previously reported that mRNA levels of synaptophysin decrease in response to high-fat feeding and hyperleptinemia (Page et al., 2014), and in this study we confirmed that a significant reduction in synaptophysin's protein level also occurs. Diminished expression of SNAP25 and synaptophysin in our animals is particularly interesting, since studies have shown that these two vesicle membrane proteins play a critical role in plasticity as well as associated cognitive and behavioral functions (Schmitt et al., 2009). For example, the marked reduction in SNAP-25 expression in our study is associated with increased immobility during the FST and reduced open arm time during EPM testing. Our studies support results generated from SNAP-25 knock-out mice which show that these mice exhibit a dramatic increase in body weight, adiposity, hyperleptinemia, leptin resistance and impaired stress-coping behavior (Valladolid-Acebes et al., 2015).

Brain effector molecules, such as GDNF, also exert regulatory influence in the CNS. In fact, GDNF has been shown to promote neuronal development and differentiation and to play an important neuroprotective role during neuronal and glial cell maturation, growth, and survival (Tsybko et al., 2017). Moreover, severe mood disorders such as MDD and bipolar affective disorder have been characterized by dysregulation of neurotrophic signaling. Both GDNF and leptin exert neurotrophin-like actions in the hippocampus (Mainardi et al., 2017), and disturbances in the availability and function of either effector has been implicated in the development of mood disorders. Recent evidence supports the hypothesis that GDNF is critical to hippocampal dendrite development and results from *in vitro* and *in vivo* experiments show that GDNF and its receptor are required for the proper growth and morphology of dendritic arbors and spines (Irala et al., 2016). Reduced GDNF mRNA and serum levels have been detected in patients with major depression (Otsuki et al., 2008) and serum GDNF level was negatively correlated with the severity of depressive symptoms. Conversely, an increase in GDNF has been shown to act as a neuroprotectant which prevents oxidative injury in neural cells (Chen et al., 2005; Ortiz-Ortiz et al., 2011). Our study indicates that GDNF expression is significantly reduced following exposure to excess saturated fat in the maternal and/or post-weaning diet. These findings confirm that early-life programming persists into adulthood and that continuous exposure to excess saturated fat during early life and adulthood intensifies the adverse effect on an animal's emotional balance.

As noted above, our results indicate that significant reductions in the expression of key proteins mediating synaptic function are also associated with decreased stress coping strategies and

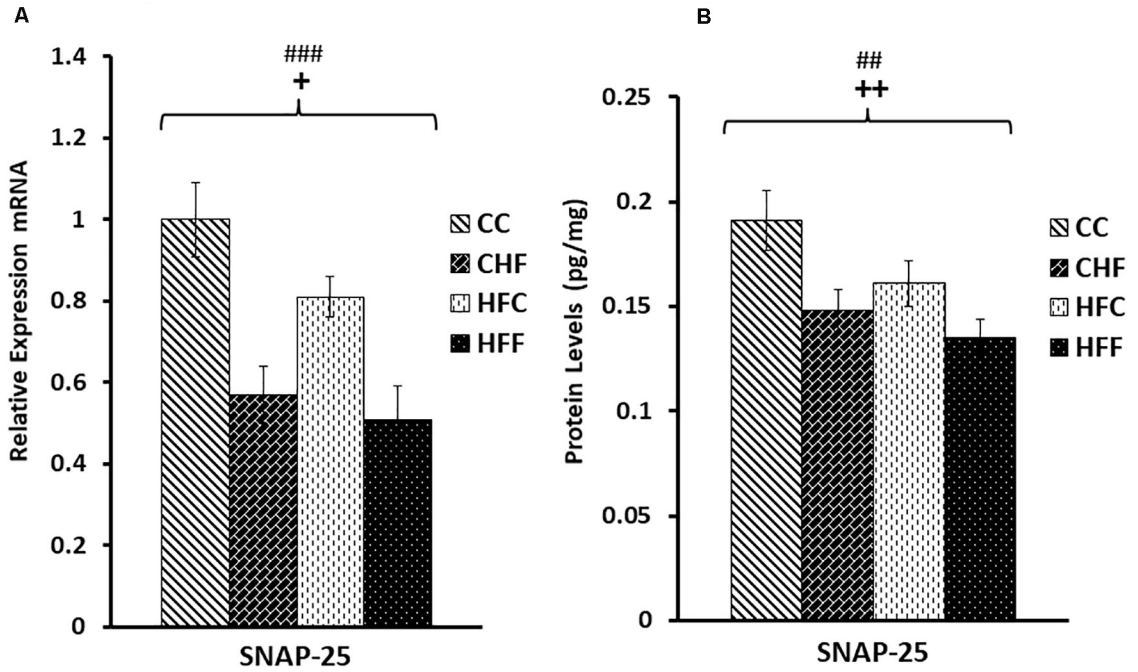


**FIGURE 5 |** Relative levels of SNAP25 mRNA and protein expression in the hippocampus. **(A)** Using qRT-PCR, hippocampal synaptosomal-associated protein 25 (SNAP25) mRNA expression was significantly reduced in response to both maternal ($F_{(3,57)}$ = 5.11, $P$ = 0.038) and post-weaning high-fat diets (post-weaning diet effect; $F_{(3,57)}$ = 12.71, $P$ = 0.001). Values are expressed in relative units where CC group is set to 1. **(B)** Significant reduction in SNAP25 protein levels (pg/mg) was also detected in response to both the maternal ($F_{(3,57)}$ = 8.36, $P$ = 0.007) and post-weaning HF diets ($F_{(3,57)}$ = 10.53, $P$ = 0.003) using ELISA. No significant interactions were detected using two-way ANOVA. Values are means ± SEM ($n$ = 16 for each group). $^{+}P \leq 0.05$,$^{++}P \leq 0.01$ (significant maternal diet effect); $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ (significant post-weaning diet effect).

increased anxiety-like behavior in animals exposed to HF diets during gestation, lactation and/or post-weaning. We investigated these disturbances further by examining the interdependent relationship among neurotrophins, serotonin receptors, and emotionality. Both animal and human studies have implicated abnormal serotonergic function in the pathophysiology of anxiety and depression (Zmudzka et al., 2018). Our results provide further evidence for serotonergic dysfunction since a significant reduction in expression of the serotonin 5HT1A receptor was detected in animals exposed to maternal and/or post-weaning HF diets. In contrast, 5HT2A receptor expression was increased only in response to the maternal HF diet. The relationship between the reduced expression of both GDNF and serotonin 5HT1A receptor in the hippocampus may, in part, be due to GDNF's role in the survival and maintenance of monoaminergic neuronal function (Pascual et al., 2011) and, in turn, hippocampal 5-HT1A receptor's mediation of GDNF's antidepressant effects (Naumenko et al., 2013). Disturbances in the 5HT1A and 5HT2A receptors have been implicated in the development of stress-related illness such as major depressive disorder (MDD) (Stockmeier, 2003; Noro et al., 2010), and enhancing 5-HT1A receptor gene expression and activity predicts a favorable outcome for anti-depressant action (Albert and Francois, 2010).

Additional studies have demonstrated a more defined link between 5HT1A, 5HT2A, and emotional disturbances. For example, increased 5HT2A receptor binding is associated with depressed mood, impulsivity, and suicidality (Bilbo and Tsang, 2010); whereas, developmental deficiencies in 5HT1A receptor expression increase an individual's susceptibility to anxiety disorders (Donaldson et al., 2014). Interestingly, a recent report suggests that a reciprocal competitive interaction exists between the two receptors. Data from this study indicates that inhibition of 5HT1A receptor expression results in a significant increase in 5HT2A receptor protein. Conversely, activation of 5HT2A receptors suppresses the expression of 5HT1A receptor and promotes anxiety-like behavior (Xiang et al., 2017, 2019). Our data provide further evidence to support the hypothesis that serotonin receptors 5HT1A and 5HT2A are engaged in a competitive interaction which is associated with anxiety-like behavior and affective disorders.

To further explore the possibility that exposure to excess saturated fat during development is involved in the impairment of synaptic functions and emotional stability, we analyzed the expression of two key subunits of glutamate's AMPA receptor, GluA1 and GluA2. These subunits are critical for glutamate's effective interaction with the AMPA receptor and, in concert with additional neurotrophic factors, they regulate developmental and adult neuroplasticity (Duman, 2014b). Evidence suggests that, in addition to changes in serotonergic function, disturbances in glutamatergic neurotransmission may be central to mood disorders (Hashimoto, 2011; Tokita et al., 2012; Hashimoto et al., 2013). Loss of coping ability and depression have been

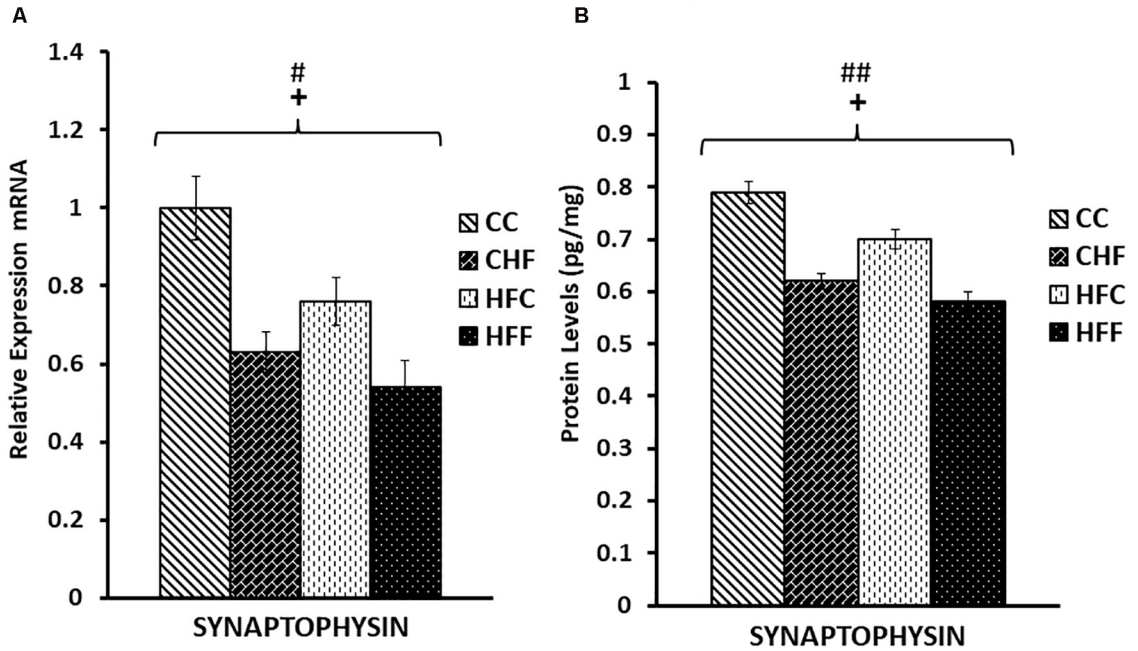


**FIGURE 6 |** Relative levels of Synaptophysin mRNA and protein expression in the hippocampus. **(A)** Using real-time PCR, hippocampal synaptophysin mRNA expression was significantly reduced in response to both maternal ($F_{(3,39)}$ = 4.14, $P$ = 0.038) and post-weaning high-fat diets (post-weaning diet effect; $F_{(3,39)}$ = 7.47, $P$ = 0.014). Values are expressed in relative units where CC group is set to 1. **(B)** Significant reductions in synaptophysin protein levels (pg/mg) were also detected in response to both the maternal ($F_{(3,39)}$ = 6.65, $P$ = 0.032) and post-weaning HF diets ($F_{(3,39)}$ = 8.64, $P$ = 0.004) using ELISA. No significant interactions were detected using two-way ANOVA. Values are means ± SEM ($n$ = 16 for each group). $^{+}P \leq 0.05$ (significant maternal diet effect); $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ (significant post-weaning diet effect).

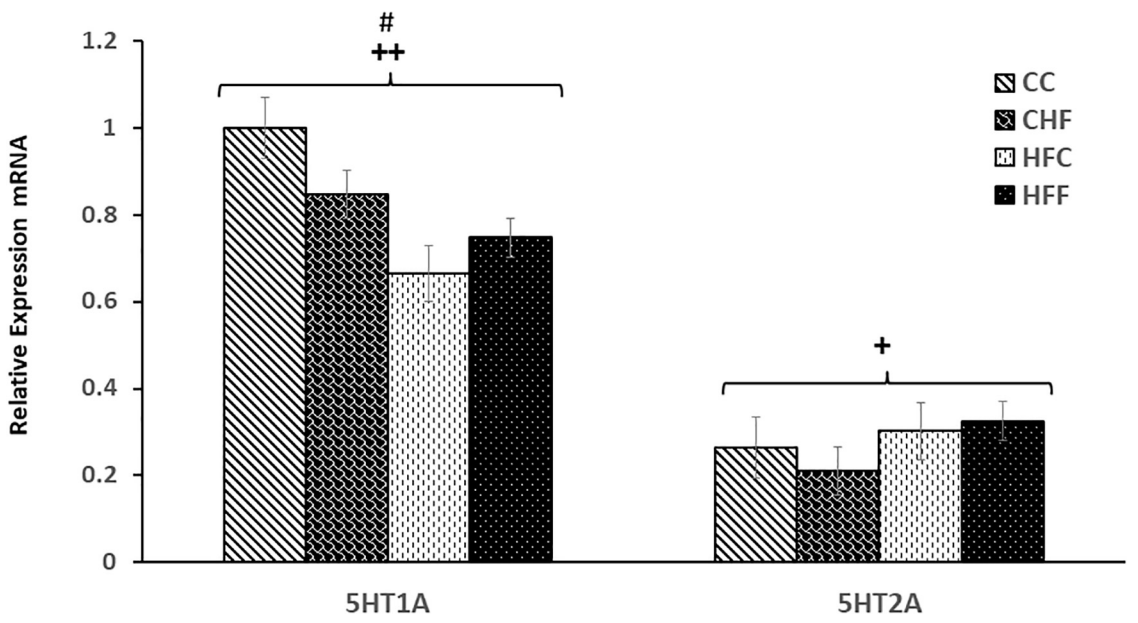


**FIGURE 7 |** Relative levels of serotonin receptor expression in the Hippocampus. Hippocampal serotonin receptor, 5HT1A and 5HT2A, mRNA expression in the four treatment groups (CC, CHF, HFC, HFF) was measured using qRT-PCR analysis. Values are expressed in relative units where the CC group is set to 1. Serotonin receptor 1A (5HT1A) mRNA expression was significantly reduced in response to both maternal diet (5HT1A, $F_{(3,39)}$ = 7.13; $P$ = 0.007) and post-weaning diet ($F_{(3,39)}$ = 6.49; $P$ = 0.027). In contrast, an increase in 5HT2A receptor was detected only in response to the maternal diet ($F_{(3,39)}$ = 7.28; $P$ = 0.039). No significant interaction was detected between maternal and post-weaning diet using two-way ANOVA. Values are means ± SEM ($n$ = 16 for each group). $^{+}P \leq 0.05$,$^{++}P \leq 0.01$ (significant maternal diet effect); $^{\#}P \leq 0.05$ (significant post-weaning diet effect).

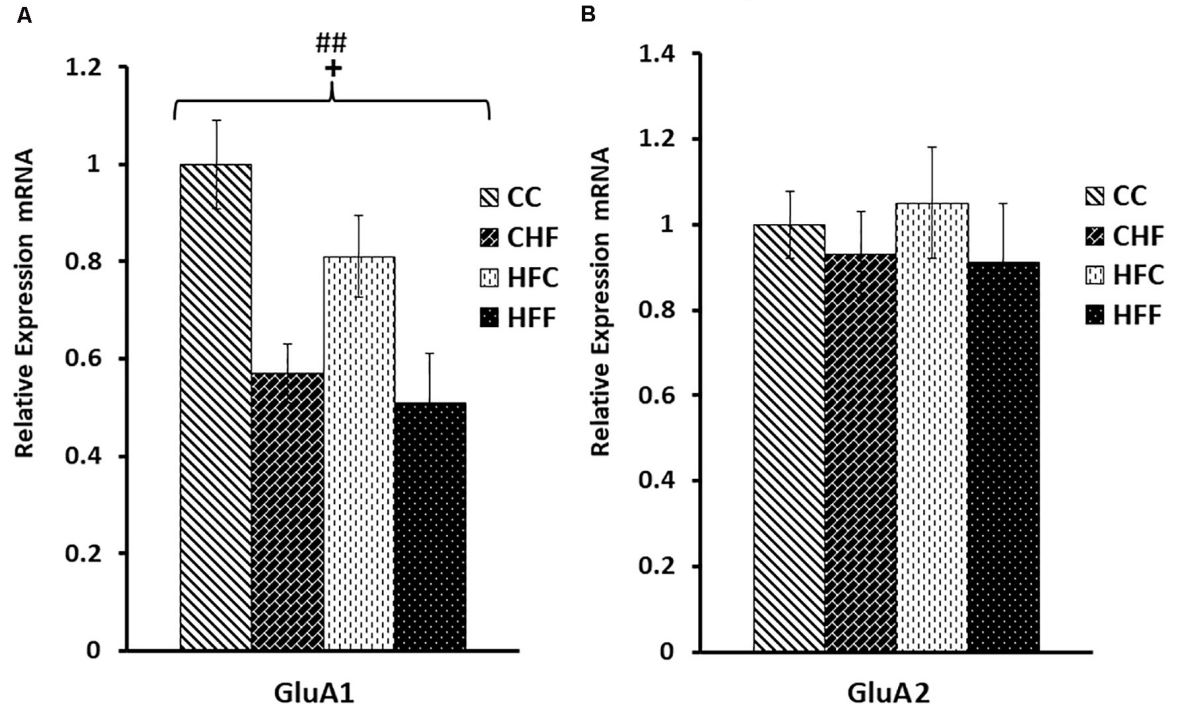


**FIGURE 8 |** Glutamate AMPA receptor subunit mRNA expression in the hippocampus. Hippocampal mRNA expression for the glutamate AMPA receptor subunits, GluA1 and GluA2, was measured in the CC, CHF, HFC, and HFF groups using qRT-PCR. Values are expressed in relative units where the CC group is set to 1. **(A)** GluA1 subunit expression was significantly reduced in response to both the maternal ($F_{(3,39)} = 4.06$, $P = 0.047$) and post-weaning HF diet ($F_{(3,39)} = 8.04$, $P = 0.008$). **(B)** No significant changes were measured for hippocampal GluA2 mRNA expression using two-way ANOVA. Values are means ± SEM ($n = 16$ for each group). $^{+}P \leq 0.05$ (significant maternal diet effect); $^{\#\#}P \leq 0.01$ (significant post-weaning diet effect).

associated with compromised AMPA receptor activation due, in part, to changes in expression of the GluA1 subunit (Duric and Duman, 2013); whereas, enhanced activation of AMPA receptors promotes an antidepressant effect (Farley et al., 2010). In fact, AMPA-receptors containing the GluA1 subunit are required for the antidepressant action of NMDA antagonists (Koike and Chaki, 2014). Our data extend support for these findings. The significant reduction in expression of the AMPA receptor GluA1 subunit in our obese animals suggests that hippocampal glutamatergic signaling is compromised by high-fat feeding and is accompanied by increased emotionality.

Together, our results show that exposure to a diet high in saturated fat during critical stages of brain development disturbs the expression of hippocampal genes involved in synaptic function and predisposes the animal to mood disorders. We detected a marked reduction in GDNF and significant decreases in the expression of genes coding for markers of synaptic function, MAP2, SNAP-25, synaptophysin, key serotonin receptors, and critical subunits of the glutamate AMPA receptor. These changes were associated with a reduction in the animal's stress-coping strategy and increased anxiety-like behaviors.

It should be noted that behavioral profiles are not directly dependent on a selection of molecular markers, rather they are a manifestation of complex molecular integrations and environmental contexts. For example, dysfunction in the central serotonergic system is prominently positioned in the pathoetiology of depression, and yet, its role in this behavioral manifestation has not been clearly elucidated. This also pertains to high-fat diet-induced neurological and cognitive dysfunctions due to neural inflammation, oxidative stress or decreased neurogenesis and plasticity in the hippocampus. In our study, we examined the expression of a few representative proteins that are likely to contribute to hippocampal plasticity changes and associated behavioral manifestations. Ultimately the results from our study and a multitude of others will provide a foundation for a more mechanistic hypothesis which will be derived from a combination of factors.

In summary, our results suggest that the levels of hippocampal proteins required for synaptic function are inadequate in rat offspring exposed to either a maternal or post-weaning high-fat diet. Moreover, exposure to the combination of maternal and post-weaning HF diets increases the magnitude of adverse consequences. Impaired neural functions resulting from the physiological stress of a diet high in saturated fat are likely to underpin significant disturbances in emotional balance.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by Bucknell University Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

KP performed the experiments and analyzed the data. KP wrote the manuscript with contributions from EA. Both authors conceived and designed the project, discussed the data, and contributed to the article and approved the submitted version.

## FUNDING

We would like to extend our gratitude to Bucknell University for generously funding this research.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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