



Maternal high fructose diet and neonatal immune challenge alter offspring anxiety-like behavior and inflammation across the lifespan



Syed Hussain F. Bukhari, Olivia E. Clark, Lauren L. Williamson*

Psychology Department, Williams College, Williamstown, MA 01267, United States

ARTICLE INFO

Keywords:

Gestational diabetes
Fructose
Anxiety
Inflammation

ABSTRACT

Aims: This study examined the interaction between maternal high fructose diet and neonatal inflammation in neonates (P7), juveniles (P26–34) and adults on measures of anxiety-like behavior and cognition. The study aimed to assess the potential synergistic effects of these two forms of early-life inflammation.

Main methods: We fed Sprague-Dawley dams with high fructose (60%) diet or normal chow. Each litter was treated with either saline or lipopolysaccharide (LPS) on postnatal day (P)3 and P5 and two pups were tested for USVs after maternal separation on P7. Post-weaning, juveniles were tested on the elevated zero maze (EZM) and in a context-object discrimination (COD) task prior to tissue harvest. Adults were tested on the EZM and the COD task as well. Immunohistochemistry and ELISA were used to assess molecular and cellular changes in the offspring.

Key findings: This study demonstrates that maternal diet and neonatal inflammation altered peripheral inflammation in neonates, altered anxiety-like behavior in juveniles, and altered anxiety-like behavior in adulthood. Maternal diet and sex increased juvenile peripheral inflammation and altered memory on the context-discrimination task.

Significance: Maternal diet has a profound impact on fetal and neonatal development, especially as obesity rates are on the rise worldwide. Together, these findings reveal enduring effects of maternal diet on offspring, support the findings on the effects of neonatal inflammation on anxiety-like behaviors in later-life periods, and add to the complex relationship between gestational and neonatal inflammation and anxiety.

1. Introduction

Gestational diabetes, characterized by a state of hyperglycemia during the gestational period, is increasingly common in the US [12]. Both the mother and fetus experience harmful conditions as maternal insulin efficacy is reduced [1,16], allowing glucose to persist in the mother's blood, increasing chances for both birth defects and late-onset diseases. Including metabolic and cardiovascular diseases, these disorders co-occur alongside the nervous system's development, which may be disrupted by the release of pro-inflammatory cytokines, triggered by gestational diabetes [26]. Recent rodent research suggests that exposure to gestational diabetes promotes overactive microglia in brain areas relevant to learning and memory tasks [34]. The release of pro-inflammatory cytokines during development can lead to adult anxiety-like behavior and an increased reactivity to stress through alteration of the hypothalamic-pituitary-adrenal axis [6,15,18]. Similar disruptions in behavior, mediated by neuroinflammation, are caused by the activation of the immune system during developmental critical periods

[31].

In rodents, lipopolysaccharide (LPS) treatment during early life leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, and results in an HPA hyper-responsiveness in adulthood and an altered glucocorticoid responsiveness to stress. In humans, this dysfunctional glucocorticoid response is linked to anxiety disorders, depression, schizophrenia and other neuropsychiatric diseases [2]. In rodents, models of both neonatal inflammation and maternal immune activation result in abnormal behavior across the lifespan [9,14,18].

Anxiety disorders are the most common neuropsychiatric disorders within the United States and gestational diabetes is increasingly common. Therefore, modeling a combined immune system disruption alongside gestational diabetes is critical given prior evidence suggesting a role of neuroinflammation in determining anxiety-like behavior throughout the lifespan. We used pregnant rats fed a fructose enriched (60%) diet, and then induced neonatal inflammation. The diet (adapted from [30]) was used to create a state of overnutrition for the extent of the gestational and postnatal period, which mirrors the course of

* Corresponding author at: Psychology Department, 25 Stetson Ct, Williams College, Williamstown, MA 01267, United States.
E-mail address: 07llw@williams.edu (L.L. Williamson).

gestational diabetes within a pregnant human.

Following neonatal immune challenges, we tested for changes in anxiety-like and learning behaviors. We assessed ultrasonic vocalizations (USVs) to test anxiety-like behavior of neonates [8]. In juvenile and adult rats, the Elevated Zero Maze (EZM) paradigm was used to assess anxiety-like behaviors [7]. To test effects on learning and memory, both juveniles and adults were tested on a context-object discrimination (COD) task. The COD paradigm is dependent upon hippocampal memory processes, linking differing contexts and the objects within them. Systemic neuroinflammation interferes with hippocampal-dependent memory processes through the release of inflammatory cytokines. As a result, performance in this task is impaired. [11,37,38].

In this study, we examined if neuroinflammation caused by a neonatal immune challenge interacts with or augments the negative phenotypes caused by gestational diabetes. We hypothesized that the increase in neuroinflammation would affect anxiety-like behavior and impair cognitive function throughout development and into adulthood. We also expected to observe molecular changes in the central and peripheral immune systems, especially in cytokines such as IL-1 β , TNF α and IL-10, and microglial morphology and activation as a result of neuroinflammation. Increased understanding of the maternal and neonatal inflammatory mechanisms of early-life immune challenges and gestational diabetes will help shape predictions for human health and behavior.

2. Materials and methods

2.1. Animals

Adult female Sprague-Dawley rats were singly housed in transparent plastic cages (45 cm L \times 25 cm W \times 15 cm H) in a temperature-controlled colony room with a 12 h light: 12 h dark cycle (0700 lights on). Female rats were divided into experimental cohorts, and given ad libitum access to water and either fructose diet (Envigo Teklad Diet 89,247) or normal chow diet (Envigo Teklad Diet 7012). After 1 week, male rats were placed in the cages for a period of 7 days for mating to induce pregnancy. Male rats were then removed, and female rats were allowed to carry to full term. Lactating mothers were also maintained on these diets following delivery up to postnatal day (PD) 12 in order to mimic the duration and physiological effects of gestational diabetes as seen in previous studies [24], and to overlap high fructose diet exposure with hippocampal development during the early lactation period for Sprague-Dawleys [13]. On postnatal day (PD) 12, all rats were returned to normal chow diet. Male and female juvenile rats from the resulting litters were weaned on PD25. During the juvenile period, rats were housed three-to-a-cage in same-sex groupings in the same colony room with ad libitum access to food and water. On P40, all rats were pair-housed. The Institutional Animal Care and Use Committee at Williams College approved all procedures.

2.2. Diet

The rats were allowed to acclimatize to the high-fructose diet for a period of a week before pregnancy was induced. The normal chow diet (Envigo Teklad Diet 7012) contained 44.3% carbohydrate, 19.1% protein, 13.7% neutral detergent fiber, 5.8% fat, and 4.6% crude fiber. The fructose-enriched diet (Envigo Teklad Diet 89247) contained 60% fructose, 21% casein, 8% fiber, 5% lard, and approximately 7% of a mineral and vitamin mix. The diet regimen was maintained through pregnancy until pups reached P12 to expose pups to the diet during gestation and lactation [35,40]. All rats were fed normal chow (Diet 7012) after P12 until weaning and thereafter.

2.3. Neonatal treatments

During the neonatal period, litters on both diets were treated with either saline or lipopolysaccharide (LPS, membrane component of Gram negative bacteria) on P3 and P5. For injections, all pups in a given litter were separated from the dam and placed in a plastic cage (18.4 cm \times 29.2 cm \times 12.7 cm) with bedding. To minimize heat loss, each cage was rested on a heating pad during the separation from the dam. All injections were done between 10:00 and 14:00. Each pup received a subcutaneous injection of either endotoxin-free saline (SAL) or *E. coli*-derived LPS (strain 0111-B4, Sigma-Aldrich, St. Louis, MO) dissolved in endotoxin-free saline. All pups in a given litter were given the same treatment and each litter was randomly assigned to a condition. Pups in the SAL group received 0.1 mL of saline, while pups in the LPS group received a dose of 50 μ g/kg of LPS. Following injections, all pups were returned to the dams.

2.4. Ultrasonic vocalization (USV) testing and tissue harvest

On P7, 1 female and 1 male pup were randomly chosen from each litter and placed into a plastic cage with bedding, separated from each other. The pups were transported into the habituation room, where they habituated for 10 min on a heating pad set to low. After this brief maternal separation, pups were individually placed in a darkened chamber housing a circular glass dish (20 cm D \times 10 cm H), with no bedding. This glass dish was located beneath an S-25 ultrasound bat detector (Ultra Sound Advice, London) set to detect signals at 50 \pm 5 kHz. The ultrasonic vocalizations (USVs) for each pup were recorded for two minutes by listening through headphones attached to the detector and every individual vocalization was counted using LabChart to produce a total number of vocalizations for each pup ($n = 42$).

Pups were returned to small plastic containers on the heating pad until tissue collection that followed USV testing. Following rapid decapitation, trunk blood was collected in microcentrifuge tubes at the time of decapitation, spun down at 16.1g for 10 min, and then serum was collected and frozen at -20° C.

2.5. Elevated zero maze

The elevated zero maze (EZM) behavioral testing apparatus is a black wooden ring, raised to 0.51 m above the ground with two opposite with tall walls (the closed portion), and two opposite quarters with low walls (the open portion) (Fig. 1D). During their respective testing, juvenile and adult rats were individually transported in a transfer cage to the EZM testing room, which was illuminated by two lights aimed away from the testing apparatus. At the time of testing, rats were placed in the center of the open arm, and the experimenter left the testing room to allow the rat to freely explore the maze. Test sessions lasted 5 min. Following testing, the rat was placed back in the transfer cage and returned to the colony room. The maze was cleaned and disinfected with Quatricide between animals. All behavior was automatically and immediately scored using AnyMaze software (Wood Dale, IL, USA). Amount of time spent in the closed arms of the maze is correlated with anxiety-like behavior in Sprague-Dawley rats [7].

2.6. Context-object discrimination

Both juvenile and adult rats underwent the COD memory task following five days of handling (2 min per day). This paradigm consists of two training days, during which the animal experiences each of two context-object sets, and one test day, on which an animal is exposed to a familiar context containing one in-context object and one out-of-context object (image and schematic of testing, Fig. 1A–C).

COD involves two apparatuses, identified as Context A and Context B. For juveniles, Context A is a white plastic cylinder, measuring 48 cm

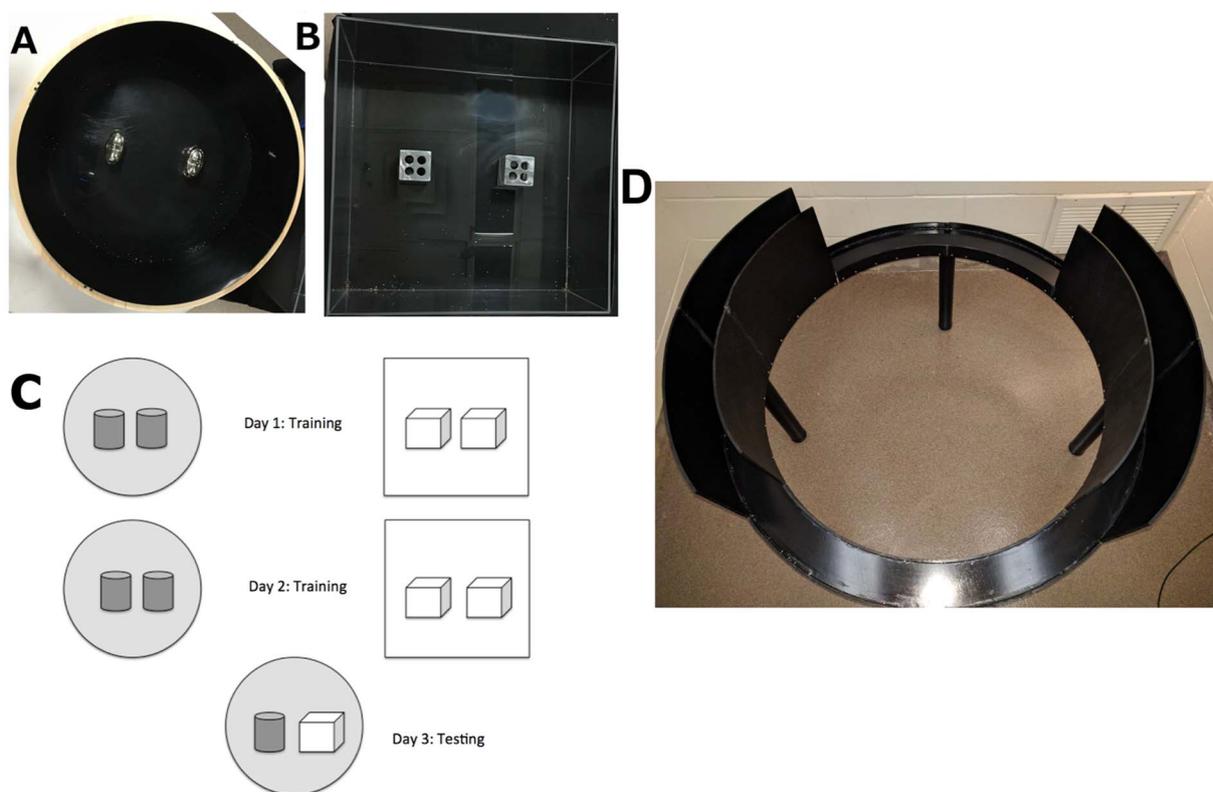


Fig. 1. Behavioral apparatuses for context-object discrimination (COD) testing and elevated zero maze (EZM). (A) Context A for adult rats containing 2 identical glass objects. (B) Context B for adult rats containing 2 identical marble cubes. (C) Schematic for COD training and testing following 5 days of gentle handling. (D) Elevated zero maze with 2 sections with high walls and 2 sections with low walls.

in diameter and 22 cm tall, and containing two identical gray, inverted bowls, measuring approximately 10 cm in diameter at the widest point, 7 cm at the base, and 5 cm tall (not shown). Context B is a white plastic square, measuring approximately 46 cm in length and width and 20 cm tall, and containing two identical blue, ceramic, dog-shaped salt-shakers, measuring approximately 9 cm tall and 6 cm wide at the base (not shown). In each context, the objects were placed on opposite sides of the context, approximately 13 cm apart and 8 cm away from the edge in Context A, and 10 cm away from the nearest edge in Context B. Context A was cleaned with Formula 409 All Purpose Cleaner, and Context B was cleaned with Quatricide between animals. The test context was identified as Context A', and was identical to Context A, except one of the bowls was replaced with a dog object from Context B. Context A' was also cleaned with Formula 409 All Purpose Cleaner between animals. Heads of the juvenile animals were marked with black sharpie in order to facilitate accurate tracking during testing.

For adults, context A was a black cylinder 70 cm in diameter and 40 cm high (Fig. 1A). Context B was a 59 cm long × 59 cm wide × 30 cm high Plexiglas container covered on the outside by black paper and a black cloth (Fig. 1B). To further differentiate the contexts, the contexts were cleaned between subjects with different solutions, context A with Formula 409 All Purpose Cleaner and context B with Quatricide. Both contexts were located in the same room. Each context had a pair of associated objects placed in the center of the apparatus, approximately 25 cm apart and at least 10 cm from the edge of the 16context. The associated objects for context A were shiny silver glass ovals, approximately 5.5 cm wide × 11 cm long × 11 cm high. The associated objects for context B were 7.5 cm wide × 7.5 cm long × 7.5 cm high gray marble cubes with holes in the sides. The objects were cleaned using the same solution as their associated contexts. For both age groups on both training and test days, rats were placed in the testing room to acclimatize for 1 h prior to training start. On Training Days 1 and 2, respectively, rats were allowed to freely explore both

Context A and Context B for 5 min each. Rats were returned to home cages between exposure to each context, and male rats were always run through the paradigm prior to females. The order of context exposure for each animal was counterbalanced across training days. On test day, each rat was placed in Context A' in order to test for context discrimination memory and learning, again for 5 min.

On training and test days, rat exploration was recorded and analyzed using AnyMaze software (Wood Dale, IL, USA). The amount of time the rat spent with its nose within two centimeters of an object was equated to exploration time of that specific object. Context discrimination memory was determined by calculation of a discrimination score:

$$\frac{[(\text{time spent exploring out-of-context object}) - (\text{time spent exploring in context object})]}{[(\text{time spent exploring out-of-context object}) + (\text{time spent exploring in context object})]}$$

with this formula, there was a minimum score of -1 and a maximum score of 1, with a negative value indicating a majority of time being spent with the in-context object, and a positive value indicating a majority of time being spent with the out-of-context object. Positive values indicate intact context discrimination memory.

2.7. Tissue collection

Following COD testing, juveniles were deeply anesthetized with a cocktail of ketamine/xylazine in preparation for perfusion. Approximately 2 mL of blood was collected from the heart muscle, which was then centrifuged to isolate serum. Serum, once separated, was frozen at -20 °C. The rat was then transcardially perfused with 0.9% saline, and the brain was removed. One hemisphere was preserved in 4% paraformaldehyde for immunohistochemistry (IHC), and

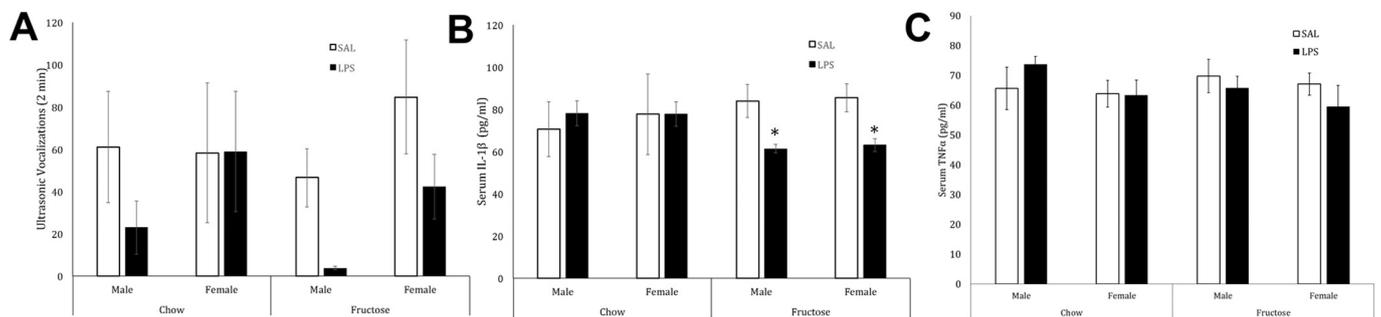


Fig. 2. Pups did not show behavioral changes after maternal diet and inflammatory challenge, but had altered peripheral IL-1 β . (A) Mean USV production was not affected by maternal diet or neonatal treatment. Sex, diet, and treatment did not interact to affect rat pups' USVs production ($n = 42$; $p > 0.05$). (B) IL-1 β serum levels were significantly reduced in rats exposed to both a high fructose maternal diet and neonatal LPS treatment ($n = 29$; $*p < 0.05$, lower than all other groups). (C) TNF α serum levels were not significantly different across groups ($n = 29$, $p > 0.05$).

the hippocampus was isolated from the opposite hemisphere. The hippocampus was flash-frozen in isopentane and stored at -80°C for later molecular analysis. For IHC, brains were kept in 4% paraformaldehyde in 0.1 M PB for 48 h and then cryoprotected in 30% sucrose for at least 48 h prior to tissue processing.

2.8. Interleukin-1 β and tumor necrosis factor α measurements

Interleukin (IL)-1 β was measured in serum and hippocampal tissue using a commercially available ELISA kit (R & D Systems, Minneapolis, MN). The ELISA was run according to the manufacturer instructions with one exception. In the standard curve that is generated using each set of samples assayed, the highest concentration point (2000 pg/mL) was excluded in favor of including one point of lower concentration on the curve. Tumor necrosis factor (TNF) α was measured in the same serum and brain tissue using a commercially available ELISA kit (R & D Systems, Minneapolis, MN). The TNF α ELISA was run according to manufacturer instructions. Results are expressed as picograms (pg) per 1 mL of serum or pg/mg of protein in hippocampus samples.

2.9. Tissue processing and immunohistochemistry

Using a freezing cryostat, the juvenile brains were sliced into 40 μm thick coronal slices. 5 series of brain sections through the anterior-posterior plane were collected, and stored at 4°C in 0.1% sodium azide solution until IHC was performed.

For immunohistochemistry, sections from each brain were rinsed in 0.01 phosphate buffered saline (PBS) and mounted on gel-coated slides. Staining for the microglia marker ionized calcium binding adaptor molecule 1 (Iba1) was performed. All sections were rinsed in PBS, incubated at room temperature in 50% methanol plus 0.3% H_2O_2 for 30 min. Following another PBS wash, sections were incubated in blocking buffer (5% normal goat serum and 0.3% Triton-X in PBS). Sections were then incubated overnight in primary antibody (Iba1 antibody, 1:1000, rabbit polyclonal, Wako Chemical Industries, Ltd.) in blocking buffer. The next day, sections were washed with PBS again, following a 2 h incubation in anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) in blocking buffer and then washed in PBS. The Avidin-Biotin Complex (ABC) method was used to bind a complex to the secondary antibody. Slides were washed in 0.1 M phosphate buffer (PB). Lastly, slides were incubated with 3,5-diaminobenzidine (DAB, Sigma-Aldrich, USA) for up to 25 min to produce a colorimetric stain. Sections were then mounted onto gel-coated slides, dehydrated with ethanol washes, xylenes and coverslipped with Permount.

2.10. Densitometry

Using a Nikon 4550L microscope, stained slices were imaged at

10 \times magnification with the NIS Elements BR 3.0 software (Nikon Instruments). For each brain, 5 slices had the CA1, CA3, and DG of the hippocampus photographed. Areas with myelin tracts and tears were ignored. Densitometry analysis was performed with ImageJ64 to identify cells positive for Iba1. A signal pixel darkness value of more than three standard deviations darker than the threshold value was used to obtain the integrated area density in each photo. A previously known measure was used to calculate the integrated area density measurement by multiplying the area of the analyzed area with average pixel darkness [36].

2.11. Statistics

For all behavior paradigms as well as molecular and cellular measures, statistics were calculated using SPSS software (IBM, Armonk, NY). 2(sex) \times 2(fructose diet/normal chow) \times 2(LPS/SAL) ANOVAs were performed.

3. Results

3.1. USV production in neonatal P7 rats was not changed by treatment or maternal diet

Average number of USVs was not affected by gestational diet or neonatal treatment. No significant difference was detected between sexes (Fig. 2A; $F(1,41)$, $p > 0.05$).

3.2. Neonatal treatment and gestational diet altered IL-1 β , but not TNF α levels in the serum of neonatal P7 rats

A significant difference in the levels of the cytokine IL-1 β was seen in the serum of neonatal P7 rats, regardless of sex. Pups treated with neonatal LPS injections and exposed to high fructose diet during the gestational and early neonatal periods had lower IL-1 β levels in serum ($F(1,28) = 5.35$, $p = 0.028$), compared to all other treatment groups (Fig. 1B). However, peripheral TNF α levels were not changed by maternal diet or neonatal treatment in P7 pups ($n = 34$, $p > 0.05$, data not shown).

3.3. Sex and gestational diet improved performance in context object discrimination tasks for juvenile P34 rats. Neonatal treatment and sex improved performance in COD for adult rats, especially saline-treated females

Juvenile male rats exposed to high fructose maternal diet performed better on tests for context object discrimination (COD), compared to all other groups (Fig. 3A; $F(1,41) = 6.224$, $p = 0.017$). However, none of the groups demonstrated discrimination scores above chance, indicating a possible inability to perform in the task. In adults, neonatal

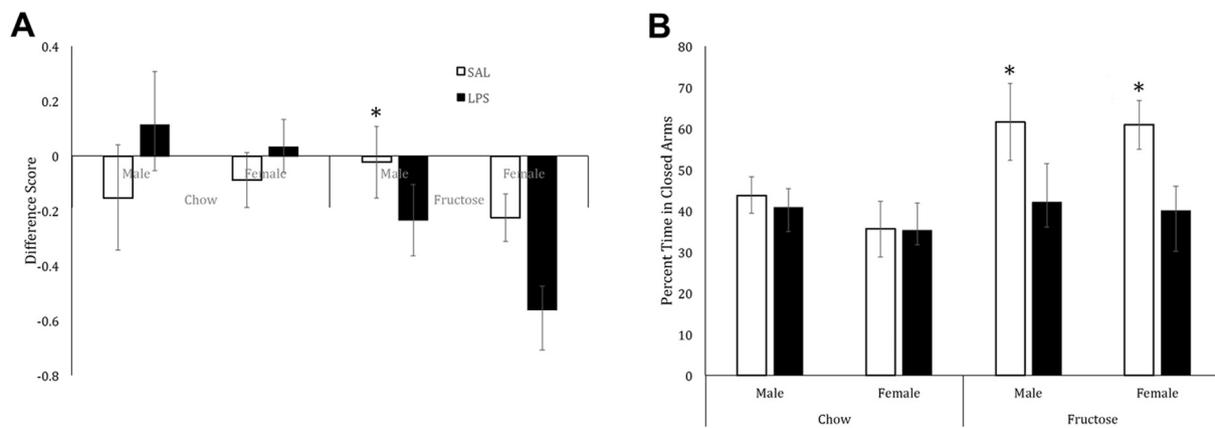


Fig. 3. Learning and anxiety-like behaviors were differentially affected in the juvenile period. (A) No groups demonstrated discrimination scores above chance, but the juvenile male rats exposed to a maternal diet high in fructose performed significantly better than all other groups in the COD task ($n = 42$; $*p < 0.05$). (B) High fructose maternal diet increased anxiety-like behaviors in pups treated with saline on the elevated zero maze (EZM). This increase did not occur in offspring treated with LPS ($n = 40$; $*p = 0.056$).

treatment and sex interacted to alter COD performance (Fig. 5A; $F(1,57) = 7.67$, $p = 0.008$). Regardless of maternal diet, females treated with saline as neonates performed significantly better than saline-treated males. Neonatal treatment with LPS reduced performance to similar levels for both sexes.

3.4. Juvenile anxiety-like behaviors were increased by an interaction of gestational diet and neonatal treatment, while adult anxiety-like behavior was reduced by maternal high fructose diet

Juvenile rats exposed to high fructose maternal diet and saline as neonates spent significantly more time in the closed arms of the elevated zero maze (EZM) task (Fig. 3B; $F(1,39) = 3.88$, $p = 0.056$). LPS treatment during the neonatal period combined with exposure to high fructose diet attenuated this anxiety-like behavior. The maternal diet significantly affected time spent in the closed arm of the EZM task for adult rats. Adult rats born to fructose-fed dams spent significantly less time in the closed arms compared to other groups (Fig. 5B; $F(1,57) = 3.86$, $p = 0.054$).

3.5. Interactions between neonatal treatment, sex, and diet affected peripheral IL-1 β levels of juvenile rats

Gestational diet combined with neonatal treatment altered IL-1 β serum levels ($F(1,39)$, $p = 0.026$), such that pups exposed to high fructose maternal diet and saline had a similar level of IL-1 β in serum to LPS-treated groups. However, pups that experienced normal maternal chow and neonatal saline treatment had significantly lower IL-1 β serum levels. Sex and gestational diet also interacted to alter IL-1 β in the serum of P34 rats ($F(1,39) = 4.69$, $p = 0.037$), such that male juveniles exposed to high fructose diet during the gestational period had higher levels of IL-1 β compared to males born to chow-fed dams. However, serum IL-1 β in female juveniles was unaffected by maternal diet (Fig. 4A). Serum TNF α was not significantly different between any juvenile treatment groups (Fig. 4C; $p > 0.05$).

3.6. Interleukin-1 β and TNF α proteins in the hippocampus were not changed by maternal diet or neonatal treatment in juvenile rats

Neither neonatal treatment nor diet during the gestational period significantly affected the concentration of IL-1 β (Fig. 4B) or TNF α (Fig. 4D) proteins within the hippocampus of juvenile rats (p 's > 0.05).

3.7. Microglial density was altered by neonatal treatment and maternal diet within hippocampal sub-regions in juvenile rats

Neonatal inflammation reduced microglial integrated area density within the dentate gyrus (DG) ($F(1,27) = 11.34$, $p = 0.002$), CA1 ($F(1,28) = 15.04$, $p = 0.0006$) and CA3 ($F(1,29) = 6.49$, $p = 0.016$), respectively. Furthermore, maternal high fructose diet reduced microglial density overall in the CA3, but no other hippocampal sub-regions ($F(1,29) = 5.96$, $p = 0.021$). (See Fig. 6)

4. Discussion

The results of this study highlight the complex relationships between gestational diet, neonatal inflammation, and anxiety-like behavior throughout the rodent lifespan. While no differences in anxiety-like behavior in the neonatal period were detected, the combination of neonatal LPS treatment and a maternal high fructose diet led to significantly higher levels of IL-1 β in the serum of P7 rats, indicating prolonged inflammation stemming from both maternal diet and neonatal treatment. In the juvenile period, male rats born to dams on a high fructose diet also showed significantly higher levels of IL-1 β in serum. No alterations in the level of TNF- α were found in either serum or hippocampus across the various testing stages. A high fructose maternal diet led to increased anxiety-like behavior in the juvenile period, which occurred regardless of sex or neonatal treatment. In adulthood, this effect on anxiety reversed with all adult rats exposed to a high fructose diet showing lowered levels of anxiety-like behavior as opposed to their chow-fed counterparts, irrespective of sex and neonatal immune challenges. Together, these findings reveal enduring effects of maternal diet on offspring, further support the extensive literature on the longitudinal effects of neonatal inflammation on behaviors in later-life periods, and add to the complex relationship between gestational and neonatal inflammation and anxiety.

Although the effects of a fructose diet on anxiety-like behavior have been tested [4,17,18,27], our study is one of the first to show the effects of maternal high-fructose diet on anxiety through multiple stages of the rodent lifecycle. We found that both juvenile and adult rats exposed to a high-fructose maternal diet display aberrant anxiety-like behavior. In juveniles, the diet increases anxiety-like behavior, while an overall decrease in anxiety-like behavior was observed in adults. The reversal of anxiety-like behavior is not likely due to proximity to maternal high fructose diet, as the diet was ended at least 2 weeks prior to behavioral testing. These opposing changes in anxiety-like behavior may be consequences of interactions between maternal and neonatal immune activation. Previous work showed that hyperglycemia, as a result of over-nutrition, can lead to immune activation in the mother [3]. This

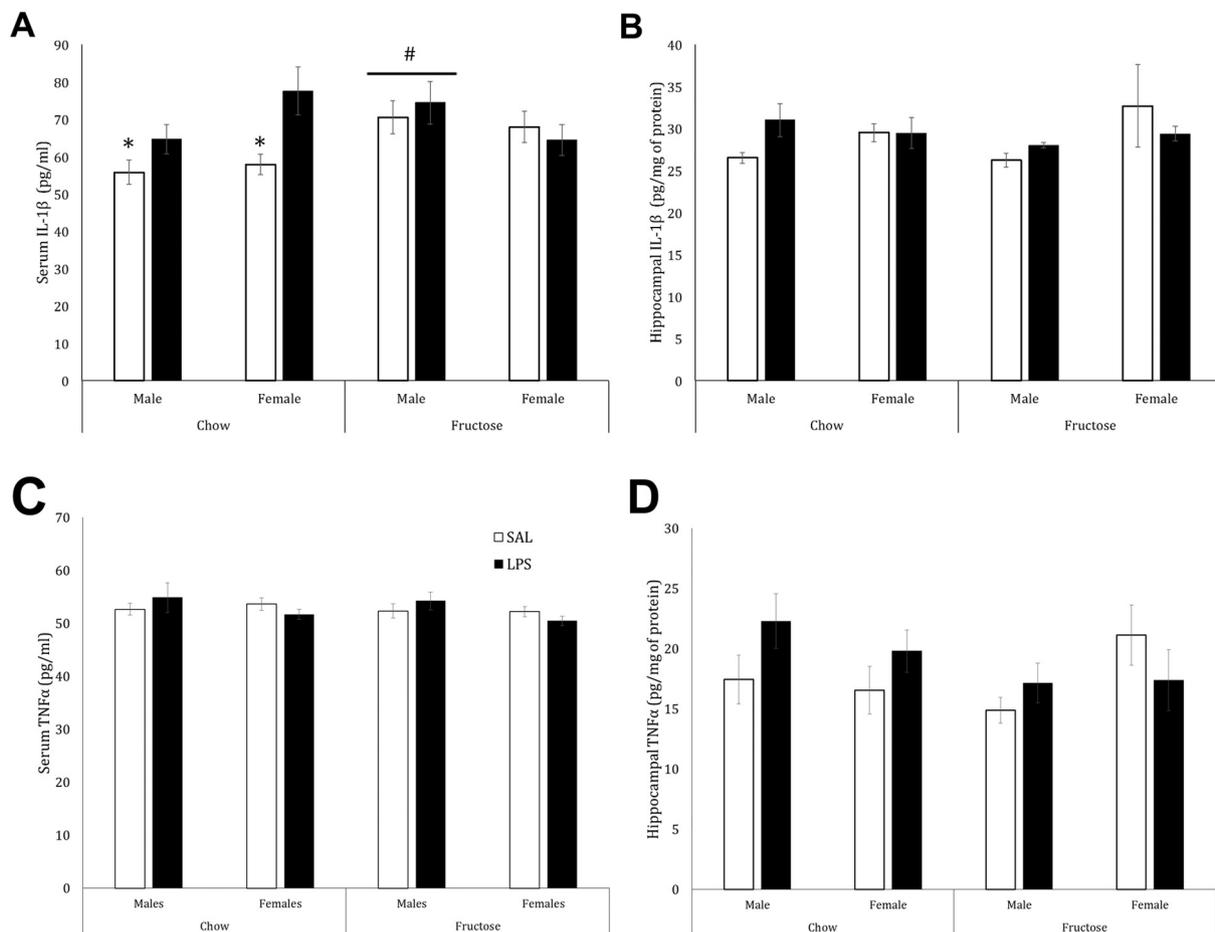


Fig. 4. While serum IL-1β is altered by treatment, sex and diet, hippocampal IL-1β protein levels remain unaltered. TNFα protein is not altered in any groups in either serum or hippocampus. (A) Saline-treated pups exposed to maternal regular chow had significantly lower levels of IL-1β than LPS-treated pups exposed to maternal regular chow. However, a high fructose maternal diet eliminated any differences established by neonatal treatment ($n = 40$: $^*p < 0.05$, significantly lower than Chow-LPS rats). Male juveniles, but not females, exposed to high fructose diet had higher levels of serum IL-1β compared to dietary controls ($n = 40$: $^#p < 0.05$, significantly higher than all Chow rats). (B) Hippocampal IL-1β protein levels were not affected by treatment, diet or sex ($n = 47$: not significant). (C) TNFα protein levels in juvenile serum were not significantly different between groups. ($n = 40$, $p > 0.05$). (D) TNFα protein levels in juvenile hippocampal tissue were not significantly different between groups. ($n = 40$, $p > 0.05$).

maternal immune activation occurs due to hormonal changes and difficulty with glucose processing resulting from hyperglycemia, and can lead to the upregulation of inflammatory cytokines in offspring [23,31]. During this critical period of development for offspring in utero, maternal immune activation and thus exposure to chronic inflammation as a result of gestational diabetes may be disruptive to developing neural

circuitry of offspring. With gestational diabetes the inflammatory profile of the in utero environment is altered, in turn contributing to pro-inflammatory changes in offspring [23].

Interleukin (IL)-1β and tumor necrosis factor (TNF)α are especially potent pro-inflammatory signals. We measured them in this study because they are known to increase within the brain, and most notably the

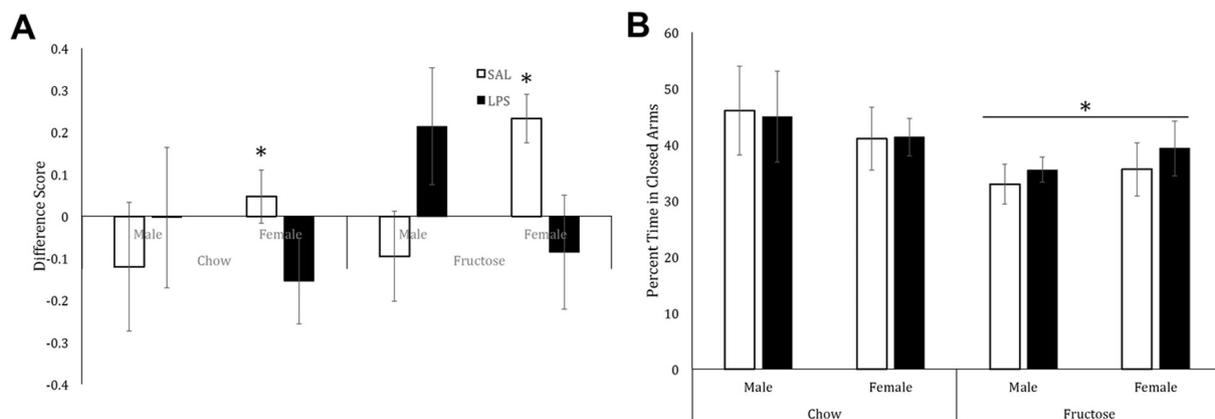


Fig. 5. In adulthood, anxiety-like behaviors were affected by maternal diet, but not learning behaviors. (A) Neonatal treatment and sex altered COD performance, with saline-treated females performing better regardless of dietary treatment ($n = 58$, $^*p < 0.01$). (B) Maternal diet decreased anxiety-like behaviors, such that rats exposed to high fructose maternal diet spent significantly less time in the closed arms. ($n = 58$, $^*p = 0.05$).

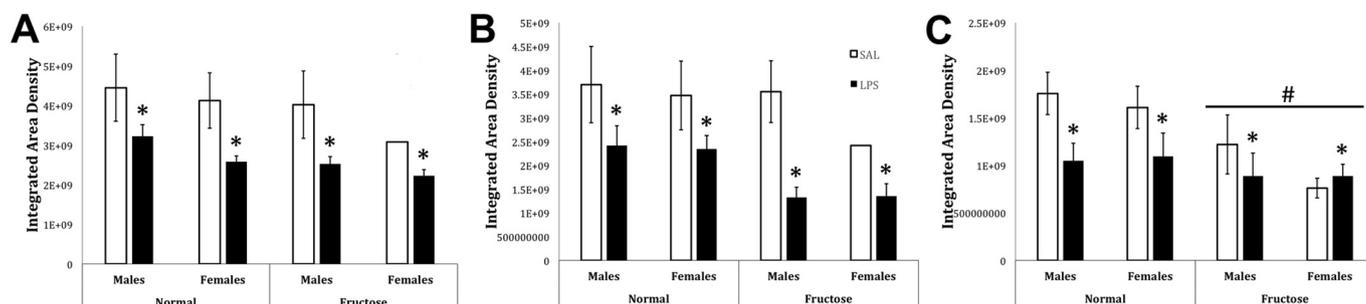


Fig. 6. Microglial density was altered by neonatal inflammation and maternal diet in hippocampal sub-regions of juvenile rats. (A) Neonatal LPS treatment reduced microglial density within the dentate gyrus (DG). (B) Neonatal LPS treatment reduced microglial density within the CA1. (C) Neonatal LPS treatment reduced microglial density. Furthermore, maternal high fructose diet reduced microglial density overall. * significantly lower than saline-treated animals, $p < 0.05$ # significantly lower than maternal normal chow exposure, $p < 0.05$.

hippocampus, following LPS treatment [20,32]. Although we found no differences in TNF α , increased pro-inflammatory cytokine signaling via IL-1 β in the neonatal brain may alter anxiety-like behaviors by affecting the amygdala. For instance, neonatal inflammation, induced through injections of LPS, has been shown to lower cannabinoid receptor 1 (CB1) binding in the amygdala in adulthood [39]. This lowered CB1 binding influences anxiety-like behavior because CB1 receptors modulate both excitatory and inhibitory neurotransmitter release in different parts of amygdala. Thus, these alterations may produce either an anxiolytic or an anxiogenic response, depending on which region of the amygdala is affected, [28]. However, the amygdala's connectivity matures from the juvenile period to adulthood [19]. By affecting the amygdala's endocannabinoid receptors, maternal and neonatal inflammation caused by high-fructose diet could lead to the differential anxiety-like behavior between juveniles and adults. The anxiety-like behavior may be age-dependent due to amygdala connectivity. Moreover, differential modulation of inhibitory and excitatory endocannabinoid receptors may also explain the differences in the anxiety-like behavior between the juveniles on a high fructose diet that were injected with either saline or LPS. As noted above, neonatal LPS injections alter endocannabinoid binding [39]. The alterations in CB1 binding brought on by the high fructose diet could be opposed by the binding changes induced via LPS, a hypothesis which merits further study.

While anxiety-like behavior was modulated by diet during two separate life periods, diet had no significant effect on learning in any of the rats we tested. In the juvenile period, all rats were unable to demonstrate intact memory in the COD paradigm. This inability is a likely result of still-developing spatial cognition. Although other work has shown that juvenile rats (PD26) can perform successfully in COD tasks for working memory (i.e., testing session 5 min after training), performance on short term memory COD tasks (i.e., testing 24 h post-training) similar to ours has yet to be investigated [25]. Developing connections between the hippocampus and perirhinal cortex may be responsible for the failure of this task at the juvenile stage [5]. In adulthood, gestational diet did not affect learning. The CA1 layer of the hippocampus is imperative for learning the COD paradigm, and is sensitive to inflammation [10]. On its own, the high-fructose maternal diet may not have caused a sufficient inflammation in the CA1 to cause impairment in the task during adulthood. Nevertheless, previous studies have suggested that a hyperglycemia-causing maternal diet conditions CA1 cells for further damage by other inflammatory agents [34]. In this case, neonatally injected LPS could possibly be acting as the inflammatory agent that causes this further damage to the primed cells within the hippocampus. As a result, adult performance in the COD task could be significantly reduced from control levels. It can be argued that the damage caused to the primed cells in the hippocampus by LPS did not show an affect during the neonatal period. USV levels were unaffected by diet or treatment, however, pups are capable of producing USVs even when the hippocampus is removed using lesion techniques [29].

Although ours is not the first study to measure cytokine levels in response to a high-fructose diet, it is the first study to include female offspring in its measurements [34]. Though maternal diet did not alter COD performance, we observed sex differences in adult COD performance. Previous findings from our laboratory demonstrate that sex differences in COD occur in adulthood, with adult females performing better on the task than adult males (unpublished data). This study validates this sex difference within a new cohort of animals. Moreover, our study supports the relationship between maternal diet and offspring sex [33]. Female rats exposed to a high-fructose diet do not experience a prolonged elevation in IL-1 β serum levels during the juvenile period like their male counterparts (Fig. 4A). The higher IL-1 β levels observed in males is a likely result of neonatal inflammation. While previous research shows that inflammation caused by neonatal *E. coli* administration and juvenile LPS injections decrease the level of circulating neutrophils, the inflammation caused by the high-fructose diet could lead to the IL-1 β surge [22]. The migration of circulating neutrophils is strongly induced by IL-1 β [21]. Further testing of white blood cell levels in the juvenile animals could provide further evidence of the differential effects of neonatal inflammatory mechanisms, like a high-fructose diet or bacterial infection, but also increase understanding about sex-specific differences. It is critical to measure inflammation and behavioral outcomes in both sexes because the increased sensitivity of male offspring may be an important focus in interventions.

5. Conclusion

Our study used an animal model to track the effects of the co-occurrence of inflammation stemming from maternal high fructose diet and neonatal LPS treatment, with results showing that anxiety-like behaviors and pro-inflammatory cytokine levels are affected across multiple stages of the rodent lifecycle. Offspring anxiety was altered during the juvenile and adult time points as a result of maternal diet and neonatal inflammation. Interleukin-1 β was reduced in neonates exposed to both stimuli and increased in juvenile males exposed to maternal fructose, demonstrating a change in inflammatory cytokines as a result of maternal diet, as well as microglial morphology changes as a result of neonatal LPS and maternal diet. Overall, this study reveals the aversive impact that gestational diabetes may have on human behavior. Through both maternal and neonatal immune activation, the behavioral and molecular changes induced by gestational diabetes can strongly affect the quality of life throughout the lifespan.

Acknowledgements and funding

The authors would like to acknowledge the tireless work of Young Cho, Anna Leonard and Anna Frey that went into data collection for this manuscript. We could not run the lab without you. We also acknowledge the critical funding provided by the Groff Foundation for the completion of this work.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

- [1] What is Gestational Diabetes? American Diabetes Association, 2017.
- [2] B. Abazyan, J. Nomura, G. Kannan, K. Ishizuka, K.L. Tamashiro, F. Nucifora, et al., Prenatal interaction of mutant DISC1 and immune activation produces adult psychopathology, *Biol. Psychiatry* 68 (2010) 1172–1181.
- [3] M. Alfaradhi, S. Ozanne, Developmental programming in response to maternal overnutrition, *Front. Genet.* 2 (2011).
- [4] R.A. Anderson, B. Qin, F. Canini, L. Poulet, A.M. Roussel, Cinnamon counteracts the negative effects of a high fat/high fructose diet on behavior, brain insulin signaling and Alzheimer-associated changes, *PLoS One* 8 (2013) e83243.
- [5] J. Bachevalier, M. Beauregard, Maturation of medial temporal lobe memory functions in rodents, monkeys, and humans, *Hippocampus* 3 (1993) Spec No:191–201.
- [6] S.D. Bilbo, J.M. Schwarz, Early-life proinflammation of later-life brain and behavior: a critical role for the immune system, *Front. Behav. Neurosci.* 3 (2009) 14.
- [7] A.A. Braun, M.R. Skelton, C.V. Vorhees, M.T. Williams, Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague–Dawley rats: effects of anxiolytic and anxiogenic agents, *Pharmacol. Biochem. Behav.* 97 (2011) 406–415.
- [8] S.A. Brunelli, Selective breeding for an infant phenotype: rat pup ultrasonic vocalization (USV), *Behav. Genet.* 35 (2005) 53–65.
- [9] L.D. Claypoole, B. Zimmerberg, L.L. Williamson, Neonatal lipopolysaccharide treatment alters hippocampal neuroinflammation, microglia morphology and anxiety-like behavior in rats selectively bred for an infantile trait, *Brain Behav. Immun.* 59 (2017) 135–146.
- [10] J. Czerniawski, T. Miyashita, G. Lewandowski, J.F. Guzowski, Systemic lipopolysaccharide administration impairs retrieval of context-object discrimination, but not spatial, memory: evidence for selective disruption of specific hippocampus-dependent memory functions during acute neuroinflammation, *Brain Behav. Immun.* 44 (2015) 159–166.
- [11] J. Czerniawski, T. Miyashita, G. Lewandowski, J.F. Guzowski, Systemic lipopolysaccharide administration impairs retrieval of context-object discrimination, but not spatial, memory: evidence for selective disruption of specific hippocampus-dependent memory functions during acute neuroinflammation, *Brain Behav. Immun.* 44 (2015) 159–166.
- [12] C.L. DeSisto, S.Y. Kim, A.J. Sharma, Prevalence estimates of gestational diabetes mellitus in the United States, Pregnancy Risk Assessment Monitoring System (PRAMS), 2007–2010, *Prev. Chronic Dis.* 11 (2014) E104.
- [13] J. Dobbing, J. Sands, Comparative aspects of the brain growth spurt, *Early Hum. Dev.* 3 (1979) 79–83.
- [14] L.W. Fan, A. Kaizaki, L.T. Tien, Y. Pang, S. Tanaka, S. Numazawa, et al., Celecoxib attenuates systemic lipopolysaccharide-induced brain inflammation and white matter injury in the neonatal rats, *Neuroscience* 240 (2013) 27–38.
- [15] M. Furtado, M.A. Katzman, Neuroinflammatory pathways in anxiety, posttraumatic stress, and obsessive compulsive disorders, *Psychiatry Res.* 229 (2015) 37–48.
- [16] M.K. Georgieff, The effect of maternal diabetes during pregnancy on the neurodevelopment of offspring, *Minn. Med.* 89 (2006) 44–47.
- [17] C.S. Harrell, J. Burgado, S.D. Kelly, Z.P. Johnson, G.N. Neigh, High-fructose diet during periadolescent development increases depressive-like behavior and remodels the hypothalamic transcriptome in male rats, *Psychoneuroendocrinology* 62 (2015) 252–264.
- [18] G. Hava, L. Vered, M. Yael, H. Mordechai, H. Mahoud, Alterations in behavior in adult offspring mice following maternal inflammation during pregnancy, *Dev. Psychobiol.* 48 (2006) 162–168.
- [19] T.T.Y. Lee, M.N. Hill, F.S. Lee, Developmental regulation of fear learning and anxiety behavior by endocannabinoids, *Genes Brain Behav.* 15 (2016) 108–124.
- [20] A.M. Lynch, C. Walsh, A. Delaney, Y. Nolan, V.A. Campbell, M.A. Lynch, Lipopolysaccharide-induced increase in signalling in hippocampus is abrogated by IL-10—a role for IL-1 beta? *J. Neurochem.* 88 (2004) 635–646.
- [21] S.H. Oliveira, C. Canetti, R.A. Ribeiro, F.Q. Cunha, Neutrophil migration induced by IL-1beta depends upon LTβ4 released by macrophages and upon TNF-alpha and IL-1beta released by mast cells, *Inflammation* 31 (2008) 36–46.
- [22] B.F. Osborne, J.I. Caulfield, S.A. Solomotis, J.M. Schwarz, Neonatal infection produces significant changes in immune function with no associated learning deficits in juvenile rats, *Dev Neurobiol.* (2017) 1221–1236.
- [23] T. Radaelli, A. Varastehpour, P. Catalano, S. Hauguel-de Mouzon, Gestational diabetes induces placental genes for chronic stress and inflammatory pathways, *Diabetes* 52 (2003) 2951–2958.
- [24] M.T. Ramirez-Lopez, M. Vazquez, L. Bindila, E. Lomazzo, C. Hofmann, R.N. Blanco, et al., Exposure to a highly caloric palatable diet during pregestational and gestational periods affects hypothalamic and hippocampal endocannabinoid levels at birth and induces adiposity and anxiety-like behaviors in male rat offspring, *Front. Behav. Neurosci.* 9 (2015).
- [25] A.I. Ramsaran, S.R. Westbrook, M.E. Stanton, Ontogeny of object-in-context recognition in the rat, *Behav. Brain Res.* 298 (2016) 37–47.
- [26] R. Rao, S. Sen, B. Han, S. Ramadoss, G. Chaudhuri, Gestational diabetes, pre-eclampsia and cytokine release: similarities and differences in endothelial cell function, in: L. Zhang, C.A. Ducusay (Eds.), *Advances in Fetal and Neonatal Physiology: Proceedings of the Center for Perinatal Biology 40th Anniversary Symposium*, Springer New York, New York, NY, 2014, pp. 69–75.
- [27] B.R. Reddy, S. Maitra, P. Jhelum, K.P. Kumar, P.K. Bagul, G. Kaur, et al., Sirtuin 1 and 7 mediate resveratrol-induced recovery from hyper-anxiety in high-fructose-fed prediabetic rats, *J. Biosci.* 41 (2016) 407–417.
- [28] S. Ruehle, A.A. Rey, F. Remmers, B. Lutz, The endocannabinoid system in anxiety, fear memory and habituation, *J. Psychopharmacol. (Oxford, England)*. 26 (2012) 23–39.
- [29] R.K. Schwarting, M. Wöhr, On the relationships between ultrasonic calling and anxiety-related behavior in rats, *Braz. J. Med. Biol. Res. = Rev. Bras. Pesqui. Med. Biol.* 45 (2012) 337–348.
- [30] L.M. Shortliffe, O. Hammam, X. Han, E. Kouba, P.S. Tsao, B. Wang, Dietary fructose in pregnancy induces hyperglycemia, hypertension, and pathologic kidney and liver changes in a rodent model, *Hypertens. Pregnancy* 5 (2015) 308–314.
- [31] S.E. Smith, J. Li, K. Garbett, K. Mirnic, P.H. Patterson, Maternal immune activation alters fetal brain development through interleukin-6, *J. Neurosci.* 27 (2007) 10695–10702.
- [32] D. Srinivasan, J.H. Yen, D.J. Joseph, W. Friedman, Cell type-specific interleukin-1beta signaling in the CNS, *J. Neurosci. Off. J. Soc. Neurosci.* 24 (2004) 6482–6488.
- [33] C.R. Toop, B.S. Muhlhäuser, K. O’Dea, S. Gentili, Impact of perinatal exposure to sucrose or high fructose corn syrup (HFCS-55) on adiposity and hepatic lipid composition in rat offspring, *J. Physiol.* 595 (2017) 4379–4398.
- [34] B. Vuong, G. Otero, S. Rozbacher, M. Stevenson, S.M. Kereliuk, T.J. Pereira, et al., Exposure to gestational diabetes mellitus induces neuroinflammation, derangement of hippocampal neurons, and cognitive changes in rat offspring, *J. Neuroinflammation* 14 (2017) 80.
- [35] C.L. Williams, J.S. Rosenblatt, W.G. Hall, Inhibition of suckling in weaning-age rats: a possible serotonergic mechanism, *J. Comp. Physiol. Psychol.* 93 (1979) 414–429.
- [36] L.L. Williamson, A. Chao, S.D. Bilbo, Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus, *Brain Behav. Immun.* 26 (2012) 500–510.
- [37] L.L. Williamson, P.W. Sholar, R.S. Mistry, S.H. Smith, S.D. Bilbo, Microglia and memory: modulation by early-life infection, *J. Neurosci. Off. J. Soc. Neurosci.* 31 (2011) 15511–15521.
- [38] R. Yirmiya, I. Goshen, Immune modulation of learning, memory, neural plasticity and neurogenesis, *Brain Behav. Immun.* 25 (2011) 181–213.
- [39] K. Zavitsanos, V.S. Dalton, A.K. Walker, C.S. Weickert, L. Sominsky, D.M. Hodgson, Neonatal lipopolysaccharide treatment has long-term effects on monoaminergic and cannabinoid receptors in the rat, *Synapse (New York, NY)* 67 (2013) 290–299.
- [40] M. Zou, E.J. Arentson, D. Teegarden, S.L. Koser, L. Onyskow, S.S. Donkin, Fructose consumption during pregnancy and lactation induces fatty liver and glucose intolerance in rats, *Nutr. Res.* 32 (2012) 588–598.