

Developmental Programming of Brain and Behavior:  
A Role for the Innate Immune System of the Placenta and Brain?

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
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ABSTRACT

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## Abstract

The field of “perinatal programming” has increasingly implicated an adverse early-life environment in the etiology of many chronic health problems and mental disorders. The following dissertation research is based on the hypothesis that the programming of brain and behavior by an altered early-life environment is propagated by inflammatory mechanisms in the placenta and developing brain. Offspring outcomes of two different maternal environmental exposures—air pollution and a “Western diet” (both highly relevant for the modern world)—were assessed in a mouse model in order to identify mechanisms common to developmental programming more generally.

The first set of experiments characterized the long-term behavioral and metabolic consequences of prenatal air pollution exposure in adult offspring. The male offspring of diesel exhaust particle (DEP)-exposed dams were predisposed to obesity, insulin resistance, and increased anxiety following placement on a high-fat diet (HFD) in adulthood. Furthermore, DEP/HFD male offspring exhibited evidence of macrophage priming, both in microglia and peripheral macrophages. The next experiment examined whether prenatal air pollution exposure could also synergize with a simultaneous “second hit” (i.e., maternal stress) during gestation. The offspring of mothers exposed to both air pollution and stress during gestation were more anxious as adults, but only the male offspring of this group also exhibited impaired cognition, in conjunction with

neuroinflammatory changes. A further experiment revealed that prenatal air pollution exposure altered microglial maturation in a TLR4- and sex-dependent manner, consistent with the previous results. However, we found limited evidence of a placental immune response to DEP, potentially due to analysis too late in gestation.

The second set of experiments characterized the enduring behavioral and metabolic consequences of maternal consumption of a “Western diet” (HFD in combination with BCAA supplementation) prior to and during gestation and lactation. The adult offspring of HFD-fed dams were more anxious in adulthood, despite being placed on a low-fat diet at weaning. Male HFD offspring were also hyperactive, whereas female HFD offspring exhibited more severe metabolic disturbances. Furthermore, there was evidence of microglial priming and peripheral macrophage priming in male HFD offspring, similar to the prenatal air pollution model. The next experiment also found evidence of altered microglial development due to maternal HFD, in conjunction with widespread, sex-specific immune gene regulation in the placenta in response to maternal diet. Moreover, maternal HFD decreased placental serotonin production, and also programmed long-term alterations in serotonergic function in the prefrontal cortex of adult HFD offspring. Taken together, these experiments define sexually dimorphic innate immune mechanisms in the placenta and developing brain that may underlie the long-term metabolic and behavioral consequences of maternal environmental exposures.

## Dedication

This work is dedicated to my family. To my maternal grandparents, Micki and Lou Tichacek, who taught me about nature, teaching me the names of trees and baby animals, and forever instilling in me a passion for science. You took me traveling with you and taught me about the world we live in, giving me a hunger for lifelong learning and exploration. You taught me the strategy for playing all kinds of different games, which instilled in me a competitive spirit that has driven me to always pursue excellence in all of my endeavors. Thank you for teaching me the importance of family, and for always being willing to host the entire family at your home for Christmas (no matter how crazy it gets). You, along with my aunts, uncles, and cousins, have always been there to support me in all of my academic achievements, and I enjoy that you still send me newspaper articles that you think are relevant to my research. Having a grandfather with a PhD has served as a shining example of success that encouraged me to also pursue the highest degree in my field.

All of this work is especially dedicated to my immediate family—my mother Janet, my father Steve, and my sister Kimberly. With parents who both have technical computer expertise, I believe I inherited their nerdiness (and intelligence). To my sister, who tried to escape this genetic nerdiness—you have provided a much-needed balance in all our lives and a reminder of what is truly important in life, while maintaining your

own creative genius of which we are all secretly jealous. To my father, with whom I have always loved talking about science, and who encouraged me to “work hard, play hard,” to always aim high and never give up on my dreams. To my mother, who has been a shining example of boundless enthusiasm, genuine kindness, and selfless generosity in my life—I am always extremely proud and humbled to hear others say I remind them of you, in both appearance and demeanor, and hope that I can continue to live up to your excellent example. To all three of you—I could not have gotten through the past 5 years without your help, support, and love. Thank you for cheering me on through this whole process.

Finally, this work is dedicated to my best friend and partner, Nathan Hedrick, who has always been there for me from the beginning of graduate school. Thank you for the privilege of always getting to talk about science at home with you, and for your advice and expertise that you offer selflessly in response to my every question, whether in reference to science or life in general. You are a model scientist and teacher—so intelligent, driven, and full of amazing ideas—I sincerely hope to have the privilege of collaborating with you in the future. Thank you for your endless patience, love, and support that kept me afloat during the difficult times and extremely happy the rest of the time.

# Contents

|  |        |
|--|--------|
| Abstract .....   | iv     |
| Dedication .....   | vi     |
| List of Tables .....   | xviii  |
| List of Figures .....  | xix    |
| Acknowledgements .....   | xxxiii |
| 1. Introduction .....  | 1      |
| 1.1 Developmental Programming by Maternal Environmental Toxin Exposures.....   | 4      |
| 1.2 Developmental Programming by Maternal Diets.....                           | 7      |
| 1.3 Mechanisms Underlying the Enduring Consequences of Maternal Exposures..... | 8      |
| 1.3.1 Role of the Innate Immune System in the Developing Brain.....            | 10     |
| 1.3.1.1 Microglial priming.....  | 11     |
| 1.3.2 Role of the Innate Immune System in the Placenta .....                   | 16     |
| 1.3.2.1 The innate immune cells of the placenta .....                          | 21     |
| 1.3.2.2 Evidence for the priming of placental immune cells .....               | 24     |
| 1.3.3 Role of the Placenta in Fetal Brain Development.....                     | 25     |
| 1.3.3.1 The placenta as the first neuroimmune organ .....                      | 26     |
| 1.3.3.2 Placental neuroimmune function during maternal immune challenge.....   | 28     |
| 1.3.4 Role of the Postnatal Environment .....                                  | 29     |
| 1.3.5 Classes of Behavioral Outcomes of Maternal Immune Challenges .....       | 31     |
| 1.3.6 Sex Differences.....   | 33     |

|   |    |
|---|----|
| 1.4 Summary.....  | 36 |
| 2. Prenatal Air Pollution Exposure Induces Neuroinflammation and Predisposes Offspring to Weight Gain in Adulthood in a Sex-Specific Manner .....       | 39 |
| 2.1 Introduction.....   | 39 |
| 2.2 Materials and Methods .....   | 41 |
| 2.2.1 Animals and Prenatal Exposures .....  | 41 |
| 2.2.2 Fetal Brain Cytokine Analysis .....   | 42 |
| 2.2.3 Adult Diets and Food Intake .....   | 43 |
| 2.2.4 Open Field Behavioral Testing.....  | 44 |
| 2.2.5 Serum and Tissue Collection .....   | 45 |
| 2.2.6 Metabolic and Endocrine Measures.....   | 46 |
| 2.2.6.1 Leptin assessment .....   | 46 |
| 2.2.6.2 Insulin assessment .....  | 46 |
| 2.2.6.3 Corticosterone assessment.....  | 46 |
| 2.2.7 Microglial Immunohistochemistry .....   | 47 |
| 2.2.8 Iba1 Quantification.....  | 48 |
| 2.2.9 Data Analysis and Statistics.....   | 49 |
| 2.3 Results .....   | 50 |
| 2.3.1 Prenatal DE exposure induces a fetal brain cytokine response .....  | 50 |
| 2.3.2 DE male, but not female, offspring weigh more and are less active at baseline .....   | 51 |
| 2.3.3 DE female offspring gain more weight than FA female offspring on HFD, whereas DE and FA male offspring gain similar amounts of weight on HFD..... | 52 |

|  |    |
|--|----|
| 2.3.4 DE male and female offspring consume more kilocalories than FA offspring on HFD .....  | 54 |
| 2.3.5 Male, but not female, offspring become less active on HFD, independent of prenatal exposure .....  | 57 |
| 2.3.5 Male, but not female, offspring become more anxious on HFD, independent of prenatal exposure .....   | 58 |
| 2.3.6 Both male and female offspring on HFD have elevated CORT levels, independent of prenatal exposure .....  | 60 |
| 2.3.7 DE female offspring on HFD have higher leptin levels than FA female offspring on HFD, whereas both DE and FA male offspring on HFD have elevated leptin levels, consistent with increased body weights ..... | 60 |
| 2.3.8 DE male, but not female, offspring on HFD have elevated insulin levels .....   | 61 |
| 2.3.9 Microglial activation is increased in DE offspring on HFD in a sex- and brain region-specific manner .....   | 63 |
| 2.4 Discussion.....  | 68 |
| 3. Prenatal Air Pollution Exposure Induces Sexually Dimorphic Fetal Programming of Metabolism, Peripheral Immune Responses, and Neuroinflammation in Adult Offspring .....   | 76 |
| 3.1 Introduction.....  | 76 |
| 3.2 Methods .....  | 78 |
| 3.2.1 Animals.....   | 78 |
| 3.2.2 DEP Exposures.....   | 79 |
| 3.2.3 Pup Weights.....   | 80 |
| 3.2.4 Maternal Care Observations .....   | 80 |
| 3.2.5 Experiment 1: Prenatal DEP Exposure and Adult HFD .....  | 81 |
| 3.2.5.1 Adult diets and food intake.....   | 81 |

|  |    |
|--|----|
| 3.2.5.2 Open field activity testing.....   | 82 |
| 3.2.5.3 Elevated zero-maze anxiety testing.....  | 82 |
| 3.2.5.4 Insulin sensitivity testing.....   | 83 |
| 3.2.5.5 Tissue collection .....  | 84 |
| 3.2.5.6 qRT-PCR.....   | 85 |
| 3.2.5.7 Flow cytometry.....  | 86 |
| 3.2.6 Experiment 2: Prenatal DEP Exposure and P30 SAL/LPS Injections.....  | 87 |
| 3.2.6.1 Injections .....   | 87 |
| 3.2.6.2 IL-1 $\beta$ ELISA.....  | 88 |
| 3.2.7 Data Analysis .....  | 88 |
| 3.3 Results .....  | 90 |
| 3.3.1 Maternal care and pup weights do not differ between prenatal treatment groups .....  | 90 |
| 3.3.2 Experiment 1: Prenatal DEP Exposure and Adult HFD .....  | 91 |
| 3.3.2.1 DEP male offspring gain more weight than VEH male offspring on HFD, whereas DEP and VEH female offspring gain similar amounts of weight on HFD ..... | 91 |
| 3.3.2.2 Offspring on HFD consume more kilocalories than offspring on LFD, regardless of DEP exposure and sex .....   | 93 |
| 3.3.2.3 Male, but not female, offspring are less active on HFD than offspring on LFD, regardless of DEP exposure.....  | 94 |
| 3.3.2.4 DEP male, but not female, offspring are more anxious on HFD.....   | 96 |
| 3.3.2.5 DEP male, but not female, offspring are more insulin resistant and hyperinsulinemic on HFD than VEH offspring.....                                   | 97 |

|  |     |
|--|-----|
| 3.3.2.6 HFD induces evidence of macrophage infiltration of the adipose tissue, but only DEP/HFD males, not females, express higher levels of a macrophage activation marker .....              | 99  |
| 3.3.2.7 DEP male, but not female, offspring on HFD express elevated levels of microglial/macrophage activation markers in HIPP.....  | 101 |
| 3.3.2.8 HFD appears to cause a greater infiltration of peripheral macrophages into HYP and HIPP, and these cells appear more activated in the HYP of DEP/HFD male offspring.....               | 103 |
| 3.3.3 Experiment 2: Prenatal DEP Exposure and P30 SAL/LPS Injections.....  | 107 |
| 3.3.3.1 DEP male offspring mount an exaggerated peripheral IL-1 $\beta$ response to an LPS challenge, whereas their central IL-1 $\beta$ response does not differ from VEH male offspring..... | 107 |
| 3.4 Discussion.....  | 108 |
| 4. Maternal Stress Worsens Effects of Prenatal Air Pollution on Offspring Mental Health Outcomes in Mice .....   | 117 |
| 4.1 Introduction.....  | 117 |
| 4.2 Materials and Methods.....   | 118 |
| 4.2.1 Animals.....   | 118 |
| 4.2.2 Prenatal Stressors .....   | 119 |
| 4.2.2.1 DEP exposures.....   | 119 |
| 4.2.2.2 Maternal resource deprivation.....   | 121 |
| 4.2.3 Neonatal Outcomes and Maternal Behavior .....  | 121 |
| 4.2.3.1 Birth weights.....   | 121 |
| 4.2.3.2 Neonatal corticosterone (CORT) measurement.....  | 122 |
| 4.2.3.3 Maternal behavior assessment .....   | 122 |

|  |     |
|--|-----|
| 4.2.4 Fetal Brain Cytokine Analysis .....  | 123 |
| 4.2.5 P30 Neuroimmune Gene Expression.....   | 124 |
| 4.2.6 Behavioral Procedures .....  | 125 |
| 4.2.6.1 Contextual and auditory cue fear conditioning.....   | 125 |
| 4.2.6.2 Elevated zero-maze.....  | 127 |
| 4.2.6.3 Forced swim test .....   | 127 |
| 4.2.7 Adult Brain Cytokine Analysis .....  | 128 |
| 4.2.8 Microglial Isolation and Gene Expression Analysis.....   | 128 |
| 4.2.9 Data Analysis .....  | 130 |
| 4.3 Results .....  | 131 |
| 4.3.1 Neonatal Outcomes and Maternal Behavior .....  | 131 |
| 4.3.2 Fetal Brain Cytokine Analysis .....  | 133 |
| 4.3.3 P30 Neuroimmune Gene Expression.....   | 134 |
| 4.3.4 Memory.....  | 135 |
| 4.3.5 Anxiety- and Depressive-Like Behavior .....  | 136 |
| 4.3.6 Adult Brain Cytokine Analysis .....  | 139 |
| 4.3.7 Gene Expression of Isolated CD11b+ and CD11b- Cells.....   | 141 |
| 4.4 Discussion.....  | 143 |
| 5. Prenatal Air Pollution Exposure Modulates Placental Immune Responses and<br>Microglial Maturation in a TLR4- and Sex-Dependent Manner ..... | 148 |
| 5.1 Introduction.....  | 148 |
| 5.2 Methods .....  | 150 |

|  |     |
|--|-----|
| 5.2.1 Experiment 1: E18 Cohort .....                                 | 150 |
| 5.2.1.1 Animals .....  | 150 |
| 5.2.1.2 Diesel Exhaust Particle (DEP) Exposures .....                | 151 |
| 5.2.1.3 Tissue Collection .....                                      | 151 |
| 5.2.1.4 SRY Genotyping.....  | 152 |
| 5.2.1.5 Cytokine Protein Measurements .....                          | 153 |
| 5.2.1.6 Immunohistochemical detection of Iba1 protein.....           | 153 |
| 5.2.1.7 Stereology.....  | 154 |
| 5.2.1.8 Data Analysis.....   | 160 |
| 5.2.2 Experiment 2: P30 Cohort .....                                 | 160 |
| 5.2.2.1 Animals .....  | 160 |
| 5.2.2.2 DEP Exposures .....  | 161 |
| 5.2.2.3 P30 Injections .....   | 161 |
| 5.2.2.4 Tissue Collection .....                                      | 161 |
| 5.2.2.4 Microglial Immunohistochemistry .....                        | 162 |
| 5.2.2.5 Stereology.....  | 163 |
| 5.2.2.6 Data Analysis and Statistics.....                            | 165 |
| 5.3 Results .....  | 165 |
| 5.3.1 Experiment 1: E18 Cohort .....                                 | 165 |
| 5.3.1.1 Placenta and Brain Weights.....                              | 165 |
| 5.3.1.2 Placental Cytokines.....                                     | 166 |
| 5.3.1.3 Placental Macrophage Counts and Morphological Analysis ..... | 167 |

|  |     |
|--|-----|
| 5.3.1.4 Fetal Brain Cytokines.....   | 168 |
| 5.3.1.5 Fetal Brain Microglia Counts and Morphological Analysis.....   | 169 |
| 5.3.1.6 P30 Brain Microglia Counts and Morphological Analysis.....   | 176 |
| 5.4 Discussion.....  | 182 |
| 6. Developmental Programming of Metabolism, Microglial Activation, and Behavior by<br>“Western Diets” .....        | 190 |
| 6.1 Introduction.....  | 190 |
| 6.2 Methods .....  | 193 |
| 6.2.1 Animals.....   | 193 |
| 6.2.2 Diets.....   | 193 |
| 6.2.3 Experimental Timeline.....   | 194 |
| 6.2.4 Experimental Procedures .....  | 196 |
| 6.2.4.1 Behavioral Testing .....   | 196 |
| 6.2.4.2 Insulin Sensitivity Testing .....  | 198 |
| 6.2.4.3 Tissue Collection for Flow Cytometry Analysis.....   | 198 |
| 6.2.4.4 Tissue collection for all other analyses.....  | 200 |
| 6.2.5 Data Analysis and Statistics.....  | 205 |
| 6.3 Results .....  | 205 |
| 6.3.1 Diets high in fat and BCAA generate an obese phenotype in dams.....  | 205 |
| 6.3.2 HFD dams display an anxious phenotype, whereas BCAA dams exhibit<br>postpartum depressive-like behavior..... | 206 |
| 6.3.3 HFD and BCAA dams show altered maternal care behaviors in the early<br>postpartum period .....               | 209 |

|   |     |
|---|-----|
| 6.3.4 Pups born to HFD and BCAA dams show changes in body weight and metabolism that endure into adulthood .....  | 212 |
| 6.3.5 Maternal HFD induces sex-specific behavioral changes in adult offspring ...   | 215 |
| 6.3.6 Maternal HFD induces sex- and brain region-specific changes in neuroimmune gene expression in the HYP and HIPP of adult offspring.....                    | 218 |
| 6.3.7 Maternal HFD increases microglial antigen density in the hippocampus in adulthood, and primes microglia for over-activation by an adult LPS challenge ..  | 221 |
| 6.3.8 Maternal HFD causes an exaggerated IL-1 $\beta$ response following an adult LPS challenge both in the hippocampus and periphery of male offspring .....   | 222 |
| 6.3.9 Maternal HFD results in increased monocyte infiltration of the brains of male offspring, and these infiltrating cells are more activated in the HYP ..... | 224 |
| 6.4 Discussion.....   | 226 |
| 7. Interaction of the Placental Immune Response with Serotonin Production and Microglial Maturation in Developmental Programming by “Western Diets” .....       | 234 |
| 7.1 Introduction.....   | 234 |
| 7.2 Methods .....   | 235 |
| 7.2.1 Animals.....  | 235 |
| 7.2.2 Diets.....  | 235 |
| 7.2.3 Breeding.....   | 236 |
| 7.2.4 E14.5 Tissue Collection .....   | 236 |
| 7.2.5 P1 Tissue Collection .....  | 237 |
| 7.2.6 P8 Tissue Collection .....  | 238 |
| 7.2.7 Adult Tissue Collection .....   | 238 |
| 7.2.7 PCR Array .....   | 239 |

|  |     |
|--|-----|
| 7.2.8 qRT-PCR.....   | 240 |
| 7.2.9 High-performance liquid chromatography with electrochemical detection ..   | 241 |
| 7.2.10 Data Analysis and Statistics.....   | 242 |
| 7.3 Results.....   | 243 |
| 7.3.1 PCR array analysis reveals multiple sex-specific immune changes in the E14.5 placenta due to maternal diet .....                             | 243 |
| 7.3.2 Maternal HFD decreases serotonin production in the E14.5 placenta.....   | 246 |
| 7.3.3 Maternal HFD and BCAA alters gene expression in the E14.5 forebrain.....   | 248 |
| 7.3.4 Litters of HFD/BCAA dams show increased pup mortality, especially in males .....   | 249 |
| 7.3.5 Maternal HFD increases P1 pup corticosterone (CORT), whereas maternal BCAA suppresses CORT levels.....                                       | 251 |
| 7.3.6 Pups of HFD dams exhibit decreased expression of microglia markers at P1251  |     |
| 7.3.7 Pups of HFD and BCAA dams exhibit altered expression of immune and serotonergic genes at P8 in a brain region- and sex-specific manner ..... | 253 |
| 7.3.8 Adult offspring of HFD and BCAA dams exhibit altered expression of serotonergic genes in a brain region- and sex-specific manner .....       | 257 |
| 7.3.9 Maternal HFD and BCAA results in increased 5-HT turnover and altered dopaminergic metabolism in the PFC of adult offspring.....              | 259 |
| 7.4 Discussion.....  | 262 |
| 8. Summary and Conclusions.....  | 269 |
| References .....   | 277 |
| Biography.....   | 301 |

## List of Tables

|  |     |
|--|-----|
| Table 1: Forward and reverse primer sequences for qRT-PCR .....  | 85  |
| Table 2: Maternal care, litter characteristics, maternal weights, and pup weights by prenatal treatment. All values are mean $\pm$ SEM. .... | 91  |
| Table 3: Forward and reverse primers for qRT-PCR analysis. ....  | 129 |
| Table 4: Litter sizes and sex ratios by maternal treatment. All values are mean $\pm$ SEM. ....  | 132 |
| Table 5: Forward and reverse primer sequences for qRT-PCR analysis .....   | 204 |
| Table 6: Forward and reverse primer sequences for qRT-PCR analysis .....   | 241 |

## List of Figures

Figure 1: Schematic depicting PRRs as coincidence detectors for the convergence of 2 “hits” (danger signals) within an innate immune cell. The first hit shown here consists of bacterial LPS (PAMP) binding to TLR4 on the surface of the cell, which initiates a downstream signaling pathway that results in the activation of NF- $\kappa$ B, a transcription factor that then translocates to the nucleus and initiates transcription of proinflammatory cytokines, such as pro-IL-1 $\beta$ . However, pro-IL-1 $\beta$  is biologically inactive until it is cleaved by caspase-1, an enzyme that forms part of the NLRP3 inflammasome (a large multimolecular complex). Thus, the second hit, consisting of hyaluronan (DAMP) released by the injured or dying cells, is required to activate the NLRP3 inflammasome in the cytoplasm of the same cell and cleave pro-IL-1 $\beta$  into its active form, IL-1 $\beta$ , which can then be released from the cell to initiate a robust inflammatory response. Note that the PRR ligands denoted here are just examples, and there can be multiple different combinations of various ligands, including XAMPs, such as environmental toxins, or DAMPs, released following psychological stress or toxin exposure. .... 13

Figure 2: Programming of brain and behavior by maternal high-fat diet involves inflammatory pathways, which interact with both the intrauterine and postnatal environments. (Many of the mechanisms portrayed here also apply to programming by prenatal air pollution exposure.) In the placenta (inset depicts a schematic of a chorionic villus), these inflammatory mechanisms include the passage of elevated maternal cytokines through the placenta into the fetal circulation, the production of proinflammatory cytokines by fetal placental macrophages (i.e., Hofbauer cells), and the increased passage of circulating maternal glucocorticoids across the placenta, due to decreased 11- $\beta$ -HSD2. Collectively, these conditions are associated with placental insufficiency, as well as intrauterine growth restriction. In the developing brain, the inflammatory mechanisms include the microglial production of proinflammatory cytokines (as a result of activation of the TLR4-mediated signaling cascade by saturated fatty acids), and the incorporation of saturated fatty acids and trans fats into the myelin of developing neurons. Thus, there is the promotion of a neuroinflammatory microenvironment, which may lead to oxidative stress, cell death, and an overall altered trajectory of brain development. Increased levels of proinflammatory cytokines lead to decreased serotonin synthesis by the placenta during fetal development, and by the brain during postnatal development, which may contribute to the alteration of brain development and an increased risk of psychiatric disorders in adulthood (a risk that has

been shown to be greater in males in humans and animal models). [Figure originally published in Bolton & Bilbo, 2014]. ..... 20

Figure 3: Prenatal DE exposure causes a significant reduction in the levels of the anti-inflammatory cytokine IL-10 in the fetal brain at E18. No significant changes in the proinflammatory cytokine IL-1 $\beta$  were detected. Data are expressed as means  $\pm$ SE; n=10-13/group. \* $P$ <0.02 vs. FA controls. .... 51

Figure 4: A) Baseline adult body weight is increased in DE male, but not female offspring. B) Baseline activity levels are decreased in DE male, but not female, offspring. C) DE offspring do not differ from FA offspring in their baseline anxiety levels. Data are mean  $\pm$  SEM, N=10-14/group. \* $P$ <0.05 vs. FA males. .... 52

Figure 5: A) DE and FA male offspring gain weight on HFD at the same rate (*left*), whereas DE female offspring gain weight at a faster rate than FA female offspring on HFD (*left*). B) DE and FA male offspring overall gained similar amounts of weight on HFD (*left*), whereas DE female offspring gained more weight than all other groups (*right*). Data are mean  $\pm$  SEM, N=4-8/group. # $P$ <0.05, HFD vs. LFD; \* $P$ <0.05 vs. all other groups; line indicates at what time point the groups diverge..... 54

Figure 6: A) DE male and female offspring consume more kilocalories than FA offspring on HFD, although consumption decreases overall across the diet. Note that male and female consumption data are combined here. B) DE offspring consumed overall more total kilocalories over the 6 weeks of diet. Data are mean  $\pm$  SEM, N=11-13/group. \* $P$ <0.05 vs. all other groups; # $P$ <0.05, FA/HFD vs. LFD groups..... 56

Figure 7: A) DE male (*left*), but not female (*right*), offspring are less active in the open field on HFD. Activity is represented here as total meters traveled each week of the diet. B) DE male (*left*), but not female (*right*), offspring display more anxiety-like behavior on HFD, as they spend more time in the surround of the open field. Anxiety is represented here as percent time spent in the surround zone of the open field during the 30 min. test for each week of the diet. Data are mean  $\pm$  SEM, N=4-8/group. # $P$ <0.05, HFD vs. LFD; line indicates at what time point the groups diverge..... 59

Figure 8: A) Both DE and FA male (*left*) and female (*right*) offspring have elevated basal CORT levels after 6 weeks of HFD. B) Both DE and FA male offspring on HFD have elevated leptin levels after 6 weeks of HFD (*left*), whereas DE female offspring on HFD have higher leptin levels than FA female offspring on HFD (*right*). C) DE male (*left*), but not female (*right*), offspring have elevated insulin levels after 6 weeks of HFD. Data are

mean  $\pm$  SEM, N=4-8/group. Data are mean  $\pm$  SEM, N=4-8/group.  $^{\#}P<0.05$ , HFD vs. LFD;  $^{*}P<0.05$  vs. all other groups. .... 62

Figure 9: Microglia appear more activated in DE offspring on HFD. A) Regions of interest for Iba1 density analysis [adapted from (George Paxinos, 2005)]. B) Representative Iba1 staining (10x magnification) in the hypothalamus of mice from each treatment group. C) Representative Iba1 staining (40x magnification) in the boxed regions in (B) of FA/HFD vs. DE/HFD mice. Scale bars = 100  $\mu$ m (B); 25  $\mu$ m (C). .... 64

Figure 10: Denser Iba1 staining, a constitutive microglial marker, is observed in DE offspring on HFD in a sex- and brain region-specific manner. A, C) Both male (left) and female (right) DE offspring have denser Iba1 staining in the hypothalamus (A) and dentate gyrus of the hippocampus (C) after 6 weeks of HFD. B) Male DE offspring have denser Iba1 staining in the amygdala after 6 weeks of HFD (left), whereas female DE offspring do not (right). D) Male DE offspring have denser Iba1 staining in area CA1 of the hippocampus after 6 weeks of HFD (left), whereas female DE and FA offspring both have denser Iba1 staining in CA1 after 6 weeks of HFD (right). E) There are no significant differences between FA and DE offspring in Iba1 staining in area CA3 of the hippocampus after 6 weeks of HFD. Data are mean  $\pm$  SEM, N=4-8/group.  $^{*}P<0.05$  vs. all other groups;  $^{**}P<0.05$  vs. DE/LFD and FA/HFD;  $^{\#}P<0.05$ , HFD vs. LFD. .... 67

Figure 11: (A) DEP male offspring gained weight on HFD at a faster rate than VEH male offspring (left), whereas DEP and VEH female offspring gained weight on HFD at the same rate (right). (B) DEP male offspring gained more total weight on HFD than all other groups (left), whereas DEP and VEH female offspring gained similar amounts of weight. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{*}p<0.05$  vs. all other groups;  $^{\#}p<0.05$  vs. LFD groups;  $^{##}p<0.05$ , HFD vs. LFD. .... 92

Figure 12: (A) Offspring on HFD consumed more kilocalories than offspring on LFD, although consumption decreased overall across the diet. (B) Total kilocalories consumed over the 9 weeks of diet were overall higher in HFD groups than LFD groups, regardless of prenatal treatment. Male and female consumption data are combined here, due to a lack of sex differences. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{##}p<0.05$ , HFD vs. LFD. .... 94

Figure 13: (A) Offspring on HFD decreased their activity levels over the 9 weeks of diet, whereas offspring on LFD did not. (B) Overall total distance traveled during the weekly open field tests was lower in HFD males than LFD males, whereas females did not differ. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{##}p<0.05$ , HFD vs. LFD. .... 95

Figure 14: (A) Prior to initiation of the diet, there were no differences in time spent in the closed arm of the elevated zero-maze between VEH and DEP groups. However, females spent more time in the closed arm than males overall. (B) There were also no significant differences in head dips performed on the zero-maze prior to diet assignment. (C) After 4 weeks on the diet, DEP/HFD males tended to spend more time in the closed arm than other groups, whereas females did not differ. (D) DEP/HFD males also tended to perform fewer head dips as a result of an additive effect of DEP and HFD, whereas there were again no differences among females. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{##}p<0.05$ , HFD vs. LFD;  $^{**}p=0.07$ , trend for DEP  $\times$  HFD interaction;  $^{*}p<0.05$ , VEH vs. DEP;  $^{#}p=0.08$ , HFD vs. LFD. .... 97

Figure 15: (A) Following insulin challenge after 5 weeks on the diet, DEP/HFD males were significantly more insulin resistant than all other groups, as they failed to decrease their blood glucose levels at the time of peak glucose response (30 min. post-insulin injection). On the other hand, DEP/HFD females did not differ from VEH/HFD females. (B) In agreement with their insulin resistance, DEP/HFD males also had significantly higher levels of fasting insulin in serum collected immediately prior to insulin injection, whereas HFD females had higher insulin levels than LFD females, regardless of prenatal treatment. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{*}p<0.05$  vs. all other groups;  $^{**}p=0.06$ , HFD vs. LFD;  $^{#}p<0.05$  vs. LFD groups;  $^{##}p<0.05$ , HFD vs. LFD. .... 99

Figure 16: (A) At the end of the 9 weeks of diet, both male and female offspring on HFD expressed significantly higher levels of CCR2 mRNA in adipose tissue than offspring on LFD, independent of prenatal treatment. (B) Offspring on HFD also exhibited higher TLR4 mRNA expression in adipose tissue, regardless of prenatal treatment or sex. (C) DEP/HFD males exhibited significantly higher CD11b mRNA expression than all other groups, whereas HFD females had overall higher CD11b mRNA expression than LFD females, independent of prenatal treatment. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{##}p<0.05$ , HFD vs. LFD;  $^{#}p<0.05$  vs. LFD groups;  $^{*}p<0.05$  vs. all other groups. .... 101

Figure 17: At the end of 9 weeks of diet, DEP/HFD male offspring exhibited increased mRNA expression of microglia/macrophage activation markers CD11b (A) and TLR4 (B), and of specific microglia marker CX3CR1 (C), in HIP, whereas female offspring did not. (D) There was a small decrease in GFAP mRNA in brains of females exposed to HFD, independent of prenatal treatment, but no differences among males. (D). Data are mean  $\pm$  SEM, n=4-6/group/sex.  $^{**}p<0.05$  vs. VEH/HFD;  $^{*}p<0.05$  vs. DEP/LFD and VEH/HFD groups;  $^{#}p<0.05$  vs. VEH/LFD and VEH/HFD;  $^{##}p<0.05$ , HFD vs. LFD. .... 103

Figure 18: (A) Following flow cytometry, cells were initially gated based on forward and side scatter to obtain only live, single cells for further analysis. (B) Cells were subsequently gated into CD11b+CD45<sup>low</sup> cells (microglia), CD11b+CD45<sup>high</sup> cells (infiltrating macrophages), and CD11b-CD45<sup>high</sup> cells (lymphocytes). (C) 9 weeks of HFD caused a significant increase in the percent of CD11b+CD45<sup>high</sup> cells found in the HYP and HIPP (analyzed together), regardless of prenatal treatment or sex. (D) DEP/HFD males possessed CD11b+CD45<sup>high</sup> cells in the HYP with a significantly higher MFI of CD11b protein, whereas females did not exhibit any differences. (E) CD11b+CD45<sup>low</sup> cells of the HYP did not display any significant differences between groups. (F) HFD caused a significant increase in the percent of CD11b-CD45<sup>high</sup> cells in the HYP of females, but not males. Data for A and B are diagrams of gating procedures from representative samples, data for C represents the mean  $\pm$  SEM of n=12/group (sexes and brain regions combined for analysis), and data for D, E and F represent the mean  $\pm$  SEM of n=3/group/sex. <sup>#</sup>*p*<0.05, HFD vs. LFD; \**p*<0.05 vs. DEP/LFD..... 106

Figure 19: (A) 2 h following LPS challenge, P30 DEP male offspring had significantly higher levels of IL-1 $\beta$  in their serum than VEH male offspring, whereas female offspring did not significantly differ due to prenatal treatment. (B) The brains of the same animals displayed a robust IL-1 $\beta$  response, but did not differ due to prenatal treatment or sex. Data are mean  $\pm$  SEM, n=6-8/group/sex. \**p*<0.05 vs. SAL groups; \**p*<0.05 vs. all other groups; <sup>#</sup>*p*<0.05, LPS vs. SAL..... 108

Figure 20: Effects of prenatal DEP and NR on neonatal outcomes and maternal behavior. (A) Prenatal NR decreased P1 pup weights, but weights normalized by P8. (B) Prenatal NR increased P1 serum CORT in male, but not female pups. (C-E) Neither prenatal DEP nor NR altered the percent of time dams spent On Nest, Nursing, or LG their pups during P2-P9. (F) Prenatal DEP and NR did not have enduring effects on maternal anxiety-like behavior 60 days post-partum. Data are mean of n=8-10/group  $\pm$  SEM for pup weights, mean of n=3-8/group  $\pm$  SEM for P1 CORT, and mean of n=3-7/group  $\pm$  SEM for maternal behavior (\*\**p*<0.05, NR vs. Control groups). ..... 133

Figure 21: Effects of prenatal DEP and NR on the fetal brain cytokine response. (A) No significant group differences were detected in IL-1 $\beta$  protein levels in the E18 brain. (B) Maternal DEP exposure elicited a sexually dimorphic IL-10 response in the fetal brain, such that male brains exhibited a downregulation of IL-10, whereas female brains exhibited an upregulation. Data are mean of n=7-8/group  $\pm$  SEM (\**p*=0.1 DEP vs. VEH groups, and significant Sex  $\times$  DEP interaction, *p*<0.05)..... 134

Figure 22: Effects of prenatal DEP and NR on P30 neuroimmune gene expression. The brains of DEP/NR males displayed increased TLR4 (A) and caspase-1 (B) expression, whereas DEP/NR female brains did not. Data are mean of n=5-13/group  $\pm$  SEM (\*\* $p$ <0.05 vs. DEP/Control and VEH/NR; \* $p$  <0.05 vs. all other groups). ..... 135

Figure 23: Effects of prenatal DEP and NR on cognitive and affective behavior of adult offspring. (A) DEP/NR males displayed decreased freezing to the fear context, which is indicative of a hippocampal-dependent memory deficit, whereas DEP/NR females did not. (B) Both male and female DEP/NR offspring spent more time in the closed arm, which is indicative of anxiety-like behavior. Data are mean of n=7-9/group  $\pm$  SEM (\* $p$ <0.05 vs. all other groups; \*\* $p$ <0.05 vs. DEP/Control and VEH/Control,  $p$ =0.07 vs. VEH/NR; ## $p$ <0.05 vs. DEP/Control). ..... 137

Figure 24: (A) Male offspring exposed to prenatal NR exhibited a decreased level of serum CORT immediately following the elevated zero maze test, whereas there were no differences in female offspring. (B) Neither males nor females displayed any significant changes in depressive-like behavior in the forced swim test. Data are mean of n=7-9/group  $\pm$  SEM (\*\* $p$ <0.05, NR vs. Control). ..... 138

Figure 25: Effects of prenatal DEP and NR on adult brain cytokine levels. (A) Male offspring exhibited a significant increase in brain IL-1 $\beta$  due to prenatal DEP exposure, whereas females did not. (B) Male offspring exhibited a significant decrease in brain IL-10 in response to both DEP and NR, whereas females did not. (C) Overall, DEP/NR males exhibited a greater proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) than DEP/NR females. Data for A-C are mean of n=5-8/group  $\pm$  SEM (\* $p$ <0.05 DEP vs. VEH groups; \*\* $p$ <0.05 NR vs. Control groups; ## $p$ =0.08, DEP/NR males vs. DEP/NR females, and significant Sex $\times$ DEP and Sex $\times$ NR interactions,  $p$ <0.05). (D-E) Brain IL-1 $\beta$  was significantly correlated with memory and anxiety measures in both males and females, though in opposite directions. (F) Brain IL-10 was not correlated with memory performance in males or females. (G) Brain IL-10 was negatively correlated with anxiety-like behavior in males, but not in females. Data for D-G are correlated measures for individual animals from the whole cohort (n=24-27 total/sex). ..... 140

Figure 26: Isolated CD11b+ cells (microglia) expressed markedly higher levels of CD11b (A), IL-1 $\beta$  (D), IL-10 (E), TLR4 (F), and caspase-1 (G) than did CD11b- cells (neurons, astrocytes). CD11b- cells expressed significantly higher levels of BDNF (B) and GFAP (C). Data are mean of n=5/group  $\pm$  SEM (\*\* $p$ <0.05, CD11b+ vs. CD11b- cells). ..... 142

Figure 27: A) The drawing of the placenta counting contour, encompassing the fetal labyrinth. B) The measurement of the cell area, defined as the contour surrounding all

dark edges of the cell. C) The measurement of the long axis, defined as the longest length of the cell. D) The measurement of the short axis, defined as the line crossing the nucleus and perpendicular to the long axis. .... 157

Figure 28: A-C) Example contours drawn around the E18 parietal cortex (A) and hippocampus (B) in the StereoInvestigator software. Hippocampal regions going clockwise, starting from the bottom left: CA3, CA1, dentate gyrus. Photos were taken of Iba1-stained section at the 4X objective. D-G) The four primary microglial morphological states on E18. During development, microglia possess round/amoeboid morphology (D) initially and gradually progress to a morphology with thin, ramified processes (G). If activated in adulthood, their morphology once again assumes the round/amoeboid shape. Photos were taken of Iba1-labeled microglia in the mouse CA1 at the 100X objective..... 159

Figure 29: A) Example contours drawn around the P30 parietal cortex (top) and dentate gyrus (bottom) in the StereoInvestigator software. Photos were taken of Iba1-stained section at the 4X objective. C-D) The two primary microglial morphological states on P30. Representative pictures of microglia with thick, long processes (C) and thin, ramified processes in the mouse parietal cortex on P30. Photos were taken of Iba1-labeled microglia at the 100X objective..... 164

Figure 30: A) E18 placenta weights were significantly greater in male DEP TLR4<sup>-/-</sup> fetuses than DEP TLR4<sup>+/-</sup>, whereas female placenta weights did not differ. B) No differences among E18 brain weights were detected. Data are mean ± SEM, n= 5-15/group/sex. \**p*<0.05 vs. DEP TLR4<sup>+/-</sup>; \*\**p*<0.05, Males vs. Females..... 166

Figure 31: A) Levels of IL-1β tended to be higher in DEP TLR4<sup>-/-</sup> placentas than VEH TLR4<sup>-/-</sup> placentas at E18. B) Levels of IL-10 were significantly higher in DEP TLR4<sup>-/-</sup> compared to all other groups. C) The proinflammatory bias (IL-1β/IL-10 ratio) was significantly higher in TLR4<sup>+/-</sup> placentas than TLR4<sup>-/-</sup> placentas. Data are mean ± SEM, n= 10-14/group (note that sexes are combined here). #*p*=0.09, DEP TLR4<sup>-/-</sup> vs. VEH TLR4<sup>-/-</sup>; \**p*<0.05 vs. all other groups; \*\**p*<0.05, TLR4<sup>+/-</sup> vs. TLR4<sup>-/-</sup>..... 167

Figure 32: A) Levels of IL-1β tended to be higher in E18 brains due to prenatal DEP exposure. B) Levels of IL-10 did not significantly differ in the E18 brain. C) The proinflammatory bias (IL-1β/IL-10 ratio) was significantly greater in DEP TLR4<sup>+/-</sup> E18 brains than in VEH TLR4<sup>+/-</sup> E18 brains. Data are mean ± SEM, n= 6-13/group (note that sexes are combined here). #*p*=0.07, DEP vs. VEH; \**p*<0.05 vs. VEH TLR4<sup>+/-</sup>. .... 168

Figure 33: A) DEP TLR4<sup>-/-</sup> females possess a significantly larger PVN volume than DEP TLR4<sup>+/-</sup> females at E18, whereas males do not differ. B) DEP TLR4<sup>+/-</sup> males tend to have more total microglia than VEH TLR4<sup>+/-</sup> males in the PVN, whereas TLR4<sup>+/-</sup> females have overall more total microglia than TLR4<sup>-/-</sup> females. C) DEP TLR4<sup>+/-</sup> males have more round microglia than VEH TLR4<sup>+/-</sup> males in the PVN, whereas DEP females have overall fewer round microglia than VEH females. In addition, TLR4<sup>+/-</sup> females have overall more round microglia than TLR4<sup>-/-</sup> females. Data are mean ± SEM, *n* = 2-4/group/sex. \**p*<0.05 vs. DEP TLR4<sup>+/-</sup> females for volume; #*p*=0.09, DEP TLR4<sup>+/-</sup> vs. VEH TLR4<sup>+/-</sup>; ##*p*<0.005, TLR4<sup>+/-</sup> vs. TLR4<sup>-/-</sup>; \*\**p*<0.05, DEP vs. VEH; \**p*<0.05 vs. VEH TLR4<sup>+/-</sup> males for round cells. .... 170

Figure 34: A) DEP TLR4<sup>+/-</sup> animals possess a significantly larger PCX volume than VEH TLR4<sup>+/-</sup> animals and DEP TLR4<sup>-/-</sup> animals at E18. Data are mean ± SEM, *n* = 8-9/group (note that sexes are combined here). \**p*<0.05 vs. VEH TLR4<sup>+/-</sup> and DEP TLR4<sup>-/-</sup>. B) DEP TLR4<sup>+/-</sup> males have significantly more round microglia in the PCX than DEP TLR4<sup>-/-</sup> males, and tend to have more than VEH TLR4<sup>+/-</sup> males also. In contrast, VEH TLR4<sup>-/-</sup> females have significantly more round microglia than DEP TLR4<sup>-/-</sup> females in the PCX at E18. Data are mean ± SEM, *n* = 3-5/group/sex. \**p*<0.05 vs. DEP TLR4<sup>-/-</sup>; #*p*=0.08 vs. VEH TLR4<sup>+/-</sup>. .... 172

Figure 35: A) DEP TLR4<sup>+/-</sup> animals possess a significantly greater DG volume at E18 than VEH TLR4<sup>+/-</sup> animals. Data are mean ± SEM, *n* = 4-8/group (note that sexes are combined here). \**p*<0.05 vs. VEH TLR4<sup>+/-</sup>. B) DEP TLR4<sup>+/-</sup> males have significantly more stout microglia than DEP TLR4<sup>-/-</sup> males in the E18 DG, whereas DEP females have significantly fewer stout microglia overall than VEH females. In addition, TLR4<sup>+/-</sup> females have overall more stout microglia than TLR4<sup>-/-</sup> females. Data are mean ± SEM, *n* = 2-5/group/sex. \**p*<0.05 vs. DEP TLR4<sup>-/-</sup>; ##*p*<0.05, TLR4<sup>+/-</sup> vs. TLR4<sup>-/-</sup>; \*\**p*<0.05, DEP vs. VEH. .... 173

Figure 36: A) No significant differences were detected in CA3 volume at E18. Data are mean ± SEM, *n* = 5-7/group (note that sexes are combined here). B) DEP TLR4<sup>+/-</sup> males have significantly more stout microglia in the CA3 at E18 than VEH TLR4<sup>+/-</sup> males, whereas DEP females have significantly fewer stout microglia overall than VEH females. Data are mean ± SEM, *n* = 2-4/group/sex. \**p*<0.05 vs. VEH TLR4<sup>+/-</sup>; \*\**p*<0.05, DEP vs. VEH. .... 174

Figure 37: A) No significant differences were detected in CA1 volume at E18. Data are mean ± SEM, *n* = 5-8/group (note that sexes are combined here). B) DEP TLR4<sup>+/-</sup> males have significantly more stout microglia in the CA1 at E18 than DEP TLR4<sup>-/-</sup> males,

whereas VEH TLR4+/- females have significantly more stout microglia than DEP TLR4+/- females. Data are mean  $\pm$  SEM,  $n= 2-5/\text{group}/\text{sex}$ .  $*p<0.05$  vs. DEP TLR4+/- for males;  $*p<0.05$  vs. DEP TLR4+/- for females. .... 175

Figure 38: A) VEH/LPS females possess a significantly smaller PVN volume than DEP/LPS and VEH/SAL females at P30, whereas males do not differ. B) DEP males have microglia with larger soma volumes in the P30 PVN than do VEH males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n= 4-6/\text{group}/\text{sex}$ .  $*p<0.05$  vs. DEP/SAL and VEH/SAL;  $**p<0.05$ , DEP vs. VEH. .... 177

Figure 39: A) DEP males possess a significantly smaller PCX volume at P30 than do VEH males, whereas females do not differ. B) DEP males tend to have more total microglia in the PCX at P30 than do VEH males, whereas DEP females tend to have fewer. C) DEP/LPS males have significantly more thick, long microglia in the PCX at P30 than do VEH/LPS males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n= 5-7/\text{group}/\text{sex}$ .  $**p<0.05$ , DEP vs. VEH;  $\#p=0.1$ , DEP vs. VEH;  $*p<0.05$  vs. VEH/LPS. .... 179

Figure 40: A) No significant differences in DG volume were detected. B) LPS males had significantly more thick, long microglia in the DG at P30 than did SAL males. DEP/LPS females had significantly more thick, long microglia than did VEH/LPS and DEP/SAL females. C) LPS males tended to have fewer thin, ramified cells than SAL males in the P30 DG, whereas DEP/LPS females had significantly fewer thin, ramified cells than did VEH/LPS and DEP/SAL females. D) DEP males have thin, ramified microglia with larger soma volumes in the DG at P30 than do VEH males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n= 5-7/\text{group}/\text{sex}$ .  $\#p<0.05$ , LPS vs. SAL;  $*p<0.05$  vs. VEH/LPS and DEP/SAL;  $\#p=0.06$ , LPS vs. SAL;  $**p=0.05$ , DEP vs. VEH. .... 181

Figure 41: HFD and BCAA generate an obese phenotype in adult females. A) After six weeks on diet, body weight was increased in both HFD and BCAA groups, with a synergistic effect in HFD/BCAA females. B) HFD and BCAA groups consumed more kilocalories than controls. C) HFD/BCAA dams gained more weight than other groups, even after controlling for kilocalories of food consumed. Data are mean  $\pm$  SEM,  $n= 35-60$  animals/group for weight gain;  $n=15-21$  cages/group for food consumption.  $*p<0.001$  vs. all other groups;  $\#p<0.05$  vs. LFD/Control;  $**p<0.001$ , HFD vs. LFD;  $\#p<0.001$ , BCAA vs. Control. .... 206

Figure 42: HFD dams showed increased postpartum anxiety-like behavior, whereas BCAA dams exhibited a postpartum depression-like phenotype. A) At 3 days postpartum, HFD dams spent less time in the open arm of the EZM than LFD controls. B) At 8 days postpartum, BCAA dams spent less time in the open arms than controls,

and HFD dams tended to show a similar pattern. C) At 28 days postpartum, HFD dams performed more stretch-attend postures in the EZM than LFD controls. D) At P8, BCAA dams were more immobile in the FST than controls. E) At P28, HFD dams were less immobile than LFD controls. Data are mean  $\pm$  SEM,  $n=7-10$ /group.  $**p<0.05$ , HFD vs. LFD;  $\#p=0.08$  HFD vs. LFD;  $\#\#p<0.05$ , BCAA vs. Control. .... 208

Figure 43: HFD and BCAA dams showed altered maternal care during the early postpartum period (P2-P9). A) HFD/BCAA dams spent more overall time on the nest than LFD groups. B) However, HFD and BCAA dams both spent less time eating than controls, likely due to the higher caloric content of their diets, which may have contributed to this effect. C) HFD dams spent more time licking and grooming their pups during the dark cycle, but not the light cycle (when most LG is typically observed), than LFD controls. D) HFD dams also spent more time self-grooming during the dark cycle than LFD controls. E) BCAA dams, especially LFD/BCAA dams, had nests of lower quality than controls. F) LFD/BCAA dams spent more of their total time nursing during the light cycle (when most nursing occurs) in efficient or passive postures (i.e., blanket and passive nursing as opposed to arched-back nursing). Data are mean  $\pm$  SEM,  $n=17-20$ /group (note that dams sacrificed at P8 and P28 are combined here).  $*p<0.05$  vs. LFD/Control and LFD/BCAA for panel A;  $**p<0.05$ , HFD vs. LFD;  $\#\#p<0.05$ , BCAA vs. Control;  $\#p=0.05$ , HFD vs. LFD;  $*p<0.05$  vs. LFD/Control for panel F. .... 211

Figure 44: Pups born to HFD and BCAA dams exhibited changes in body weight and metabolism that endured into adulthood. A) HFD and BCAA both decreased pup weight at P1 compared to controls ( $n=15-21$  litter-average weights/group). B) BCAA pups weighed less at weaning than controls at weaning, whereas HFD/Control pups weighed more than LFD/Controls ( $n=9$  litter-average weights/group). C) The decrease in body weight due to maternal BCAA endured into adulthood ( $n=19-30$  animals/group/sex). D) Male HFD and BCAA offspring exhibited increased insulin sensitivity (i.e., a greater glucose decrease to insulin), whereas female HFD and BCAA offspring tended to be more insulin-resistant ( $n=17-29$ /group/sex). E) Female HFD offspring had more visceral fat as a percentage of their total body weight relative to LFD controls, whereas males did not differ ( $n=7-21$ /group/sex). Data are mean  $\pm$  SEM.  $**p<0.05$ , HFD vs. LFD;  $\#\#p<0.05$ , BCAA vs. Control;  $*p<0.05$  vs. LFD/Control;  $\#p=0.08$ , BCAA vs. Control;  $\wedge p=0.06$ , HFD vs. LFD. .... 214

Figure 45: Adult offspring of HFD dams exhibited long-term behavioral changes. A) Both male and female offspring of HFD dams spent less time in the open arms of the EZM than LFD controls. B) HFD offspring also performed more stretch-attend postures, a cautious behavior at the interface of the closed arms in the EZM, than LFD controls. C)

Male, but not female, offspring of HFD dams were hyperactive in the OFT. D) Both male and female HFD offspring spent less time actively struggling in the FST, and BCAA further exacerbated this indication of depressive-like behavior. Data are mean  $\pm$  SEM,  $n=46-49$ /group for A & B (sexes combined),  $n=18-28$ /group/sex for C & D (sexes separate). \*\* $p<0.05$ , HFD vs. LFD; # $p<0.05$ , BCAA vs. Control..... 217

Figure 46: Maternal HFD and BCAA induced sex- and brain region-dependent alterations in neuroimmune gene expression. A) Female HFD offspring expressed higher levels of Iba-1 in the HYP, whereas males did not differ. B) Female offspring of HFD dams also tended to increase TLR4 expression in the HYP relative to LFD controls. C) BCAA decreased GFAP expression in both males' and females' HYP, and HFD further decreased GFAP expression in males. D) HFD tended to decrease leptin receptor expression in the male HYP, whereas HFD tended to do the exact opposite in the female HYP. E) In the HIP, both HFD and BCAA increased expression of GFAP in males, whereas females did not differ. F) HFD/Control offspring expressed higher levels of leptin receptor in the HIP than LFD/Control offspring, regardless of sex. Data are mean  $\pm$  SEM,  $n=5-9$ /group/sex for A-E (sexes separate),  $n=14-16$ /group for F (sexes combined). \*\* $p<0.05$ , HFD vs. LFD; # $p<0.05$ , BCAA vs. Control; # $p=0.1$ , HFD vs. LFD; \* $p<0.05$  vs. LFD/Control..... 220

Figure 47: Maternal HFD increased microglial antigen density in the hippocampus. A) Adult offspring of HFD/Control dams exhibited increased Iba-1 antigen density in the DG of the hippocampus relative to offspring of LFD/Control dams, whereas BCAA groups did not differ. B) Maternal HFD increased Iba-1 staining density at baseline, and LPS caused an exaggerated increase in the HFD offspring only. Data are mean  $\pm$  SEM,  $n=28-32$ /group for A (injections combined),  $n=14-17$ /group for B (injections separate). Note sexes are combined here, due to a lack of significant sex differences. \* $p<0.05$  vs. LFD/Control; \*\* $p<0.05$ , HFD vs. LFD; # $p<0.05$ , LPS vs. SAL..... 222

Figure 48: HFD/Control male offspring exhibited an exaggerated IL-1 $\beta$  response to LPS in both the hippocampus (A) and plasma (B) relative to LFD groups. HFD/BCAA female offspring exhibited a significantly blunted IL-1 $\beta$  response in the periphery (B) relative to other diet groups. IL-1 $\beta$  response is calculated here as the % IL-1 $\beta$  in the LPS-injected diet groups relative to their respective SAL-injected diet group (e.g., percent increase in IL-1 $\beta$  of HFD/LPS animals over HFD/SAL animals). Data are mean  $\pm$  SEM,  $n=6-8$ /group/sex. \* $p<0.05$  vs. LFD/Control and HFD/BCAA; # $p<0.05$  vs. all other groups. .... 224

Figure 49: Maternal HFD altered monocytes infiltration of the brain in male offspring long-term. A) Gating strategy for flow cytometry: cells were first gated to exclude

doublets and dead cells, then gated to select the CD11b+CD45high population (infiltrating cells, not resident microglia). From this population, neutrophils were excluded, and the CCR2+ cells of the remaining non-polymorphonuclear cells were selected to end up with the population of infiltrating monocytes. B) HFD/Control and LFD/BCAA male offspring exhibited increases in the percent of CCR2+ infiltrating cells in their brains (regardless of brain region), whereas females did not differ. C) This population of CCR2+ cells in the HYP expressed higher cell-surface levels of CD14 in male HFD/Control offspring relative to LFD/Control offspring, whereas it was the opposite in female HFD/Control offspring. Data are mean  $\pm$  SEM,  $n=14-16$ /group/sex for B (brain regions HYP and FB analyzed together),  $n=7-8$ /group/sex for C (HYP only). \* $p<0.05$  vs. LFD/Control. .... 226

Figure 50: Maternal diet altered immune gene expression in the E14.5 placenta. A-B) Maternal HFD caused an immune response, as shown here by an increase in IL-18, a proinflammatory cytokine, and Kng1, which is involved in coagulation and inflammation. C) Maternal BCAA also caused alterations in immune gene expression, as shown here by an increase in CCR2, a marker for infiltrating monocytes. D) There was also interaction between maternal HFD and BCAA in the regulation of immune gene expression, as shown here by decreased CD14 in HFD/Controls placentas, but not in HFD/BCAA placentas. E-F) There were sex-specific alterations in the placenta in response to maternal diet, and most were more pronounced in females than males, as shown here by decreased IL-6, an important cytokine for placental growth, in HFD female placentas, and increased CCL20 in HFD/BCAA female placentas. Data are mean  $\pm$  SEM,  $n=5-6$ /group for A-D (sexes combined),  $n=3$ /group/sex for E-F (sexes separate). \*\* $p<0.05$ , HFD vs. LFD; ## $p=0.06$ , BCAA vs. Control; \* $p<0.05$  vs. HFD/BCAA,  $p=0.07$  vs. other groups for D; \* $p<0.05$  vs. all other groups for F..... 245

Figure 51: Maternal HFD decreased measures of serotonin production in the E14.5 placenta. A) Maternal HFD decreased 5-HT levels in the E14.5 placenta, but especially for HFD/Control placentas for male fetuses, and HFD/BCAA placentas for female fetuses. B) Similarly, HFD/Control males and HFD/BCAA females exhibited increased serotonin turnover (as measured by the 5-HIAA/ 5-HT ratio). C) Placental tryptophan content was overall decreased in BCAA fetuses, and tended to be further decreased in HFD fetuses, so that HFD/BCAA fetuses had the lowest tryptophan levels. Data are mean  $\pm$  SEM,  $n=6-8$ /group/sex for A-B (sexes separate),  $n=15-16$ /group for C (sexes combined). \* $p<0.05$  vs. LFD/Control for males, and vs. LFD/BCAA and HFD/Control for females; # $p=0.09$  vs. LFD/Control in A, and HFD vs. LFD in C; ## $p<0.05$ , BCAA vs. Control..... 247

Figure 52: Maternal HFD and BCAA altered gene expression in the E14.5 forebrain. A) Fetuses of BCAA dams exhibited decreased Nestin expression, a marker of neural stem cells, in their forebrains at E14.5. B) Male fetuses of BCAA dams had decreased TLR2 expression in their forebrains, whereas female fetuses of HFD dams showed decreased TLR2 expression at E14.5. Data are mean  $\pm$  SEM,  $n=13-16$ /group for A (sexes combined),  $n=7-8$ /group/sex for B (sexes separate).  $^{\#}p<0.05$ , BCAA vs. Control;  $^{**}p<0.05$ , HFD vs. LFD..... 249

Figure 53: Litters of HFD/BCAA dams exhibited increased pup mortality, especially in males. A) The number of pups born per litter did not differ, but the number of pups surviving to weaning was significantly decreased in the HFD/BCAA group. B) The sex ratio does not differ for all pups born, but the % of live male pups at P1 is significantly decreased in HFD litters. Data are mean  $\pm$  SEM,  $n=17-32$  litters/group.  $^{*}p<0.05$  vs. all other groups;  $^{**}p<0.05$ , HFD vs. LFD. .... 250

Figure 54: Pups of HFD dams exhibited increased CORT levels on P1, whereas BCAA suppressed this effect, regardless of pup sex. Data are mean  $\pm$  SEM,  $n=15-18$ /group (sexes combined).  $^{**}p<0.05$ , HFD vs. LFD;  $^{\#}p<0.05$ , BCAA vs. Control..... 251

Figure 55: Maternal HFD caused decreased expression of microglial markers in P1 pup brains, including Iba-1, TLR2, and IL-1 $\beta$  (A-C). D) CD11b exhibited a similar pattern, except that BCAA blunted the effect of HFD, such that only HFD/Control brains expressed lower CD11b than LFD controls. E) GFAP, an astrocyte marker, exhibited a different pattern, in which HFD/BCAA brains expressed the highest amount of GFAP. Data are mean  $\pm$  SEM,  $n=9-18$ /group (sexes combined).  $^{**}p<0.05$ , HFD vs. LFD;  $^{*}p<0.05$  vs. LFD/Control for D, vs. HFD/Control and LFD/BCAA for E..... 253

Figure 56: Maternal HFD and BCAA altered immune and serotonergic gene expression in a sex- and brain region-dependent manner at P8. A) LFD/BCAA pups exhibited lower CD11b expression in the HYP than LFD/Control and HFD/BCAA pups, regardless of sex. B) GFAP tended to be decreased by maternal BCAA in the P8 HYP. C) BCAA pups exhibited decreased 5-HT1A expression relative to Controls in the HYP as well. D) 5-HT2CR tended to be decreased by maternal HFD in the HYP relative to LFD controls. E) HFD/BCAA male pups had decreased Iba-1 expression in the HIPPP at P8, whereas females did not differ. F) TLR2 expression was increased in HFD/Control and LFD/BCAA males in the HIPPP relative to controls, whereas females did not differ. G) 5-HT1A expression was decreased in HFD/BCAA males in the HIPPP, but females were not different. H) HFD/Control males exhibited increased 5-HTT expression in the HIPPP relative to LFD controls, but females did not differ. Data are mean  $\pm$  SEM,  $n=12-18$ /group

(sexes combined) for HYP,  $n=6-8/\text{group}/\text{sex}$  (sexes separate) for HIP.  $*p<0.05$  vs. LFD/Control and HFD/BCAA for A, F, and H, vs. LFD/BCAA for E and G;  $\#p=0.07$ , BCAA vs. Control in B, HFD vs. LFD in D;  $\#\#p<0.05$ , BCAA vs. Control. .... 256

Figure 57: Maternal HFD and BCAA altered serotonergic gene expression in the HIP of adult offspring in a sex-specific manner. A) HFD females exhibited decreased expression of 5-HTT in the HIP in adulthood relative to LFD controls, in conjunction with a tendency for BCAA to increase expression, whereas male offspring did not differ. B) Adult HFD/Control males exhibited a tendency for increased 5-HT1A expression in the HIP relative to LFD/Control males, whereas females did not differ. Data are mean  $\pm$  SEM,  $n=6-9/\text{group}/\text{sex}$ .  $**p<0.05$ , HFD vs. LFD;  $\#p=0.06$ , BCAA vs. Control in A,  $\#p=0.1$  vs. LFD/Control in B. .... 258

Figure 58: Maternal HFD and BCAA caused increased serotonin turnover and altered dopaminergic metabolism in the PFC of adult offspring. A) HFD/Control, LFD/BCAA, and HFD/BCAA animals all exhibited an increase in 5-HIAA levels in the PFC relative to LFD/Control, regardless of sex, which is indicative of increased serotonin release at the synapse. B) The same groups also showed increased serotonin turnover (as measured by the 5-HIAA/5-HT ratio) in the PFC relative to LFD/Control, which is consistent with a hyperserotonergic phenotype. C) LFD/BCAA animals had increased tryptophan in the PFC relative to LFD/Control and HFD/BCAA animals, and HFD/Control tended to also have more tryptophan relative to LFD/Control. D) Male, but not female, HFD offspring exhibited decreased levels of HVA, a dopamine metabolite. E) Male HFD offspring also exhibited increased dopamine turnover (as measured by the DOPAC/DA ratio; DOPAC is a different dopamine metabolite) relative to LFD offspring, whereas female BCAA offspring had increased dopamine turnover relative to Controls. Data are mean  $\pm$  SEM,  $n=12-16/\text{group}$  for A-C (sexes combined),  $n=5-8/\text{group}/\text{sex}$  (sexes separate) for D-E.  $*p<0.05$  vs. LFD/Control;  $\#p<0.1$  vs. LFD/Control in A and C,  $\#p<0.09$ , HFD vs. LFD in D-E;  $\#\#p<0.05$ , BCAA vs. Control. .... 261

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# 1. Introduction<sup>1</sup>

An adverse early-life environment, whether due to maternal infection, stress, diet, or toxin exposures, has been increasingly implicated in the etiology of many chronic health problems and mental disorders (Lanphear, Vorhees, & Bellinger, 2005; Seckl & Holmes, 2007). This epidemiological evidence has fostered the emergence of the field of “perinatal programming”, which is based on the idea that alterations of the intrauterine and/or early postnatal environment during this sensitive period may “program” or shift the normal trajectory of development and the “set point” of multiple homeostatic systems, such as the immune and nervous systems, ultimately resulting in significant changes in adult physiology and behavior (Bilbo & Schwarz, 2009). **My dissertation work is based on the hypothesis that the programming of brain and behavior by an altered early-life environment is propagated by inflammatory mechanisms in the placenta and developing brain**, as immune molecules (e.g., cytokines, chemokines) have increasingly gained recognition as having a dual role in developmental processes and host defense [reviewed in (Boulangier, 2009a; Deverman & Patterson, 2009)].

Previous work from my laboratory has implicated microglia, the resident innate immune cells of the brain and the primary producers of cytokines and chemokines, as

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the key mediators of the long-term effects of early-life infection on brain function and behavior (Williamson, Sholar, Mistry, Smith, & Bilbo, 2011). A large amount of research now indicates that normal neural patterning clearly depends on the complex interactions of microglia with other cells found in the developing brain, including neurons and astrocytes [reviewed in (Schwarz & Bilbo, 2011)]. Similarly, placental macrophages, known as Hofbauer cells, are highly involved in the normal development and function of the placenta, which has important consequences for brain development as well (Hsiao & Patterson, 2012). For example, the placenta serves as the sole source of serotonin for the developing fetal brain throughout most of gestation (Bonnin et al., 2011), and inflammation is a negative regulator of serotonin production (Kannan et al., 2010).

The role of inflammation in the placenta and developing brain remains relatively unexplored in the context of two of the most pressing public health concerns for present and future generations—maternal exposure to environmental toxins and high-fat diets, which are an increasing concern in the developed world, just as our fear of more traditional immune challenges, such as parasites and bacteria, diminishes. The innate immune system, which is fully present in the placenta and developing brain, plays a key role in the body's recognition of and response to these harmful substances. This response occurs via broad-specificity pattern recognition receptors (PRRs), much like in the response to more conventional pathogens, as the body works to defend the organism

against a wide range of threats. Immune responses can result in inflammation, which is critical for repair and to fight pathogens, but may be harmful if prolonged, e.g., when the threat is chronic, such as is usually the case with environmental toxin and dietary exposures. The developing brain is especially vulnerable to the effects of such exposures and the resultant inflammation, as any disruption of the delicate balance between immune molecules and growth factors or neurotransmitters (e.g., serotonin) early in life can have long-term cognitive consequences (Sutton et al., 2012), including lower IQ (Needleman, Schell, Bellinger, Leviton, & Allred, 1990) and increased risk of neurodevelopmental disorders, such as autism (Volk, Lurmann, Penfold, Hertz-Picciotto, & McConnell, 2013).

The involvement of microglia in mediating the effects of early-life events on later-life brain function is likely due to their capacity, along with other macrophages, for a form of long-term immunological “memory”, referred to as “priming”, in which their response to a current immune challenge is modulated by prior challenges they have encountered (Pace, Russell, Torres, Johnson, & Gray, 1983). For example, members of my laboratory have demonstrated that an early-life bacterial infection on postnatal day (P)4 predisposes rats to memory impairment in adulthood following a subsequent immune challenge (Bilbo et al., 2005), which is mediated by the early-life priming of microglia and their exaggerated cytokine response to the “second hit” (Williamson et al., 2011). These effects of microglial activation on adult cognitive function are possible because

cytokines and chemokines are not just important during development—they are also critical for brain homeostasis and normal function in adulthood, including mood regulation, learning, and memory [reviewed in (Yirmiya & Goshen, 2011)]. Importantly, we believe that the mechanisms of microglial priming are not limited to the context of neonatal infection and may also apply to other types of immune challenges, such as environmental toxins and high-fat diets.

The goal of my dissertation is to characterize the long-term changes in brain and behavior that are programmed by maternal environmental exposures during the perinatal period, and to determine whether priming of the innate immune system in the placenta and developing brain also occurs and may play a role in the observed phenotypes. In the sections that follow in this chapter, I will review the literature in the areas of perinatal programming by maternal environmental toxin exposures and maternal diets, which are the focus of my dissertation experiments. Moreover, I will introduce key innate immune mechanisms in the placenta and brain that I hypothesize could play a role in developmental programming by maternal exposures, and which I will examine extensively in my dissertation experiments in Chapters 2-7.

## ***1.1 Developmental Programming by Maternal Environmental Toxin Exposures***

Environmental toxins/pollutants are becoming nearly unavoidable in the world we live in, as they are found in the food we eat, the water we drink, and the air we breathe. Perera (2008) warned that our children are going to inherit the most severe

consequences of our fossil fuel addiction, not only in terms of climate change and ecosystem destruction, but also in terms of the health risks that result from pollution exposure during the sensitive period of fetal development (Perera, 2008). Multiple environmental toxins, such as the polycyclic aromatic hydrocarbons (PAHs) found in cigarette smoke and air pollution, ethanol in alcoholic beverages, and heavy metals, such as lead and mercury, are able to cross the placenta and gain access to the fetal compartment (Bearer, 1995). Furthermore, heavy metals, alcohol, and components of cigarette smoke have been well characterized as neurotoxins that adversely affect early brain development and can cause long-term cognitive deficits and learning disorders (Needleman et al., 1990; Weiss & Landrigan, 2000). For this reason, the human placenta has recently begun to be used as a biomarker to assess the level of maternal toxin exposures, including to mercury, cadmium, and lead, during pregnancy, and to attempt to predict subsequent impairments in cognitive development (Esteban-Vasallo, Aragonés, Pollán, López-Abente, & Pérez-Gómez, 2012).

In contrast, the effects of maternal air pollution exposure during gestation on fetal brain development and later-life cognitive and behavioral outcomes are just beginning to attract attention. Recent epidemiological evidence suggests that exposure to air pollution during pregnancy is associated with poorer academic performance of children in high school (Sanders, 2012), an increased incidence of autism (Raz et al., 2014; Volk et al., 2013), and an increased risk of childhood obesity (Rundle et al., 2012)—

a disorder in which the brain clearly plays a central role via its neuroendocrine regulation of food intake, metabolism and body fat content (Schwartz & Porte Jr, 2005).

One goal of my dissertation research is to characterize the effects of prenatal air pollution exposure on later-life offspring cognitive and metabolic outcomes in a mouse model, in order to better explore the underlying mechanisms (see Chapters 2-3).

An intriguing new area of research that remains relatively unexplored is the interaction of maternal exposure to environmental “physical toxins”, such as air pollution, with maternal exposure to “social toxins” (i.e., maternal psychosocial stress) during gestation, which may result in a synergistic effect on offspring outcomes (Wright, 2009). An understanding of this interaction is important, because environmental exposures are typically not experienced in isolation in the real world. Notably, expectant mothers living in areas of low socioeconomic status experience the greatest burden of toxins and pollutants (Evans & Kantrowitz, 2002a), along with fewer resources and high psychological stress (Seguin, Potvin, St-Denis, & Loiselle, 1995), the combined effects of which may exaggerate the deleterious consequences for their unborn children. For example, parental stress has been shown to increase the effect of *in utero* tobacco smoke exposure on childhood asthma risk (Shankardass et al., 2009). Another goal of my dissertation research is to explore the mechanisms by which maternal stress during gestation may increase the vulnerability of offspring, thus enabling a toxin to initiate

significant injury to developing physiological systems when it would have been insufficient to do so in isolation (see Chapter 4).

## **1.2 Developmental Programming by Maternal Diets**

Non-communicable diseases such as heart disease and diabetes now account for the majority of morbidity and mortality worldwide (Alwan, 2011). Obesity and overweight are at epidemic proportions and contribute to most if not all of these diseases (Alwan, 2011). Increasing evidence suggests the perinatal environment may be especially important in shaping lifelong health and disease outcomes. Maternal obesity is associated with gestational diabetes, stillbirth, and preeclampsia leading to preterm birth (Cedergren, 2004). Beyond these acute consequences, and perhaps most troubling, maternal obesity may also “program” offspring for lifelong obesity and associated metabolic disorders, setting in motion a vicious cycle of propagating health problems (Grattan, 2008; McGuire, Dyson, & Renfrew, 2009). For instance, children of obese women exhibit increased body mass index (Simmons, 2008), body fat percentage, and insulin resistance (Freeman, 2009), and high-fat-fed rodent dams produce offspring with increased fat, leptin, and body length at birth (Chen, Simar, Lambert, Mercier, & Morris, 2008; Dunn & Bale, 2009), and insulin resistance and obesity later in life (Armitage, Khan, Taylor, Nathanielsz, & Poston, 2004).

Though it has received less attention, obesity, metabolic syndrome, and insulin resistance are also increasingly linked to mental health dysfunction, including impaired

cognition and increased anxiety (de la Monte, 2009; Pasinetti & Eberstein, 2008).

Multiple animal studies demonstrate links between high- fat diet exposure and/or obesity and increased anxiety. Notably, this is true for direct exposure during adulthood as well as in development –i.e., the *offspring* of mothers fed a high-fat diet during pregnancy and lactation show increased anxiety, across multiple species including non-human primates (Sullivan et al., 2010), even in the absence of direct high-fat feeding. The latter case is a classic example of perinatal programming – i.e., the capacity for events occurring during the perinatal period to alter or “program” the normal course of development, with the result that adult outcomes, including behavior, are significantly and often permanently altered (Bennet & Gunn, 2006). My dissertation research will characterize the effects of maternal “Western diet” consumption during gestation and lactation on later-life metabolic and mental health outcomes in offspring (see Chapter 6).

### ***1.3 Mechanisms Underlying the Enduring Consequences of Maternal Exposures***

An understanding of the mechanisms by which maternal diets and toxin exposures during the perinatal period result in long-term effects on brain function and behavior, though not yet defined, will be instrumental in the development of effective interventions and therapies to prevent or mitigate their effects. In pursuit of this goal, the larger body of literature surrounding other “programming” factors, such as early-life infections, is a helpful guide, as they share important similarities with maternal toxin exposures and diets. For example, both infectious agents and environmental toxins, as

well as saturated fatty acids, can activate innate immune pathways via PRRs, which are “sensors” used by cells to recognize a wide range of threats to the organism, including foreign pathogen-associated molecular patterns (PAMPs), endogenous danger/damage-associated molecular patterns (DAMPs; also known as alarmins) that are released by host tissues during cellular stress or injury (as may occur following toxin or psychosocial stress exposure), and even xenobiotic-associated molecular patterns (XAMPs), a newly identified class of PRR ligands that may range from drugs of abuse to dietary substances and environmental toxins (e.g., the HFD and DEP used in our studies described below) (Frank, Watkins, & Maier, 2011; Hutchinson et al., 2012; Jalleh et al., 2012). Activation of PRRs initiates an inflammatory response, characterized by the secretion of soluble immune proteins, called cytokines and chemokines, that mobilize and activate immune cells in an attempt to clear the body of the threat and initiate repair mechanisms if necessary. However, these same innate immune molecules (i.e., PRRs, cytokines, and chemokines) that are critical for the defense of an organism against invading pathogens also orchestrate the coordinated movements and interactions of specific cells that are instrumental for the normal developmental processes of the organism, including in the placenta and brain [reviewed in (Boulangier, 2009b; Deverman & Patterson, 2009; Hunt, Pace, & Gill, 2010)]. Thus, inflammation during pregnancy is a double-edged sword, in that inflammation can resolve an infection that could damage the developing fetus, but inflammation in itself can also alter the trajectory of fetal development, resulting in long-

term changes in brain function and behavior. This is particularly a risk if the inflammatory event is chronic or not able to be targeted and resolved by immune cells, as is typically the case for maternal exposure to environmental toxins and diets.

### **1.3.1 Role of the Innate Immune System in the Developing Brain**

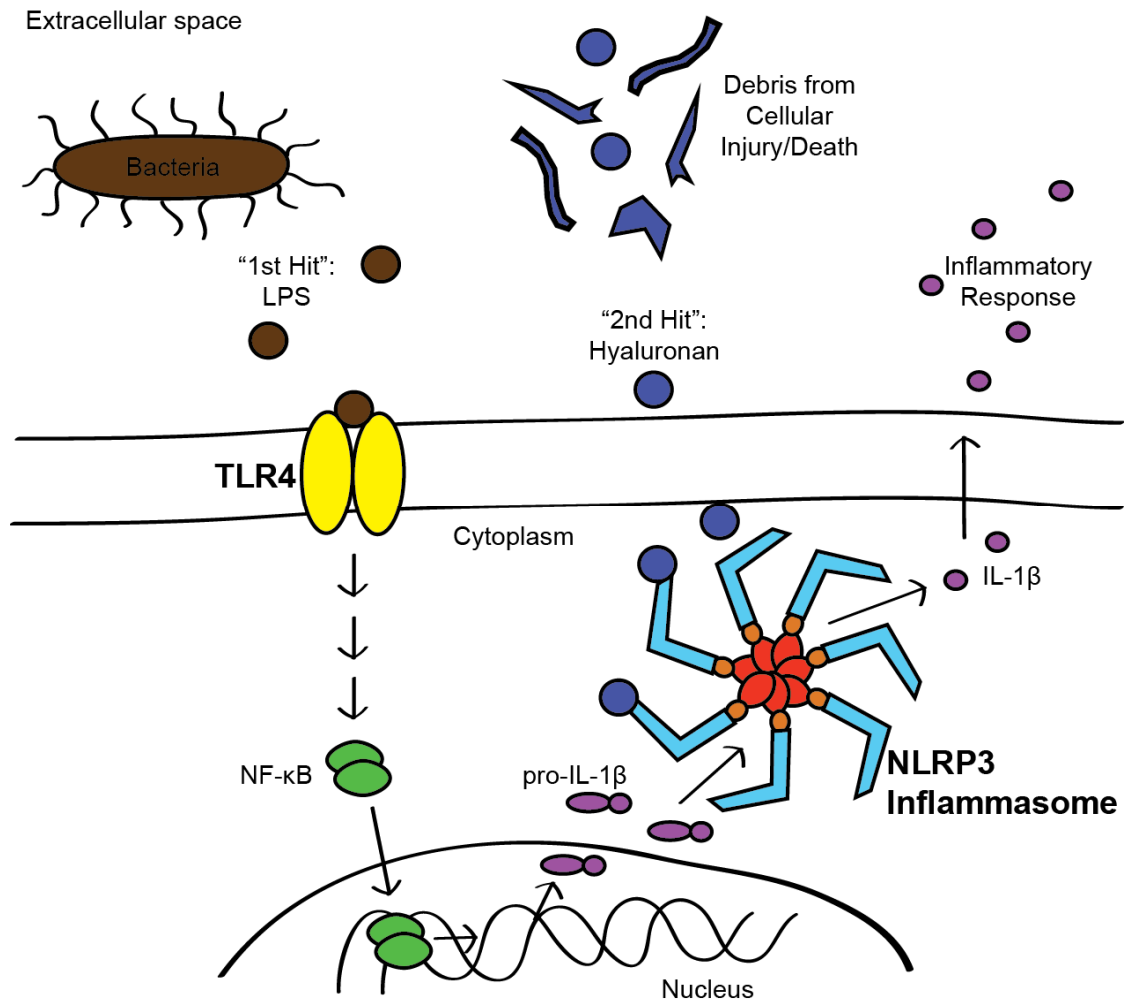
As mentioned above, previous work from my laboratory has implicated microglia as the key mediators of the long-term effects of early-life infection on brain function and behavior (Williamson et al., 2011). Importantly, microglia, in addition to their cytokine/chemokine products, play critical roles in brain development, including the promotion of neurogenesis and neuronal/glial migration, regulation of apoptosis in surplus neural cells, phagocytosis of these apoptotic cells, and pruning of superfluous synapses between neurons [reviewed in (Schwarz & Bilbo, 2011)]. Microglia are of the myeloid lineage, but unlike other macrophages, do not derive from the fetal bone marrow. Instead, recent studies have revealed that adult microglia derive entirely from primitive erythromyeloid progenitors that arise in the yolk sac prior to E8 (Ginhoux et al., 2010; Kierdorf et al., 2013). Microglia first begin to colonize the developing brain at E9.5, entering from the blood or ventricles, and then migrating throughout the brain (Cuadros & Navascués, 1998; Ginhoux et al., 2010). In addition, microglia maintain an amoeboid morphology during their initial colonization of the brain during fetal development, and only after birth do they begin to progress to the ramified, “quiescent” state characteristic of the adult brain. Notably, this amoeboid morphology is strikingly

similar to that observed in activated microglia of the adult brain after an immune challenge, which we have hypothesized makes these cells more vulnerable to perinatal immune challenges and therefore the likely cellular substrates of long-term changes in brain function and behavior, due to their relatively low turnover and long life in the adult brain (Bilbo & Schwarz, 2009; Schwarz & Bilbo, 2011).

### **1.3.1.1 Microglial priming**

The involvement of microglia in mediating the effects of early-life events on later-life brain function is likely due to their capacity, along with other macrophages, for a form of long-term immunological “memory”, referred to as “priming”, in which their response to a current immune challenge is modulated by prior challenges they have encountered (Pace et al., 1983). For example, macrophages can be primed by a low dose of lipopolysaccharide (LPS, a component of bacterial walls that activates PRRs on immune cells; referred to here as the priming stimulus), such that a subsequent LPS exposure (referred to as the triggering stimulus or “second hit”) results in an exaggerated immune response (Deng, Maitra, Morris, & Li, 2013). Following the second hit, macrophages are fully activated, such that they are capable of robust cytokine and chemokine secretion, reactive oxygen species production, antigen presentation to T cells (via upregulation of MHC class I and II molecules on their surface), and more efficient phagocytosis (Janeway, Travers, Walport, & Shlomchik, 2005), all of which better allow them to clear the body of a pathogen or other threat. Although in this scenario the first

signal may not be sufficient for cytokine production, there are certain enduring changes that occur following the priming stimulus alone, including upregulation of PRRs on the surface of or inside the cell. These changes sensitize the macrophage long-term, such that it will respond rapidly and fully to the second hit, even if it is subthreshold (i.e., would not garner a response in isolation). Thus, PRRs play an important role in macrophage priming due to their capacity to serve as “coincidence detectors” for the convergence of the priming stimulus and the second hit (Figure 1).



**Figure 1: Schematic depicting PRRs as coincidence detectors for the convergence of 2 "hits" (danger signals) within an innate immune cell. The first hit shown here consists of bacterial LPS (PAMP) binding to TLR4 on the surface of the cell, which initiates a downstream signaling pathway that results in the activation of NF-κB, a transcription factor that then translocates to the nucleus and initiates transcription of proinflammatory cytokines, such as pro-IL-1β. However, pro-IL-1β is biologically inactive until it is cleaved by caspase-1, an enzyme that forms part of the NLRP3 inflammasome (a large multimolecular complex). Thus, the second hit, consisting of hyaluronan (DAMP) released by the injured or dying cells, is required to activate the NLRP3 inflammasome in the cytoplasm of the same cell and cleave pro-IL-1β into its active form, IL-1β, which can then be released from the cell to initiate a robust inflammatory response. Note that the PRR ligands denoted here are just examples, and there can be multiple different combinations of various ligands, including**

**XAMPs, such as environmental toxins, or DAMPs, released following psychological stress or toxin exposure.**

Traditionally, immunological memory has been associated solely with the adaptive immune system, an idea that necessitated revision following the discovery of macrophage priming. However, unlike the adaptive immune system, the memory of the innate immune system is not stimulus-specific (i.e., “cross-priming” can occur), which is inherently due to the broad recognition of a variety of threats by PRRs. We believe that this “two-hit” model for the priming of macrophage activation, mediated by the PRRs as coincidence detectors, is an excellent candidate mechanism underlying the long-term programming of brain function and behavior by perinatal immune challenges. In support of this hypothesis, it has often been found in our work and that of others that, in the absence of outright brain damage (such as occurs in cerebral palsy), the more subtle, but enduring, consequences of early-life events may not be observed until a second hit is encountered later in life, which serves to “unmask” the fundamental changes in brain function, and resultant effects on behavior, that were incurred in response to the initial challenge during the sensitive period of development (Bolton et al., 2012; Williamson et al., 2011). Specifically, members of my laboratory have demonstrated that an early-life bacterial infection on postnatal day (P)4 predisposes rats to memory impairment in adulthood following a subsequent immune challenge (Bilbo et al., 2005), which is mediated by the early-life priming of microglia and their exaggerated cytokine response to the second hit (Williamson et al., 2011). These effects of microglial activation on adult

cognitive function are possible because cytokines and chemokines are not just important during development—they are also critical for brain homeostasis and normal function in adulthood, including mood regulation, learning, and memory [reviewed in (Yirmiya & Goshen, 2011)].

However, in our recent work involving maternal exposure to air pollution alone or in combination with maternal stress, either one or both “hits” occur *in utero*, prior to neural patterning, when microglia may not yet be present in the fetal brain to undergo priming, and even after they arrive, it may not be possible for them to be directly activated by maternal immune challenges because they are located behind the protective barrier of the placenta. Thus, our previous hypothesis concerning microglial priming as the mechanism for the enduring behavioral consequences of early-life (i.e., early postnatal period) events is not sufficient by itself to explain our findings of long-term behavioral effects following *prenatal* immune challenges. Therefore, I propose that these mechanisms extend even further back in development, to the priming of innate immune cells in the first neuroimmune organ, the placenta. I will present evidence in the coming sections that the placenta is ideally positioned to mount the initial fetal immune response to maternal immune challenges, and that placental innate immune cells, like microglia, play an important role in development and are capable of being primed by prenatal exposures.

### **1.3.2 Role of the Innate Immune System in the Placenta**

The placenta is the interface between the maternal and intrauterine environments and the fetus and the primary means of nutrient acquisition; thus, it is likely that the placenta plays a critical role in the fetal programming of offspring physiology by maternal environmental exposures. Furthermore, in the context of a maternal immune challenge, such as high-fat diet, the placenta is fully immunocompetent and prepared to mount an inflammatory response to defend the fetus, as it possesses macrophages (Wynn, 1967) with pattern recognition receptors [e.g., toll-like-receptor (TLR)4 (Abrahams, 2008)] that can bind saturated fatty acids (Milanski et al., 2009). However, inflammation during pregnancy is a double-edged sword, in that inflammation can resolve an infection that could damage the developing fetus, but inflammation itself, especially when chronic, as is typically the case for maternal high-fat diet, can also alter the trajectory of fetal development, including the fetal brain, resulting in long-term changes in brain function and behavior (Hsiao & Patterson, 2012).

Epidemiological studies in humans have shown that placental dysfunction is associated with later-life psychiatric disorders. For example, low placental weight, in conjunction with low birth weight, is associated with an increased incidence of schizophrenia in adulthood (Wahlbeck, Forsén, Osmond, Barker, & Eriksson, 2001). This type of intrauterine growth restriction, commonly associated with maternal obesity as well as undernutrition, is caused by “placental insufficiency,” or inadequate placental

blood flow (Jansson & Powell, 2007), which is accompanied by a placental proinflammatory cytokine response (Bartha, Romero-Carmona, & Comino-Delgado, 2003; Wang, Athayde, & Trudinger, 2003) (Figure 2). Notably, the balance between the fetal demand and placental supply of nutrients, which can be modulated by epigenetic mechanisms, is critical for normal development and can even have consequences for later-life behavior. For example, a placenta-specific knockout of the maternally imprinted gene *Igf2*, which causes reduced placental supply relative to fetal demand, leads to intrauterine growth restriction, followed in adulthood by increased anxiety and a corresponding decrease in GABA and serotonin receptor expression in the hippocampus of adult offspring (Mikaelsson, Constância, Dent, Wilkinson, & Humby, 2013). Considering the role of inflammatory processes in intrauterine growth restriction, it is likely that immune changes also play a role in the long-term behavioral consequences of this manipulation, but this remains to be tested directly.

Although the placenta was originally imagined to act as a protective barrier between mother and fetus, it is becoming clear that it is actually more of an interface than a barrier, as it mediates the complex interactions of maternal and fetal cells and the exchange of various substances between the two compartments (Mor, Romero, Aldo, & Abrahams, 2005; Robinson, Atkinson, Jones, & Sibley, 1988). The limited “barrier” capacity of the placenta is further disrupted in the case of perturbations in maternal diet. For example, placentas from protein-restricted rats exhibit a marked reduction of 11- $\beta$ -

hydroxysteroid dehydrogenase 2 enzyme (11- $\beta$ -HSD2), which normally protects the fetus from elevated maternal corticosteroids, thus resulting in fetal exposure to abnormally high glucocorticoid levels during gestation and later hypertension in the adult offspring (Langley-Evans et al., 1996). This finding is representative of fetal programming of the HPA axis, and has been shown in other contexts to be associated with increases in anxiety and the incidence of other neuropsychiatric disorders in adulthood (Seckl & Holmes, 2007), although the ultimate mechanism remains undefined. Proinflammatory cytokines can cause decreased activity of 11- $\beta$ -HSD2 (Kossintseva et al., 2006), and thus may play a role in programming by maternal diet (see Figure 2).

Proinflammatory cytokines found in the placenta may come from two sources: 1) Certain maternal cytokines (e.g., IL-6), resulting from the systemic inflammation associated with obesity, may be able to cross the placenta into the fetal compartment (Zaretsky, Alexander, Byrd, & Bawdon, 2004), and/or 2) the placenta itself is also capable of mounting an inflammatory response to maternal immune challenges (Hsiao & Patterson, 2011; Wynn, 1967), including maternal high-fat diet. During a normal pregnancy, both maternal decidual macrophages and fetal placental macrophages (also known as Hofbauer cells) exhibit an anti-inflammatory phenotype in order to maintain maternal immunotolerance of the fetal allograft (Kim et al., 2012). Thus, the coordinated interaction of maternal and fetal cells, orchestrated by immune molecules like cytokines

and chemokines, is necessary for the normal progression of pregnancy (Hunt et al., 2010). However, during maternal obesity, in parallel with increased maternal monocyte differentiation and inflammatory markers in the maternal circulation, there is also the accumulation of fetal Hofbauer cells in the placenta and the increased placental production of proinflammatory cytokines (Challier et al., 2008; Roberts et al., 2011) (Figure 2). Moreover, due to the dual role of innate immune molecules in host defense and developmental processes (as discussed above), the creation of an inflammatory milieu *in utero* can have significant consequences for later-life brain function and behavior.

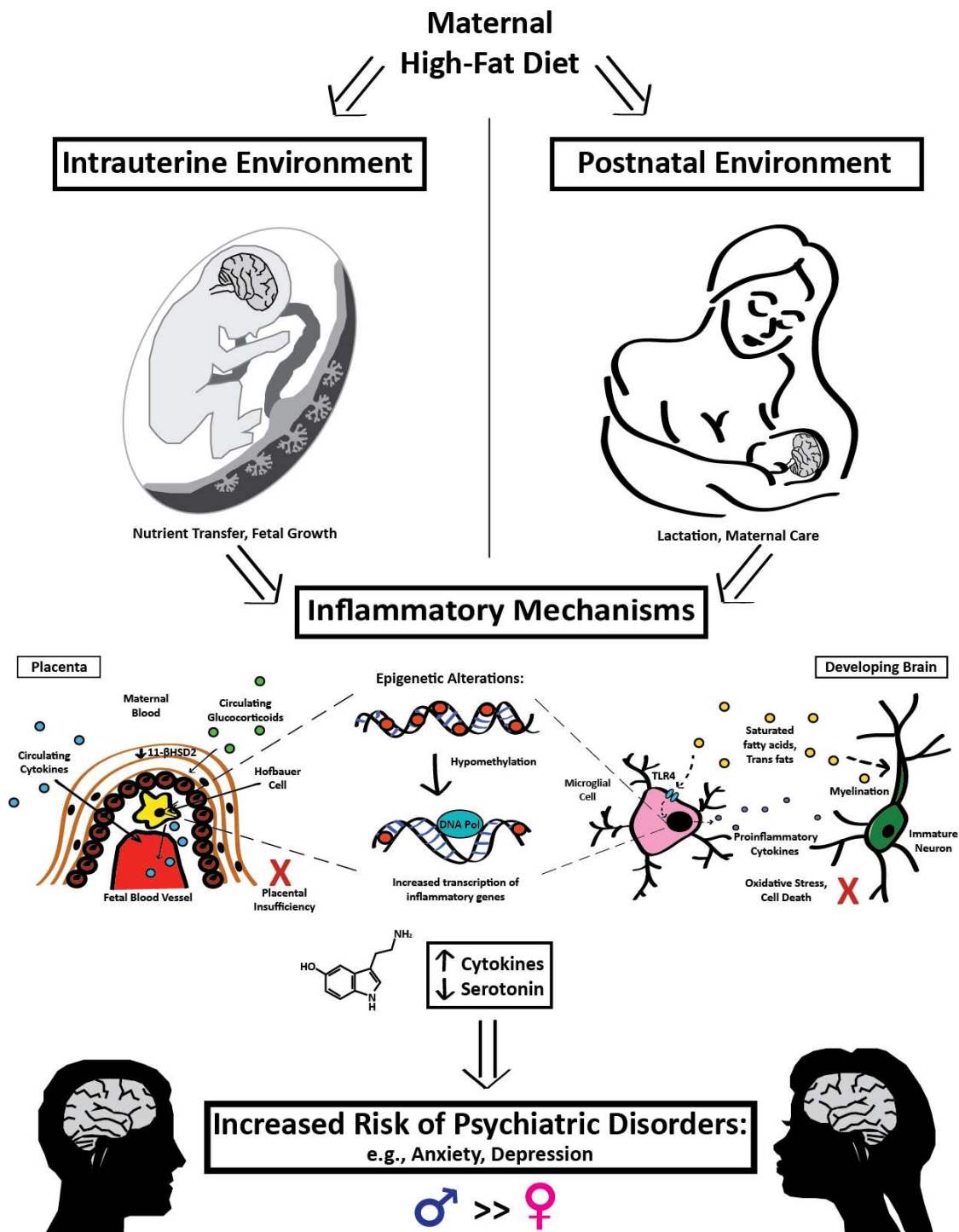


Figure 2: Programming of brain and behavior by maternal high-fat diet involves inflammatory pathways, which interact with both the intrauterine and postnatal

environments. (Many of the mechanisms portrayed here also apply to programming by prenatal air pollution exposure.) In the placenta (inset depicts a schematic of a chorionic villus), these inflammatory mechanisms include the passage of elevated maternal cytokines through the placenta into the fetal circulation, the production of proinflammatory cytokines by fetal placental macrophages (i.e., Hofbauer cells), and the increased passage of circulating maternal glucocorticoids across the placenta, due to decreased 11- $\beta$ -HSD2. Collectively, these conditions are associated with placental insufficiency, as well as intrauterine growth restriction. In the developing brain, the inflammatory mechanisms include the microglial production of proinflammatory cytokines (as a result of activation of the TLR4-mediated signaling cascade by saturated fatty acids), and the incorporation of saturated fatty acids and trans fats into the myelin of developing neurons. Thus, there is the promotion of a neuroinflammatory microenvironment, which may lead to oxidative stress, cell death, and an overall altered trajectory of brain development. Increased levels of proinflammatory cytokines lead to decreased serotonin synthesis by the placenta during fetal development, and by the brain during postnatal development, which may contribute to the alteration of brain development and an increased risk of psychiatric disorders in adulthood (a risk that has been shown to be greater in males in humans and animal models). [Figure originally published in Bolton & Bilbo, 2014].

#### 1.3.2.1 The innate immune cells of the placenta

Both immune and traditionally “non-immune” cells are thought to play a key role in the innate immune function of the placenta. Maternal contributions include macrophages, dendritic cells, uterine natural killer cells, T cells, and stromal cells of the decidua. These decidual immune cells have been implicated in fetal tolerance, development of the uterine vasculature, extravillous trophoblast invasion, and the progression of parturition (e.g., cervical ripening) (Hsiao & Patterson, 2012; Nagamatsu & Schust, 2010). Fetal components of the placental innate immune system include epithelial and stromal cells of the placenta and fetal membranes, such as the trophoblast giant cells and syncytiotrophoblasts, in addition to fetal macrophages, known as Hofbauer cells. These cells are the first line of defense against pathogens and toxins

gaining entry into the fetal compartment from the maternal circulation. However, they also play important roles in the normal development of the placenta and fetus.

Like microglia, Hofbauer cells derive from the myeloid cell lineage. Because they appear prior to the establishment of fetal bone marrow, their origin is controversial. However, it is clear that they are fetal-derived (Wynn, 1967). Hofbauer cells have been proposed to have different anatomical origins throughout gestation, such that they may represent a heterogeneous group of cells (Castellucci et al., 1987). Very early in pregnancy, Hofbauer cells may differentiate directly from mesenchymal cells in the villous stroma (Demir, Kaufmann, Castellucci, Erben, & Kotowski, 1989). Alternatively, there is evidence that Hofbauer cells, like microglia, derive from granulocyte-monocyte progenitor cells in the fetal yolk sac, which migrate to the mesenchyme of the placental villi beginning on E10 in the mouse (Takahashi, Naito, Katabuchi, & Higashi, 1991).

Because Hofbauer cells are present in the placenta from very early on, they are thought to be important in its normal development, performing a role quite similar to microglia in the developing brain. Specifically, they have been implicated in vasculogenesis and angiogenesis, remodeling of the villous architecture that divides the maternal and fetal blood supplies, and control of trophoblast proliferation, differentiation, and apoptosis within the villous core (Benirschke, Kaufmann, & Baergen, 2006; Khan, Katabuchi, Araki, Nishimura, & Okamura, 2000). They are most commonly

observed in the stroma surrounding the villous structures where nutrient and gas exchange takes place (and where a pathogen would be most likely to gain entry; (Hofbauer, 1903)). Importantly for their role in host defense, they retain considerable motility by utilizing stromal channels throughout the villous core (Castellucci, Zaccheo, & Pescetto, 1980). Furthermore, Hofbauer cells express complement receptors, such as CD11b/CD18, that are constitutively expressed on all macrophages, in addition to multiple leukocyte Fc receptors, which may bind maternal anti-fetal antigen-antibody complexes and thus play a role in protecting the fetus from the maternal immune system (Wood, 1980). Like other macrophages, they are phagocytic, but they surpass typical adult macrophages in their capacity for extensive fluid uptake (i.e., macropinocytosis), as indicated by their highly vacuolated appearance. It has been suggested that they may use this ability in combination with their motility through stromal channels to remove excess fetal serum proteins and maintain water balance in the placenta, thus serving as a sort of primitive lymphatic system (Enders & King, 1970). Notably, trophoblast cells, though not professional phagocytes, are also capable of phagocytosis, which may facilitate their invasion of maternal tissues and provide an early source of nutrition before the placenta is fully formed, in addition to playing a role in host defense (Bevilacqua, Hoshida, Amarante-Paffaro, Albieri-Borges, & Zago-Gomes, 2010).

### 1.3.2.2 Evidence for the priming of placental immune cells

In support of my hypothesis, there is evidence that placental innate immune cells, like microglia, do indeed exhibit the characteristics of priming. For example, Hofbauer cells in the placenta upregulate TLR4 during gestational insults, such as preterm chorioamnionitis (Kumazaki, Nakayama, Yanagihara, Suehara, & Wada, 2004). Furthermore, Hofbauer cells express both MHC I and II, and perform antigen presentation *in vitro* with almost the same efficiency as adult macrophages (Glover, Brownstein, Burchett, Larsen, & Wilson, 1987). Although this ability is normally suppressed *in vivo* by high levels of maternal progesterone, which has an immunosuppressive effect on fetal macrophages (Yagel, Hurwitz, Rosenn, & Keizer, 1987), it may be enhanced by priming stimuli that upregulate MHC I and II expression. In addition, interferon- $\beta$ , which can be produced by placental trophoblasts, has been shown to prime both Hofbauer cells and trophoblasts themselves, leading to enhanced interferon secretion from these cells in response to a challenge with poly(I:C), a viral mimic that binds to TLR3 (Toth, Juhl, Nørskov-Lauritsen, Mosborg Petersen, & Ebbesen, 1990; Toth, Nørskov-Lauritsen, Juhl, & Ebbesen, 1991). Therefore, it is possible that both Hofbauer cells and placental trophoblasts are capable of being primed by immune challenges, since both express PRRs and can secrete cytokines and chemokines.

Levels of cytokines and chemokines must be in a precise balance for the proper function of the placenta, as they can act as growth factors and chemotactic factors that

facilitate the development of the fetus (Hunt et al., 2010). Thus, maternal immune challenges have the capability, via the priming and activation of placental immune cells, to alter this balance between innate immune protection and pathology at the maternal-fetal interface, with the result that the trajectory of fetal development may be permanently altered. For example, Hsiao and Patterson (2011) found that maternally-derived IL-6 following injection of poly(I:C) (a viral mimic) activated immune signaling in the placenta that resulted in disruption of placental growth factors and endocrine factors that are important for fetal development. The authors hypothesized that this was the ultimate cause of the IL-6 dependent alterations in fetal brain development and autism- and schizophrenia-related behaviors in the adult offspring that they had previously characterized in this maternal immune activation model. However, further work needs to be done to directly link the immune changes in the placenta and later-life behavior.

### **1.3.3 Role of the Placenta in Fetal Brain Development**

Because the placenta is the gateway for nutrients from the mother to the fetus as well as the source of essential developmental signals, its proper function does not just have consequences for its own development, but also for the development of the rest of the fetus, especially the fetal brain. The prenatal period is a critical period for brain development, as it is when the majority of the basic architecture of the neural circuitry is laid down. Furthermore, the molecular and cellular components of the innate immune

system are critical for orchestrating the complexities of both placental and fetal brain development. Therefore, I hypothesize that the placenta, as the first neuroimmune organ and the source of multiple substances critical in brain development, plays a key regulatory role in a number of neurodevelopmental processes, and thus has the power to alter the trajectory of brain development in response to changes in the intrauterine environment, such as those that may result from maternal immune challenges.

### **1.3.3.1 The placenta as the first neuroimmune organ**

Because cytokines and chemokines are so critical for the early development of the placenta, it is likely that the placenta is the first source of these immune signals in the developing fetus. Furthermore, these placental products can travel to the developing brain, where they may influence early neuroimmune development and function, such as early neural patterning and microglial colonization. Importantly, the blood-brain barrier is much more permeable in the fetus than in the adult, as dyes injected into the bloodstream of newborn mice readily accumulate in the CNS, often in association with large plasma proteins, which does not occur in the intact adult (Adinolfi, 1985). Furthermore, because cytokines have been shown to cross the intact blood-brain barrier even in normal adult mice, it is highly probable that cytokines from the periphery (i.e., the placenta) can access the developing brain (Dammann & Leviton, 1997). Therefore, it is possible that placental cytokines and chemokines play a role in early brain development, before any neural cells are capable of secreting these critical

developmental guidance cues, although future studies are necessary to test this hypothesis.

In addition to immune mediators, the placenta is also capable of secreting molecules that have traditionally been considered to be produced solely by neurons, such as neurotransmitters. For example, Bonnin and colleagues (2011) recently demonstrated using a novel *ex vivo* placental perfusion technique that the placenta, specifically the syncytiotrophoblast, is capable of synthesizing serotonin from maternal tryptophan (Bonnin et al., 2011). Serotonin is a critical modulator of neurogenesis and axon growth in the developing forebrain, especially during the period of E10.5-E15.5 in the mouse (corresponding to the first and early second trimester in humans), but prior to this study, the source of this serotonin was unclear, due to the lack of serotonergic innervation at this stage (Buznikov, Lambert, & Lauder, 2001). Importantly, Bonnin et al. also ruled out the possibility that serotonin in the fetal brain *in vivo* is maternally-derived by demonstrating that serotonin transporter knockout (SERT<sup>-/-</sup>) dams bred with SERT<sup>+/-</sup> males produce SERT<sup>+/-</sup> fetuses that have normal levels of serotonin in the embryonic forebrain at E12.5. Finally, Bonnin et al. demonstrated that there is a progressive switch from the exogenous placental source of serotonin to a brain-intrinsic source by E18.5 (i.e., just before birth), when serotonergic neurons from the dorsal raphe are fully functional and have innervated the forebrain. Therefore, this study provided clear evidence for a novel mechanism by which placental function can directly impact

fetal brain development, which has clear implications for the programming of later-life brain function and behavior.

### **1.3.3.2 Placental neuroimmune function during maternal immune challenge**

Notably, the above-mentioned neural and immune products (i.e., neurotransmitters and cytokines) of the placenta have the potential for interaction and reciprocal regulation, especially following a maternal immune challenge. Specifically, the enzyme indoleamine 2,3-dioxygenase (IDO), which causes tryptophan degradation, is upregulated by high levels of proinflammatory cytokines, resulting in decreased serotonin synthesis and a potentially damaging by-product, quinolinic acid, which is a helpful defense against a pathogen, but also a potent NMDA receptor agonist that is capable of causing excitotoxicity and oxidative stress in the brain. For example, maternal endotoxin exposure has been shown to cause decreased serotonin synthesis, increased cell death, and diminished serotonergic innervation of the somatosensory cortex in the brains of newborn rabbits (Kannan et al., 2010). Furthermore, IDO is expressed in the normal placenta and is upregulated in placentas from preterm pregnancies associated with intrauterine infection. This upregulation was associated with an increase in quinolinic acid in the fetal bloodstream, which suggests that it may circulate to the fetal brain, where it can induce brain damage (Manuelpillai et al., 2005). Therefore, I propose that there is a delicate balance between the cytokine- and serotonin-producing

capabilities of the placenta that is critical for normal brain development and neuroimmune function.

### **1.3.4 Role of the Postnatal Environment**

Most maternal diets are not simply a potential programming factor during gestation, as they are likely initiated prior to pregnancy and continue after birth. Thus, maternal diet can exert influence on offspring during the postnatal period, at least throughout lactation and up until weaning. Most components of the maternal diet, including saturated and trans fats, influence the composition of the breast milk (Wolff, 2003), which makes up the sole source of an infant's nutrients, both in animals and in humans that choose to breast feed, during the critical period of postnatal brain development that includes extensive myelination and synaptic pruning (Andersen, 2003). As such, maternal dietary fats, such as trans fats, are incorporated into neuronal myelin sheaths, synaptic terminals, and capillaries of an infant's brain (Grandgirard et al., 1994). Furthermore, the ability of these dietary fats to traverse the BBB (Spector, 1988; Strosznajder, Chalimoniuk, Strosznajder, Albanese, & Alberghina, 1996) suggests it's possible that maternal high-fat diet can continue to foster an inflammatory environment in the brains of offspring, potentially disrupting the postnatal period of brain development (as discussed in the previous section for the fetal period; see Figure 2). Gorski and colleagues (Gorski, Dunn-Meynell, Hartman, & Levin, 2006) demonstrated that cross-fostering the pups born to lean dams to obese dams resulted in

impaired insulin sensitivity and a predisposition to obesity, whereas the converse was true for the pups of obese dams cross-fostered to lean dams. Future studies must determine if exposure to maternal high-fat diet during the early postnatal period alone is also sufficient to program behavioral changes in adulthood.

In addition to the direct consumption of maternal diet by offspring, maternal diet can influence maternal behavior and the quality of maternal-offspring interactions after birth. The quality of maternal care during the early postnatal period has significant consequences for offspring neural development and behavior in adulthood (Weaver et al., 2004), which is linked in part to the epigenetic modification of immune genes in microglia within the brain (Schwarz, Hutchinson, & Bilbo, 2011). However thus far, only a small number of studies have examined changes in maternal care due to alterations in maternal diet. Smart and Preece (Smart & Preece, 1973) showed that rat mothers that were malnourished throughout pregnancy and lactation licked and groomed their pups significantly less than control mothers, but did not examine consequences for the offspring. On the other hand, Purcell and colleagues (Purcell et al., 2011) found that dams consuming a high-fat diet throughout gestation and lactation spent more time nursing their pups and more time overall caring for their pups, although no difference in time spent licking and grooming, during the first week of postnatal life. However, even in the absence of the mother, the pups of high-fat diet dams consumed more milk in tests of independent ingestion, suggesting that an increase in pup appetite may be

driving the observed change in maternal behavior. It remains to be shown whether alterations in maternal care due to high-fat diet play a role in the changes in offspring behavior that are seen in adulthood, and whether these changes are accompanied by epigenetic modifications in immune genes as we have previously demonstrated in another model (Schwarz et al., 2011). However, the strong link between maternal high-fat diet and an increase in anxiety-like behavior in adulthood, in rats as well as other species, at least suggests that the observed increase in nursing (in the absence of increased licking and grooming) may be insufficient to *decrease* offspring stress-responsiveness in adulthood (Weaver et al., 2004).

### **1.3.5 Classes of Behavioral Outcomes of Maternal Immune Challenges**

There are two distinct categories of long-term effects on brain function and behavior that can result from the alteration of placental and/or microglial neuroimmune function by maternal immune challenges. First, there is the class of disorders that are present in an organism at birth or immediately following the insult, due to significant alterations of fetal brain development and/or outright brain damage, and thus do not require the occurrence of a second hit later in life to unmask its effects. An example of this is cerebral palsy, a disabling childhood neurological disorder that has been associated with severe placental infection [i.e., chorioamnionitis (McAdams & Juul, 2012)]. In this case, the enduring effects on brain function and behavior are due to a

single, but severe, perinatal insult that causes robust placental- and/or neuro-inflammation.

The second class of disorders that can result from maternal immune challenges during gestation are not present at baseline in the offspring, but require a “second hit” in adulthood to be revealed. A classic example of this is schizophrenia, which is thought to be linked to maternal infection during gestation in combination with a second challenge, such as infection or stress, during young adulthood, that precipitates the sudden clinical onset of the disorder (Giovannoli et al., 2013). In this example, it is possible that the priming of placental immune cells occurs in response to the maternal infection, but how can this priming be maintained into adulthood, when the second hit is received, if the placenta is lost following birth? Importantly, the induction of IDO and quinolinic acid release from microglia in response to infection or stress has been implicated in the etiology of schizophrenia (Anderson, 2011). I hypothesize that placental cytokines and chemokines that are released into the fetal circulation following maternal infection (or any other maternal immune challenge) likely cross the blood-brain barrier more readily in the presence of fetal inflammation, and thus have the potential to induce IDO in microglia, resulting in the propagation of neurotoxic damage due to quinolinic acid and causing the release of DAMPs, which can bind to microglial PRRs and result in their priming. Therefore, I propose that a maternal immune challenge that primes placental innate immune cells can also subsequently prime their cousins in the developing brain,

the immature, amoeboid microglia, thereby ensuring that susceptibility to a second hit, and thus vulnerability to neuropsychiatric disorders, following birth (and separation from the placenta) will remain permanently programmed via these long-lived brain cells.

### **1.3.6 Sex Differences**

Sex differences in offspring outcomes due to perinatal events, including maternal diet and toxin exposures, are gradually gaining recognition in the field of perinatal programming. For example, male newborns of women with pregestational diabetes suffer worse perinatal outcomes, including preterm birth and birth defects (García-Patterson et al., 2011), and male babies born to women with gestational diabetes are more likely to be delivered by caesarean section and to have a higher risk of neonatal hypoglycemia than female babies (Tundidor et al., 2012). Similarly, male offspring of asthmatic mothers are more likely to suffer severe complications, such as premature birth or stillbirth, than female offspring in response to an acute asthma exacerbation during pregnancy (Murphy, Gibson, Talbot, & Clifton, 2005). At the same time, there is a well-documented, but poorly understood, male bias in the prevalence of neurodevelopmental disorders, including learning disabilities (Flannery, Liederman, Daly, & Schultz, 2000) and autism (Stone et al., 2004), that have been linked to maternal immune challenges. Maternal diet and toxin exposures, now recognized to modulate the immune system, have increasingly been found to be the common factor linking certain

neonatal outcomes and later-life psychiatric disorders, often with a male bias. For example, SGA male, but not female, infants have a greater risk of depression as adults (C. Thompson, Syddall, Rodin, Osmond, & Barker, 2001). Furthermore, only the male offspring of high-fat diet rat dams exhibit elevated body weight and increased anxiety in adulthood (Bilbo & Tsang, 2010). However, the mechanisms underlying these intriguing sex differences remain poorly defined and theoretical.

One of the potential factors underlying the greater vulnerability of males to early-life programming of brain and behavior may lie in sex differences in the immune responsiveness of the developing brain and placenta. For example, there are marked sexual dimorphisms in microglial colonization of the rat brain across development, such that males have a greater number of microglia than females shortly after birth, which has been hypothesized to render them more vulnerable to early-life immune challenges (Schwarz, Sholar, & Bilbo, 2012). This type of microglial “priming”, or long-term alteration of microglial neuroimmune function, has clear consequences for cognition and behavior in adulthood (Williamson et al., 2011).

Alternatively, Clifton (Clifton, 2010) suggests that sex differences in offspring outcome may be due to the sexually dimorphic strategies males and females employ in a compromised intrauterine environment. Male placentae respond to maternal inflammation with few changes in gene expression in order to allow for accelerated or continued growth, but at the cost of increased risk for adverse outcomes. On the other

hand, female placentae respond with multiple gene changes that enhance the fetal immune response to the maternal immune challenge, which leads to a minor decrease in growth, but has adaptive value overall in promoting survival, especially in the face of further maternal insults (Murphy et al., 2003; Murphy et al., 2005; Scott et al., 2009). In a striking example of the interaction between maternal diet and placental sex, Mao and colleagues (Mao et al., 2010) demonstrated that maternal high-fat diet causes more dramatic alterations in the gene expression of female placentae than male placentae, thus providing experimental support for Clifton's hypothesis in the context of maternal diet.

I hypothesize that the sex differences observed in microglia may extend further back in development to the first neuroimmune organ, the placenta. In support of this hypothesis, there is evidence that normal human term placentae from males contain more macrophages than placentae from females (Scott et al., 2009), and that cultured placental trophoblasts from normal male fetuses possess higher levels of TLR4 than trophoblasts from female fetuses (Yeganegi et al., 2009). Importantly, these sex differences are likely not dependent on gonadal sex hormones [as significant testosterone production is not apparent until E18 in the mouse; (O'Shaughnessy et al., 1998)], but may instead be determined by X-linked genes, many of which encode proteins with immune functions, including several TLRs (Fish, 2008). Of particular interest may be how maternal stress and environmental toxins interact differently in the

male and female placenta, potentially with divergent effects on macrophage priming and PRRs, as sex differences have previously been noted in the placental response to maternal stress alone (Mueller & Bale, 2008).

Future studies aimed at exploring the range of sex differences will advance our understanding of the mechanisms by which perinatal programming occurs, particularly how the same maternal immune challenge can lead to disparate brain and behavior outcomes in males and females. The goal of my dissertation research is to not only characterize the long-term programming of brain and behavior by maternal exposures, but to do this thoroughly in both male and female offspring, and explore whether the corresponding immune changes in the placenta and developing brain are also sex-specific (see Chapters 5 and 7). Importantly, this knowledge will better inform social or medical interventions that must be tailored to the sex of the at-risk child.

## **1.4 Summary**

*The overarching thesis for my dissertation work is that innate immune mechanisms, such as microglial priming and placental immune responses, alter normal brain and placental function during critical windows of development in order to result in the long-term programming of brain and behavior by early-life environmental exposures. My dissertation addresses the following research questions:*

- 1) Can prenatal air pollution exposure predispose offspring to obesity and neuroinflammation following high-fat diet consumption in adulthood?
- 2) Is this neuroinflammation the result of microglial priming specifically or is there also an involvement of the peripheral innate immune system in the offspring of air pollution-exposed mothers?
- 3) Can prenatal air pollution exposure synergize with a simultaneous “second hit” (maternal stress) during gestation to program long-term changes in adult offspring behavior and neuroimmune function?
- 4) Does prenatal air pollution exposure alter placental immune responses and microglial colonization/maturation via TLR4 (a specific pattern recognition receptor), thus resulting in long-term changes in microglial morphology?
- 5) Can maternal consumption of a “Western diet” prior to and during gestation and lactation program offspring for long-term changes in behavior and CNS immune reactivity?
- 6) Does maternal consumption of a “Western diet” alter placental innate immune responses, serotonin production, and microglial colonization/maturation in order to effect long-term changes in offspring serotonergic function, immune reactivity, and behavior?

With the following 6 experiments, I seek to answer these questions and further elucidate the innate immune mechanisms in the placenta and developing brain that underlie the programming of later-life behavior and neuroinflammation, often in a sex-specific manner.

## **2. Prenatal Air Pollution Exposure Induces Neuroinflammation and Predisposes Offspring to Weight Gain in Adulthood in a Sex-Specific Manner<sup>1</sup>**

### **2.1 Introduction**

Obesity is an escalating public health concern with complex genetic, behavioral, and environmental origins. There is growing consensus that over-nutrition and lack of exercise alone cannot fully account for the sharp rise in obesity prevalence over the past two decades (Keith et al., 2006). Emerging evidence suggests that environmental chemical exposures may contribute to obesity by altering several aspects of metabolism, including adipose tissue biochemistry and insulin sensitivity, as well as by impacting behavior such as food intake (Heindel & vom Saal, 2009). For example, air pollution has been shown to worsen inflammation and insulin resistance in a mouse model of obesity, and obesity can exaggerate the systemic inflammatory response to air pollution (Dubowsky, Suh, Schwartz, Coull, & Gold, 2006; Sun et al., 2009; Xu et al., 2010), implying a bidirectional association. However, the mechanisms underlying these relationships remain unknown. Even more importantly, the impact of so-called “obesogens” such as air pollution on endocrine set points may be greatest during critical windows of development (Grün & Blumberg, 2009; Lawlor & Chaturvedi, 2006).

Whether prenatal air pollution exposure can alter the later-life response to diet

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composition (e.g., fat content) has not been explored. We believe these questions are critical, as there are well-documented health disparities due to socioeconomic status (SES) (Evans & Kantrowitz, 2002b), and air pollution and high-fat diets are both more prevalent in low SES neighborhoods due to proximity to highways and fast-food restaurants (Hurvitz, Moudon, Rehm, Streichert, & Drewnowski, 2009).

One primary mechanism by which air pollution may create enduring changes in health outcomes is via neuroinflammation mediated by the activation of microglia, and their cytokine and chemokine products that lead to adverse neural adaptations and/or neurotoxicity (Block & Calderón-Garcidueñas, 2009; Levesque et al., 2011a). Obesity is also characterized by low-grade inflammation within the body and microglial activation within the brain (Kennedy, Martinez, Chuang, LaPoint, & McIntosh, 2009; Milanski et al., 2009). Moreover, toll-like-receptor (TLR) 4, one of the innate immune system's PRRs present on all innate immune cells, including microglia, has been implicated as critical in the cross-talk that occurs between inflammatory and metabolic signals (Saber et al., 2009; Tsukumo et al., 2007). For instance, TLR4 signaling on microglia within the hypothalamus is key to saturated fatty acid-induced cytokine release, and the subsequent development of insulin resistance in a rat model of obesity (Milanski et al., 2009). Thus, microglia may be uniquely positioned to mediate interactions between the immune, nervous, and endocrine/metabolic systems in response to environmental signals, both during development and in adulthood.

We tested the hypothesis that fetal/maternal inflammation in response to prenatal air pollution exposure would predispose the offspring to greater weight gain upon exposure to a high-fat diet in adulthood. Pregnant mice were exposed to filtered air or concentrations of diesel exhaust relevant to human exposures [as fully characterized in (Sharkhuu et al., 2010)] throughout the second half of gestation. This method of maternal diesel exposure provokes a maternal inflammatory response, as indicated by increased leukocyte infiltration of the lungs (Auten et al., 2012), without significantly impacting litter size or composition (Sharkhuu et al., 2010). The male and female offspring were assessed as adults for body weight and metabolic hormones, food intake, activity, anxiety behavior and endpoint microglial activation within the brain in response to low- and high-fat diet exposure. We predicted that air pollutant-induced fetal neuroinflammatory responses during gestation would impact offspring responses to a high-fat diet in adulthood, characterized by changes in metabolic, brain, and behavioral outcomes.

## **2.2 Materials and Methods**

### **2.2.1 Animals and Prenatal Exposures**

C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC, USA) and housed in pathogen-free vivarium conditions with *ad libitum* access to food and filtered water at the U.S. Environmental Protection Agency (Research Triangle Park, NC, USA). Time-mated females were exposed to filtered air (FA; n=6) or 2.0 mg/m<sup>3</sup> of

diesel exhaust (DE; n=6) in exposure chambers housed in an isolated animal exposure room for 4 h/day for 9 consecutive days from embryonic day 9 to 17 (E9-E17) as previously described (Auten et al., 2012). Dams and their offspring were housed on specialized bedding (AlphaDri) until euthanasia to avoid exposure to potentially confounding antigens that can be found in typical bedding. After the final exposure to DE on E17, dams were transported 6 miles from the U.S. EPA inhalation exposure facilities to a Duke University satellite animal facility, where they gave birth between E18 and E20. Pups were weighed on postnatal day (P) 0 in order to acquire a litter average for birth weight. Offspring were weaned at 4 weeks of age, from which point they were fed standard laboratory chow (Rodent Diet 5001; Lab-Diet, Philadelphia, PA, USA) until the beginning of the diet manipulation. Once the offspring reached adulthood, 2-3 animals/sex/litter were transferred to the main animal facility, housed in same-sex groups of 2-3 in individually ventilated, microisolator polypropylene cages, and maintained on a reverse 12:12-h light-dark cycle (lights off at 9 AM) in order to initiate metabolic and behavioral monitoring at 4-5 months of age. All experiments were conducted with protocols approved by both the U.S. EPA and Duke University Animal Care and Use Committees.

### **2.2.2 Fetal Brain Cytokine Analysis**

A separate group of FA(n=3)- and DE(n=2)-exposed dams were treated in the same way as described above, but euthanized at E18 for analysis of fetal brain cytokine

levels at the end of the prenatal DE exposure period. Fetuses were obtained by hysterotomy under sodium pentobarbital anesthesia (250 mg/kg i.p.), placed on ice, and decapitated. Whole fetal brains were snap-frozen under liquid nitrogen and stored at -80°C until assayed. Measurements of protein levels of the cytokines interleukin [IL]-1 $\beta$  and IL-10 were made using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) performed on fetal brain homogenates (200  $\mu$ g/well; n=3-5/litter). For data analysis purposes, samples that had undetectable levels of IL-1 $\beta$  or IL-10 were assigned a value of one-half the lowest detectable value in the assay (A. L. Thompson et al., 2012).

### **2.2.3 Adult Diets and Food Intake**

Adult male (n=23) and female (n=24) offspring 4-5 months of age in 3 cohorts were given either: (1) a **low-fat diet [LFD]**; 20% of calories from protein, 70% from carbohydrate, and 10% from fat (44:56% lard: soybean oil); Research Diets, New Brunswick, NJ, USA, cat. no. D12450B; n=23], or (2) a choice between the LFD and a **high-fat diet** (50 g of each, divided into alternate sides of the food hopper) [**HFD**; 20% protein, 35% carbohydrate, and 45% fat (88:12% lard: soybean oil); Research Diets D12491; n=24]. The LFD provided 3.85 kcal/g, and the HFD provided 4.73 kcal/g. All other nutritional parameters of the two diets were identical. The complete nutritional profiles of each diet are available online (<http://www.researchdiets.com>). Animals were weighed immediately prior to diet assignment, and every 3 days thereafter at the same

time each day for 6 weeks until sacrifice. After beginning the diet, the animals' food was weighed every 3 days. Food consumption data was normalized for the different energy content of the diets by converting grams to kilocalories of food consumed. Of the animals that were given a choice between diets, all of the animals consumed the HFD almost exclusively; thus these animals will be referred to simply as HFD.

#### **2.2.4 Open Field Behavioral Testing**

Adult male and female offspring were tested for baseline activity and anxiety levels in the open field the day before diet assignment, as well as weekly thereafter at the same time each week for the 6 weeks of diet. Animals were tested in groups of four, each within its own open field, with only one sex in the testing room at one time. Tests lasted 30 min during the animals' dark cycle between 12-5 PM in the afternoon. The open field apparatus consisted of four custom-made black Plexiglas boxes (each 40 cm x 45.1 cm x 34.9 cm) elevated on a gray platform approximately 53 cm off the ground, with a camera attached to the ceiling overhead, positioned to record four mice in the four distinct enclosures of the apparatus at once. ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA) was used to track the movement of each mouse, and distance traveled (m) by each animal was used as a measure of activity level. Total time spent mobile during the 30 min test was used as a secondary measure of activity levels to corroborate the distance results. In addition, virtual zones were created using the ANY-maze software in order to define a "Center" zone (approximately 30 cm x 30 cm

square in the center) and a “Surround” zone (the remaining space surrounding the center zone) in each of the boxes. Percent time spent in the Surround zone of the box was considered to be a measure of anxiety level, as more anxious mice will spend more time in the darker regions close to the walls of the apparatus than in the open center space (P. Simon, Dupuis, & Costentin, 1994). As a secondary measure of anxiety that controls for activity levels, percent distance traveled in the Surround zone (distance traveled in the Surround zone / total distance traveled x 100) was also analyzed. In this case, a higher percentage of distance traveled in the Surround zone indicates that the animal is more anxious because it prefers to travel more in the safer surround zone, even if it is overall moving less.

### **2.2.5 Serum and Tissue Collection**

At the end of the 6 weeks of diet, mice were deeply anesthetized with a ketamine/xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine i.p.), and a non-fasting blood sample was collected from each animal by penetrating the retro-orbital sinus with a sterile heparinized capillary tube. Immediately afterwards, mice were transcardially perfused with ice-cold saline for 2 min, followed by 4% paraformaldehyde for 2 min to fix the brain tissue. Whole brains were rapidly extracted and post-fixed by 4 successive changes of fresh 4% paraformaldehyde daily, and then stored in 0.1% sodium azide in PBS at 4°C until cryo-sectioned for histological analyses. Blood was centrifuged at 16,100 x g at 4°C for 10 min, and serum was removed and stored in microcentrifuge

tubes at -20°C until assayed. All serum and tissue collection occurred during the dark cycle between 10 AM-1 PM.

## **2.2.6 Metabolic and Endocrine Measures**

### **2.2.6.1 Leptin assessment**

Leptin was measured in the serum using a commercially available enzyme-linked-immunosorbent assay (ELISA) kit (cat. no. MOB00; R&D Systems, Minneapolis, MN, USA). Samples were diluted 1:40 in the manufacturer's calibrator diluent and run in duplicate. The detection limit of the assay was 22 pg/ml, and the intra-assay coefficient of variation was <10%.

### **2.2.6.2 Insulin assessment**

Non-fasting insulin levels were measured in the serum using a commercially available enzyme-linked-immunosorbent assay (ELISA) kit (cat. no. 90080; Crystal Chem, Inc., Downers Grove, IL, USA). The high range assay (1-64 ng/ml) was performed according to the manufacturer's instructions. Samples were run in duplicate, and the intra-assay coefficient of variation was <10%.

### **2.2.6.3 Corticosterone assessment**

Total serum corticosterone (CORT) concentrations were assessed using a colorimetric enzyme immunoassay (EIA) kit from Assay Designs, Inc. (cat. no. ADI-901-097; Ann Arbor, MI, USA) as described previously (Bilbo et al., 2007). Samples were diluted 1:50 in 0.05% steroid displacement-modified assay buffer provided with the kit.

The detection limit of the assay is 27 pg/ml, and the intra-assay coefficient of variation was <10%.

### **2.2.7 Microglial Immunohistochemistry**

Fixed whole brains were cryoprotected in 30% sucrose plus 0.1% sodium azide for 3 days prior to slicing, then quickly frozen in -30°C isopentane and sliced coronally from the anterior hypothalamus through the ventral hippocampus in a 1:6 series at 40 µm in a -20°C cryostat. Floating sections were stored in a 0.1% sodium azide solution until immunohistochemistry was performed. All sections were stained at the same time to minimize any differences in background staining due to differences in antibody preparation and binding across days.

The ionized calcium-binding adaptor molecule (Iba)-1 protein was used because it is specific to microglia, and its expression is constitutive throughout the cell body and processes (Ito et al., 1998). Free-floating sections were washed with PBS, incubated in 50% methanol for 30 min to quench blood vessels, and then washed in PBS again. They were then incubated for 1 h in PBS with 0.03% H<sub>2</sub>O<sub>2</sub>, 5% normal goat serum, and 0.3% Triton X in order to quench endogenous peroxidase, block, and permeabilize, respectively. Sections were incubated with primary antibody (rabbit anti-Iba1, 1:10,000; Wako Chemicals, Richmond, VA, USA) overnight at room temperature on an orbital shaker. On day 2, sections were washed and incubated with a biotinylated secondary antibody (goat anti-rabbit IgG, 1:1,000; Vector Laboratories, West Grove, PA, USA) for 2

h at room temperature. Sections were washed, and immunostaining was identified by the streptavidin/HRP technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as the chromagen. Sections were mounted on gelatinized slides, dehydrated, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

### **2.2.8 Iba1 Quantification**

Quantification of Iba-1 positive cells was performed as described previously (Bilbo & Tsang, 2010) by trained observers blind to experimental treatment conditions. Briefly, digitized images of tissue sections (10x) were analyzed using US National Institutes of Health Image J software (<http://rsbweb.nih.gov/ij/>). Sections were imaged using a Nikon Eclipse 80i microscope and digital camera (Nikon, Tokyo, Japan) with PictureFrame software (version 2.3, Optronics, Goleta, CA, USA). Signal pixels of a region of interest were defined as having a gray value of 3 SD above the mean gray value of a cell-poor area of the stained tissue section close to the region of interest. The number of pixels and the average gray values above the set background were then computed and multiplied together to give an integrated density measurement. All values across each region were averaged to obtain a single integrated density value per region for each animal. A mouse brain atlas (Franklin & Paxinos, 1997) was used to identify the anatomical location of each of the 5 regions of interest in both brain hemispheres for Iba1 quantification: hypothalamus (HYP, 12 sections, bregma +0.62 mm

to -2.30 mm), amygdala (AMY, 12 sections, bregma -0.58 mm to -2.30 mm), dentate gyrus of hippocampus (DG, 8 sections, bregma -1.34 mm to -2.30 mm), CA1 region of hippocampus (CA1, 8 sections, bregma -1.34 mm to -2.30 mm), and CA3 region of hippocampus (CA3, 8 sections, bregma -1.34 mm to -2.30 mm).

### **2.2.9 Data Analysis and Statistics**

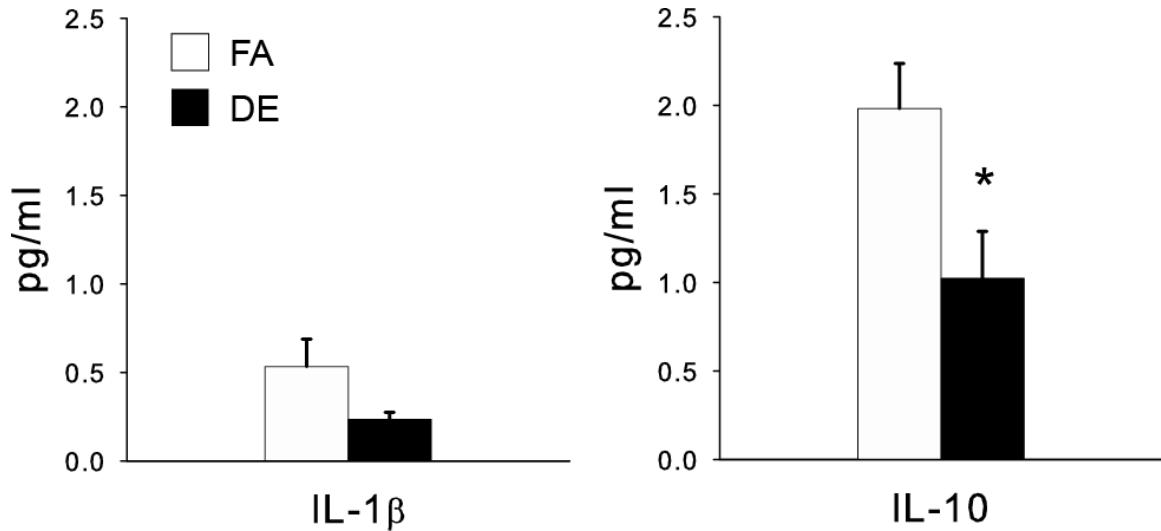
All data were analyzed with SPSS statistical software (IBM, Armonk, NY, USA). All body weight data were log-transformed for use in statistical analysis because of heterogeneous variance (especially due to inherent biological sex differences in the absolute values of body weight), but figures represent the actual values. Fetal cytokine responses and birth weights were assessed using independent *t*-tests to compare prenatal treatment groups. Baseline measures in adulthood (body weight, activity, anxiety) were assessed using 2-way (sex x prenatal treatment) ANOVAs, and significant interactions were followed up with *post hoc* comparisons (Tukey's HSD) to further distinguish among groups. Three-way (sex x prenatal treatment x adult diet) repeated-measures (time on diet) ANOVAs were used to assess changes in weight, food consumption, activity, and anxiety levels over the course of the diet. Significant interactions with sex justified subdivision of the data by sex for further analysis with 2-way (prenatal treatment x adult diet) repeated measures (time on diet) ANOVAs, and significant interactions with time justified *post hoc* tests (Tukey's HSD) at individual time points. For repeated-measures ANOVAs, a Greenhouse-Geisser correction for sphericity

was applied to all reported *P*-values. Endpoint measures (metabolic and endocrine measures, microglial activation) were assessed using 3-way (sex x prenatal treatment x adult diet) ANOVAs. Significant interactions with sex justified subdivision of the data by sex for further analysis with 2-way (prenatal treatment x adult diet) ANOVAs, followed up with *post hoc* tests (Tukey's HSD) to further distinguish among groups. For complex factorial ANOVAs, only the highest-order significant interactions are reported because they qualify all of the lower-order effects. All differences were considered statistically significant if  $P < 0.05$ .

## **2.3 Results**

### **2.3.1 Prenatal DE exposure induces a fetal brain cytokine response**

We assessed the cytokine response in fetal brain at E18 to prenatal DE exposure, and found a significant decrease in the anti-inflammatory cytokine IL-10 in DE compared to FA [ $t(21)=2.58$ ,  $P < 0.02$ ; Figure 3, *right*]. However, no significant differences were found for the pro-inflammatory cytokine IL-1 $\beta$ , as fewer than 40% of the samples were detectable by this measure (Figure 3, *left*).

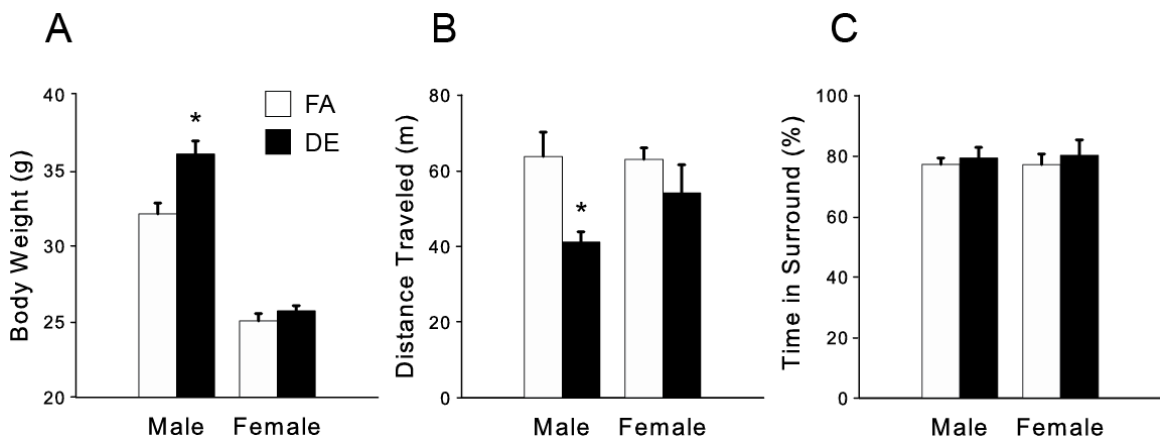


**Figure 3: Prenatal DE exposure causes a significant reduction in the levels of the anti-inflammatory cytokine IL-10 in the fetal brain at E18. No significant changes in the proinflammatory cytokine IL-1 $\beta$  were detected. Data are expressed as means  $\pm$ SE; n=10-13/group. \* $P$ <0.02 vs. FA controls.**

### **2.3.2 DE male, but not female, offspring weigh more and are less active at baseline**

First, we assessed the influence of prenatal DE exposure on birth weight (P0) of offspring, and found no differences between FA and DE litters (AVG: FA= 1.35  $\pm$  0.02 g, DE= 1.31  $\pm$  0.02 g). Next, we analyzed the influence of prenatal DE exposure on body weights of adult offspring (4-5 months old) before the initiation of the diet manipulation and found a significant sex  $\times$  prenatal treatment interaction [ $F(1,43)=4.28$ ,  $P$ <0.05]. *Post hoc* tests revealed that DE male offspring weighed more than FA offspring ( $P$ <0.003), but DE and FA female offspring did not differ in baseline body weight (Figure 4A). In addition, we analyzed the influence of maternal DE exposure on baseline activity levels in an open field prior to the initiation of the diet manipulation and found a significant

main effect of prenatal treatment [ $F(1,43)=10.05$ ,  $P<0.004$ ]. However, this main effect is driven by a significant difference between DE and FA males ( $P<0.02$ ), whereas there is no significant difference between DE and FA females ( $P>0.5$ ). Therefore, in agreement with their heavier weight, DE males are also less active at baseline than FA males (Figure 4B). Similarly, we assessed baseline anxiety levels in an open field before the initiation of the diet, but found no significant differences in baseline anxiety levels due to maternal DE exposure or its interaction with sex (Figure 4C).

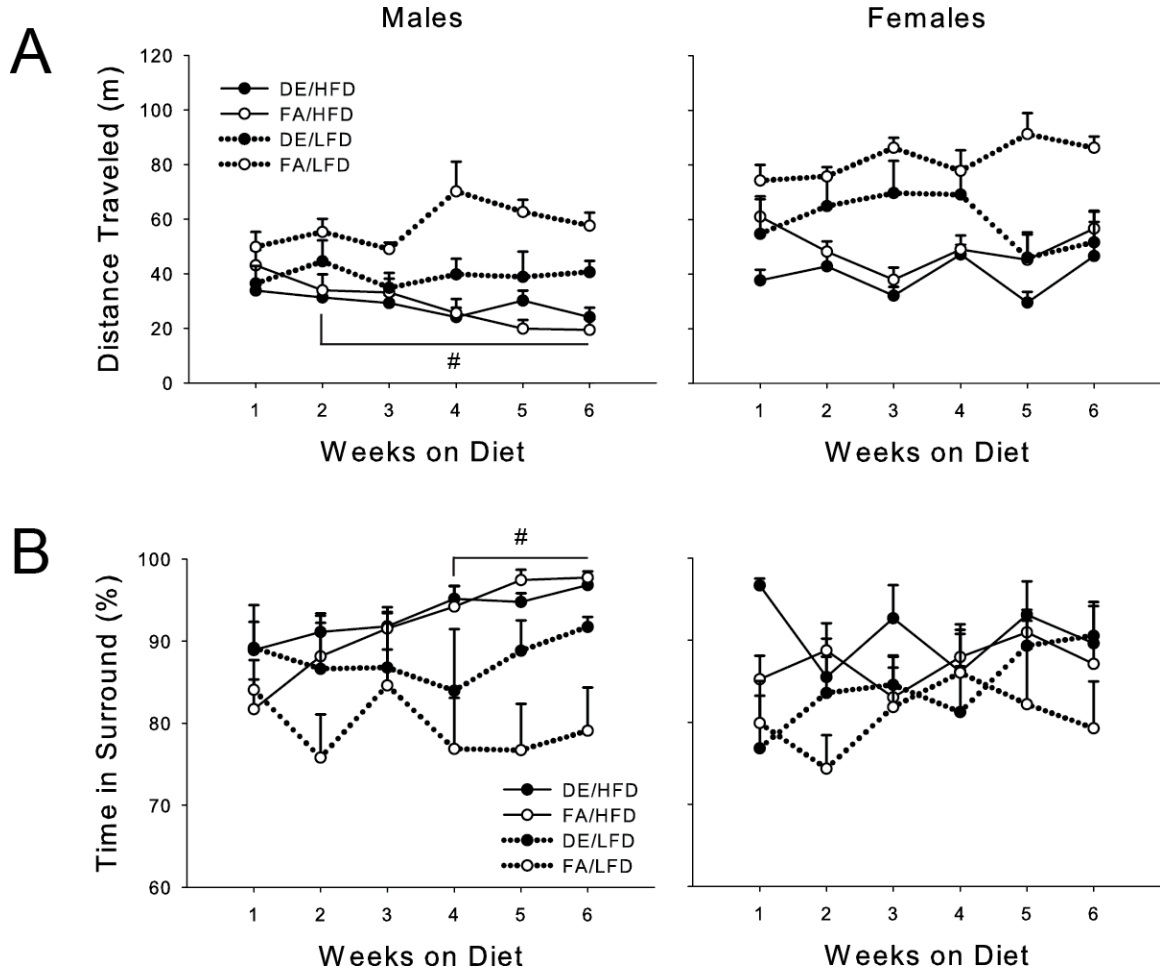


**Figure 4:** A) Baseline adult body weight is increased in DE male, but not female offspring. B) Baseline activity levels are decreased in DE male, but not female, offspring. C) DE offspring do not differ from FA offspring in their baseline anxiety levels. Data are mean  $\pm$  SEM, N=10-14/group. \* $P<0.05$  vs. FA males.

### 2.3.3 DE female offspring gain more weight than FA female offspring on HFD, whereas DE and FA male offspring gain similar amounts of weight on HFD

We assessed weight gain of DE or FA adult offspring over 6 weeks of LFD or HFD, and found a significant sex  $\times$  prenatal treatment  $\times$  adult diet  $\times$  time interaction [ $F(12,468)=2.63$ ,  $P<0.04$ ]. *Post hoc* tests revealed that male offspring, regardless of prenatal

treatment, gained more weight on HFD than LFD [adult diet x time interaction,  $F(12,228)=30.24, P<0.001$ ], as HFD groups diverged from LFD diet groups as early as day 6 of the diet ( $P<0.01$ ) and maintained this elevation in body weight over the rest of the 6 weeks of diet (Figure 5A, *left*). Of note, the pre-existing difference in weight between DE and FA males (DE > FA) was maintained across the 6-week diet [between-subjects main effect of prenatal treatment,  $F(1,19)=10.54, P<0.005$ ]. In contrast, only DE female offspring on HFD gained more weight than LFD groups [prenatal treatment x adult diet x time interaction,  $F(12,240)=2.83, P<0.04$ ], as DE females on HFD diverged from all other groups by day 15 of the diet ( $P<0.03$ ) and maintained this elevation in body weight over the rest of the 6 weeks of diet (Figure 5A, *right*). Total weight change (final - initial weight) by the end of the diet was also assessed in males and females separately. In agreement with the above analysis, we found that HFD males gained more weight than LFD males overall [main effect of adult diet,  $F(1,19)=142.29, P<0.001$ ] (Figure 5B, *left*), and that DE females on HFD gained significantly more weight than all other groups [significant prenatal treatment x adult diet interaction,  $F(1,20)=15.07, P<0.002$ ; *post hoc*,  $P<0.001$ ] (Figure 3B, *right*).



**Figure 5: A) DE and FA male offspring gain weight on HFD at the same rate (left), whereas DE female offspring gain weight at a faster rate than FA female offspring on HFD (left). B) DE and FA male offspring overall gained similar amounts of weight on HFD (left), whereas DE female offspring gained more weight than all other groups (right). Data are mean  $\pm$  SEM, N=4-8/group. # $P$ <0.05, HFD vs. LFD; \* $P$ <0.05 vs. all other groups; line indicates at what time point the groups diverge.**

### **2.3.4 DE male and female offspring consume more kilocalories than FA offspring on HFD**

To investigate energy intake, we assessed food consumption of DE vs. FA offspring over the 6 weeks of diet. There was a significant prenatal treatment x adult diet x time interaction [ $F(12,468)=2.46$ ,  $P$ <0.05], which justified post hoc tests at individual

time points. However, there was no apparent effect of sex on energy consumption, so males and females were kept together for further analysis. We found that DE offspring on HFD consumed more kilocalories than FA offspring on HFD and both LFD groups ( $P < 0.05$ ) for most of the diet (Figure 6A), whereas consumption in DE and FA offspring on LFD was similar. In addition, FA offspring on HFD consumed more than both LFD groups ( $P < 0.05$ ) on several days of the diet, but did not differ for most of the diet. By the end of the 6 weeks of diet (day 36), DE offspring on HFD had decreased their food intake to near LFD levels. We also examined total kilocalories consumed by each group over the course of the 6-week diet (summing across days) and found a significant prenatal treatment x adult diet interaction [ $F(1,43)=19.80, P < 0.001$ ]. In agreement with the above analysis, DE offspring on HFD consumed more total kilocalories than all other groups ( $P < 0.001$ ), but FA offspring on HFD still consumed more total kilocalories than both LFD groups ( $P < 0.001$ ) (Figure 6B).

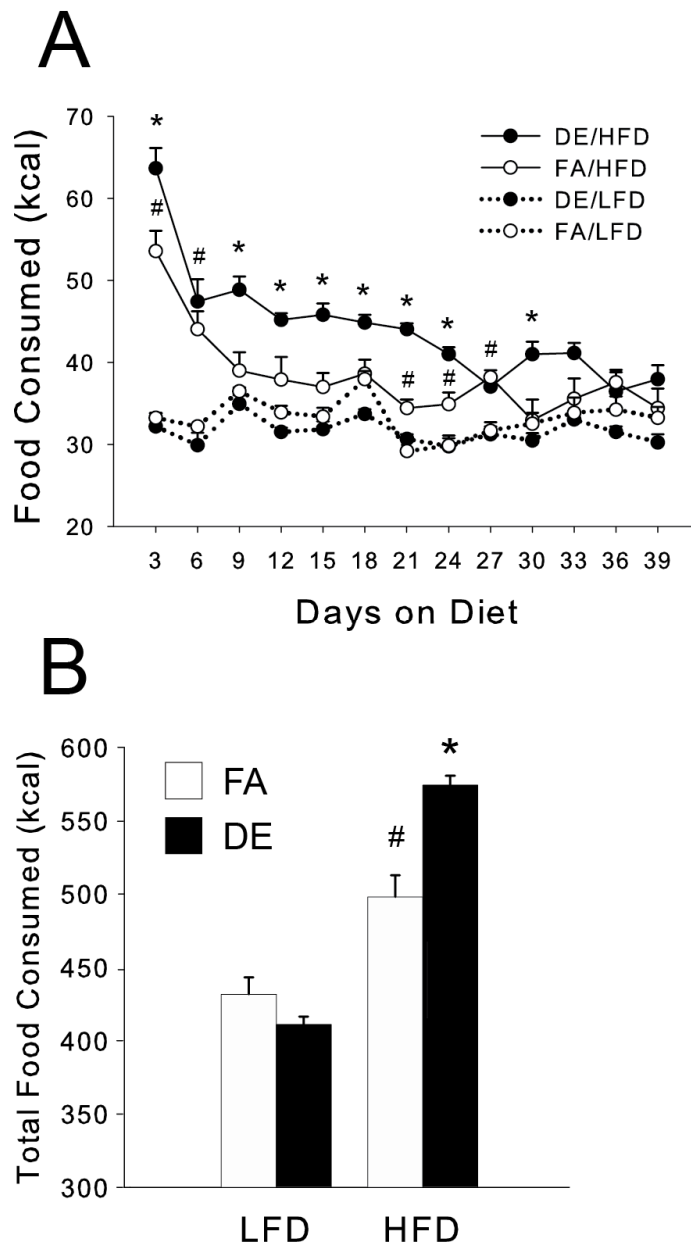


Figure 6: A) DE male and female offspring consume more kilocalories than FA offspring on HFD, although consumption decreases overall across the diet. Note that male and female consumption data are combined here. B) DE offspring consumed overall more total kilocalories over the 6 weeks of diet. Data are mean  $\pm$  SEM, N=11-13/group. \* $P$ <0.05 vs. all other groups; # $P$ <0.05, FA/HFD vs. LFD groups.

### **2.3.5 Male, but not female, offspring become less active on HFD, independent of prenatal exposure**

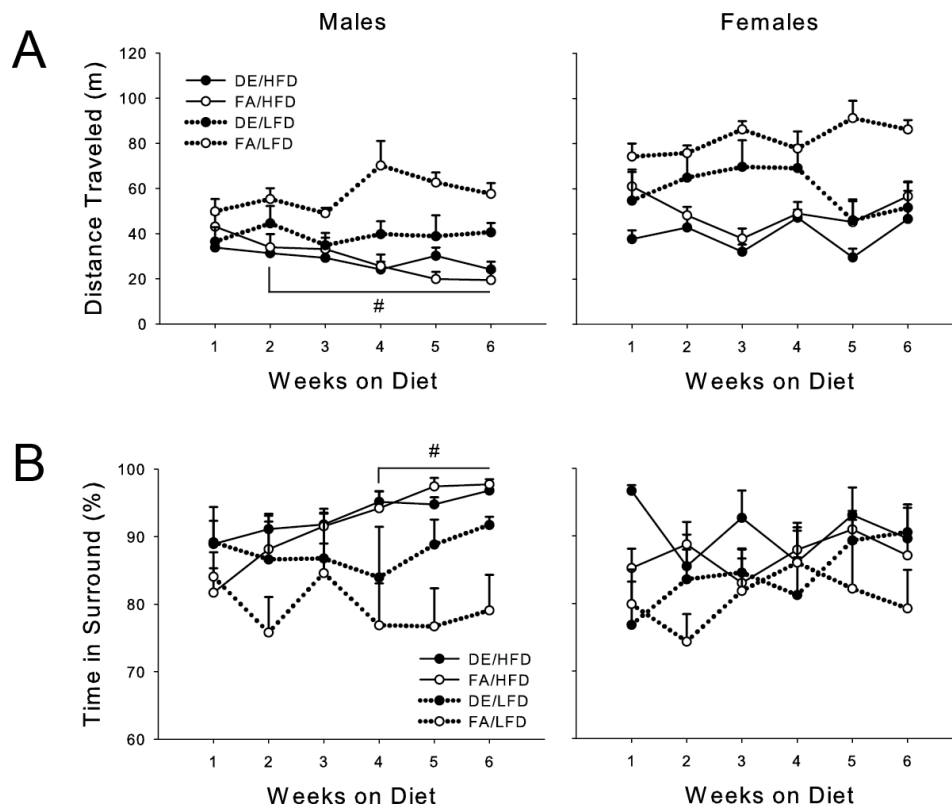
To investigate energy output, we assessed changes in activity levels (using total distance traveled in the open field) over 6 weeks of HFD or LFD and found a significant sex x adult diet x time interaction [ $F(5, 195)=3.06, P<0.03$ ]. *Post hoc* tests revealed that male offspring, regardless of prenatal treatment, became less active on HFD compared to LFD [adult diet x time interaction,  $F(5,95)=6.04, P<0.002$ ], as HFD groups diverged from LFD groups by week 2 of the diet ( $P<0.003$ ) and maintained this decrease in activity over the rest of the 6 week-diet (Figure 7A, *left*). Of note, the pre-existing difference in activity levels (DE<FA) in males persisted during weeks 1 and 3 of the diet ( $P<0.05$ ), but disappeared by week 4 when the effect of adult diet became more prominent. In contrast, we found that activity levels of female offspring did not significantly change with time on the diet in any of the treatment groups (Figure 7A, *right*). We also analyzed the time animals spent mobile during the open field test over 6 weeks of diet as a secondary measure of activity levels, and found the same pattern of results: Male offspring, regardless of prenatal treatment, became less mobile on the HFD [adult diet x time interaction,  $F(5,95)=4.81, P<0.005$ ], whereas female offspring showed no significant changes in time spent mobile over the course of the diet (data not shown). Although not as comprehensive a measure of locomotor activity as home-cage activity tracking, the open field measure of activity used here provided a “snapshot” in time that was sufficient to characterize a decrease in activity over multiple weeks of high-fat diet

consumption in the male offspring. Furthermore, open field measures of locomotion have been found to correlate significantly with home-cage spontaneous activity (Tang & Sanford, 2005).

### **2.3.5 Male, but not female, offspring become more anxious on HFD, independent of prenatal exposure**

Next, we assessed changes in anxiety levels (using percent time spent in the “Surround zone” of the open field) over 6 weeks of HFD or LFD, and found a significant sex x adult diet x time interaction [ $F(5,195)=4.51, P<0.004$ ]. *Post hoc* tests revealed that male offspring, regardless of prenatal treatment, became more anxious on HFD compared to LFD [adult diet x time interaction,  $F(5,95)=5.30, P<0.003$ ], as HFD groups diverged from LFD groups by week 4 of the diet ( $P<0.006$ ) and maintained this increase in anxiety over the rest of the 6-week diet (Figure 7B, *left*). In contrast, we found that anxiety levels of female offspring did not significantly change with time on the diet in any of the female groups (Figure 7B, *right*). To ensure that the increase in time spent in the surround zone was not merely due to a decrease in activity in the males, we also analyzed percent distance traveled in the Surround zone. We found exactly the same pattern of results with this second measure of anxiety: Male offspring, regardless of prenatal treatment, preferred to travel more in the Surround zone on the HFD compared to the LFD [adult diet x time interaction,  $F(5,95)=6.78, P<0.001$ ], whereas there were no significant changes in the percentage of distance traveled in the Surround zone in females (data not shown). Repeated weekly exposures to the open field may lead to

habituation to the testing environment, which could affect the interpretation of anxiety-like behaviors. However, habituation would be expected to lead to decreased anxiety-like behavior (i.e., greater exploration of the center zone), which is the opposite of the pattern of results observed here. Therefore, we feel confident that the anxiety test we used was sufficient to reveal an increase in anxiety with high-fat diet in the male offspring.



**Figure 7:** A) DE male (*left*), but not female (*right*), offspring are less active in the open field on HFD. Activity is represented here as total meters traveled each week of the diet. B) DE male (*left*), but not female (*right*), offspring display more anxiety-like behavior on HFD, as they spend more time in the surround of the open field. Anxiety is represented here as percent time spent in the surround zone of the open field during the 30 min. test for each week of the diet. Data are mean  $\pm$  SEM, N=4-8/group. <sup>#</sup> $P < 0.05$ , HFD vs. LFD; line indicates at what time point the groups diverge.

### **2.3.6 Both male and female offspring on HFD have elevated CORT levels, independent of prenatal exposure**

We analyzed the influence of diet on the basal serum CORT levels of DE and FA offspring at the end of the 6-week diet. Both male (Figure 8A, *left*) and female (Figure 8A, *right*) offspring that were on HFD for 6 weeks had higher CORT than offspring on LFD, regardless of prenatal treatment [main effect of diet,  $F(1, 39)=19.85$ ,  $P<0.001$ ].

### **2.3.7 DE female offspring on HFD have higher leptin levels than FA female offspring on HFD, whereas both DE and FA male offspring on HFD have elevated leptin levels, consistent with increased body weights**

We analyzed the influence of diet on the non-fasting serum leptin levels of DE and FA offspring at the end of the 6-week diet and found a significant sex x prenatal treatment x adult diet interaction [ $F(1, 39)=6.14$ ,  $P<0.02$ ]. *Post hoc*s revealed that males on HFD had overall higher leptin levels than males on LFD, regardless of prenatal treatment, in agreement with their increased body weight at the end of the diet [main effect of adult diet,  $F(1,19)=69.36$ ,  $P<0.001$ ] (Figure 8B, *left*). In addition, we found that DE females on HFD had higher leptin levels than FA females on HFD and both LFD groups, which, like males, is also in agreement with their higher body weight at the end of the 6-week diet [trend for an interaction of prenatal treatment x adult diet [ $F(1,20)= 3.82$ ,  $P<0.07$ ; *post hoc*,  $P<0.03$ ] (Figure 8B, *right*). As leptin was assayed in non-fasting animals, feeding behavior may have influenced the results. However, previous studies have shown a high correlation between fasting and non-fasting leptin measures (Hancox &

Landhuis, 2011), suggesting that non-fasting leptin is sufficient for the analyses required here.

### **2.3.8 DE male, but not female, offspring on HFD have elevated insulin levels**

We analyzed the influence of diet on the non-fasting serum insulin levels of DE and FA offspring at the end of the 6-week diet and found a significant sex x prenatal treatment x adult diet interaction [ $F(1,39)=4.73, P<0.04$ ]. *Post hoc*s revealed that DE males on HFD had significantly higher insulin levels than FA males on HFD and both LFD groups [prenatal treatment x adult diet interaction,  $F(1,19)=5.66, P<0.03$ ; *post hoc*,  $P<0.05$ ] (Figure 8C, *left*). In contrast, there were no significant differences in insulin levels in females due to DE exposure or HFD (Figure 8C, *right*). Again, feeding behavior may have had some influence on the observed differences in non-fasting insulin levels, but previous observations of moderate correlations between fasting and non-fasting insulin levels (Hancox & Landhuis, 2011) suggest that non-fasting insulin levels are sufficient for this initial analysis.

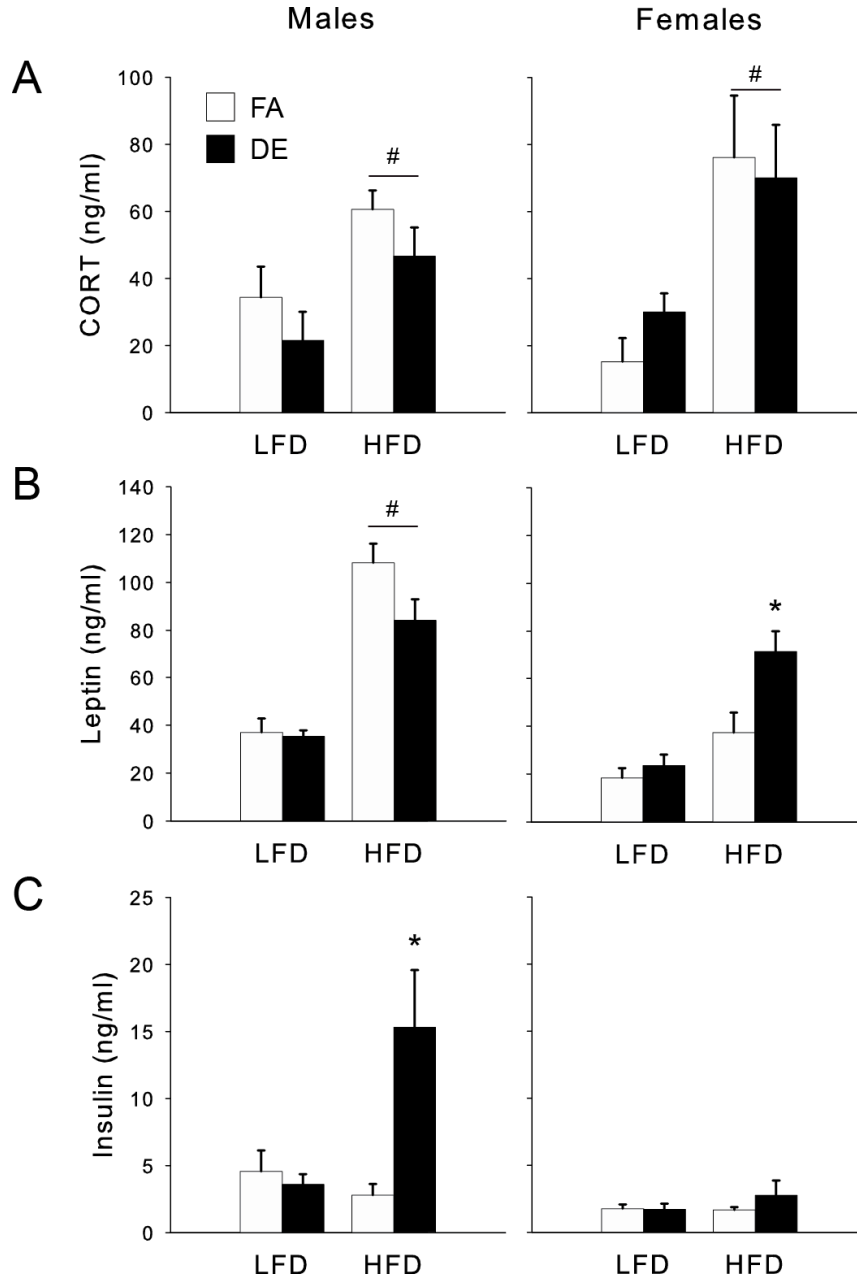
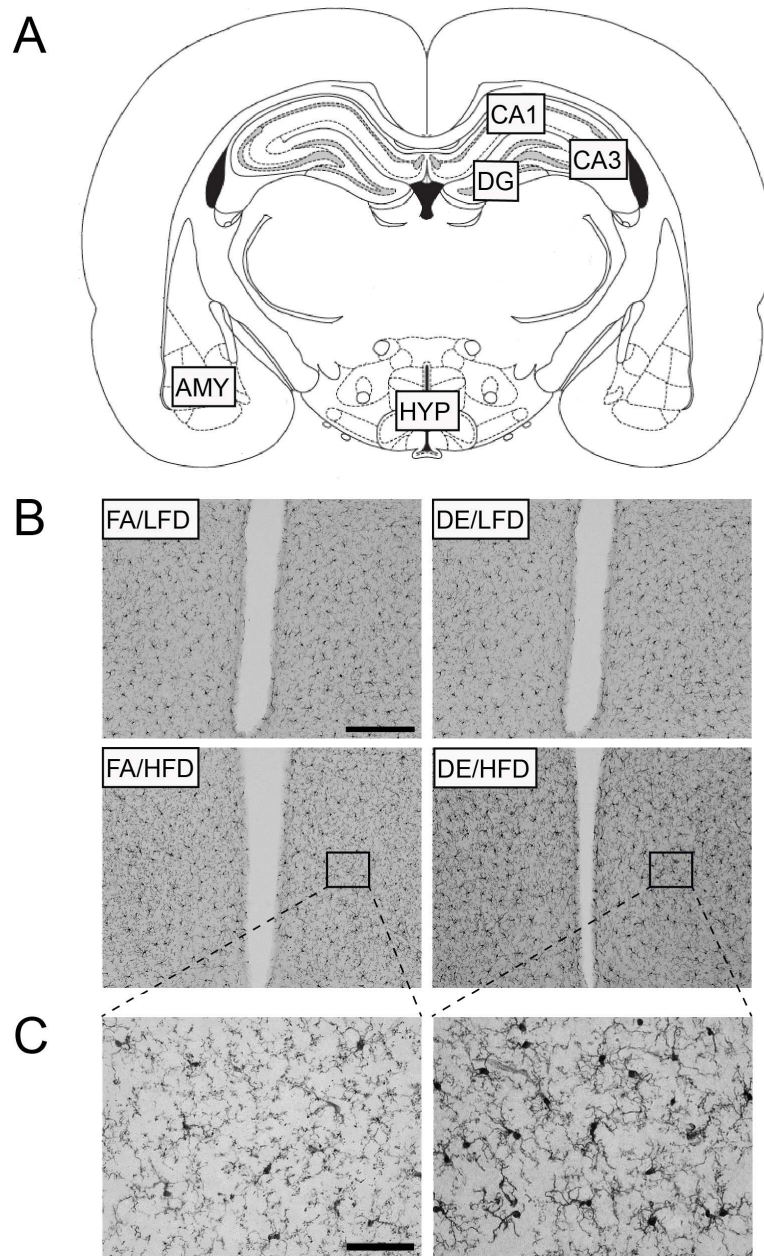


Figure 8: A) Both DE and FA male (*left*) and female (*right*) offspring have elevated basal CORT levels after 6 weeks of HFD. B) Both DE and FA male offspring on HFD have elevated leptin levels after 6 weeks of HFD (*left*), whereas DE female offspring on HFD have higher leptin levels than FA female offspring on HFD (*right*). C) DE male (*left*), but not female (*right*), offspring have elevated insulin levels after 6 weeks of HFD. Data are mean  $\pm$  SEM, N=4-8/group. Data are mean  $\pm$  SEM, N=4-8/group. # $P$ <0.05, HFD vs. LFD; \* $P$ <0.05 vs. all other groups.

### **2.3.9 Microglial activation is increased in DE offspring on HFD in a sex- and brain region-specific manner**

We analyzed the influence of diet on microglial cell surface antigen (Iba1) expression as a marker of microglial activation in the hypothalamus (HYP) of DE and FA offspring at the end of the 6-week diet and found a significant prenatal treatment x adult diet interaction [ $F(1,39)=20.22, P<0.001$ ] and a significant sex x adult diet interaction [ $F(1,39)=4.17, P<0.05$ ]. *Post hoc*s revealed that DE males on HFD had significantly higher levels of Iba1 expression than FA males on HFD and both LFD groups, which did not differ from each other [prenatal treatment x adult diet interaction,  $F(1,19)=18.88, P<0.001$ ; *post hoc*,  $P<0.006$ ] (Figures 9 and 10A, *left*). Similarly, DE females on HFD had significantly higher levels of Iba1 expression than all other groups [prenatal treatment x adult diet interaction [ $F(1,20)=4.50, P<0.05$ ; *post hoc*,  $P<0.006$ ], (Figures 9 and 10A, *right*).



**Figure 9: Microglia appear more activated in DE offspring on HFD. A) Regions of interest for Iba1 density analysis [adapted from (George Paxinos, 2005)]. B) Representative Iba1 staining (10x magnification) in the hypothalamus of mice from each treatment group. C) Representative Iba1 staining (40x magnification) in the boxed regions in (B) of FA/HFD vs. DE/HFD mice. Scale bars = 100  $\mu\text{m}$  (B); 25  $\mu\text{m}$  (C).**

We analyzed the influence of diet on Iba1 expression in the amygdala (AMY) of DE and FA offspring and found a trend for a sex x prenatal treatment x adult diet interaction [ $F(1,39)=2.53, P=0.1$ ] that guided us to subdivide the data by sex for further analysis. We found that DE males on HFD had significantly higher levels of Iba1 expression than FA males on HFD and both LFD groups, which did not differ from each other [prenatal treatment x adult diet interaction [ $F(1,19)=4.56, P<0.05$ ; *post hoc, P<0.05*] (Figure 10B, *left*). However, in females we found no significant differences in Iba1 expression (Figure 10B, *right*).

We analyzed the influence of diet on Iba1 expression in the hippocampus of DE and FA offspring and found a significant subregion x sex x adult diet interaction [ $F(2,78)=6.06, P<0.005$ ], a significant subregion x sex x prenatal treatment interaction [ $F(2,78)=4.39, P<0.02$ ], and a significant subregion x prenatal treatment x adult diet interaction [ $F(2,78)=19.78, P<0.001$ ], which justified subdividing the data by subregion for further analysis.

Within dentate gyrus (DG), there was a significant sex x adult diet interaction [ $F(1, 39)=6.00, P<0.02$ ], a significant sex x prenatal treatment interaction [ $F(1,39)=5.04, P<0.02$ ], and a significant prenatal treatment x adult diet interaction [ $F(1,39)=13.81, P<0.002$ ]. *Post hoc*s revealed that DE males on HFD had greater Iba1 expression than FA males on HFD and DE males on LFD [prenatal treatment x adult diet interaction,  $F(1,19)=11.13, P<0.004$ ; *post hoc, P<0.05*] (Figure 10C, *left*). Similarly, DE females on HFD

had greater Iba1 expression than FA females on HFD and both LFD groups [prenatal treatment x adult diet interaction,  $F(1,20)=4.79$ ,  $P<0.05$ ; *post hoc*,  $P<0.01$ ] (Figure 10C, *right*).

Within CA1, there was a significant sex x prenatal treatment x adult diet interaction [ $F(1,39)=11.70$ ,  $P<0.002$ ]. *Post hoc*s revealed that DE males on HFD had greater Iba1 expression than FA males on HFD and DE males on LFD [prenatal treatment x adult diet interaction,  $F(1,19)=12.62$ ,  $P<0.003$ ; *post hoc*,  $P<0.05$ ] (Figure 10D, *left*). In contrast, we found that females on HFD had overall greater Iba1 expression than LFD females, regardless of prenatal treatment [main effect of adult diet,  $F(1,20)=4.92$ ,  $P<0.04$ ] (Figure 10D, *right*).

Within CA3, there were no significant effects or interactions (Figure 10E, *left* and *right*).

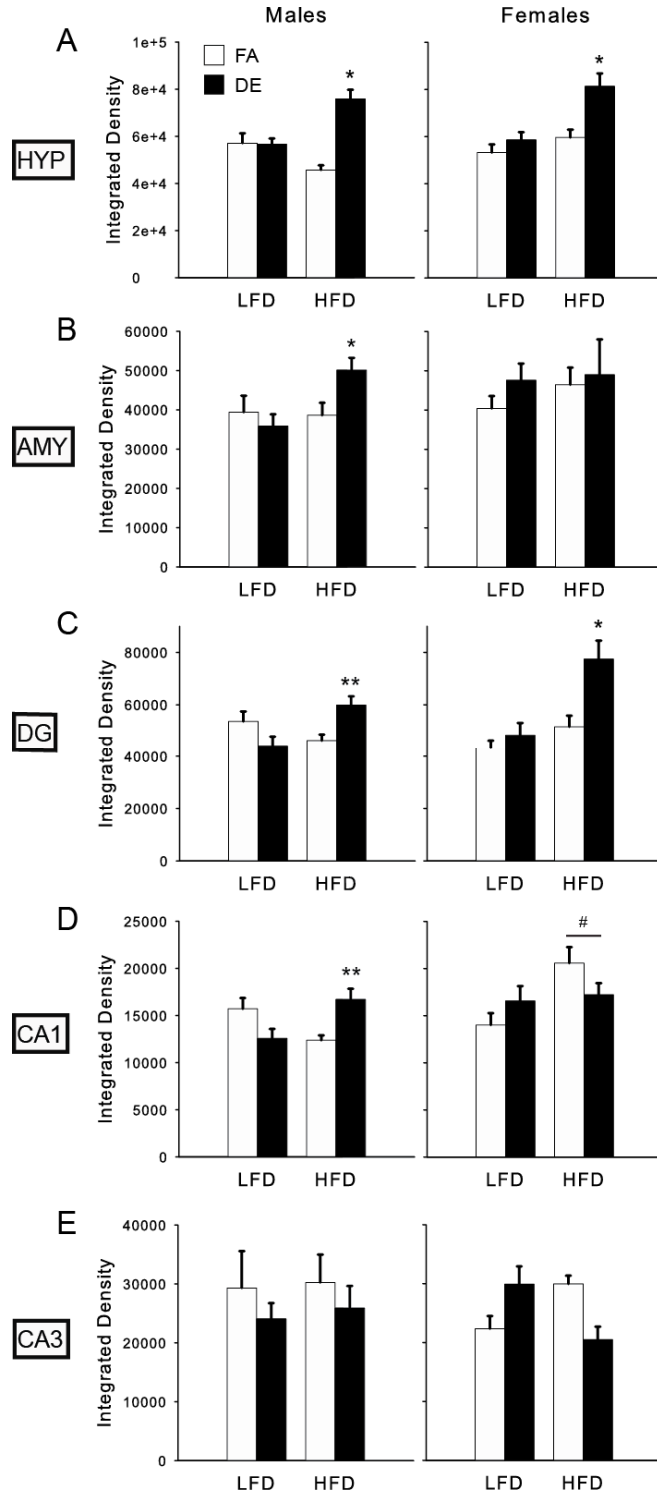


Figure 10: Denser Iba1 staining, a constitutive microglial marker, is observed in DE offspring on HFD in a sex- and brain region-specific manner. A, C) Both male (left)

and female (*right*) DE offspring have denser Iba1 staining in the hypothalamus (A) and dentate gyrus of the hippocampus (C) after 6 weeks of HFD. B) Male DE offspring have denser Iba1 staining in the amygdala after 6 weeks of HFD (*left*), whereas female DE offspring do not (*right*). D) Male DE offspring have denser Iba1 staining in area CA1 of the hippocampus after 6 weeks of HFD (*left*), whereas female DE and FA offspring both have denser Iba1 staining in CA1 after 6 weeks of HFD (*right*). E) There are no significant differences between FA and DE offspring in Iba1 staining in area CA3 of the hippocampus after 6 weeks of HFD. Data are mean  $\pm$  SEM, N=4-8/group. \* $P$ <0.05 vs. all other groups; \*\* $P$ <0.05 vs. DE/LFD and FA/HFD; # $P$ <0.05, HFD vs. LFD.

## **2.4 Discussion**

Emerging evidence suggests that environmental chemical exposures may contribute to the escalating prevalence of obesity by altering several aspects of metabolism. We have demonstrated that environmentally relevant concentrations of diesel exposure *in utero* markedly impacts offspring body weight, metabolic hormones, activity, food intake, and microglial activation in ways that are adult diet- and sex-dependent. The field of “metabolic programming” has increasingly shown that the intrauterine environment has important metabolic consequences for offspring throughout the lifespan. However, the vast majority of these studies have manipulated maternal diet in isolation. For example, maternal high-fat diet during gestation induces greater susceptibility to diet-induced obesity and exacerbates metabolic problems (hyperleptinemia, hyperinsulinemia, etc.) in adult offspring (Tamashiro, Terrillion, Hyun, Koenig, & Moran, 2009). Notably, maternal high-fat diet also alters basal microglial morphology within the offspring brain and increases their reactivity to an adult inflammatory challenge (Bilbo & Tsang, 2010), indicative of long-term changes in

microglial function that may underlie changes in neural function and behavior. Our data demonstrate for the first time that the interaction between inflammation and obesity is likely bidirectional, as prenatal DE exposure results in a neuroinflammatory environment within the fetal brain, and increased vulnerability to the HFD later in life across a number of measures. Notably, prenatal cytokine expression has also been strongly linked to long-term programming of neural function and behavior, for instance in the context of maternal viral infection (Bilbo & Schwarz, 2009; Deverman & Patterson, 2009).

Correlational studies in humans have demonstrated that maternal exposure to tobacco smoke (Pryor et al., 2011) or pesticides (Wohlfahrt-Veje et al., 2011) is associated with elevated BMI trajectory in offspring, but the effects of diesel exposure have been less well studied. Recently, Rundle *et al.* (Rundle et al., 2012) demonstrated that maternal exposure to ambient air polycyclic aromatic hydrocarbons (PAHs), a component of diesel exhaust, is associated with childhood obesity, as well as lower IQ at age 5 (Perera et al., 2009). Air pollution during pregnancy has been associated with fetal growth restriction (Pereira, Nassar, Cook, & Bower, 2011; van den Hooven et al., 2012), which in turn is associated with later insulin resistance in offspring [reviewed in (Ornoy, 2011)]. However, in the current study, we did not find a difference in birth weight due to prenatal diesel exposure [in agreement with (Sharkhuu et al., 2010)], which suggests that our findings are not simply a result of low birth weight followed by compensatory

growth (i.e., thrifty phenotype), a phenomenon that has been well described in the metabolic programming literature [reviewed in (Godfrey & Barker, 2001)]. Therefore, when taken together, it is becoming clear that exposure to environmental endocrine disruptors *in utero*, including air pollution, plays a role in fetal metabolic programming, although the mechanisms are still being explored.

Microglia are the primary producers of cytokines within the brain and likely mediators of risk or resilience to inflammatory challenges, including air pollution and HFD. These cells colonize the fetal brain during the latter half of gestation, during which time they play an active role in several developmental processes, including the phagocytosis of apoptotic cells and synaptic pruning (Paolicelli et al., 2011; Wakselman et al., 2008). Because microglia and their cytokine products are critical for normal brain development, and because microglia are long-lived and capable of changing their function long-term in response to activation (known as sensitization or “priming”), we have hypothesized that early development is a sensitive period for impacting their function and thus long-term brain and behavioral function (Bilbo & Schwarz, 2009). Previous studies have demonstrated that DE exposure in adult rats increases microglial activation and cytokine production within the CNS (Levesque et al., 2011a). Notably, the current study is the first to demonstrate that DE exposure *in utero* can cause enduring changes in the microglia of adult offspring. Other experiments have illustrated that DE impacts macrophage function in mice specifically via TLR4 (Hofer et al., 2004; Inoue et

al., 2006), which is compelling given that microglia share a myeloid lineage with macrophages. Moreover, fatty acids bind directly to TLR4 on microglia (Milanski et al., 2009), resulting in their activation. Thus, DE and HFD may converge to impact microglia within the brain via a common innate immune pathway, although this remains to be tested. Overall, we observed increased microglial activation only in the brains of animals that received two “hits” (prenatal DE and adult HFD), with the exception of area CA1 in females that was increased by HFD alone. This may signify that microglia were primed during gestation by the neuroinflammation caused by prenatal DE exposure, and only displayed morphological changes (perhaps indicative of increased reactivity) following HFD in adulthood. An important caveat is that morphology alone cannot fully describe or predict changes in microglial function. Moreover, whether changes in microglial function underlie changes in metabolism or behavior remains to be explored.

One of the most interesting findings of this study was the sex difference we observed in almost every measured outcome. These data are consistent with increasing recognition within the field of child health that there are sex differences in multiple outcomes following maternal inflammatory insults during pregnancy. For example, male newborns are 20% more likely to experience a poorer outcome following complications during pregnancy, such as preeclampsia, preterm delivery, and intrauterine growth restriction (Vatten & Skjærven, 2004), and males also have a higher

risk for poor perinatal outcome when their mothers are diabetic (García-Patterson et al., 2011). Furthermore, Alonso-Magdalena *et al.* (Alonso-Magdalena et al., 2010) recently demonstrated that Bisphenol A (BPA) exposure during pregnancy disrupts glucose homeostasis specifically in adult male, but not female, offspring. Our data are consistent with a male disadvantage, because overall we saw that female offspring developed fewer adverse consequences than did male offspring, both in response to prenatal DE alone as well as following HFD in adulthood. Specifically, DE female offspring failed to develop insulin resistance, in contrast to male offspring, despite gaining comparable weight on the HFD to DE males. Insulin resistance is considered to be more serious than increased body weight alone, as it can lead to the cluster of medical conditions that make up metabolic syndrome, including type 2 diabetes, hypertension, dyslipidemia, and cardiovascular disease (Haffner et al., 1992). In addition, DE female offspring did not show the marked behavioral changes (increases in anxiety and decreases in activity) that male offspring exhibited on HFD, nor did they exhibit as global an increase in microglial activation as males. Previous work from our laboratory (Schwarz et al., 2012) has demonstrated that male rats have overall more microglia than females early in neural development [and we have replicated this finding in mice as well (unpublished data)], which may contribute to the greater vulnerability of males to glial priming by inflammatory challenges early in life.

Furthermore, there may be an inherent sex difference in the response to a high-fat diet. Although DE female offspring gained as much weight on HFD as their male counterparts, FA female offspring gained far less weight on HFD than did matched males, even though they had comparable energy intake. This suggests that females exhibit some protection from the extent of the adverse consequences of high-fat diet observed in males. Similarly, Hwang *et al.* (Hwang *et al.*, 2009) found that female mice put on a high-fat diet at weaning failed to develop any obvious weight gain compared to control females until they had been on the diet for 3 months. Even after 9-12 months of high-fat diet, females still had far lower weight gains and showed less hyperinsulinemia and hypercholesterolemia than males. Interestingly, they also failed to show the deficits in learning and hippocampal synaptic plasticity that male mice on a high-fat diet exhibited, which is comparable to the lack of change in activity or anxiety levels observed in females in the current study. In addition, estrogen has been shown to promote the accumulation of subcutaneous fat deposits, instead of central (intra-abdominal) fat deposits, which are more common in males and have been linked with increased risk for metabolic syndrome and cardiovascular disease (Kannel *et al.*, 1991; Krotkiewski, Björntorp, Sjöström, & Smith, 1983). Considering the relative lack of responsiveness of normal female mice to a high-fat diet, it is remarkable that we observed such marked weight gains in the DE female offspring in just 6 weeks of HFD

(much shorter than many other diet studies), which suggests that the inherent protection females typically have was abolished by prenatal diesel exposure.

Alternatively, it is possible that females are simply slower to respond to HFD than males, as normal females typically gain weight more gradually than normal males on HFD (Gold, 1970; Hwang et al., 2009). Therefore, prenatal DE exposure may accelerate body weight gain compared to normal females, and with a longer diet, DE females may have shown more serious metabolic and behavioral consequences comparable to DE males. Interestingly, female offspring on HFD did exhibit increased CORT (similar to males) at the end of the 6-week diet, which may precede changes in anxiety-like behavior. High-fat diets have been previously shown to cause increased basal glucocorticoid production (Tannenbaum et al., 1997), prompting the authors to suggest that high fat intake can serve as a type of chronic stressor. High-fat diets also cause increases in anxiety-like behavior (Souza et al., 2007), but studies in this area commonly use only male rats or mice, so the female behavioral response to a high-fat diet is less well-characterized.

In summary, prenatal diesel exhaust exposure during pregnancy causes fetal brain cytokine responses, followed by an increased susceptibility to diet-induced weight gain, and metabolic and behavioral changes in adult offspring. Microglia primed by air pollutant-induced neuroinflammation during gestation may undergo exaggerated activation in response to a high-fat diet in adulthood, which may in turn mediate the

changes in body weight regulation and metabolic function. We also demonstrated that fetal programming due to air pollution exposure *in utero* is sexually dimorphic, with more global detrimental effects in male offspring, both basally and in response to a high-fat diet. Further research is needed to determine the mechanism by which these marked sex differences in outcome are caused in response to the same prenatal and adult challenges. These findings may ultimately have implications for environmental policy regulations, as well as for social and clinical interventions.

### **3. Prenatal Air Pollution Exposure Induces Sexually Dimorphic Fetal Programming of Metabolism, Peripheral Immune Responses, and Neuroinflammation in Adult Offspring<sup>1</sup>**

#### **3.1 Introduction**

Our previous study (see Chapter 2) demonstrated that maternal exposure to diesel exhaust in mice exacerbates weight gain in offspring following exposure to a high-fat diet in adulthood (Bolton et al., 2012). The mechanisms by which this long-term programming of metabolic set points occurs remains unclear, but a large body of evidence suggests it critically involves both central and peripheral inflammation, which can lead to endocrine disruption, changes in adipose tissue development, and long-term neuroendocrine and neuroimmune dysregulation (Dahlgren, Samuelsson, Jansson, & Holmång, 2006; Das, 2007; Heindel & vom Saal, 2009). Because we had observed evidence of neuroinflammation at E18 and increases in microglial antigen density in adult DE/HFD animals suggestive of microglial priming, we next examined whether there were any corresponding changes in microglial activation markers and function in adult these “two-hit” animals. In order to address the contribution of peripheral inflammatory signals to this neuroinflammation, we also aimed to characterize the peripheral inflammatory response and the population of peripheral macrophages in the

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<sup>1</sup> This chapter was originally published as: Bolton, J. L., Auten, R. L., Bilbo, S. D. (2014). Prenatal air pollution exposure induces sexually dimorphic fetal programming of metabolic and neuroinflammatory outcomes in adult offspring. *Brain, Behavior, and Immunity*, 37, 30-44.

brain itself—a population that is known to increase in size due to peripheral inflammation and blood-brain barrier breakdown (D'Mello, Le, & Swain, 2009; Stamatovic et al., 2005).

In the current study, we tested the hypothesis that exposure to air pollution during fetal development would prime microglia long-term, such that later exposure to a high-fat diet would result in exacerbated adverse outcomes, specifically for body weight regulation as we have observed previously, as well as for affective behavior. Obesity is associated with higher rates of affective disorders (G. E. Simon et al., 2006), and prenatal exposure to air pollution, beyond its association with childhood obesity, is also associated with cognitive difficulties and mood dysregulation in early childhood (Lovasi et al., 2013; Perera et al., 2012), further highlighting the inextricable link between physical and mental health. We also sought to determine if sex differences in the degree of adverse outcomes following prenatal DEP exposure would continue from what we have observed in previous studies, which indicated that males were more vulnerable than females (Bolton et al., 2012). To this end, we exposed time-mated mouse dams to intermittent respiratory instillations of either vehicle (VEH) or diesel exhaust particles (DEP) throughout gestation. In the first experiment, adult male and female offspring were fed either a low-fat diet (LFD) or high-fat diet (HFD) for 9 weeks and assessed for behavioral, metabolic, and neuroimmune changes. In the second experiment, postnatal day (P)30 male and female offspring were injected with saline (SAL) or

lipopolysaccharide (LPS) and assessed for both central and peripheral interleukin (IL)-1 $\beta$  response in order to assess the inflammatory output of microglia vs. peripheral immune cells and their potential priming by prenatal DEP exposure, prior to adult diet manipulation.

## **3.2 Methods**

### **3.2.1 Animals**

Adult male and female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC, USA) and housed in individually ventilated, microisolator polypropylene cages with specialized bedding (AlphaDri; Shepherd Specialty Papers, Milford, NJ, USA; used to avoid exposure to potentially confounding antigens that can be found in typical bedding) and *ad libitum* food (PicoLab Mouse Diet 5058; Lab-Diet, Philadelphia, PA, USA) and filtered water. The colony was maintained at 22°C on a 12:12-hr light-dark cycle (lights off at 9 AM). Following acclimation to laboratory conditions for 1 week, males were paired with 2 females each for timed mating, for a maximum of 2 weeks. Females were examined twice daily for evidence of a vaginal plug [confirmation of successful mating, considered to be embryonic day (E)0], at which point they were separated from the male and housed with another pregnant female. Females were bred in two separate cohorts (n=12 dams for cohort 1, n=8 dams for cohort 2), and each cohort was used for a separate experiment: 1) prenatal VEH or DEP exposure followed by LFD or HFD exposure for 9 weeks in adulthood, and 2) prenatal VEH or

DEP exposure followed by SAL or LPS injections on P30 and sacrifice 2 h later. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

### **3.2.2 DEP Exposures**

Beginning on E2, time-mated females were lightly anesthetized with 2% isoflurane for ~1 min and treated with DEP *via* oropharyngeal aspiration (the method and detailed analysis of DEP have been previously described) (Auten et al., 2012; Bolton et al., 2013). Females received 50 µg DEP suspended in 50 µl vehicle (DEP group; n=5 dams from cohort 1, n=3 dams from cohort 2) or vehicle alone (VEH group; n=7 dams from cohort 1, n=5 dams from cohort 2) every 3 days E2-E17 for a total of 6 doses, as a model of intermittent exposure. This dose and route of delivery induces maternal lung inflammation (e.g. leukocyte infiltration) comparable to levels observed following intermittent maternal inhalation of diesel exhaust at environmentally relevant concentrations (Auten et al., 2012). Moreover, both routes of delivery result in similar levels of particle deposition within the lung (Foster, Walters, Longphre, Macri, & Miller, 2001). Importantly, the oropharyngeal aspiration technique used in this study yielded a similar weight gain phenotype to that of our previous study, which utilized chamber inhalation of diesel exhaust at the Environmental Protection Agency (Bolton et al., 2012). This replication suggests that inflammation occurring in utero rather than route of exposure is the critical variable for the offspring outcomes we are assessing.

### **3.2.3 Pup Weights**

All animals included in this study were delivered spontaneously on gestational days 18-21 (with the day of birth defined as P0). Dams were weighed at E17 prior to the final DEP exposure, and again following the weaning of their pups at P28. Pups were weighed and sexed on P1 (n=13-21/group/sex, 3-5 litters/group represented from cohort 2), P9, P19, and P28 (n=27-42/group/sex, 8-12 litters/group represented from both cohorts). Offspring were weaned at 4 weeks of age into clean cages of 2-5 same-sex siblings, and provided *ad libitum* access to standard chow and filtered water.

### **3.2.4 Maternal Care Observations**

We observed dams with their litters P2-P9 once daily: cohort 1 was observed during the dark cycle (5-6 PM), and cohort 2 was observed during the light cycle (8-9 AM). During each session, we scored dams (n=3-6/group for cohort 1; n=3-5/group for cohort 2) for 'on nest', nursing, and licking and grooming (LG) behavior every 5 min for 1 hr (Myers, Brunelli, Squire, Shindeldecker, & Hofer, 1989). As previously described, the frequency of occurrence for some of the behaviors (particularly LG) was rare, so we summed data for each behavior across all 8 observation sessions and calculated a percent of time spent performing each behavior for each dam. We took care not to disturb dams during observation sessions, and changed their cages only upon completion of the final observation on P9.

## 3.2.5 Experiment 1: Prenatal DEP Exposure and Adult HFD

### 3.2.5.1 Adult diets and food intake

Adult male (n=34) and female (n=40) offspring 4-5 months of age from cohort 1 were given either: (1) a low-fat diet [LFD; 20% of calories from protein, 70% from carbohydrate, and 10% from fat (44:56% lard: soybean oil); Research Diets, New Brunswick, NJ, USA, cat. no. D12450B], or (2) a choice between the LFD and a high-fat diet (50 g of each, divided into alternate sides of the food hopper) [HFD; 20% protein, 35% carbohydrate, and 45% fat (88:12% lard: soybean oil); Research Diets D12491], resulting in n=7-10 animals/group/sex. Half of the offspring of each sex from each litter were assigned to LFD, and half were assigned to the diet choice, thus allowing within-litter comparisons between diets. The LFD provided 3.85 kcal/g, and the HFD provided 4.73 kcal/g. All other nutritional parameters of the two diets were identical. The complete nutritional profiles of each diet are available online (<http://www.researchdiets.com>). Animals were weighed immediately prior to diet assignment, and housed in same-sex cages of 2-3 littermates. After beginning the diet, the animals and their food were weighed every 3 days at the same time each day (9-10 AM) for 9 weeks until sacrifice. Food consumption data was normalized for the different energy content of the diets by converting grams to kilocalories of food consumed and was divided by the number of animals in the cage. Of the animals that were given a choice between diets, all of the animals consumed the HFD almost exclusively; thus these animals will be referred to simply as HFD.

### **3.2.5.2 Open field activity testing**

Adult offspring were tested for baseline activity in the open field the day before diet assignment, as well as weekly thereafter at the same time each week for 8 weeks of diet (not on the week of sacrifice). Animals were tested in groups of four, each within its own open field, with only one sex in the testing room at one time. Tests lasted 30 min during the animals' dark cycle between 10 AM-5 PM. The open field apparatus consisted of four custom-made black Plexiglas boxes (each 40 cm × 45.1 cm × 34.9 cm) elevated on a gray platform approximately 53 cm off the ground, with a camera attached to the ceiling overhead, positioned to record four mice in the four distinct enclosures of the apparatus at once. ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA) was used to track the movement of each mouse, and distance traveled (m) by each animal during the test was used as a measure of activity level. The apparatus was cleaned between animals with QTB disinfectant (Caltech Industries, Inc., Midland, MI, USA) followed by water. The estrous cycle of the female mice was monitored following each behavioral test; however, the females' stage of estrous cycle did not significantly affect their behavior in any of the tests (data not shown).

### **3.2.5.3 Elevated zero-maze anxiety testing**

Adult offspring were tested for baseline anxiety levels in the elevated zero-maze 1-2 weeks before diet assignment, and subsequently following 4 weeks of diet. We adapted a method that measures time spent in the closed vs. open arms of a circular

maze and is widely used as a test for anxiety-like behavior in rodents (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). The maze has an elevated (49.5 cm high) circular lane (4.5 cm wide) divided into four quadrants. Two opposite quadrants are enclosed by walls (15.9 cm high), whereas the remaining two quadrants are left exposed. Each mouse was placed onto the center of an open arm and was scored for the total time they spent in the closed arms out of 5 min and the total number of head dips (an exploratory behavior) they performed over the side of the open arm. An increase in time spent in the closed arms and a reduced number of head dips are indicative of increased anxiety (Shepherd et al., 1994). The maze was cleaned between animals with 70% ethanol followed by water.

#### **3.2.5.4 Insulin sensitivity testing**

Following 5 weeks of diet, adult offspring were assessed for blood glucose reductions after insulin challenge as a measure of insulin sensitivity. In order to assess the basal metabolic state, animals were fasted for 6 h prior to receiving an i.p. injection of insulin (0.75 U/kg; Humulin R; Eli Lilly, Indianapolis, IN, USA) (Holland et al., 2011). Whole-blood glucose was assessed using a glucometer (TRUE2go; CVS Pharmacy, Inc., Woonsocket, RI, USA) at 0, 30, 60, and 120 min post-insulin injection, and the data are expressed as a percentage of the baseline levels (0 min) (McClung et al., 2004). Additionally, fasting insulin levels were measured in serum collected immediately prior

to insulin injection using a commercially available enzyme-linked-immunosorbent assay (ELISA) kit (Crystal Chem, Inc., Downers Grove, IL, USA).

### **3.2.5.5 Tissue collection**

On week 9 of the diet, mice were deeply anesthetized with a ketamine/xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine i.p.) and transcardially perfused with ice-cold saline for 2 min to clear brains of blood. Approximately 100 mg sections of epididymal fat pads were collected from each animal just prior to perfusion and snap-frozen for later quantitative real-time PCR (qRT-PCR) analysis. Brains were rapidly extracted and dissected into hypothalamus (HYP) and hippocampus (HIP) on ice. These regions were selected for their known roles in metabolism, as well as mood regulation (Davidson, Kanoski, Schier, Clegg, & Benoit, 2007; Kishi & Elmquist, 2005). Tissue collections were spread across week 9 of the diet so that the HYP and HIP (n=3/group/sex/brain region) could be processed and stained for flow cytometry analysis of cellular composition (maximum of 8 samples/day to minimize time for tissue processing prior to fixation, for a total of 6 days, with one sample from each group and sex per day). In addition, the isolated HIPs from 4-6 animals/group/sex were snap-frozen and stored at -80°C for later qRT-PCR analysis. Animals included in each group for each of the different applications were selected in order to best represent the average weight of that group, and in order to include only 1-2 animals/sex/litter in each group. All tissue collection occurred during the dark cycle between 9 AM-11 AM.

### 3.2.5.6 qRT-PCR

Total RNA was isolated from frozen HIPP or fat samples using TRIzol (Chomczynski & Sacchi, 1987), and cDNA was synthesized from 100 ng of RNA using the QuantiTect reverse transcription kit (Qiagen, Inc., Valencia, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using a QuantiFast SYBR Green PCR kit (Qiagen) on a Mastercycler ep *realplex* (Eppendorf, Hauppauge, NY, USA). For each reaction, 1  $\mu$ l of cDNA was added to 12  $\mu$ l of master mix containing specific primers for the gene of interest. Primers for CD11b and CX3CR1 were purchased from Qiagen, and we designed primer pairs for OAZ-1, TLR4, GFAP, and CCR2 (see Table 1) as previously described (Williamson et al., 2011). Designed primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Optimal annealing temperatures for each primer pair were determined by running a temperature gradient, and specificity was verified by melt-curve analysis. We selected OAZ-1 as a novel housekeeping gene because we have found it to be more stably expressed than traditional housekeeping genes like GAPDH in response to our treatments (de Jonge et al., 2007). Threshold cycle ( $C_T$ ) was determined for each reaction, and relative gene expression was calculated using the  $2^{-\Delta\Delta C_T}$  method (Livak & Schmittgen, 2001; Pfaffl, 2001; Williamson et al., 2011).

**Table 1: Forward and reverse primer sequences for qRT-PCR**

| <b>Gene</b>  | <b>Forward Primer (5'-3')</b> | <b>Reverse Primer (5'-3')</b> |
|--------------|-------------------------------|-------------------------------|
| <i>OAZ-1</i> | gctgtagtaacctgggtccg          | gtcacattcagccgctcgtc          |
| <i>TLR4</i>  | cagcagaggagaaagcat            | caccaggaataaagtctctg          |
| <i>GFAP</i>  | tggagagaaaggttgaatcg          | gtattgagtgcgaatctctc          |

### 3.2.5.7 Flow cytometry

Immediately following tissue collection, dissected brain regions (HYP or HIP) were brought to a single-cell suspension and demyelinated using Miltenyi's Neural Tissue Dissociation Kit (P) and anti-myelin microbeads (Miltenyi Biotec, Inc., Auburn, CA, USA) as previously described (Williamson et al., 2011). Demyelinated cells were washed once in PBS and centrifuged at  $350 \times g$  for 5 min, after which the PBS was decanted. Cells were incubated with 5  $\mu$ l anti-mouse Fc receptor block (CD16/CD32; BioLegend, San Diego, CA, USA) for 5 min at 4°C, followed by incubation for 20 min at 4°C in 100  $\mu$ l of antibody mixture, containing phycoerythrin-cyanine dye 5 (PECy5)-conjugated anti-mouse CD11b and allophycocyanin (APC)-conjugated CD45.2 (eBioscience, Inc., San Diego, CA, USA), both diluted 1:500. Cells were washed in 2 ml PBS supplemented with 0.5% BSA and 2 mM EDTA, spun down at  $350 \times g$ , and fixed in 200  $\mu$ l 1.5% paraformaldehyde before analysis using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar, Inc., Ashland, OR, USA). For each sample, 10,000 events were collected and doublets, debris, and dead cells were excluded from the analysis based on properties of cell size and granularity (forward scatter and side scatter).

## 3.2.6 Experiment 2: Prenatal DEP Exposure and P30 SAL/LPS Injections

### 3.2.6.1 Injections

P30 offspring (n=6-8/group/sex from cohort 2) received an i.p. injection of either sterile saline or 165 µg/kg LPS derived from *Escherichia coli* (serotype 0111:B4; Sigma, St. Louis, MO, USA) between 8 AM and 10 AM, and blood and brain tissues were collected 2 h later. This dose and time point were selected based on previous studies (Bilbo et al., 2005; Godbout et al., 2005) and initial pilot experiments from our lab demonstrating mild sickness behavior and a robust but submaximal IL-1 $\beta$  response under these conditions in the brains of control mice (unpublished data). Animals were anesthetized and perfused as above, and brains were rapidly extracted and dissected into hypothalamus (HYP), prefrontal cortex (PFC), hippocampus (HIP), and parietal cortex (PCX) on ice. These brain regions were selected for their known roles in the cognitive and affective behaviors that we have observed to be affected by DEP in previous studies (Bolton et al., 2013). Dissected regions were pooled prior to homogenization for each animal in order to obtain enough total protein for cytokine analysis as previously described (Bolton et al., 2013), then snap-frozen and stored at -80°C until processing. Blood was centrifuged at 16,100  $\times$  g at 4°C for 10 min, and serum was removed and stored in microcentrifuge tubes at -20°C until assayed.

### **3.2.6.2 IL-1 $\beta$ ELISA**

Brain samples were homogenized and sonicated in a 50 mM TRIS buffer (pH 7.5) containing 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol (DTT), then lipid-depleted and normalized to total protein (400  $\mu$ g/well) in calibration buffer as previously described (Bolton et al., 2013). IL-1 $\beta$  was measured from the supernatant using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). Serum was run in a 1:5 dilution with calibration buffer as previously described (Bilbo & Tsang, 2010). The detection limit for the assay was 1 pg/ml and the intra-assay and inter-assay coefficients of variation were <10%.

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### **3.2.7 Data Analysis**

All data were analyzed with SPSS statistical software (IBM, Armonk, NY, USA). For ELISA analyses, samples that had undetectable levels of IL-1 $\beta$  were assigned a value

of half the lowest detectable value in the assay (A. L. Thompson et al., 2012). Litter effects were controlled for by using pups from multiple litters per treatment group. Moreover, analyses using litter as a variable failed to find any significant interactions of litter with the variables under study. Maternal care observations were analyzed with 2-way ANOVAs (DEP  $\times$  Time of Day Observed), and maternal weights, litter size, and sex ratio were analyzed with independent *t*-tests. Two-way (Sex  $\times$  DEP) ANOVAs were used to analyze pup weights and baseline behavioral measures. Three-way (Sex  $\times$  DEP  $\times$  HFD) repeated-measures (time on diet) ANOVAs were used to assess changes in weight, food consumption, and activity levels over the course of the diet, and three-way repeated measures (time post-injection) ANOVAs were also used to assess changes in blood glucose following insulin injection during the insulin sensitivity test. For repeated-measures ANOVAs, a Huynh-Feldt correction for sphericity was applied to all reported *p*-values. Three-way univariate (Sex  $\times$  DEP  $\times$  HFD) ANOVAs were used to assess total weight gain, total food consumed, and total distance traveled over the diet, as well as peak glucose response to insulin injection, as a means to address overall between-subjects differences. Four-way (Brain Region  $\times$  Sex  $\times$  DEP  $\times$  HFD) ANOVAs were used to analyze flow cytometry data of HYP and HIPP. Three-way (Sex  $\times$  DEP  $\times$  HFD or Sex  $\times$  DEP  $\times$  LPS) ANOVAs were used to analyze all other data from offspring in Experiment 1 and 2. Significant interactions with sex justified subdivision of the data by sex for further analysis with separate 2-way (DEP  $\times$  HFD or DEP  $\times$  LPS) ANOVAs for

males and females to identify sex-specific effects, and significant 2-way interactions within each sex were subsequently followed up with post hoc comparisons (Tukey's HSD) to identify group differences. For complex factorial ANOVAs, only the highest-order significant interactions are reported because they qualify all of the lower-order effects. All differences were considered statistically significant if  $p < 0.05$ .

### **3.3 Results**

#### **3.3.1 Maternal care and pup weights do not differ between prenatal treatment groups**

There were no significant differences between the VEH and DEP groups in any of the maternal behaviors we observed (Table 2); however, we did observe significantly greater time spent on the nest overall during the light cycle vs. the dark cycle [main effect of Time of Day,  $F(1,13)=7.47$ ,  $p < 0.05$ ], consistent with previous studies (Champagne, Francis, Mar, & Meaney, 2003). Importantly, there were no significant differences in litter size or sex ratio due to prenatal treatment (Table 2), in agreement with our previous work (Bolton et al., 2013), nor were there significant differences in maternal weights during (E17) or after pregnancy (at weaning; Table 2). Furthermore, birth weights of VEH and DEP pups did not significantly differ (Table 2), although male pups were significantly heavier than female pups [main effect of Sex,  $F(1,57)=5.48$ ,  $p < 0.05$ ], consistent with previous studies (Williams et al., 1982). P9 and P19 pup weights did not exhibit any significant differences (Table 2), and weaning weights on P28 exhibited only a significant sex difference [main effect of Sex,  $F(1,129)=65.40$ ,  $p < 0.001$ ].

**Table 2: Maternal care, litter characteristics, maternal weights, and pup weights by prenatal treatment. All values are mean  $\pm$  SEM.**

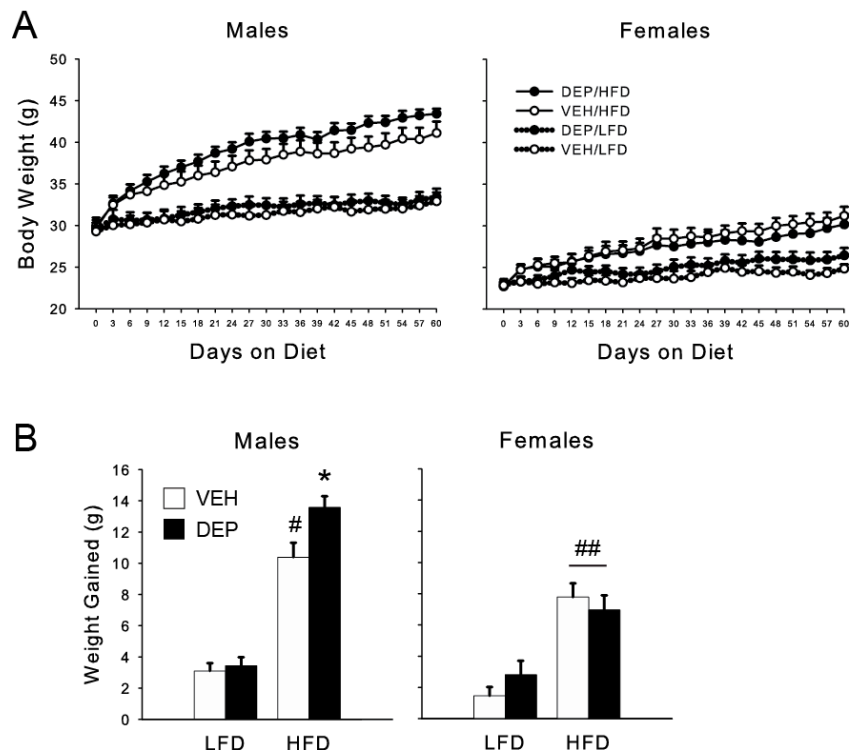
| <b>Measure</b>                | <b>VEH</b>        | <b>DEP</b>         |
|-------------------------------|-------------------|--------------------|
| <b>Maternal Care</b>          |                   |                    |
| % Time on Nest, Light         | 84.58 $\pm$ 3.67% | 89.24 $\pm$ 1.39%  |
| % Time on Nest, Dark          | 60.24 $\pm$ 9.08% | 67.71 $\pm$ 11.46% |
| % Time Nursing, Light         | 58.96 $\pm$ 3.59% | 54.86 $\pm$ 0.92%  |
| % Time Nursing, Dark          | 44.44 $\pm$ 6.73% | 52.57 $\pm$ 10.49% |
| % Time LG, Light              | 10.83 $\pm$ 1.21% | 15.97 $\pm$ 2.11%  |
| % Time LG, Dark               | 12.33 $\pm$ 1.87% | 10.76 $\pm$ 3.31%  |
| <b>Litter Characteristics</b> |                   |                    |
| Litter Size                   | 7.08 $\pm$ 0.54   | 7.63 $\pm$ 0.63    |
| Sex Ratio (% Males)           | 51.40 $\pm$ 4.18% | 42.88 $\pm$ 8.16%  |
| <b>Maternal Weights</b>       |                   |                    |
| E17 Weight (g)                | 36.02 $\pm$ 0.84  | 36.04 $\pm$ 1.05   |
| P28 Weight (g)                | 28.83 $\pm$ 0.65  | 28.06 $\pm$ 0.79   |
| <b>Pup Weights</b>            |                   |                    |
| P1 Male Weight (g)            | 1.42 $\pm$ 0.03   | 1.42 $\pm$ 0.03    |
| P1 Female Weight (g)          | 1.36 $\pm$ 0.03   | 1.33 $\pm$ 0.03    |
| P9 Male Weight (g)            | 5.14 $\pm$ 0.16   | 5.29 $\pm$ 0.12    |
| P9 Female Weight (g)          | 5.25 $\pm$ 0.14   | 5.09 $\pm$ 0.12    |
| P19 Male Weight (g)           | 8.45 $\pm$ 0.27   | 9.00 $\pm$ 0.19    |
| P19 Female Weight (g)         | 8.65 $\pm$ 0.24   | 8.55 $\pm$ 0.20    |
| P28 Male Weight (g)           | 17.28 $\pm$ 0.39  | 18.31 $\pm$ 0.38   |
| P28 Female Weight (g)         | 16.05 $\pm$ 0.24  | 16.23 $\pm$ 0.17   |

### **3.3.2 Experiment 1: Prenatal DEP Exposure and Adult HFD**

#### **3.3.2.1 DEP male offspring gain more weight than VEH male offspring on HFD, whereas DEP and VEH female offspring gain similar amounts of weight on HFD**

Prior to diet assignment, there were no significant differences in adult body weight between any of the groups within males and females, although males did weigh significantly more than females, as at P28 [main effect of Sex,  $F(1,70)=193.65$ ,  $p<0.001$ ;

Figure 11A]. By Day 3 of the diet, HFD animals already weighed significantly more than LFD animals [main effect of HFD,  $F(1,66)=11.01$ ,  $p<0.005$ ]. However, adult male offspring of DEP dams gained significantly more weight over the course of the HFD than offspring of VEH dams, whereas female DEP offspring did not differentially gain weight on HFD [Time on Diet  $\times$  Sex  $\times$  DEP  $\times$  HFD interaction,  $F(4,296)=2.36$ ,  $p<0.05$ ; Figure 11A]. Similarly, we also found that DEP/HFD males had a significantly greater overall weight change (final – initial weight) than all other groups by the end of the diet, whereas HFD females gained more than LFD females, regardless of prenatal treatment [Sex  $\times$  DEP  $\times$  HFD interaction,  $F(1,66)=4.91$ ,  $p<0.05$ ; *post hoc* $<0.05$ ; Figure 11B].

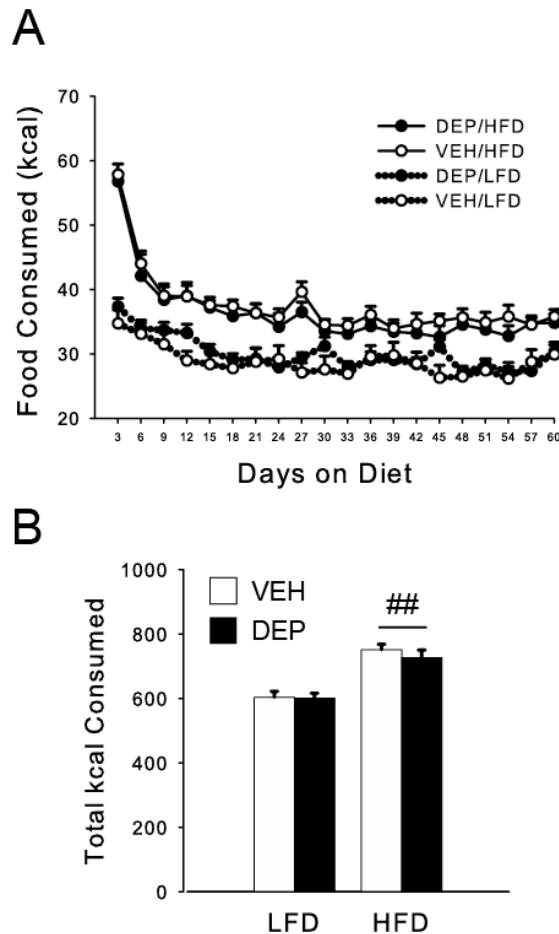


**Figure 11: (A) DEP male offspring gained weight on HFD at a faster rate than VEH male offspring (left), whereas DEP and VEH female offspring gained weight on HFD**

at the same rate (*right*). (B) DEP male offspring gained more total weight on HFD than all other groups (*left*), whereas DEP and VEH female offspring gained similar amounts of weight. Data are mean  $\pm$  SEM, n=7-10/group/sex. \* $p$ <0.05 vs. all other groups; # $p$ <0.05 vs. LFD groups; ## $p$ <0.05, HFD vs. LFD.

### 3.3.2.2 Offspring on HFD consume more kilocalories than offspring on LFD, regardless of DEP exposure and sex

Following diet assignment, animals given access to HFD consumed significantly more kilocalories than animals on LFD, although they quickly decreased their food intake over the first week to steady-state levels that persisted over the remainder of the diet [Time on Diet  $\times$  HFD interaction,  $F(16,392)=8.95$ ,  $p$ <0.001; Figure 12A]. Likewise, we also found that the total number of kilocalories consumed over the diet was higher in the HFD groups than the LFD groups, regardless of prenatal treatment and sex [main effect of HFD,  $F(1,24)=66.46$ ,  $p$ <0.001; Figure 12B].



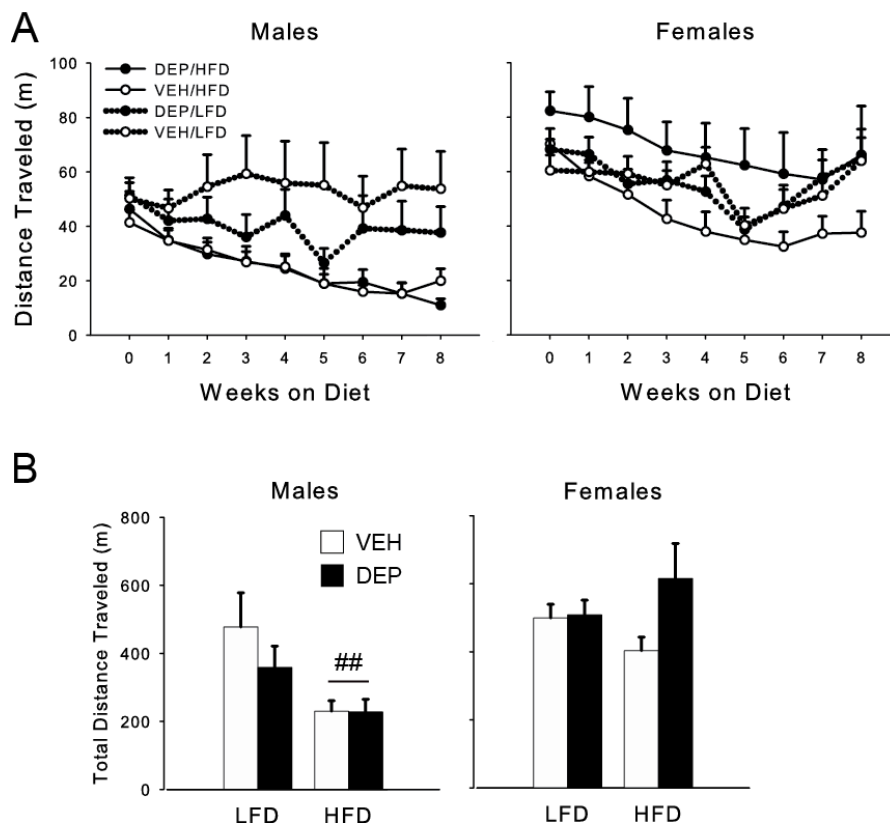
**Figure 12: (A) Offspring on HFD consumed more kilocalories than offspring on LFD, although consumption decreased overall across the diet. (B) Total kilocalories consumed over the 9 weeks of diet were overall higher in HFD groups than LFD groups, regardless of prenatal treatment. Male and female consumption data are combined here, due to a lack of sex differences. Data are mean  $\pm$  SEM,  $n=7-10/\text{group}/\text{sex}$ . ## $p<0.05$ , HFD vs. LFD.**

### 3.3.2.3 Male, but not female, offspring are less active on HFD than offspring on LFD, regardless of DEP exposure

Prior to diet assignment, there were no significant differences in activity levels in the open field between VEH and DEP groups, although females were significantly more active than males overall [main effect of Sex,  $F(1,70)=31.41$ ,  $p<0.001$ ; Figure 13A]. Over

the course of the diet, HFD animals decreased their activity levels significantly more than LFD animals, regardless of prenatal treatment [Time on Diet  $\times$  HFD interaction,  $F(5,331)=5.56, p<0.001$ ], and males tended to decrease their activity more than females [trend for Time on Diet  $\times$  Sex interaction,  $F(5,331)=1.83, p=0.1$ ; Figure 13A].

Correspondingly, the total distance traveled over the weekly open field tests by the end of the diet was significantly lower in HFD males than LFD males [main effect of HFD,  $F(1,30)=8.44, p<0.01$ ], whereas there were no significant differences among females [Sex  $\times$  HFD interaction,  $F(1,66)=4.59, p<0.05$ ; Figure 13B].

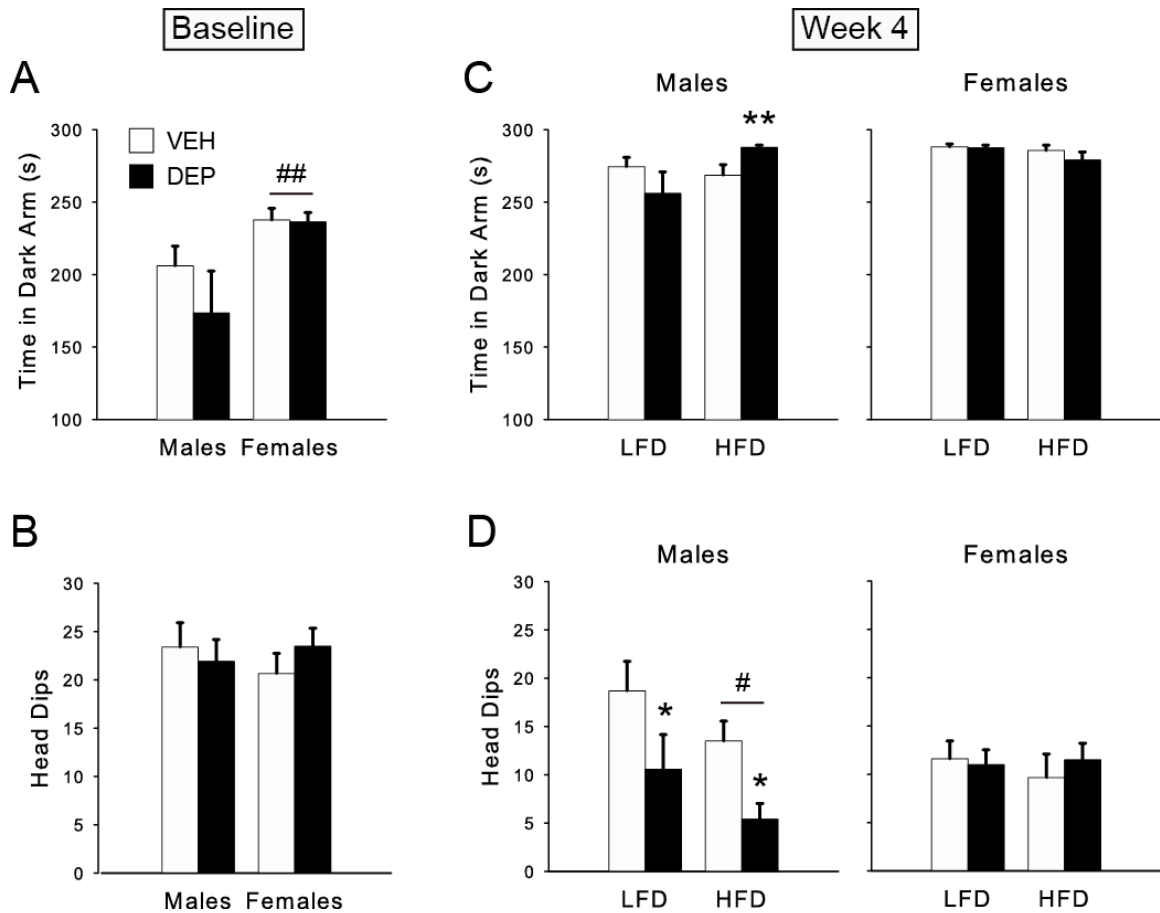


**Figure 13: (A) Offspring on HFD decreased their activity levels over the 9 weeks of diet, whereas offspring on LFD did not. (B) Overall total distance traveled during the**

**weekly open field tests was lower in HFD males than LFD males, whereas females did not differ. Data are mean  $\pm$  SEM, n=7-10/group/sex.  $^{**}p<0.05$ , HFD vs. LFD.**

#### **3.3.2.4 DEP male, but not female, offspring are more anxious on HFD**

At baseline, there were no significant differences in anxiety-like behavior in the elevated zero-maze test between VEH and DEP groups, although females spent significantly more time in the closed arms than males overall [main effect of Sex,  $F(1,54)=12.74$ ,  $p<0.005$ ; Figure 14A,B]. However, after 4 weeks on the diet, DEP/HFD males tended to spend more time in the closed arms than other groups [trend for DEP  $\times$  HFD interaction,  $F(1,24)=3.65$ ,  $p=0.07$ ], whereas females did not exhibit any significant differences [Sex  $\times$  DEP  $\times$  HFD interaction,  $F(1,59)=5.55$ ,  $p<0.05$ ; Figure 14C]. Furthermore, there was a similar pattern in the opposite direction for head dips performed over the walls of the elevated zero-maze, in that DEP/HFD males tended to be less exploratory than other groups [main effect of DEP,  $F(1,24)=8.28$ ,  $p<0.01$ ; trend for main effect of HFD,  $p=0.08$ ], whereas there were no significant differences among females [Sex  $\times$  DEP interaction,  $F(1,59)=7.16$ ,  $p<0.05$ ; Figure 14D].

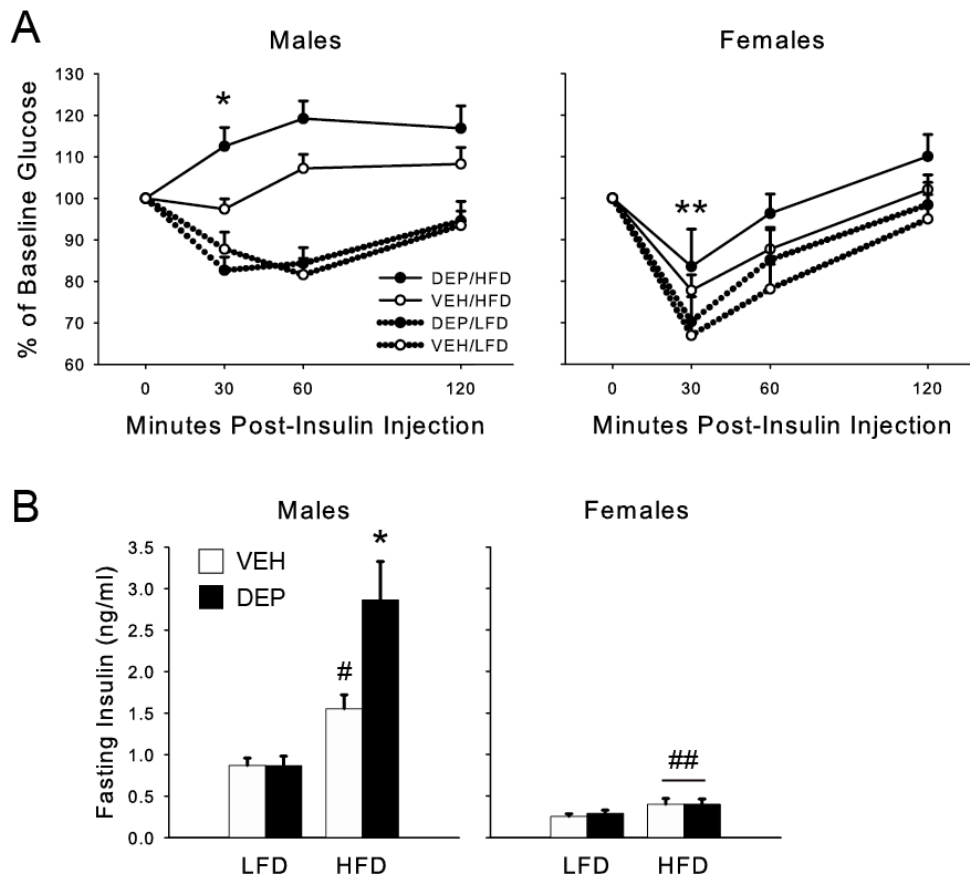


**Figure 14: (A)** Prior to initiation of the diet, there were no differences in time spent in the closed arm of the elevated zero-maze between VEH and DEP groups. However, females spent more time in the closed arm than males overall. **(B)** There were also no significant differences in head dips performed on the zero-maze prior to diet assignment. **(C)** After 4 weeks on the diet, DEP/HFD males tended to spend more time in the closed arm than other groups, whereas females did not differ. **(D)** DEP/HFD males also tended to perform fewer head dips as a result of an additive effect of DEP and HFD, whereas there were again no differences among females. Data are mean  $\pm$  SEM,  $n=7-10/\text{group}/\text{sex}$ .  $^{##}p<0.05$ , HFD vs. LFD;  $^{**}p=0.07$ , trend for DEP  $\times$  HFD interaction;  $^{*}p<0.05$ , VEH vs. DEP;  $^{#}p=0.08$ , HFD vs. LFD.

### 3.3.2.5 DEP male, but not female, offspring are more insulin resistant and hyperinsulinemic on HFD than VEH offspring

Following insulin challenge at 5 weeks on the diet, HFD animals exhibited an attenuated initial decrease in blood glucose compared to LFD animals, which was

followed by an increase back to baseline levels by 2 h post-injection [Time Post-Injection  $\times$  HFD interaction,  $F(3,174)=9.43$ ,  $p<0.001$ ; Figure 15A]. Interestingly, this effect was more pronounced in HFD males than females [trend for Time Post-Injection  $\times$  Sex  $\times$  HFD interaction;  $F(1,174)=2.07$ ,  $p=0.1$ ; significant Time Post-Injection  $\times$  Sex interaction,  $F(3,174)=9.97$ ,  $p<0.001$ ; Figure 15A]. Likewise, we found that at the time of peak glucose response (30 min. post-insulin injection), DEP/HFD males were significantly more insulin resistant than all other groups, as they exhibited an *increase* in blood glucose in response to insulin, instead of the expected *decrease* at this time point [DEP  $\times$  HFD interaction,  $F(1,24)=7.06$ ,  $p<0.05$ , *post hoc*,  $p<0.05$ ], whereas DEP/HFD females did not differ from VEH/HFD females [trend for main effect of HFD,  $F(1,35)=3.88$ ,  $p=0.06$ ]. Furthermore, analysis of fasting serum collected just prior to insulin injection revealed that DEP/HFD males had significantly higher insulin levels than all other groups [DEP  $\times$  HFD interaction,  $F(1,29)=6.81$ ,  $p<0.05$ ; *post hoc*,  $p<0.005$ ], whereas HFD females had only slightly higher insulin levels than LFD females, regardless of prenatal treatment [main effect of HFD,  $F(1,34)=6.15$ ,  $p<0.05$ ; Sex  $\times$  DEP  $\times$  HFD interaction,  $F(1,63)=7.97$ ,  $p<0.01$ ; Figure 15B].

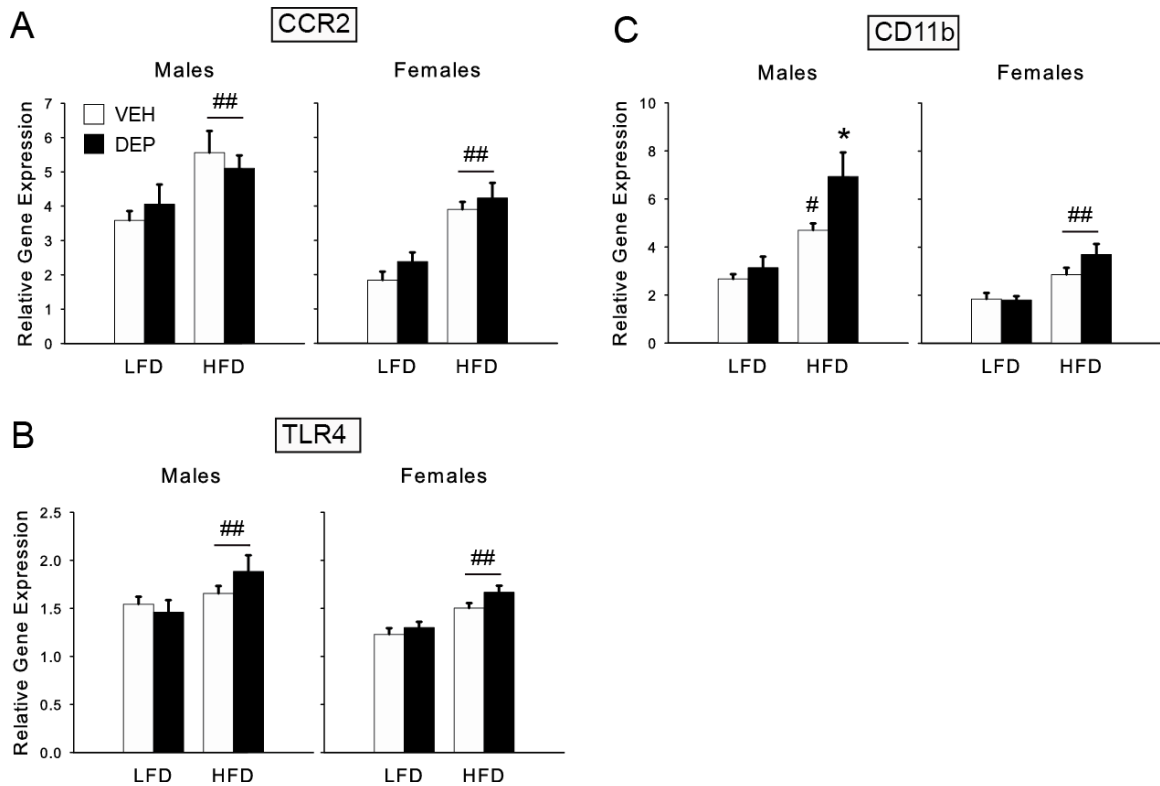


**Figure 15: (A)** Following insulin challenge after 5 weeks on the diet, DEP/HFD males were significantly more insulin resistant than all other groups, as they failed to decrease their blood glucose levels at the time of peak glucose response (30 min. post-insulin injection). On the other hand, DEP/HFD females did not differ from VEH/HFD females. **(B)** In agreement with their insulin resistance, DEP/HFD males also had significantly higher levels of fasting insulin in serum collected immediately prior to insulin injection, whereas HFD females had higher insulin levels than LFD females, regardless of prenatal treatment. Data are mean  $\pm$  SEM,  $n=7-10$ /group/sex. \* $p<0.05$  vs. all other groups; \*\* $p=0.06$ , HFD vs. LFD; # $p<0.05$  vs. LFD groups; ## $p<0.05$ , HFD vs. LFD.

### 3.3.2.6 HFD induces evidence of macrophage infiltration of the adipose tissue, but only DEP/HFD males, not females, express higher levels of a macrophage activation marker

At the end of 9 weeks of diet, CCR2 mRNA expression, a marker of infiltrating macrophages in adipose tissue that is associated with insulin resistance (Weisberg et al.,

2006), was higher in the adipose tissue of HFD groups than LFD groups, regardless of prenatal treatment [main effect of HFD,  $F(1,63)=36.18$ ,  $p<0.001$ ], and was also more highly expressed overall in males than females [main effect of Sex,  $F(1,63)=26.61$ ,  $p<0.001$ ; Figure 16A]. TLR4, an innate immune receptor critical for the response to both environmental toxins and high-fat diets (Arbour et al., 2000; Milanski et al., 2009; Shi et al., 2006), followed a similar pattern as CCR2, exhibiting higher mRNA expression in HFD animals than LFD animals [main effect of HFD,  $F(1,62)=21.43$ ,  $p<0.001$ ] and higher overall mRNA expression in males than females [main effect of sex,  $F(1,62)=11.06$ ,  $p<0.005$ ; Figure 16B]. There was also a tendency for TLR4 mRNA to exhibit a synergistic effect of DEP and HFD, but only when the sexes were combined [trend for DEP  $\times$  HFD interaction,  $F(1,62)=2.56$ ,  $p=0.1$ ]. Furthermore, CD11b mRNA, a macrophage activation marker, exhibited an additive effect of DEP and HFD in male offspring [main effect of DEP,  $F(1,30)=6.05$ ,  $p<0.05$ ; main effect of HFD,  $F(1,30)=27.96$ ,  $p<0.001$ ] and a tendency for a synergistic effect [trend for DEP  $\times$  HFD interaction,  $F(1,30)=2.54$ ,  $p=0.1$ ], as DEP/HFD males had significantly higher CD11b mRNA expression than all other groups (*post hoc*,  $p<0.05$ ; Figure 16C). However, this pattern failed to reach significance in females, as HFD females exhibited higher CD11b mRNA expression than LFD females, regardless of prenatal treatment [main effect of HFD,  $F(1,33)=23.54$ ,  $p<0.001$ ], and this difference was of a lower overall amplitude than that of males [Sex  $\times$  HFD interaction,  $F(1,63)=5.64$ ,  $p<0.05$ ; Figure 16C].

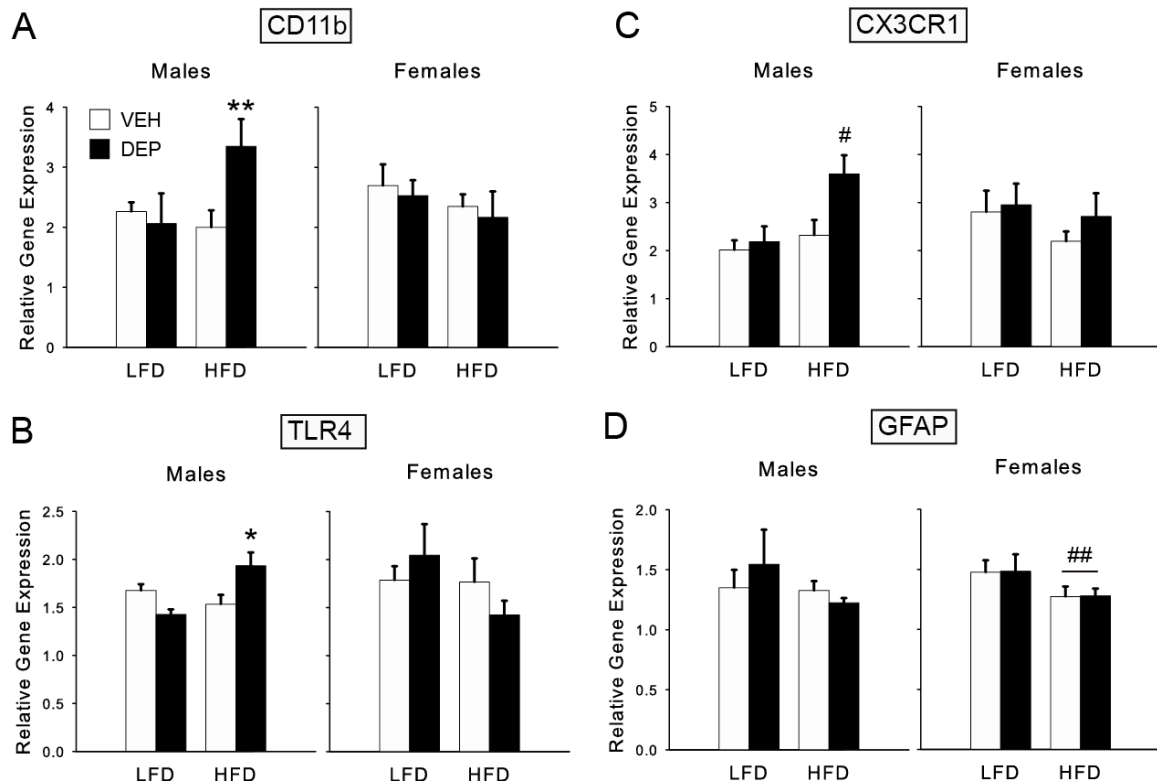


**Figure 16: (A)** At the end of the 9 weeks of diet, both male and female offspring on HFD expressed significantly higher levels of CCR2 mRNA in adipose tissue than offspring on LFD, independent of prenatal treatment. **(B)** Offspring on HFD also exhibited higher TLR4 mRNA expression in adipose tissue, regardless of prenatal treatment or sex. **(C)** DEP/HFD males exhibited significantly higher CD11b mRNA expression than all other groups, whereas HFD females had overall higher CD11b mRNA expression than LFD females, independent of prenatal treatment. Data are mean  $\pm$  SEM,  $n=7-10/\text{group}/\text{sex}$ . ## $p<0.05$ , HFD vs. LFD; # $p<0.05$  vs. LFD groups; \* $p<0.05$  vs. all other groups.

### 3.3.2.7 DEP male, but not female, offspring on HFD express elevated levels of microglial/macrophage activation markers in HIPP

At the end of 9 weeks of diet, DEP/HFD males expressed significantly higher levels of CD11b mRNA (used here as a microglial/macrophage activation marker) in the HIPP than VEH/HFD males [DEP  $\times$  HFD interaction,  $F(1,15)=4.65$ ,  $p<0.05$ ; *post hoc*,  $p<0.05$ ], whereas there were no significant differences among females [trend for Sex  $\times$

DEP × HFD interaction,  $F(1,33)=2.45$ ,  $p=0.1$ ; Figure 17A]. Likewise, DEP/HFD males expressed significantly higher levels of TLR4 mRNA in the HIPPO than both the DEP/LFD and VEH/HFD groups [DEP × HFD interaction,  $F(1,16)=9.69$ ,  $p<0.01$ ; *post hoc*,  $p<0.05$ ], whereas there were no significant differences among females [Sex × DEP × HFD interaction,  $F(1,36)=5.21$ ,  $p<0.05$ ; Figure 17B]. CX3CR1, the receptor for CX3CL1/fractalkine and a specific marker for microglia in brain tissue (Mizutani et al., 2012), followed a similar pattern, in that DEP/HFD males had significantly higher levels of CX3CR1 mRNA than VEH/HFD and VEH/LFD groups [trend for DEP × HFD interaction,  $F(1,16)=2.91$ ,  $p=0.1$ ; *post hoc*,  $p<0.05$ ] whereas females showed no significant differences [Sex × HFD interaction,  $F(1,36)=4.31$ ,  $p<0.05$ ; Figure 17C]. On the other hand, GFAP, an astrocyte activation marker, did not follow the same pattern as the microglial/macrophage markers, as there were no significant differences among males, whereas HFD decreased GFAP mRNA expression in females, regardless of prenatal treatment [main effect of HFD,  $F(1,18)=4.46$ ,  $p<0.05$ ; Figure 17D].



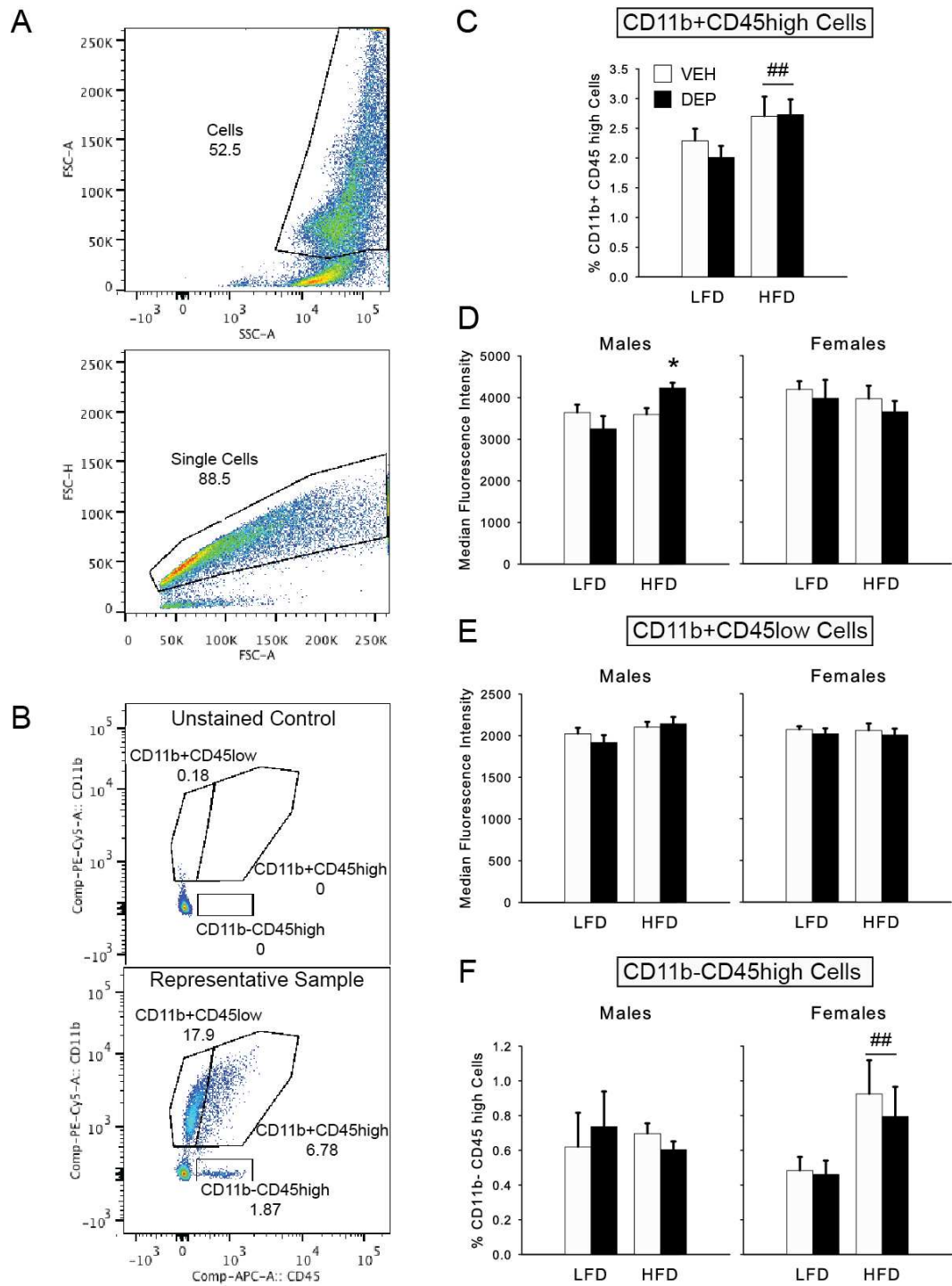
**Figure 17: At the end of 9 weeks of diet, DEP/HFD male offspring exhibited increased mRNA expression of microglia/macrophage activation markers CD11b (A) and TLR4 (B), and of specific microglia marker CX3CR1 (C), in HIPP, whereas female offspring did not. (D) There was a small decrease in GFAP mRNA in brains of females exposed to HFD, independent of prenatal treatment, but no differences among males. (D). Data are mean  $\pm$  SEM, n=4-6/group/sex. \*\* $p$ <0.05 vs. VEH/HFD; \* $p$ <0.05 vs. DEP/LFD and VEH/HFD groups; # $p$ <0.05 vs. VEH/LFD and VEH/HFD; ## $p$ <0.05, HFD vs. LFD.**

### 3.3.2.8 HFD appears to cause a greater infiltration of peripheral macrophages into HYP and HIPP, and these cells appear more activated in the HYP of DEP/HFD male offspring

For flow cytometry analysis of cellular composition of the HYP and HIPP at the end of 9 weeks of diet, cells were initially gated based on forward and side scatter to obtain only single, live cells (Figure 18A), and subsequently were gated into CD11b+CD45<sup>low</sup> cells (parenchymal microglia), CD11b+CD45<sup>high</sup> cells (infiltrating

macrophages), and CD11b-CD45<sup>high</sup> (lymphocytes; Figure 18B) (Ford, Goodsall, Hickey, & Sedgwick, 1995; Parney, Waldron, & Parsa, 2009). This analysis revealed that 9 weeks of HFD caused a significant increase in the percent of CD11b+CD45<sup>high</sup> cells found in these brain regions, regardless of prenatal treatment or sex [main effect of HFD,  $F(1,32)=5.33, p<0.05$ ; Figure 18C]. Of note, this result only reached statistical significance when HYP and HIPP were analyzed together, although there were still trends for an effect of diet when each brain region was analyzed separately [ $p's=0.1$ ]. Also of interest, the HIPP had an overall higher percentage of CD11b+CD45<sup>high</sup> cells ( $2.80 \pm 0.20\%$ ) than the HYP ( $2.07 \pm 0.12\%$ ) when expressed as a percent of all cells present in a sample [main effect of Brain Region,  $F(1,32)=8.98, p<0.01$ ], whereas the HYP had an overall greater percentage of CD11b+CD45<sup>high</sup> cells ( $23.44 \pm 1.17\%$ ) than the HIPP ( $13.31 \pm 1.18\%$ ) when expressed as a percent of all CD11b+ cells (i.e., those of a myeloid lineage) present in a sample. Importantly, we found that the CD11b+CD45<sup>high</sup> cells in the HYP of DEP/HFD males had a significantly higher median fluorescence intensity (MFI) of CD11b protein than DEP/LFD males, whereas VEH/LFD and VEH/HFD males did not differ from each other [DEP  $\times$  HFD interaction,  $F(1,8)=6.25, p<0.05$ ; *post hoc*,  $p<0.05$ ]. On the other hand, there were no significant differences among females [trend for Sex  $\times$  HFD interaction,  $F(1,16)=3.88, p=0.07$ , and trend for Sex  $\times$  DEP  $\times$  HFD interaction,  $F(1,16)=2.24, p=0.1$ ; Figure 18D]. Furthermore, this result did not reach significance in the CD11b+CD45<sup>low</sup> cells of the HYP (Figure 18E), nor was this pattern observed in the HIPP (data not

shown). Analysis of the CD11b-CD45<sup>high</sup> cells revealed that HFD caused a significant increase in the percentage of lymphocytes in the HYP of females [main effect of diet,  $F(1,8)=7.49$ ,  $p<0.05$ ], whereas there were no significant differences in the HYP of males [trend for Sex  $\times$  HFD interaction,  $F(1,16)=4.17$ ,  $p=0.06$ ; Figure 18F] or in the HIPP for either sex (data not shown).



**Figure 18: (A) Following flow cytometry, cells were initially gated based on forward and side scatter to obtain only live, single cells for further analysis. (B) Cells were**

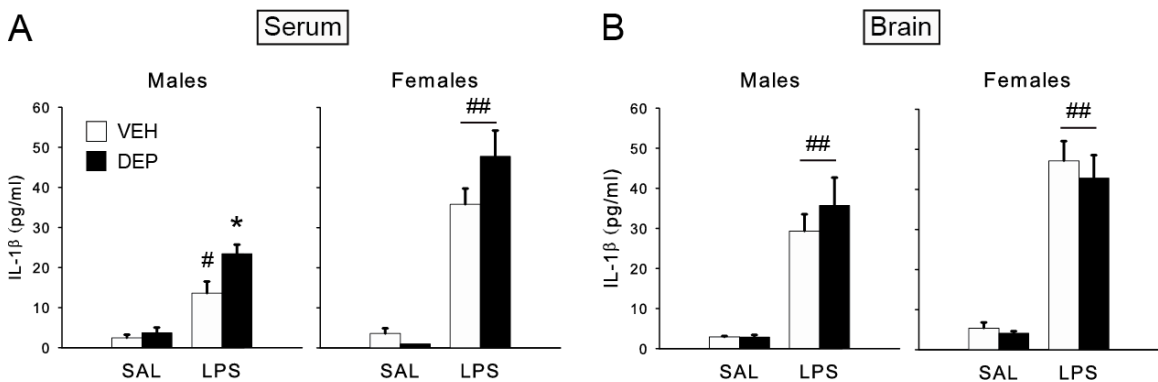
subsequently gated into CD11b+CD45<sup>low</sup> cells (microglia), CD11b+CD45<sup>high</sup> cells (infiltrating macrophages), and CD11b-CD45<sup>high</sup> cells (lymphocytes). (C) 9 weeks of HFD caused a significant increase in the percent of CD11b+CD45<sup>high</sup> cells found in the HYP and HIPP (analyzed together), regardless of prenatal treatment or sex. (D) DEP/HFD males possessed CD11b+CD45<sup>high</sup> cells in the HYP with a significantly higher MFI of CD11b protein, whereas females did not exhibit any differences. (E) CD11b+CD45<sup>low</sup> cells of the HYP did not display any significant differences between groups. (F) HFD caused a significant increase in the percent of CD11b-CD45<sup>high</sup> cells in the HYP of females, but not males. Data for A and B are diagrams of gating procedures from representative samples, data for C represents the mean  $\pm$  SEM of n=12/group (sexes and brain regions combined for analysis), and data for D, E and F represent the mean  $\pm$  SEM of n=3/group/sex. <sup>##</sup>*p*<0.05, HFD vs. LFD; \**p*<0.05 vs. DEP/LFD.

### 3.3.3 Experiment 2: Prenatal DEP Exposure and P30 SAL/LPS Injections

#### 3.3.3.1 DEP male offspring mount an exaggerated peripheral IL-1 $\beta$ response to an LPS challenge, whereas their central IL-1 $\beta$ response does not differ from VEH male offspring

Two hours following an LPS challenge, P30 DEP male offspring had significantly higher levels of IL-1 $\beta$  in their serum than VEH male offspring and both SAL-injected groups [DEP  $\times$  LPS interaction,  $F(1,19)=5.16$ ,  $p<0.05$ , *post hoc*,  $p<0.05$ ], whereas DEP female offspring did not differ from VEH female offspring following LPS injection, despite an overall greater IL-1 $\beta$  response in females than males that is consistent with previous studies (Darnall & Suarez, 2009; Lynch, Dinarello, & Cannon, 1994) [main effect of LPS,  $F(1,19)=95.23$ ,  $p<0.001$ ; main effect of Sex,  $F(1,38)=25.06$ ,  $p<0.001$ ; Sex  $\times$  LPS interaction,  $F(1,38)=29.02$ ,  $p<0.001$ ; Figure 19A]. Although there was a tendency for females to display a pattern similar to males, it did not reach significance [trend for DEP  $\times$  LPS interaction,  $F(1,19)=3.19$ ,  $p=0.09$ ]. On the other hand, although there was a robust

IL-1 $\beta$  response in the brains (HYP, HIP, PFC, and PAR pooled) of the same animals 2 h post-LPS injection [main effect of LPS,  $F(1,45)=154.07$ ,  $p<0.001$ ], there was not a differential central response to LPS due to prenatal treatment or sex (Figure 19B).



**Figure 19: (A) 2 h following LPS challenge, P30 DEP male offspring had significantly higher levels of IL-1 $\beta$  in their serum than VEH male offspring, whereas female offspring did not significantly differ due to prenatal treatment. (B) The brains of the same animals displayed a robust IL-1 $\beta$  response, but did not differ due to prenatal treatment or sex. Data are mean  $\pm$  SEM,  $n=6-8$ /group/sex.  $^{\#}p<0.05$  vs. SAL groups;  $^*p<0.05$  vs. all other groups;  $^{\#\#}p<0.05$ , LPS vs. SAL.**

### 3.4 Discussion

In agreement with emerging evidence that environmental chemical exposures may contribute to the escalating prevalence of obesity, we have demonstrated that prenatal air pollution exposure causes exaggerated weight gain, insulin resistance, anxiety-like behavior, and changes in neuroinflammatory markers following high-fat diet consumption in adulthood, which cannot be fully explained by changes in food intake and physical activity. Notably, our results are in line with previous studies of other obesogens. For example, prenatal bisphenol A (BPA) exposure causes a 10% increase in body weight gain on a high-fat diet (Wei et al., 2011), and here we report a

40% increase in the body weight gain of DEP-exposed males on a high-fat diet. These findings are also consistent with and expand upon our previous work demonstrating that offspring gain more weight, develop hyperinsulinemia, and exhibit widespread increases in microglial antigen expression as a result of maternal diesel exhaust inhalation during pregnancy and 6 weeks of high-fat diet in adulthood (Bolton et al., 2012). Our previous findings led us to hypothesize that microglia are primed long-term by prenatal air pollution exposure and subsequently overreact to high-fat diet exposure in adulthood, resulting in exacerbated metabolic and behavioral outcomes. However, the current data suggest that the observed adverse effects may instead be due to the priming of peripheral monocytes/macrophages by prenatal air pollution exposure, and their later activation and infiltration of both the adipose tissue and the brain following high-fat diet exposure. This interpretation is supported by 3 key findings of the current study: 1) HFD increased the percentage of CD11b+CD45<sup>high</sup> cells in the hypothalamus and hippocampus, as well as the expression of CCR2 in the adipose tissue, 2) DEP/HFD males had elevated surface levels of CD11b protein on these putative infiltrating macrophages in the hypothalamus and increased CD11b expression in the adipose tissue, and 3) DEP males had an exaggerated peripheral, but not central, IL-1 $\beta$  response to LPS at P30 that may be indicative of monocyte/macrophage priming.

Notably, these findings do not rule out the contribution of microglial activation to the adverse metabolic and behavioral outcomes in this model, as we also found

increased expression of microglial markers in the hippocampus of DEP/HFD males. It is important to note that the current markers we have to distinguish parenchymal microglia and infiltrating macrophages in the CNS are imperfect. For example, it is possible for microglia to acquire a CD11b+CD45<sup>high</sup> phenotype, but this is thought to occur only in a small subset of microglia in response to very high levels of neurotoxicity (Ford et al., 1995). Although TLR4 and CD11b may be expressed by either microglia or peripheral macrophages, CX3CR1 is expressed specifically by microglia in the brain, whereas infiltrating macrophages express CCR2, not CX3CR1 (Mizutani et al., 2012). In the current study, CX3CR1 expression was elevated in the hippocampus specifically of DEP/HFD males. The role of CX3CR1, the receptor for fractalkine/CX3CL1, in the CNS is complex—it has been shown to dampen microglial activation and neurotoxicity (Cardona et al., 2006), but its expression is also increased following chronic inflammation (Hughes, Botham, Frentzel, Mir, & Perry, 2002) and is critical for the development of neuropathic pain (Zhuang et al., 2007). In the current study, it may be that the microglia react to an increased infiltration of peripheral cells by upregulating CX3CR1, either in an effort to limit excessive activation and neurotoxicity, or as a byproduct of their increased activation. Alternatively, the increase in CX3CR1 expression may signify that there are simply more microglia (Harrison et al., 1998) in the hippocampus of DEP/HFD males. Either way, the marked increases in Iba1 staining in brain sections that we observed in our previous study due to the combination of

prenatal air pollution exposure and adult high-fat diet (Bolton et al., 2012) suggest that there is likely widespread microglial reactivity to the high-fat diet and the potential macrophage infiltration. Moreover, it is possible that microglia play a key role in initiating and guiding macrophage infiltration of the brain, as previous studies have shown that microglia produce markedly higher levels of monocyte chemoattractant protein (MCP-1/CCL2), the ligand that binds to CCR2 on circulating monocytes, in conjunction with an increased percentage of CNS macrophages (Wohleb et al., 2011).

A particularly novel finding of the current study is our preliminary evidence for macrophage infiltration of the brain that appears to parallel the well-documented macrophage infiltration of the adipose tissue in animals consuming high-fat diets. Leukocyte trafficking into the brain has been previously implicated in the pathogenesis of neurological diseases like multiple sclerosis and Parkinson's disease (Rezai-Zadeh, Gate, & Town, 2009), as well as in response to repeated social stress and LPS injections (Wohleb et al., 2011; Wohleb et al., 2012; Wohleb, Powell, Godbout, & Sheridan, 2013), but only very recently in response to chronic high-fat diet consumption. Buckman et al. (2013) used bone marrow chimeric male mice to demonstrate that 15 weeks of high-fat diet feeding causes increased recruitment of peripheral immune cells, primarily macrophages, into the parenchyma of the adult brain. Macrophage infiltration of the adipose tissue is key for inducing insulin resistance in the periphery; thus, macrophage infiltration of the brain may play a role in inducing central insulin resistance as well.

Milanski et al. (2009) have already implicated TLR4 in the hypothalamus as critical for the development of central insulin resistance, but did not distinguish between its expression on microglia or infiltrating macrophages. Our data in combination with those of Buckman et al. (2013) suggest the possibility that it may be TLR4 expressed on activated, infiltrating macrophages (alone or in combination with that on microglia) in the hypothalamus that initiates an inflammatory cascade leading to central insulin resistance. Furthermore, we observed an increase in TLR4 expression in the hippocampus of DEP/HFD males in conjunction with evidence of increased macrophage infiltration, which may have played a role in the development of anxiety-like behavior in these animals. In support of this idea, previous studies have shown that repeated social defeat increases anxiety-like behavior in association with an increased inflammatory profile in microglia and macrophages (Wohleb et al., 2011), and notably, the stress-induced recruitment of infiltrating macrophages to the brain is critical for the development of this anxiety-like behavior (Wohleb et al., 2013). Although our data provide evidence suggestive of an increase in the trafficking of peripheral macrophages into the brain, this remains to be demonstrated directly in our model using, for instance, bone marrow transfer experiments. However, we believe the recent experiments of this kind from Buckman et al. (2013) provide strong support for our findings.

Another important finding of this study was our observation of robust sex differences in almost every outcome. These data are consistent with our previous work

demonstrating that males are more vulnerable to the adverse effects of prenatal air pollution exposure (Bolton et al., 2012), as well as with evidence within the field of child health that males typically experience poorer outcomes following maternal inflammatory insults during pregnancy. For example, males have a higher risk of poor perinatal outcome when their mothers are diabetic during pregnancy (García-Patterson et al., 2011), and BPA exposure during pregnancy has been shown to disrupt glucose homeostasis in male, but not female, offspring (Alonso-Magdalena et al., 2010). In the current study, prenatal DEP exposure did not affect weight gain, insulin resistance, or behavioral changes in response to HFD in female offspring. Interestingly, even though high-fat diet consumption caused evidence of macrophage infiltration of the adipose tissue and brain in both males and females, DEP females did not display the increases in macrophage activation markers (CD11b, TLR4) characteristic of macrophage priming that we observed in their male littermates. We have previously shown that male brains at E18 downregulate IL-10 protein in response to prenatal DEP exposure, whereas female E18 brains upregulate IL-10 following DEP exposure, which may be protective against macrophage priming (Bolton et al., 2013). In the current study, we found that HFD females displayed a downregulation of GFAP expression in the hippocampus, which again may be suggestive of an increased anti-inflammatory tone in the CNS (Morgan et al., 1997), but could also signify a loss of astrocytes. In regard to peripheral metabolic changes, Pettersson et al. (2012) recently demonstrated that female mice are

protected from HFD-induced insulin resistance due to an increase in the regulatory T cell population of their adipose tissue compared to males, which helps maintain an anti-inflammatory environment. Interestingly, we observed an increase in the lymphocyte population specifically in the hypothalamus of females, but not males, in response to HFD. Thus, it is possible that an increase in regulatory T cells, in both the brain and the adipose tissue, could explain the apparent protection of HFD female offspring in our study. Due to the prevalence of a male disadvantage in the human and animal fetal programming literature, the mechanisms of these sex differences warrant further investigation.

The mechanisms by which prenatal events impact offspring behavioral, metabolic, and brain outcomes are usually complex, though poorly defined, and may involve several physiological pathways, as well as changes in maternal-offspring interactions after birth. In this study, we did not observe any significant alterations of maternal care due to maternal DEP exposure, consistent with our previous work (Bolton et al., 2013), in which we also found no long-term changes in maternal anxiety due to prenatal treatment. Therefore, we believe the male anxiety phenotype is likely due to the effect of DEP exposure on *in utero* development, in combination with a “second hit” later in life (adult high-fat diet). However, it is possible there were sex-specific changes in maternal-offspring interactions, which are typically male-biased (Moore & Morelli,

1979), or more subtle shifts within the normal distribution of maternal behavior, which our observation methodology was unable to detect.

Importantly, there were no significant differences in maternal weights during or after pregnancy as a result of DEP exposure that would be suggestive of maternal obesity, nor are the metabolic effects we observed in the offspring simply the long-term consequences of low birth weight, as is commonly studied in the field of metabolic programming. It is more likely that the effects of air pollution exposure *in utero* are due to the inflammatory or endocrine-disrupting effects of this obesogen during sensitive periods of brain development (Bolton et al., 2013). An interesting possibility that remains to be explored in our model is the role of the placenta, the interface between the mother and the fetus, in the fetal programming of future immune and metabolic function in response to prenatal air pollution exposure.

In conclusion, we have demonstrated that prenatal air pollution exposure causes exaggerated weight gain, insulin resistance, and anxiety-like behavior specifically in male offspring on a high-fat diet, and that these changes may be accompanied by the increased infiltration of the brain and adipose tissue by activated macrophages. Our working hypothesis is that these macrophages are primed early during development by prenatal air pollution exposure, and only following the “second hit”, exposure to the high-fat diet, are they induced to emigrate from the bone marrow or other peripheral tissues and invade the brain and adipose tissue, creating a pro-inflammatory

environment with significant consequences for behavioral and metabolic regulation. The mechanisms that allow for the specific vulnerability of males and relative protection of females following these immune challenges remain to be defined, but likely involve the promotion of an anti-inflammatory environment in the brain and adipose tissue of females. Future studies aimed at elucidating these sexually dimorphic mechanisms of fetal programming will be critical for informing environmental policy regulations, as well as identifying effective interventions.

## 4. Maternal Stress Worsens Effects of Prenatal Air Pollution on Offspring Mental Health Outcomes in Mice<sup>1</sup>

### 4.1 Introduction

An intriguing new area of research that remains relatively unexplored is the interaction of maternal exposure to environmental “physical toxins”, such as air pollution, with maternal exposure to “social toxins” (i.e., maternal psychosocial stress) during gestation, which may result in a synergistic effect on offspring outcomes (Wright, 2009). An understanding of this interaction is important, because environmental exposures are typically not experienced in isolation in the real world. Notably, expectant mothers living in areas of low socioeconomic status experience the greatest burden of toxins and pollutants (Evans & Kantrowitz, 2002a), along with fewer resources and high psychological stress (Seguin et al., 1995), the combined effects of which may exaggerate the deleterious consequences for their unborn children. For example, parental stress has been shown to increase the effect of *in utero* tobacco smoke exposure on childhood asthma risk (Shankardass et al., 2009). In such cases, stress may increase vulnerability, permitting a toxin to initiate significant injury to physiological systems when it would have been insufficient to do so in isolation. Importantly, these synergistic effects of stress and pollutants are possible because they likely act on common biological systems, such

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as innate immune pathways (Frank, Baratta, Sprunger, Watkins, & Maier, 2007; Levesque et al., 2011b) within the developing nervous system.

In the current study, we hypothesized that the addition of maternal stress during gestation to the impact of prenatal air pollution exposure would act synergistically in offspring to impair mental health outcomes, compared with the effects of either exposure alone. In order to test this hypothesis, we combined our animal model of prenatal diesel exhaust exposure (Auten et al., 2012) with an adaptation of a novel model of maternal resource deprivation (nest restriction; NR) that was limited to the prenatal period (Rice, Sandman, Lenjavi, & Baram, 2008). Pregnant mice were exposed to intermittent aspiration of vehicle or diesel exhaust particles (DEP) throughout gestation, and either lived in normal housing or had reduced nesting materials during the last third of pregnancy. We report for the first time that mice exposed prenatally to a common urban air pollutant in combination with maternal stress during gestation exhibit strikingly increased anxiety and impaired cognition as adults. Importantly, these behavioral changes in offspring of DEP/NR dams were sex-specific and associated with neuroinflammatory changes.

## ***4.2 Materials and Methods***

### **4.2.1 Animals**

We obtained adult male and female C57BL/6 mice from Charles River Laboratories (Raleigh, NC). Mice were housed in individually ventilated, microisolator

polypropylene cages with specialized bedding (AlphaDri; to avoid exposure to potentially confounding antigens that can be found in typical bedding) and *ad libitum* food (PicoLab Mouse Diet 5058, Lab-Diet, Philadelphia, PA) and filtered water. The colony was maintained at 22°C on a 12:12-hr light-dark cycle (lights off at 9 AM). Following acclimation to laboratory conditions for 1 week, we placed males with 2 females each for breeding, for a maximum of 2 weeks. We examined females twice daily for evidence of a vaginal plug [confirmation of successful mating, considered to be embryonic day (E)0], at which point we separated them from the male and paired them with another successfully mated female. Females were bred in four separate cohorts (n=46 total dams), and each cohort was used for a separate analysis: 1) fetal brain cytokine protein, 2) P30 brain gene expression, 3) adult behavior and brain cytokine protein, and 4) adult microglial isolations and gene expression. In addition, one pup was randomly selected at P1 from litters across the second and third cohorts for CORT analysis. All mice used in this study were treated humanely and with regard for alleviation of suffering, and experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

## **4.2.2 Prenatal Stressors**

### **4.2.2.1 DEP exposures**

Diesel exhaust was generated using a 4.8kW direct injection single-cylinder 320 mL displacement Yanmar L70V diesel generator operating at a constant 3.500 rpm.

Resistance heating elements were used to impose a constant 3kW load to the engine. Low sulfur diesel fuel was used. Diesel exhaust particles were collected using an electrostatic precipitator. Chemical analysis of particles yielded an organic carbon/elemental ratio = 0.63, with extractable organic matter = 39.8%. The particle size distribution ranged from 18-200  $\mu\text{m}$  [further detailed analyses may be found in (Auten et al., 2012)]. We prepared 1 mg/ml DEP suspensions by briefly sonicating in phosphate buffered saline pH 7.2 0.05% Tween-20.

Beginning on E2, we administered 50  $\mu\text{l}$  DEP in vehicle (DEP group) or vehicle alone (VEH group) to dams by oropharyngeal aspiration under 2% isoflurane anesthesia. Following brief anesthetization (~1 min), we suspended mice by frontal incisors, and upon the beginning of recovery with the onset of deeper inspirations, we gave the dose by holding the tongue with forceps and administering the suspension with a micropipettor to the posterior oropharynx. DEP treatment occurred every 3 days E2-E17 for a total of 6 doses, as a model of intermittent exposure. This dose and route of delivery induces maternal lung inflammation (e.g. white blood cell infiltration) comparable to levels observed following intermittent maternal inhalation of diesel exhaust at environmentally relevant concentrations (Auten et al., 2012). Moreover, both routes of delivery result in similar levels of particle deposition within the lung (Foster et al., 2001). Specifically, bronchoalveolar lavage two days after administration demonstrates DEP in alveolar macrophages, and pilot studies show distal alveolar

deposition at necropsy (data not shown). Furthermore, gross inspection of placentae rarely reveals any abnormalities, besides the occasional fetal resorption, and placental weights are not affected by maternal treatment (data not shown).

#### **4.2.2.2 Maternal resource deprivation**

We adapted a model of postnatal nest restriction (Rice et al., 2008) by applying it to the prenatal period, and used the degree of restriction that produced minimal phenotypic changes with NR alone. Beginning on E14 following DEP exposures, we singly housed half of the VEH- and DEP-treated dams in clean cages with a thin layer of bedding under an elevated fine-gauge aluminum mesh platform (mesh dimensions 0.4 cm × 0.9 cm; McNichols Co., Tampa, FL) and provided them with 2/3 of one square of felt-like nesting material (~1.9 g; NR group). The remaining dams we singly housed in clean cages with bedding and one full square of nesting material (~2.8 g; Control group). On E19, we placed NR dams in clean cages with normal bedding and one full square of nesting material, and from that point forward, treated them identically to Control dams. This design resulted in 4 groups of dams: VEH/Control (n=8), DEP/Control (n=10), VEH/NR (n=8), and DEP/NR (n=10).

#### **4.2.3 Neonatal Outcomes and Maternal Behavior**

##### **4.2.3.1 Birth weights**

All animals included in this study were allowed to deliver spontaneously on gestational days 19-20 [defined as postnatal day (P)0], and offspring were not cross-

fostered. We weighed pups (sex not determined) on P1 in order to acquire a litter average for birth weight (n=8-10 litters/group from 3 cohorts). Four weeks later, we weaned offspring into clean cages of 2-5 same-sex siblings, and provided them with *ad libitum* access to standard chow and filtered water.

#### **4.2.3.2 Neonatal corticosterone (CORT) measurement**

On P1, we randomly selected one pup from each litter (males: n=5-8/group; females: n=3-7/group from 2 cohorts), performed rapid decapitation, and collected trunk blood to obtain a measure of basal circulating CORT levels soon after birth. We assessed total serum CORT concentrations using an enzyme-linked-immunosorbent assay (ELISA; Enzo Life Sciences, Inc., Ann Arbor, MI) (Bilbo et al., 2007).

#### **4.2.3.3 Maternal behavior assessment**

In order to characterize the effect of prenatal stressors on maternal care, we observed dams (n=3-7/group from 2 cohorts) twice daily with their litters P2-P9, once each during the light (8-9 AM) and dark (4-5 PM) cycles. During each session, we scored dams (n=3-7/group) for 'on nest', nursing, and licking and grooming (LG) behavior every 5 min for 1 hr (Myers et al., 1989). As previously described, the frequency of occurrence for some of the behaviors (particularly LG) was rare, so we summed data for each behavior across all 16 observation sessions and calculated a percent of time spent performing each behavior for each dam. We took care not to disturb dams during

observation sessions, and changed their cages only upon completion of the final observation on P9.

#### **4.2.4 Fetal Brain Cytokine Analysis**

In order to assess the fetal brain cytokine response to DEP and/or NR, we euthanized a separate cohort of identically treated dams (n=2-3/group) at E18. Thus, we obtained fetuses by hysterotomy under sodium pentobarbital anesthesia (250 mg/kg i.p.), placed them on ice, and decapitated them. We snap-froze whole fetal brains and stored them at -80°C until processing. We also collected tail snips for later genotyping to determine the sex of each fetus. From these tail snips, we extracted genomic DNA (Kouduka, Matsuoka, & Nishigaki, 2006), and subsequently assessed SRY PCR products (Koopman, Gubbay, Vivian, Goodfellow, & Lovell-Badge, 1991) (see Table 3).

We used ELISAs (R&D Systems, Minneapolis, MN) to measure interleukin (IL)-1 $\beta$ , a proinflammatory cytokine, and IL-10, an anti-inflammatory cytokine, in fetal brain homogenates normalized to total protein (200  $\mu$ g/well) and lipid-depleted (n=7-8/sex/group). We selected these cytokines due to their important role in microglial function, brain development, and behavior (Williamson et al., 2011; Yirmiya & Goshen, 2011) We measured protein levels of IL-1 $\beta$  and IL-10 in fetal and adult brain as described previously (Williamson et al., 2011) except for minor changes. Previous studies in our lab (unpublished data) have shown that the protein measurement works

best when myelin is removed via lipid extraction (Hara & Radin, 1978) of the brain homogenates prior to analysis. Thus, we added 1 ml of hexanes (~2 × volume of homogenate; Mallinckrodt Baker, Inc., Philipsburg, NJ) to each sample following tissue homogenization and sonication. We vortexed and centrifuged samples briefly at 13,000 × g and then extracted the aqueous layer. Finally, we removed residual hexanes by decantation after freezing the aqueous fractions on dry ice for ~3 min.

#### **4.2.5 P30 Neuroimmune Gene Expression**

To determine the long-term effect of prenatal stressors on genes critical for innate immune recognition and the subsequent cytokine response in the brain, we assessed male (n=8-13/group; 2-4 litters represented/group from 1 cohort) and female offspring (n=5/group; 2-4 litters represented/group from 1 cohort) at P30. Thus, we deeply anesthetized animals with 430 mg/kg ketamine and 65 mg/kg xylazine i.p. and transcardially perfused them with ice-cold saline for 2 min to clear brains of blood. Afterwards, we extracted the brains, removed the cerebellum and hindbrain, and cut the remaining forebrain sagittally in half. We snap-froze half-brains and stored them at -80°C until analysis.

We isolated total RNA from P30 half-brains using TRIzol (Chomczynski & Sacchi, 1987). Subsequently, we synthesized cDNA from 100 ng of RNA using the QuantiTect reverse transcription kit (Qiagen, Inc., Valencia, CA) and a 15 min cDNA synthesis reaction. We performed quantitative real-time PCR (qRT-PCR) using a

QuantiFast SYBR Green PCR kit (Qiagen) on a Mastercycler ep *realplex* (Eppendorf, Hauppauge, NY). For each reaction, we added 1  $\mu$ l of cDNA to 12  $\mu$ l of master mix containing specific primers for GAPDH, TLR4, or caspase-1 (Qiagen). For analysis, we determined the threshold cycle ( $C_T$ ) for each reaction and calculated relative gene expression using the  $2^{-\Delta\Delta C_T}$  method (Livak & Schmittgen, 2001; Pfaffl, 2001; Williamson et al., 2011).

#### **4.2.6 Behavioral Procedures**

In order to assess behavioral outcomes as a result of prenatal stressors, we tested young adult (P60-P90) male and female offspring (n=7-9 animals/sex/group, 2-4 litters represented/group from 1 cohort) with the sequence of behavioral tests listed below, with 1 week between tests. We tested males and females separately and performed all testing during the animals' dark cycle between 10 AM and 4 PM. Throughout testing, we also monitored females' estrous cycles.

##### **4.2.6.1 Contextual and auditory cue fear conditioning**

We used contextual fear conditioning to assess hippocampal-dependent memory, which we have previously found to be uniquely vulnerable to early-life insults (Williamson et al., 2011) in comparison with auditory cue fear conditioning, which does not require the hippocampus (Phillips & LeDoux, 1992).

*Apparatus.* The conditioning context consisted of chambers (20 cm  $\times$  19 cm  $\times$  33 cm) made of clear Plexiglas with metal side walls (Coulbourn Instruments, Whitehall,

PA), placed inside a black Plexiglas box (41.9 cm × 41.9 cm × 45.4 cm) with an open front to allow viewing of the animals, but to prevent them from seeing each other. Each chamber has a ceiling-mounted speaker capable of emitting a phasic auditory cue (2976-Hz tone presented at 76 dB) and a white house light. A removable floor of stainless steel rods (0.5 cm diameter, spaced 1.75 cm apart) is wired to a shock generator and scrambler. The chambers were cleaned with 70% ethanol followed by water before each animal.

*Procedure.* We allowed mice to explore the chamber for 2 min before the onset of a 15 sec tone, followed immediately by a 2 sec footshock (0.8 mA). The same tone-shock pairing was repeated 90 and 180 sec later, and immediately after the termination of the third footshock, we removed mice from the chamber. We tested mice 48 hr later for contextual fear memory, as assessed by freezing behavior, in the original conditioning chamber, but in the absence of auditory cues or footshocks. Freezing represents the dominant defensive fear response of rodents and is characterized by an immediate suppression of behavior and immobility (except for respiration) (Blanchard, Fukunaga, & Blanchard, 1976) We began scoring 10 sec after placing the animal into the chamber, and continued every 10 sec for the remainder of the 6-min test. For assessment of auditory-cue fear memory (3 hr after contextual fear test), testing occurred in a novel triangular chamber (31.8 cm × 26.7 cm × 38.1 cm × 25.4 cm) with clear Plexiglas walls and floor, illuminated by a 7-W clear, red light bulb. We again assessed freezing for 6 min.

The auditory conditioned stimulus (CS) was absent for the first 3 min (pre-CS period) and present for the last 3 min (CS period). Freezing during the pre-CS period indicates generalized fear of a novel environment, whereas freezing during the CS period indicates fear specific to the auditory cue (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Paylor, Tracy, Wehner, & Rudy, 1994).

#### **4.2.6.2 Elevated zero-maze**

In order to assess anxiety-like behavior, we adapted a widely used method for rodents that measures time spent in the closed vs. open arms of a circular maze (Shepherd et al., 1994). The maze has an elevated (49.5 cm high) circular lane (4.5 cm wide) divided into four quadrants. Two opposite quadrants are enclosed by walls (15.9 cm high), whereas the remaining two quadrants are left exposed. We placed each mouse onto the center of an open arm, and scored the total time they spent in the closed arms out of 5 min. An increase in time spent in the closed arms is indicative of increased anxiety (Shepherd et al., 1994). We cleaned the maze between animals. Immediately following the test (<5 min), we collected a small blood sample (~100 µl) from the facial vein of each mouse in a separate room in order to determine CORT levels induced by the test.

#### **4.2.6.3 Forced swim test**

In order to assess depressive-like behavior, we adapted a commonly used method for mice by measuring time spent immobile in a container of water (Castagné,

Moser, Roux, & Porsolt, 2010). The apparatus is a Plexiglas cylinder (20.3 cm diameter × 49.5 cm high) filled 2/3-full with 22°C water. We lowered each mouse into the center of the water-filled cylinder, and then scored them for their duration of immobility for 6 min. Immobility was defined as the absence of all swimming, except for the subtle motions required to keep the head above water (Castagné et al., 2010).

#### **4.2.7 Adult Brain Cytokine Analysis**

In order to determine the enduring effects of prenatal stressors on brain cytokines, and their potential role in observed behavioral changes, we assessed cytokine protein levels in the brains of adult offspring 10 days following behavioral testing. Thus, we anesthetized and perfused all offspring (n=7-9/group/sex) as described above, after which we extracted brains and dissected them on ice into hypothalamus (HYP), prefrontal cortex (PFC), hippocampus (HIP), and adjacent parietal cortex (PCX). We selected these regions for their known roles in the cognitive and affective behaviors we assessed. In order to obtain enough total protein for analysis, we pooled the dissected regions for each animal, snap-froze them, and stored them at -80°C until processing. We performed ELISA protein analyses of IL-1 $\beta$  and IL-10 as described above for E18.

#### **4.2.8 Microglial Isolation and Gene Expression Analysis**

In order to determine the cellular source of the measured cytokines, we anesthetized and perfused a separate group of adult (~P60) behaviorally naïve offspring (n=5/sex/group; 2-3 litters represented/group from 1 cohort, including each of the 4

maternal treatment groups) as described above. Again, we pooled dissected HYP, PFC, HIPPP, and PCX from each animal in order to obtain enough cells for later analysis. We isolated microglia by magnetic-activated cell sorting (MACS) (Williamson et al., 2011), using Miltenyi's Neural Tissue Dissociation Kit (P), anti-myelin microbeads, and anti-CD11b (an established marker for microglia) microbeads (Miltenyi Biotec, Inc., Auburn, CA). Afterwards, we washed cells in sterile PBS and stored them at -80°C until analysis (Williamson et al., 2011).

We isolated total RNA from CD11b+ and CD11b- cells using TRIzol (Chomczynski & Sacchi, 1987), adding glycogen (Invitrogen, Grand Island, New York) to the aqueous phase to maximize RNA precipitation. Next, we synthesized cDNA from 100 ng of RNA as above, but using a 30 min cDNA synthesis reaction, followed by qRT-PCR as above. We purchased primers for CD11b, caspase-1, IL-1 $\beta$ , BDNF, and GFAP from Qiagen and designed primer pairs for 18S, IL-10, and TLR4 (Sigma Aldrich; see Table 3) as previously described (Williamson et al., 2011). We subsequently determined optimal annealing temperatures for each primer pair by running a temperature gradient, and verified specificity by melt-curve analysis.

**Table 3: Forward and reverse primers for qRT-PCR analysis.**

| Gene       | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|------------|------------------------|------------------------|
| <i>Sry</i> | TGGGCTGGACTAGGGAGGTCC  | TGCTGGGCCCAACTTGTGCCT  |
| <i>18S</i> | GAATAATGGAATAGGACCGC   | CTTTCGCTCTGGTCCGTCTT   |

|             |                        |                     |
|-------------|------------------------|---------------------|
| <i>Il10</i> | GGACAACATACTGCTAACCGAC | TGCTCCTTGATTCTGGGC  |
| <i>Tlr4</i> | GCTGGATTTATCCAGGTGTG   | AGTCCAGAGAACTTCCTGG |

#### 4.2.9 Data Analysis

We analyzed all data with SPSS statistical software (IBM, Armonk, NY). Due to heterogenous variance, we log-transformed neonatal CORT data. Furthermore, for ELISA analyses, samples that had undetectable levels of IL-1  $\beta$  or IL-10 were assigned a value of half the lowest detectable value in the assay (A. L. Thompson et al., 2012). We used three-way (Sex  $\times$  DEP  $\times$  NR) ANOVAs to analyze all data, except for the PCR data from CD11b $\pm$  isolated cells, for which we used four-way (Sex  $\times$  DEP  $\times$  NR  $\times$  cell population) ANOVAs. We followed up interactions with sex with separate 2-way (DEP  $\times$  NR) ANOVAs for males and females to identify sex-specific effects, and subsequently followed up significant DEP  $\times$  NR interactions within each sex with post hoc comparisons (Tukey's HSD) to identify group differences, assuming significance for  $p < 0.05$ . All reported  $p$  values are two-tailed, except for the correlations between behavioral measures and cytokine measures from adult brains, which utilized one-tailed  $p$  values because we had clear *a priori* hypotheses based on the apparent correspondence between observed group differences in the two measures. Finally, litter effects were controlled for by using multiple litters per treatment group, and can also be excluded due to the sex-specific effects observed in males and females from the same litters.

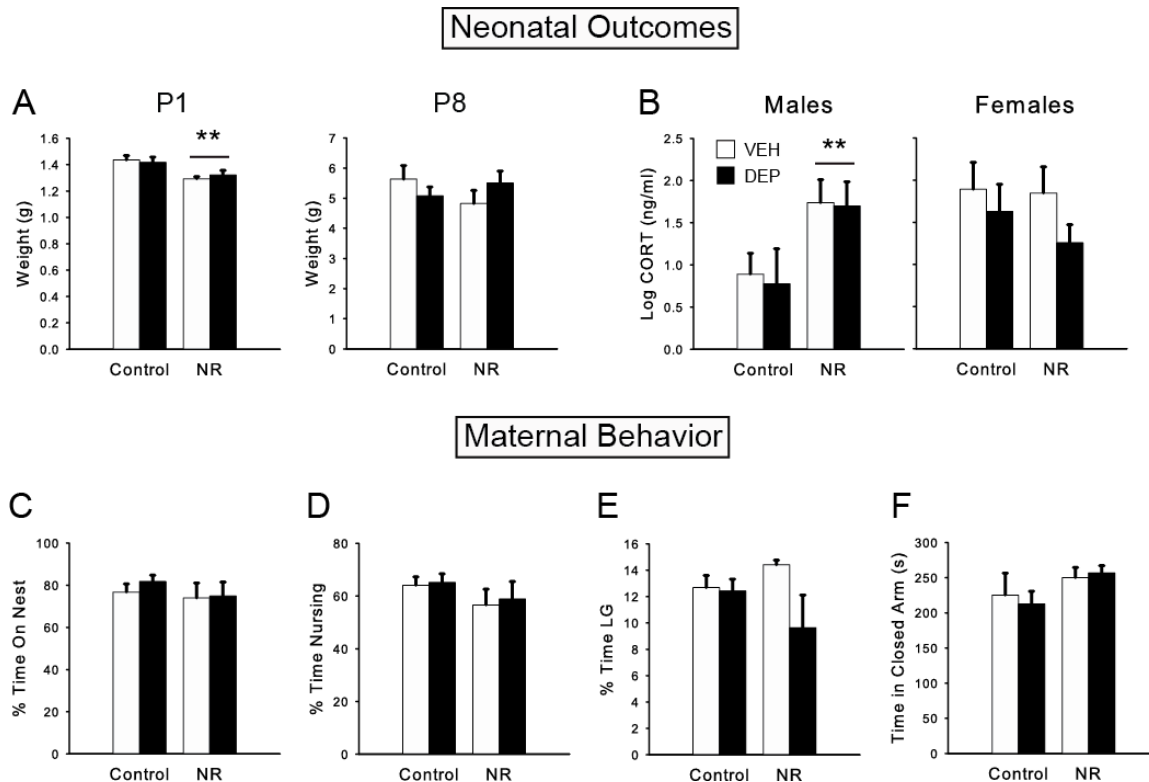
## 4.3 Results

### 4.3.1 Neonatal Outcomes and Maternal Behavior

Previous research has shown that *postnatal* NR results in more frequent dam departures from the nest, decreased pup weights and increased plasma CORT at P9 (Rice et al., 2008). In this adaptation of the model, *prenatal* NR decreased birth weights [main effect of NR,  $F(1,32)=12.09$ ,  $p<0.005$ ], regardless of prenatal DEP exposure, but weights normalized by P8 (Figure 20A). Importantly, there were no significant differences in litter size or composition due to either environmental stressor (see Table 4). In addition to the effect on birth weight, prenatal NR also increased P1 serum CORT in male pups [main effect of NR,  $F(1,20)=7.89$ ,  $p<0.05$ ], but not females [Sex  $\times$  NR interaction,  $F(1,35)=2.78$ ,  $p<0.05$ ; Figure 20B]. However, unlike postnatal NR, prenatal NR did not affect maternal behavior during P2-P9, a critical period for changes in maternal care to affect brain development (Avishai-Eliner, Eghbal-Ahmadi, Tabachnik, Brunson, & Baram, 2001). Specifically, there were no significant group differences in percent time dams spent on the nest (Figure 20C), nursing (Figure 20D), or licking and grooming (LG) their pups (Figure 20E). Furthermore, prenatal stressors did not have any enduring effects on maternal anxiety-like behavior when tested in the elevated zero-maze 60 days post-partum (Figure 20F).

**Table 4: Litter sizes and sex ratios by maternal treatment. All values are mean  $\pm$  SEM.**

| <b>Litter Size</b>         |                  |
|----------------------------|------------------|
| VEH/Control                | 9.0 $\pm$ 0.5    |
| DEP/Control                | 8.7 $\pm$ 0.4    |
| VEH/NR                     | 8.5 $\pm$ 0.8    |
| DEP/NR                     | 8.3 $\pm$ 0.5    |
| <b>Sex Ratio (% Males)</b> |                  |
| VEH/Control                | 56.9% $\pm$ 6.5% |
| DEP/Control                | 57.5% $\pm$ 7.3% |
| VEH/NR                     | 59.8% $\pm$ 3.1% |
| DEP/NR                     | 53.6% $\pm$ 5.8% |

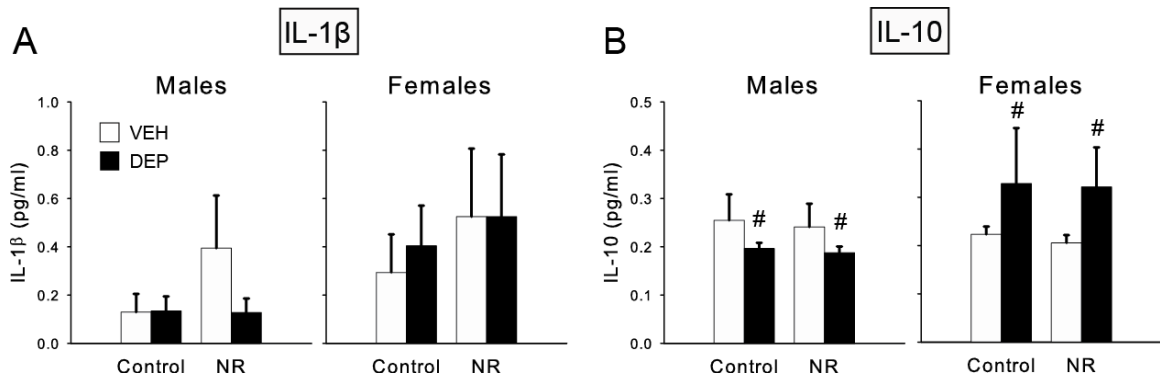


**Figure 20: Effects of prenatal DEP and NR on neonatal outcomes and maternal behavior. (A) Prenatal NR decreased P1 pup weights, but weights normalized by P8. (B) Prenatal NR increased P1 serum CORT in male, but not female pups. (C-E) Neither prenatal DEP nor NR altered the percent of time dams spent On Nest, Nursing, or LG their pups during P2-P9. (F) Prenatal DEP and NR did not have enduring effects on maternal anxiety-like behavior 60 days post-partum. Data are mean of n=8-10/group  $\pm$  SEM for pup weights, mean of n=3-8/group  $\pm$  SEM for P1 CORT, and mean of n=3-7/group  $\pm$  SEM for maternal behavior (\*\* $p$ <0.05, NR vs. Control groups).**

### 4.3.2 Fetal Brain Cytokine Analysis

The proinflammatory cytokine, IL-1 $\beta$ , was not detectable in most of the E18 brain samples, and there were no significant group differences due to sex or prenatal stressors (Figure 21A). On the other hand, the anti-inflammatory cytokine, IL-10, was detectable in a greater proportion of samples, and there was a significant Sex  $\times$  DEP interaction

[ $F(1,55)=4.24, p<0.05$ ]. Follow-up tests revealed that males tended to downregulate IL-10 in response to DEP exposure [trend for main effect of DEP,  $F(1,27)=2.43, p=0.1$ ], whereas females tended to upregulate IL-10 in response to DEP [trend for main effect of DEP,  $F(1,28)=2.395, p=0.1$ ; Figure 21B].

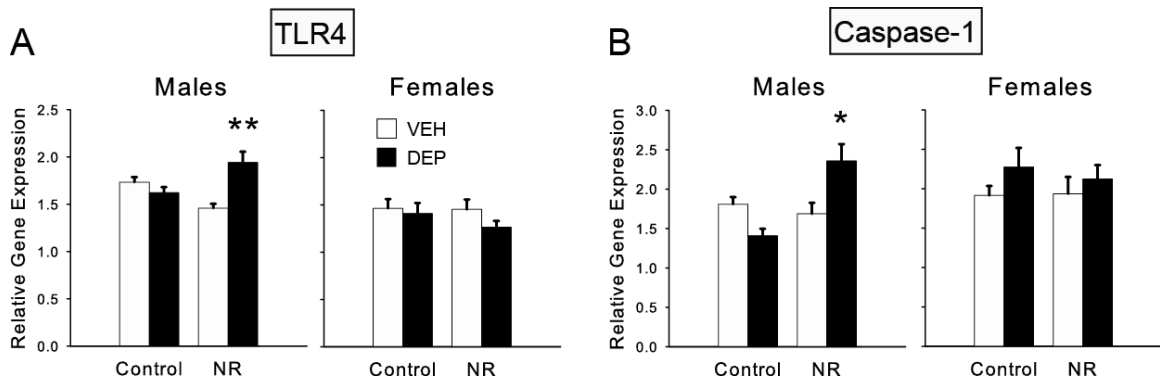


**Figure 21: Effects of prenatal DEP and NR on the fetal brain cytokine response. (A) No significant group differences were detected in IL-1 $\beta$  protein levels in the E18 brain. (B) Maternal DEP exposure elicited a sexually dimorphic IL-10 response in the fetal brain, such that male brains exhibited a downregulation of IL-10, whereas female brains exhibited an upregulation. Data are mean of  $n=7-8$ /group  $\pm$  SEM ( $\#p=0.1$  DEP vs. VEH groups, and significant Sex  $\times$  DEP interaction,  $p<0.05$ ).**

### 4.3.3 P30 Neuroimmune Gene Expression

A preliminary mouse inflammatory response 84-gene PCR array (SABiosciences/Qiagen), performed on P30 male brains, identified TLR4 as the only gene that exhibited a synergistic effect of DEP and NR (data not shown). Notably, TLR4 is an innate immune receptor critical for the response to both environmental toxins and stress (Arbour et al., 2000; Caso et al., 2008). We replicated this result with single-analyte qRT-PCR, finding a significant DEP $\times$ NR interaction [ $F(1,34)=16.79, p<0.001$ ] for P30 male brains. Specifically, the DEP/NR group had significantly higher TLR4 expression than

both the DEP/Control and VEH/NR groups ( $p < 0.05$ ; Figure 22A). Interestingly, there were no significant differences among P30 females [Sex  $\times$  DEP  $\times$  NR interaction,  $F(1,51)=9.18$ ,  $p < 0.005$ ]. In addition, expression of caspase-1, a downstream effector molecule and key enzyme for IL-1 $\beta$  production (Black et al., 1988), exhibited a similar pattern to TLR4 expression. Post hoc tests revealed that DEP/NR male brains had significantly higher expression of caspase-1 than all other groups [DEP  $\times$  NR interaction,  $F(1,37)=14.66$ ,  $p < 0.001$ ; post hoc,  $p < 0.01$ ], whereas there were no significant differences among females [Sex  $\times$  DEP  $\times$  NR interaction,  $F(1,54)=6.45$ ,  $p < 0.05$ ; Figure 22B].



**Figure 22: Effects of prenatal DEP and NR on P30 neuroimmune gene expression. The brains of DEP/NR males displayed increased TLR4 (A) and caspase-1 (B) expression, whereas DEP/NR female brains did not. Data are mean of  $n=5-13/\text{group} \pm \text{SEM}$  (\*\* $p < 0.05$  vs. DEP/Control and VEH/NR; \* $p < 0.05$  vs. all other groups).**

#### 4.3.4 Memory

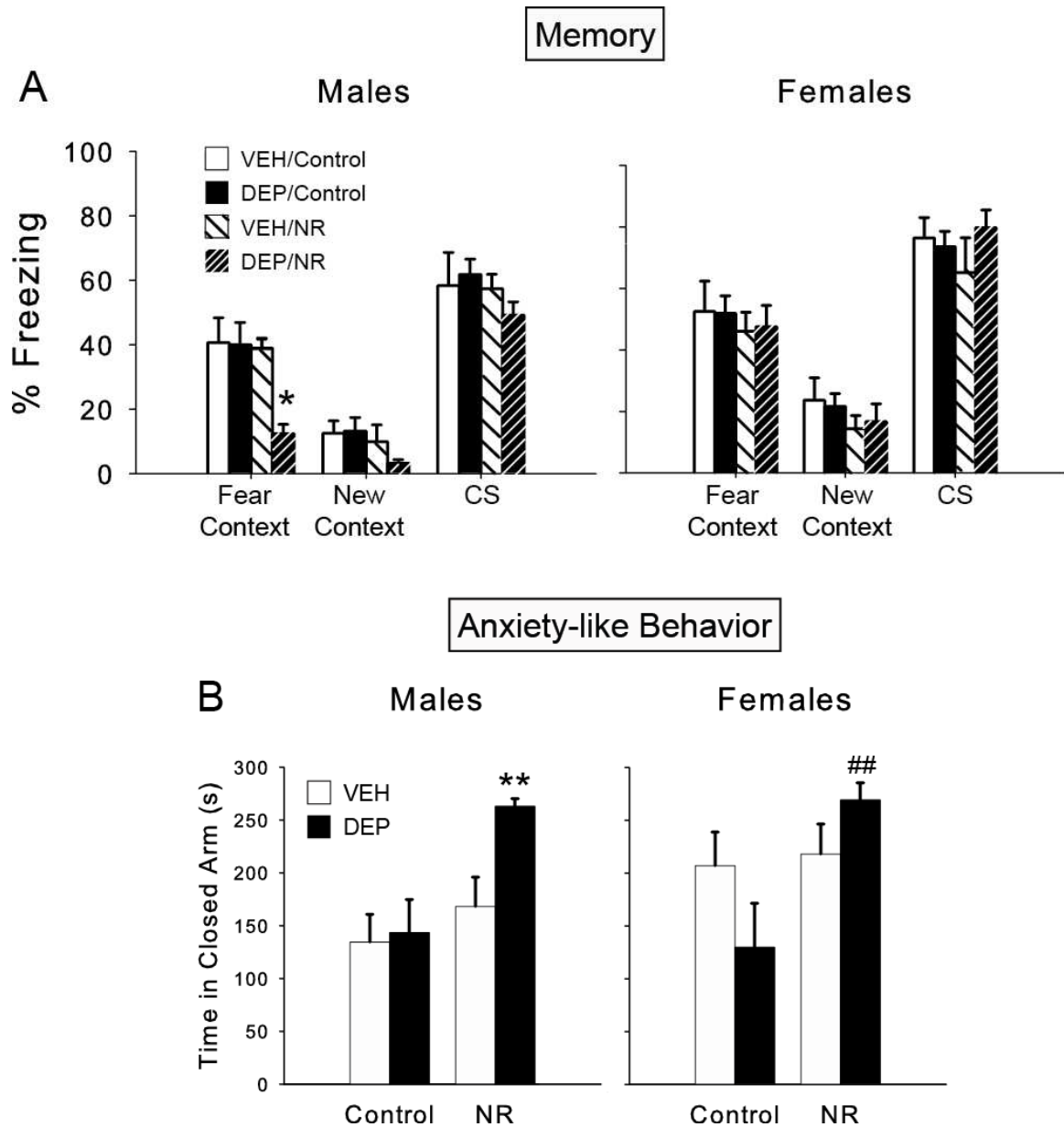
Following contextual and auditory cue fear conditioning of adult offspring, DEP/NR males froze significantly less than all other groups when assessed for contextual fear recall [DEP  $\times$  NR interaction,  $F(1,29)=5.15$ ,  $p < 0.05$ ; post hoc,  $p < 0.05$ ]. However, DEP/NR females exhibited no such hippocampal-dependent memory

impairment [trend for Sex  $\times$  DEP  $\times$  NR interaction,  $F(1,57)=2.23$ ,  $p=0.1$ ; Figure 23A].

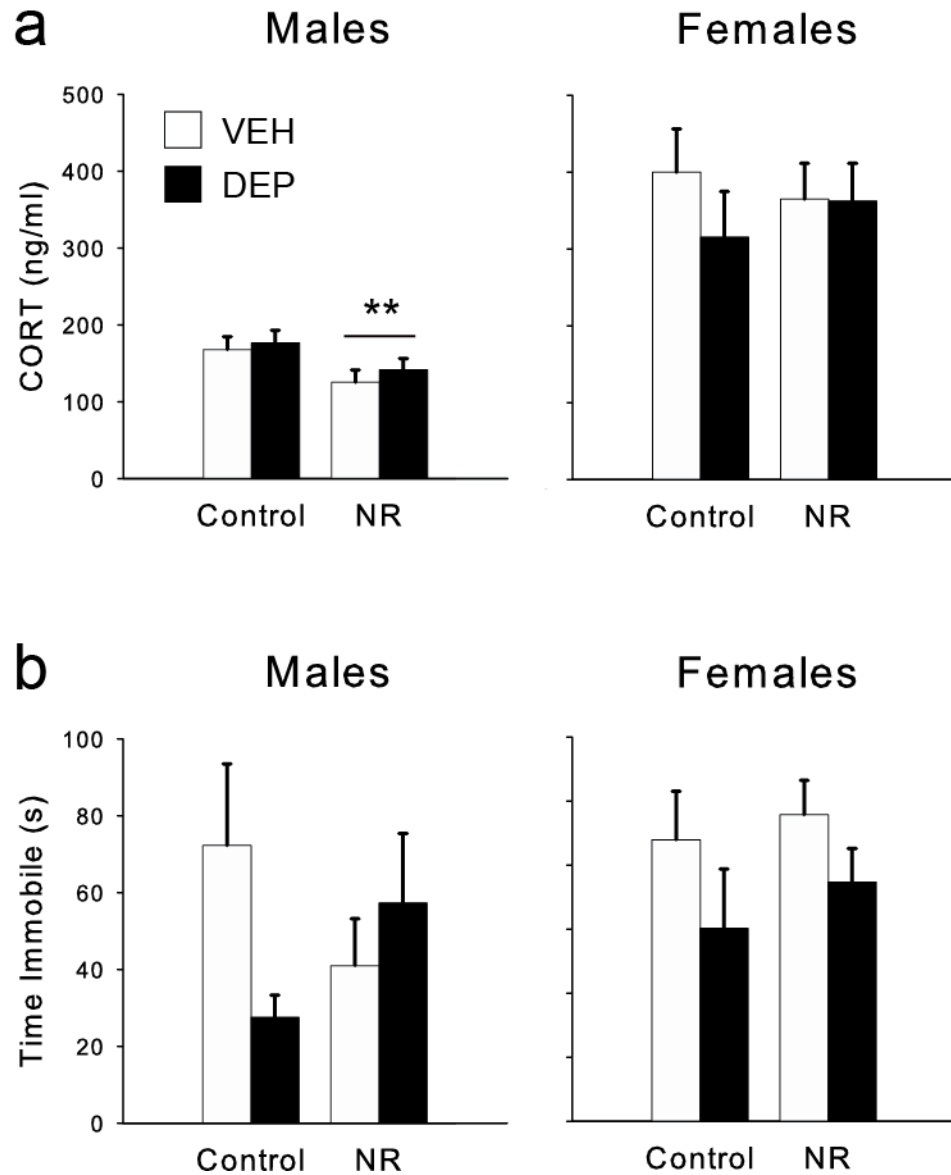
Importantly, this male-specific deficit was not due to generalized hyperactivity or an inability of the DEP/NR males to freeze, as there were no significant differences in freezing in a novel context or freezing to the auditory cue (Figure 23A). Furthermore, the females' stage of estrous cycle did not significantly affect their behavior in any of the tests (data not shown).

#### **4.3.5 Anxiety- and Depressive-Like Behavior**

In the elevated zero-maze, both adult male and female DEP/NR offspring spent more time in the closed arms, indicative of increased anxiety [DEP  $\times$  NR interaction with sexes combined,  $F(1,55)=7.350$ ,  $p<0.01$ ; Figure 23B]. Post hoc tests revealed that DEP/NR males were significantly more anxious than both VEH/Control and DEP/Control males ( $p<0.05$ ), and there was a trend for DEP/NR males to be more anxious than VEH/NR males ( $p=0.07$ ), which did not significantly differ from VEH/Control males. Furthermore, DEP/NR females were more anxious than DEP/Control females ( $p<0.05$ ), whereas VEH/control and VEH/NR females did not differ from each other. However, this increase in anxiety in the DEP/NR animals was not associated with increased serum CORT, as NR males exhibited a slight decrease in CORT immediately following the test, regardless of prenatal DEP exposure [main effect of NR,  $F(1,20)=5.286$ ,  $p<0.05$ ], and females displayed no significant differences (Figure 24A). Finally, neither males nor females displayed any significant group differences in the forced swim test (Figure 24B).



**Figure 23: Effects of prenatal DEP and NR on cognitive and affective behavior of adult offspring. (A) DEP/NR males displayed decreased freezing to the fear context, which is indicative of a hippocampal-dependent memory deficit, whereas DEP/NR females did not. (B) Both male and female DEP/NR offspring spent more time in the closed arm, which is indicative of anxiety-like behavior. Data are mean of  $n=7-9$ /group  $\pm$  SEM (\* $p<0.05$  vs. all other groups; \*\* $p<0.05$  vs. DEP/Control and VEH/Control,  $p=0.07$  vs. VEH/NR; ## $p<0.05$  vs. DEP/Control).**



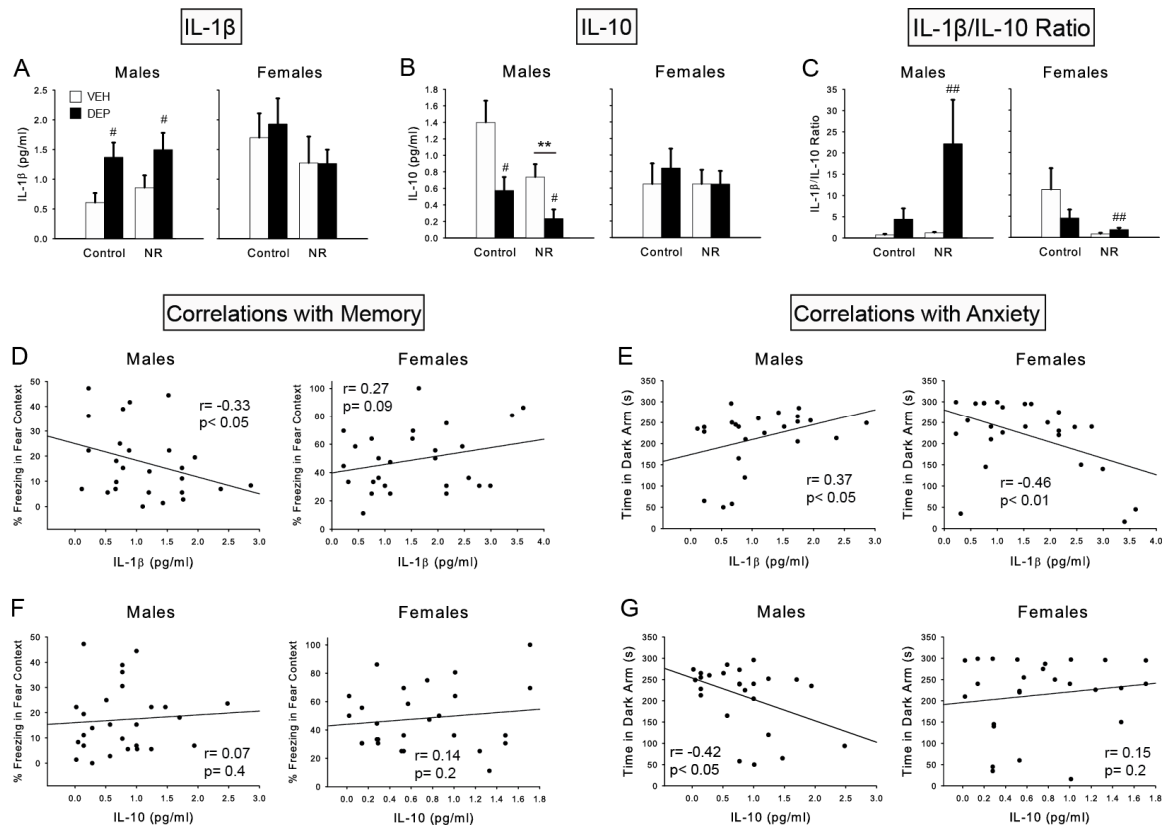
**Figure 24: (A) Male offspring exposed to prenatal NR exhibited a decreased level of serum CORT immediately following the elevated zero maze test, whereas there were no differences in female offspring. (B) Neither males nor females displayed any significant changes in depressive-like behavior in the forced swim test. Data are mean of n=7-9/group  $\pm$  SEM (\*\*p<0.05, NR vs. Control).**

#### 4.3.6 Adult Brain Cytokine Analysis

Ten days following behavioral testing, male brains from DEP groups exhibited increased levels of IL-1 $\beta$  protein [main effect of DEP,  $F(1,22)=7.84$ ,  $p<0.01$ ], whereas there were no group differences among female brains [trend for Sex  $\times$  NR interaction,  $F(1,45)=2.49$ ,  $p=0.1$ ; Figure 25A]. On the other hand, maternal DEP exposure and NR both caused decreased levels of IL-10 protein, in an additive fashion, in male brains [main effect of DEP,  $F(1,22)=12.13$ ,  $p<0.005$ ; main effect of NR,  $F(1,22)=6.89$ ,  $p<0.05$ ], whereas female brains again did not exhibit any significant group differences due to prenatal stressors [Sex  $\times$  DEP interaction,  $F(1,45)=7.10$ ,  $p<0.05$ ; Figure 25B]. Overall, DEP/NR males exhibited a greater proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) (de Wit et al., 2010) than DEP/NR females [Sex  $\times$  DEP interaction,  $F(1,39)=4.26$ ,  $p<0.05$ ; Sex  $\times$  NR interaction,  $F(1,39)=4.53$ ,  $p<0.05$ ; post hoc,  $p=0.08$ ; Figure 25C].

It was striking to us that the group differences in brain cytokine measures seemed to parallel the behavioral differences we had observed in the same animals, and indeed, further analyses revealed that the brain levels of IL-1 $\beta$  and IL-10 correlated significantly with the behavioral measures of memory and anxiety, though in a divergent manner in males and females. Males exhibited a significant negative correlation between IL-1 $\beta$  and contextual fear memory [ $r(26)=-0.33$ ,  $p<0.05$ ], such that higher levels of proinflammatory IL-1 $\beta$  were associated with decreased freezing in the fear context, whereas females showed a trend for a positive correlation [ $r(27)=0.27$ ,

$p=0.09$ ; Figure 25D]. IL-1 $\beta$  was also positively correlated with anxiety-like behavior in males [ $r(24)=0.37$ ,  $p<0.05$ ], such that higher levels of IL-1 $\beta$  were associated with increased time spent in the dark arm of the elevated zero-maze, whereas in females IL-1 $\beta$  was negatively correlated with anxiety-like behavior [ $r(26)=-0.46$ ,  $p<0.01$ ; Figure 25E]. On the other hand, anti-inflammatory IL-10 exhibited no significant correlations with memory in males or females (Figure 25F), but it was negatively correlated with anxiety-like behavior in males [ $r(25)=-0.42$ ,  $p<0.05$ ] and not females (Figure 25G).

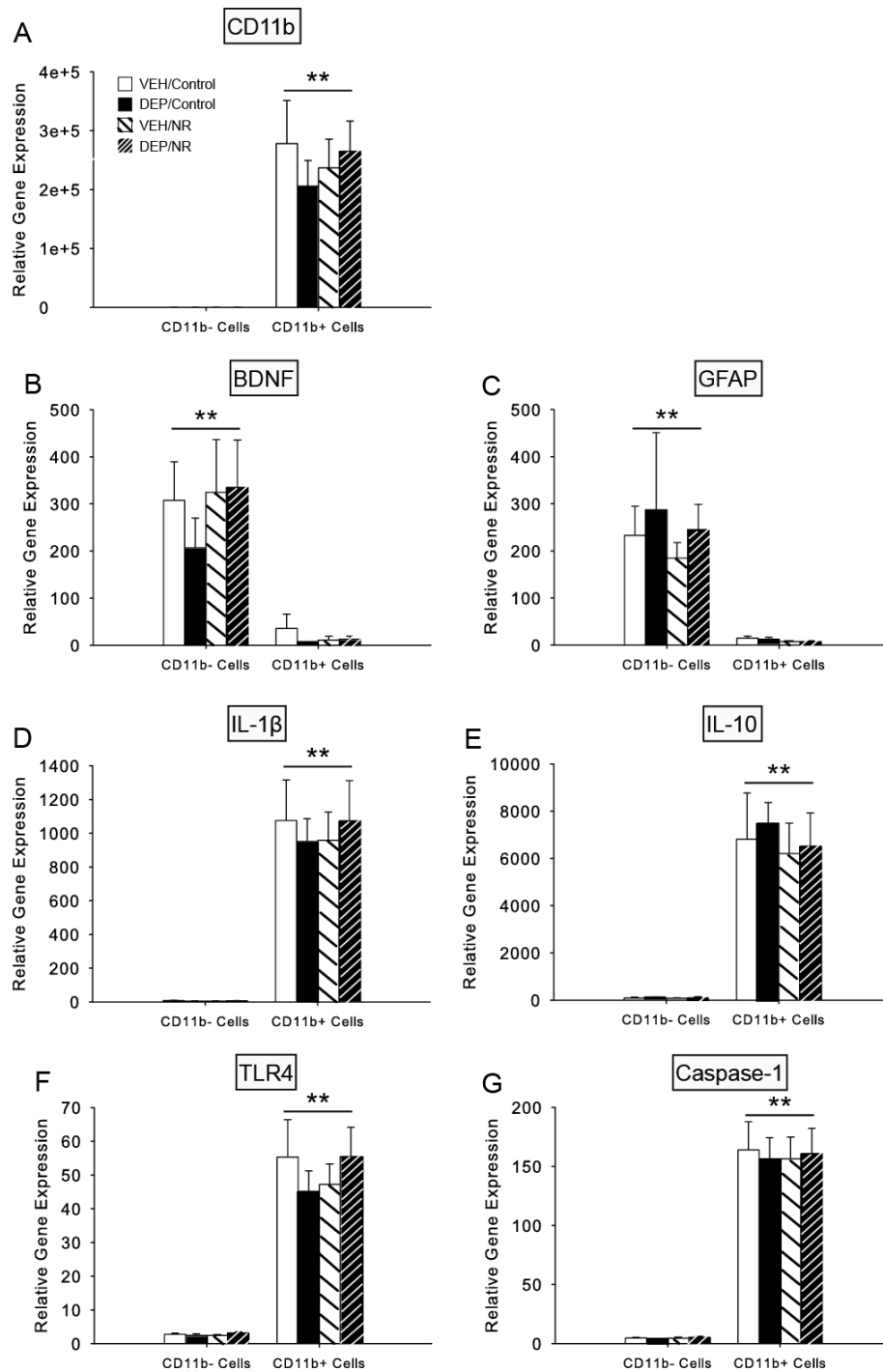


**Figure 25: Effects of prenatal DEP and NR on adult brain cytokine levels. (A) Male offspring exhibited a significant increase in brain IL-1 $\beta$  due to prenatal DEP exposure, whereas females did not. (B) Male offspring exhibited a significant decrease in brain IL-10 in response to both DEP and NR, whereas females did not. (C) Overall, DEP/NR males exhibited a greater proinflammatory bias (IL-1 $\beta$ /IL-10 ratio)**

than DEP/NR females. Data for A-C are mean of n=5-8/group  $\pm$  SEM ( $^{\#}p<0.05$  DEP vs. VEH groups;  $^{**}p<0.05$  NR vs. Control groups;  $^{\#\#}p=0.08$ , DEP/NR males vs. DEP/NR females, and significant Sex $\times$ DEP and Sex $\times$ NR interactions,  $p<0.05$ ). (D-E) Brain IL-1 $\beta$  was significantly correlated with memory and anxiety measures in both males and females, though in opposite directions. (F) Brain IL-10 was not correlated with memory performance in males or females. (G) Brain IL-10 was negatively correlated with anxiety-like behavior in males, but not in females. Data for D-G are correlated measures for individual animals from the whole cohort (n=24-27 total/sex).

#### 4.3.7 Gene Expression of Isolated CD11b<sup>+</sup> and CD11b<sup>-</sup> Cells

To assess the purity of the isolated cells, we confirmed that CD11b mRNA was expressed only in CD11b<sup>+</sup> cells, as expected [main effect of cell population,  $F(1,64)=71.68$ ,  $p<0.001$ ; Figure 26A]. In contrast, CD11b<sup>-</sup> cells expressed ~20-fold higher GFAP and BDNF mRNA than CD11b<sup>+</sup> cells (all  $p$ 's $<0.001$ ; Figure 26B,C), indicating that this cell population (~80% of total cells) contains astrocytes and neurons. Isolated CD11b<sup>+</sup> cells (microglia) had ~170-fold higher levels of mRNA for IL-1 $\beta$  and ~80-fold higher levels for IL-10 than CD11b<sup>-</sup> cells (all  $p$ 's $<0.001$ ; Figure 26D,E). In addition, CD11b<sup>+</sup> cells expressed ~40-fold more caspase-1 and ~20-fold more TLR4 than CD11b<sup>-</sup> cells (all  $p$ 's $<0.001$ ; Figure 26F,G). However, there were no significant group differences in gene expression due to sex or prenatal stressors.



**Figure 26: Isolated CD11b+ cells (microglia) expressed markedly higher levels of CD11b (A), IL-1 $\beta$  (D), IL-10 (E), TLR4 (F), and caspase-1 (G) than did CD11b- cells (neurons, astrocytes). CD11b- cells expressed significantly higher levels of BDNF (B) and GFAP (C). Data are mean of n=5/group  $\pm$  SEM (\*\* $p$ <0.05, CD11b+ vs. CD11b- cells).**

#### **4.4 Discussion**

We report for the first time that maternal stress during late pregnancy exacerbates the impact of air pollution *in utero* on mental health outcomes in adult offspring, which are associated with alterations in neuroinflammatory tone. The adult males of dams exposed to combined stressors exhibit both striking memory deficits and anxiety, whereas females display only slightly increased anxiety. Importantly, the impact of prenatal events on offspring behavior is always complex, and may involve changes in several physiological pathways, as well as potential alterations in maternal-offspring interactions after birth—both of which can profoundly modify neural development (Caldji et al., 1998). Maternal stress reduced birth weight and increased corticosterone at birth in males, independent of maternal exposure to diesel exhaust particles. However, maternal care was not significantly altered, and no changes in maternal anxiety were observed as a consequence of prenatal treatment. Furthermore, no corticosterone differences were detected in adulthood that could explain behavioral changes in the combined stressor offspring. Instead, the alterations in brain cytokines, occurring in a sexually dimorphic manner, may underlie distinct behavioral phenotypes in adult male and female offspring. An optimal balance of central proinflammatory cytokines, such as IL-1 $\beta$ , is critical for mental health, including mood regulation and hippocampal-dependent learning and memory (Yirmiya & Goshen, 2011). In contrast, high levels of anti-inflammatory IL-10 are protective against behavioral changes due to

microglial-driven neuroinflammation (Schwarz et al., 2011). Therefore, the diminished IL-10, in combination with increased IL-1 $\beta$ , in DEP/NR males could underlie their relative vulnerability to cognitive impairments and mood dysregulation, in comparison to DEP/NR females, which do not exhibit such a proinflammatory bias. The underlying mechanism of this sex difference warrants further exploration. Importantly, there is a male bias in the prevalence of neurodevelopmental disorders, including learning disabilities (Flannery et al., 2000) and autism (Stone et al., 2004), in addition to gender differences in childhood outcomes following maternal stress during pregnancy (Cao, Laplante, Brunet, Ciampi, & King, 2012; Fang et al., 2011).

Our data from isolated CD11b<sup>+</sup> and CD11b<sup>-</sup> cells demonstrate that microglia, not neurons or astrocytes, are the primary source of the measured cytokines in the brain, suggesting that they are a target of “programming” by the prenatal stressors. Microglia begin to colonize the rodent brain around E9-10 (Ginhoux et al., 2010) and critically shape several aspects of normal brain development. Importantly, microglia largely remain in an activated, amoeboid state until the early postnatal period (E. A. Ling & Wong, 2004), which we have demonstrated makes them especially sensitive to long-term functional changes by perinatal inflammatory events (Bilbo & Schwarz, 2009; Williamson et al., 2011). However, we did not detect any significant differences in gene expression of cytokines in isolated adult microglia due to prenatal stressors, despite the clear differences in cytokine protein. This discrepancy may stem from the fact that the

animals used for protein analysis underwent behavioral testing, whereas the animals used for CD11b isolation and gene expression analysis were behaviorally naïve. It is possible that behavioral testing may serve as a sufficient stressor to elicit relatively long-term increases in cytokine levels (i.e., enduring until tissue collection) in the brains of the DEP/NR animals, which would not be observed at baseline. Alternatively, there may be additional regulatory mechanisms at work. For instance, the selective increase in caspase-1 expression in DEP/NR males supports a role for the inflammasome, which is critical for the cleavage of proIL-1 $\beta$  into its mature form (Latz, 2010), although we did not examine this directly. Importantly, TLR4 signaling is required for the activation of the inflammasome (Bauernfeind et al., 2009). Furthermore, DEP and maternal stress-induced signals may converge on microglia via TLR4, which we show is predominantly expressed by microglia, consistent with our previous findings in rats (Schwarz & Bilbo, 2013), and is exaggerated in the brains of DEP/NR males. TLR4 is an important innate immune receptor that recognizes pathogen-associated molecular patterns (e.g., LPS), but also endogenous danger-associated molecular patterns released in response to cellular distress (e.g., DEP-induced hyaluronan or HMGB1) (Bianchi, 2007). Notably, glucocorticoids may upregulate TLRs on microglia, augmenting subsequent neuroinflammatory responses (Frank, Miguel, Watkins, & Maier, 2010; Garate et al., 2013). Thus, TLR4 upregulation may occur to a greater extent in males than females as a result of the significant increase in serum corticosterone in males born to stressed dams.

Although the alterations in IL-1 $\beta$  we observed in adulthood were not present in the fetal brain, we did observe a significant sex difference in the fetal brain IL-10 response to maternal DEP exposure. Specifically, DEP males exhibited a downregulation of IL-10, whereas DEP females displayed an upregulation of this anti-inflammatory cytokine, which may have been protective against the impairment of neurodevelopment by prenatal stressors. In addition to brain cytokines, the placenta has been increasingly implicated as a key player in brain development, as well as in the fetal response to prenatal insults (Hsiao & Patterson, 2012), and warrants further exploration in our model. Thus, our working hypothesis is that maternal stress-induced changes in TLR4 signaling enhance the effects of a chemical exposure such as DEP, likely involving the maternal-placental-fetal interface (Auten et al., 2009; Auten et al., 2012), and ultimately converging onto microglia within the fetal brain, resulting in the long-term alteration of brain function and behavior.

In closing, we have demonstrated for the first time that maternal psychological stress induced by resource deprivation during late pregnancy increases the vulnerability of murine offspring, particularly males, to *in utero* air pollutant exposure. Furthermore, it is clear that developmental exposure to maternal stress and air pollution, similar to other environmental compounds such as pesticides and LPS (Z. Ling et al., 2004), can have a long-lasting impact on microglial function and neuroinflammation. Future studies aimed at elucidating the complex interactions of psychosocial and chemical

stressors will be critical for informing environmental and public health policy and identifying effective interventions.

## **5. Prenatal Air Pollution Exposure Modulates Placental Immune Responses and Microglial Maturation in a TLR4- and Sex-Dependent Manner**

### ***5.1 Introduction***

We have now observed strikingly similar long-term changes in TLR4 expression in the brains of male offspring in two distinct “2-hit” models that include prenatal air pollution exposure (one involving adult HFD and one involving simultaneous maternal stress during gestation). In both models, we have also observed greater adverse outcomes in terms of behavioral, metabolic, and neuroinflammatory changes in the male compared to the female offspring. This sexual dimorphism is consistent with a growing recognition in the field of fetal programming of sex differences in offspring outcomes following maternal immune challenges during pregnancy. For example, male offspring of asthmatic mothers are more likely to suffer severe complications, such as premature birth or stillbirth, than female offspring in response to an acute asthma exacerbation during pregnancy (Murphy et al., 2005). Furthermore, there is a well-documented, but poorly understood, male bias in the prevalence of neurodevelopmental disorders, including learning disabilities (Flannery et al., 2000) and autism (Stone et al., 2004), which is consistent with our data.

Clifton (2010) has proposed that these sex differences in offspring outcome may be due to the sexually dimorphic strategies males and females employ in a compromised intrauterine environment (Clifton, 2010). Specifically, male placentae respond to

maternal inflammation with few changes in gene expression in order to allow for accelerated or continued growth, but at the cost of increased risk for adverse outcomes. On the other hand, female placentae respond with multiple gene changes that enhance the fetal immune response to the maternal immune challenge, which leads to a minor decrease in growth, but has adaptive value overall in promoting survival, especially in the face of further maternal insults (Murphy et al., 2003; Murphy et al., 2005; Scott et al., 2009). Our finding of a sexually dimorphic response during gestation to prenatal DEP exposure, in which female brains upregulate IL-10 in response to DEP, whereas male brains downregulate IL-10, is in agreement with Clifton's hypothesis.

I hypothesize that these sex differences in the innate immune response of the brain may extend further back in development to the first neuroimmune organ, the placenta. In support of this hypothesis, there is evidence that normal human term placentae from males contain more macrophages than placentae from females (Scott et al., 2009), and that cultured placental trophoblasts from normal male fetuses possess higher levels of TLR4 than trophoblasts from female fetuses (Yeganegi et al., 2009). Thus, it is possible that a different density of Hofbauer cells in the male vs. female placenta, in addition to sexually dimorphic placental TLR4 expression, could underlie the sex difference in the brain cytokine response to DEP in our model, which in turn could contribute to the observed sex differences in adult outcomes.

In order to test this hypothesis, we first analyzed the TLR4<sup>+/-</sup> and TLR4<sup>-/-</sup> male and female placentae and brains resulting from the cross of TLR4<sup>+/-</sup> females to TLR4<sup>-/-</sup> males, in which the dams were treated with VEH or DEP intermittently throughout gestation. We exhaustively counted the number of Hofbauer cells (placental macrophages) and microglia, respectively, as well as analyzed cytokine protein levels of IL-1 $\beta$  and IL-10 in each tissue. In addition, to address the underpinnings of the long-term changes in behavior that we have observed in previous studies, we also conducted unbiased stereology of P30 offspring brains to obtain counts and morphological analysis of microglia. In this final experiment, P30 wild-type offspring were treated prenatally with either VEH or DEP, and then treated with saline (SAL) or LPS just prior to sacrifice in order to assess potential priming effects of the DEP exposure.

## **5.2 Methods**

### **5.2.1 Experiment 1: E18 Cohort**

#### **5.2.1.1 Animals**

Adult male TLR4 deficient (TLR4<sup>-/-</sup>) and female TLR4 heterozygous (TLR4<sup>+/-</sup>) (C57BL/6 background) mice were housed in individually ventilated, microisolator polypropylene cages with specialized bedding (Alpha-Dri; Shepherd Specialty Papers, Milford, NJ, USA; used to avoid exposure to potentially confounding antigens that can be found in typical bedding) and given *ad libitum* access to food (PicoLab Mouse Diet 5058, Lab-Diet, Philadelphia, PA, USA) and filtered water. The colony was maintained at

22°C on a 12:12-h light-dark cycle (lights on at 7 AM). Following acclimation to laboratory conditions, males were placed with 2 females each for breeding, for a maximum of 2 weeks. TLR4<sup>-/-</sup> males were mated with TLR4<sup>+/-</sup> females, in order to produce litters of approximately 50% TLR4<sup>+/-</sup> and 50% TLR4<sup>-/-</sup> fetuses. Females were examined twice daily for evidence of a vaginal plug [confirmation of successful mating, considered to be E0], at which point they were separated from the male and caged with 1-4 other successfully mated females. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

#### **5.2.1.2 Diesel Exhaust Particle (DEP) Exposures**

Beginning on the morning of E2, time-mated females were treated with diesel exhaust particles (DEP) delivered by oropharyngeal aspiration (the method and detailed analysis of DEP have been previously described) (Auten et al., 2012; Bolton et al., 2013). Females received 50 µg DEP suspended in 50 µl vehicle (DEP group, n=9) or 50 µl vehicle (VEH group, n=7) on E2, E5, E8, E12, and E16.

#### **5.2.1.3 Tissue Collection**

Fetal brains and placentas were collected at E18. Fetuses were sexed and their *Tlr4* genotype determined by subsequent PCR analysis of the *Sry* and *Tlr4* genes from tail samples. At the time of tissue collection, pregnant mice were deeply anesthetized with sodium pentobarbital, and fetuses were removed by hysterotomy. For half of each litter (randomly chosen), each fetus's placenta and whole brain was removed and

weighed, before being placed into a 1.5-ml microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80°C until processing. For the other half of each litter, each fetus's placenta and whole head was removed and placed in 1.5-ml microcentrifuge tubes filled with 10% formalin. Twenty-four h later, fixed placentas were transferred into 70% ethanol, and heads were transferred into 0.1% sodium azide, for long-term storage at 4°C. Three days prior to slicing, heads were submerged in 10% gelatin solution. After the gelatin solidified, the gelatin-submerged heads were transferred to cold 4% paraformaldehyde for 24 h before being transferred into 30% sucrose for 48 h prior to freezing and slicing. Placentas were submerged in 30% sucrose for 2 days prior to freezing and slicing.

#### **5.2.1.4 SRY Genotyping**

DNA extraction of tail snips was performed by heating the samples for 1 h at 95°C in a basic solution (25 mM NaOH, 0.2 mM EDTA), then adding an acidic solution (40 mM Tris HCl) to neutralize the pH. Each sample was centrifuged at 400 x g for 10 min at 4°C, and the supernatant was collected. Subsequently, PCR amplification was performed, which consisted of heating samples at 94°C for 3 min, followed by 34 cycles of 94°C for 20 s, 67.5°C-64.5°C for 30 s (the first 6 cycles comprised a touchdown from 67.5°C to 64.5°C, in half-degree increments), and 72°C for 40 s, and finished at 72°C for 50 s. The PCR reaction consisted of 1 µl of sample and 24 µl of BIOLASE PCR mix (Bioline USA Inc., Taunton, MA, USA) containing forward (5'-

TGGGCTGGACTAGGGAGGTCC-3') and reverse (5'-TGCTGGGCCAACTTGTGCCT - 3') SRY primers and 3 TLR4 primers: forward (5'- CTG ACG AAC CTA GTA CAT GTG GA), reverse (5'- ACC TCT TAG AGT CAG TTC ATG GA), and Neo-rrt1 (5'- TGG CGG ACC GCT ATC AGG AC). TLR4<sup>+/-</sup> fetuses should have 2 bands, one of 280 bp and one of 186 bp, whereas TLR4<sup>-/-</sup> fetuses should have one band of 280 bp. The Y chromosome-specific product expected from the amplification of male DNA was 431 bp. Products were resolved on a 1.7% agarose gel stained with 5% GelStar Nucleic Acid Stain (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA).

#### **5.2.1.5 Cytokine Protein Measurements**

Commercial ELISA kits (R&D Systems, Minneapolis, MN) were used to measure interleukin (IL)-1 $\beta$ , a proinflammatory cytokine, and IL-10, an anti-inflammatory cytokine, in fetal placenta and brain homogenates normalized to total protein (200  $\mu$ g/well) and lipid-depleted [n=5-8/sex/genotype/treatment (Bolton et al., 2013)]. These cytokines were selected due to their important role in microglial function, brain development, and behavior (Williamson et al., 2011; Yirmiya & Goshen, 2011).

#### **5.2.1.6 Immunohistochemical detection of Iba1 protein**

Placentas and gelatin-blocked brains were sectioned exhaustively in a 1:5 series at 12  $\mu$ m and 14  $\mu$ m, respectively, on a Leica cryostat at 20°C and thaw-mounted directly onto Superfrost+ Micro Slides (VWR), where they were allowed to dry before being stored at 4°C. The ionized calcium-binding adaptor molecule Iba1 protein was selected

for staining because it is specific to macrophages, including Hofbauer cells within the placenta and microglia within the brain parenchyma, its expression is constitutive, and it labels the entire cell body including processes, thus allowing an accurate assessment of morphology. Slides were washed with phosphate-buffered saline (PBS) and incubated for 1 h in PBS with 1% H<sub>2</sub>O<sub>2</sub>, 10% normal goat serum, and 0.9% Triton X to quench endogenous peroxidase, block, and permeabilize, respectively. Slides were then incubated with 200 µL of primary antibody (rabbit anti-Iba1, 1:500; Wako Chemicals, Richmond, VA, USA) overnight at room temperature. On the next day, slides were washed and incubated with a biotinylated secondary antibody (goat anti-rabbit IgG, 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 h room temperature. Slides were washed, and immunostaining was identified by the streptavidin/horseradish peroxidase technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromagen. Afterwards, slides were dehydrated and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

#### **5.2.1.7 Stereology**

Iba1-labeled cells were counted using the optical fractionator method within StereoInvestigator software (Microbrightfield Inc., Williston, VT, USA) (Bland et al., 2010; Glaser, Greene, & Hendricks, 2007; Mouton et al., 2002). For analysis, we set an optical dissector height of 5 µm with a 1-µm guard zone on top and bottom, and

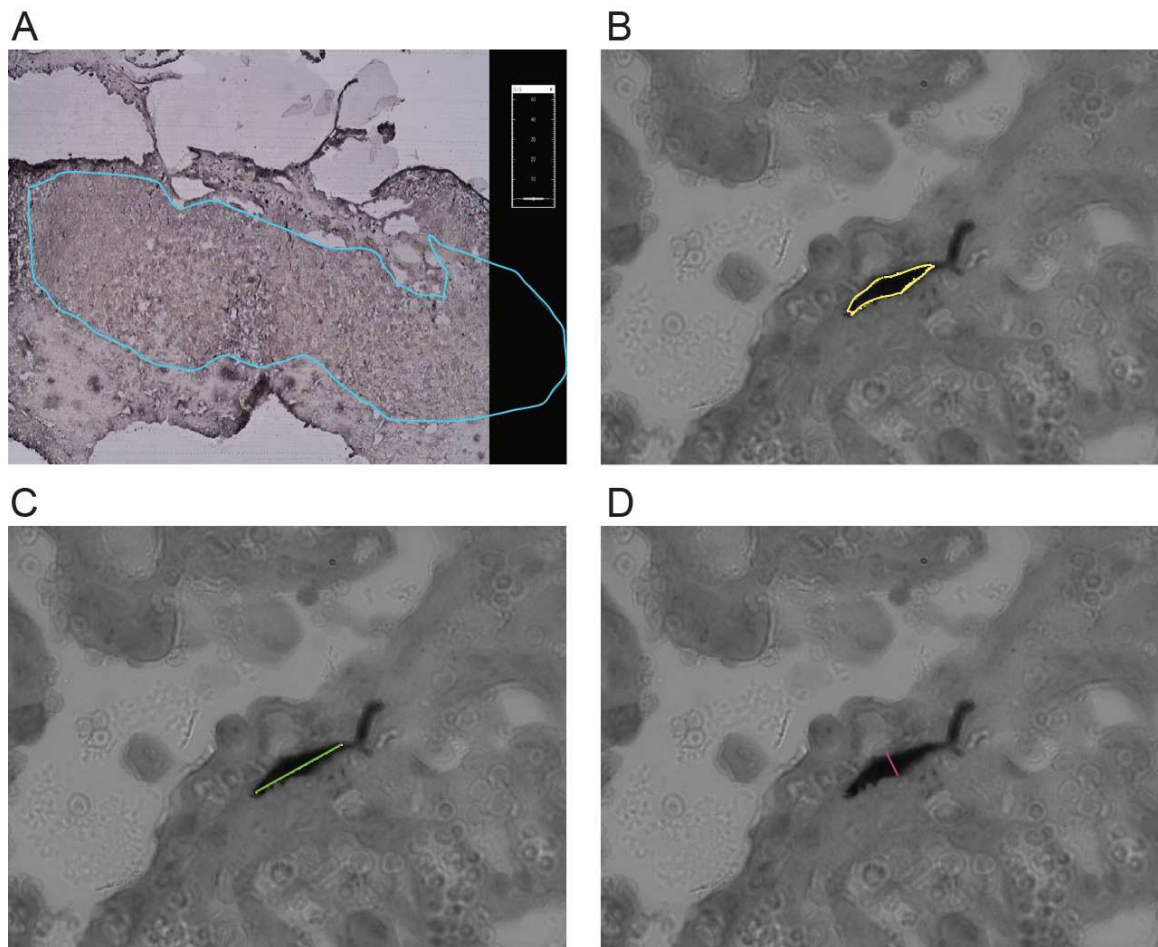
counted stained cells within each frame using a 100X oil objective lens. Cells were only counted if the entire, rounded cell body was visible (average diameter = between 15 and 25  $\mu\text{m}$ ) and the stain appeared uniformly dark and apparent throughout the cell, to avoid counting a cell fragment.

#### *5.2.1.7.1 Placental Macrophage Counts and Morphological Analysis*

A 100  $\mu\text{m}$  by 100  $\mu\text{m}$  counting frame was used to systematically divide the entire section into countable frames. The counting region was drawn to only include the fetal labyrinth and to exclude the maternal sides of the placenta (Figure 27A), with darker clumps due to inconsistency in staining omitted to keep consistent results. While the fetal side was infiltrated with macrophages, the maternal sides of placenta had very few Iba1+ cells. In addition, edges were included in the counting frame only if they were not affected by the “edge effect” in which staining was concentrated around the outer parts of the tissue. Rips in the tissue were also omitted to keep a consistent recording of the tissue area and volume.

For analysis of placental macrophage morphology, images of cells were taken at 100X oil objective lens using StereoInvestigator Software (Microbrightfield Inc., Williston, VT, USA). Previous studies have shown cell elongation to affect cytokine expression in Hofbauer cells. In the presence of inflammatory stimuli, Hofbauer cells polarize towards a rounder, less elongated state. However, in cells exposed to IL-4 and IL-13, macrophages are more elongated, reverting to a prohealing state (McWhorter, Wang, Nguyen, Chung, & Liu, 2013). We measured cellular dimensions to study the

interaction between DEP, genotype, and sex on cell elongation. The long and short axes of cells were measured using ImageJ software (National Institutes of Health). The long axis was defined as the longest length of the cell (Figure 27C), while the short axis was defined as the widest length perpendicular to the long axis and across the nucleus (Figure 27D). The elongation factor was measured by determining the ratio of long axis length to short axis length. The area was also measured by manually tracing an outline around the dark edges of the cell (Figure 27B).



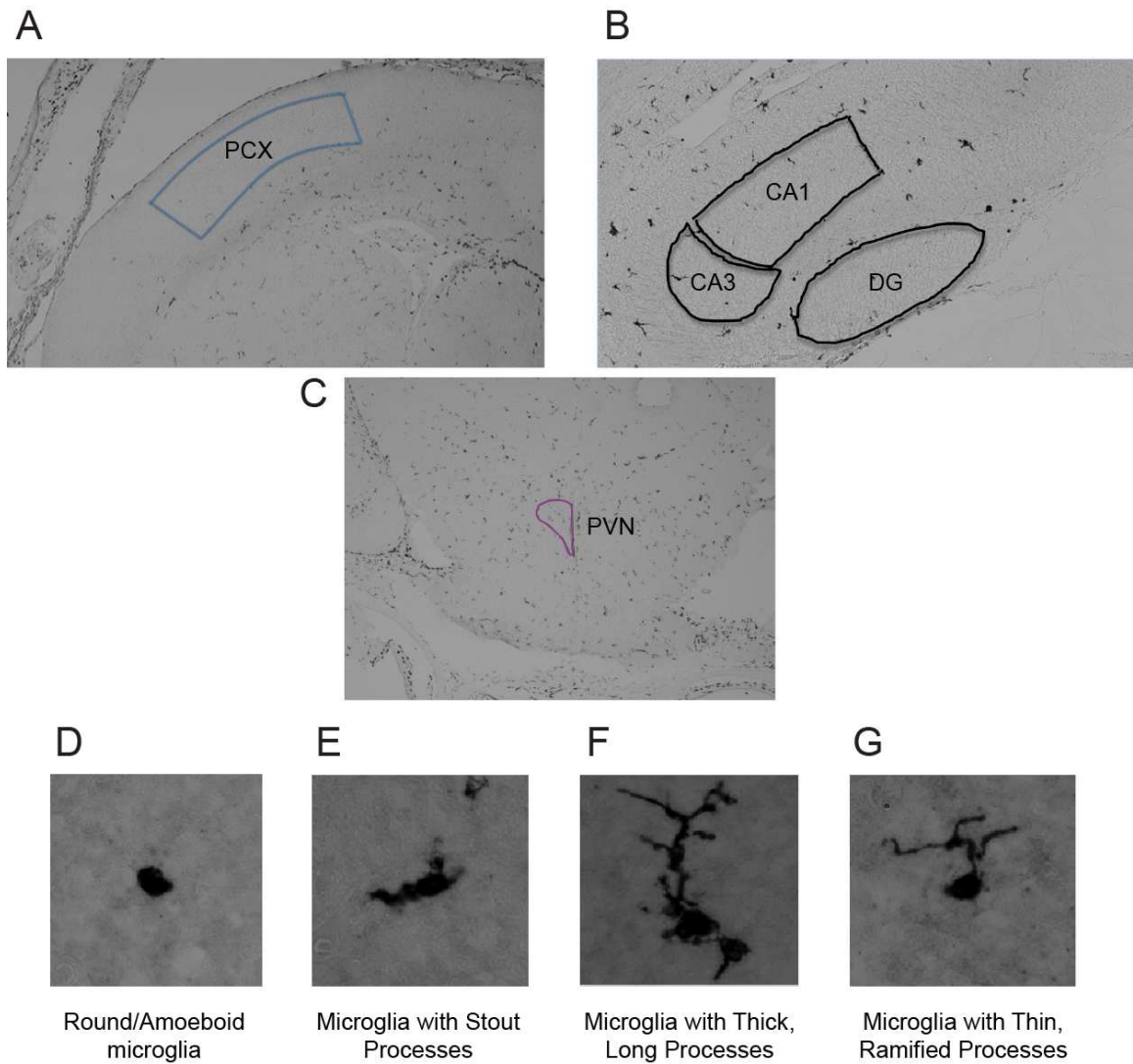
**Figure 27: A) The drawing of the placenta counting contour, encompassing the fetal labyrinth. B) The measurement of the cell area, defined as the contour surrounding all dark edges of the cell. C) The measurement of the long axis, defined as the longest length of the cell. D) The measurement of the short axis, defined as the line crossing the nucleus and perpendicular to the long axis.**

#### *5.2.1.7.2 Microglia Counts and Morphological Analysis*

We used an exhaustive 50  $\mu\text{m}$  by 50  $\mu\text{m}$  counting frame to count cells throughout each section for each section counted. For each animal, we analyzed every section throughout the paraventricular nucleus of the hypothalamus (PVN), parietal cortex and hippocampus (dentate gyrus, CA3, and CA1). Nine sections of the parietal cortex were

analyzed for every animal, 7 per dentate gyrus, 4 per CA3, 6 per CA1, and 4 per PVN. For each section examined, the area was calculated by the StereoInvestigator software based on the boundaries of the contour tracings (Figure 28A-C). Regional volume estimates were obtained by summing the areas given by the Cavalieri estimator for each section, and then multiplying this value by the product of the pre-histology thickness of each section (14  $\mu\text{m}$ ) and the number of sections examined.

Iba1-positive cells were classified into 4 morphological types based on their cell shape and the configuration of their processes (Gomez-Gonzalez & Escobar, 2010; Kreutzberg, 1996; Wu, Wen, Shieh, & Ling, 1992; Wu, Wen, Shieh, & Ling, 1993). These four cell types consisted of round/amoeboid microglia, microglia with stout processes, microglia with thicker, longer processes, and microglia with thinner, more ramified processes (Figure 28D-G). The number of subjects analyzed varied per region and group,  $n=2-5/\text{group}/\text{sex}$  for the PVN, PCX, DG, CA3, and CA1.



**Figure 28: A-C) Example contours drawn around the E18 parietal cortex (A) and hippocampus (B) in the StereoInvestigator software. Hippocampal regions going clockwise, starting from the bottom left: CA3, CA1, dentate gyrus. Photos were taken of Iba1-stained section at the 4X objective. D-G) The four primary microglial morphological states on E18. During development, microglia possess round/amoeboid morphology (D) initially and gradually progress to a morphology with thin, ramified processes (G). If activated in adulthood, their morphology once again assumes the round/amoeboid shape. Photos were taken of Iba1-labeled microglia in the mouse CA1 at the 100X objective.**

### **5.2.1.8 Data Analysis**

All data were analyzed with SigmaStat statistical software (Systat Software Inc., San Jose, CA). Three-way (DEP X Genotype X Sex) were performed first, and following a significant or trending ( $p < 0.1$ ) interaction with Sex, two-way (DEP X Genotype) ANOVAs within each sex were used to assess differences in regional volume ( $\text{mm}^3$ ), the total number of cells/ $\text{mm}^3$ , and the number of cells (of the 4 different morphologies for microglia)/ $\text{mm}^3$ . Following significant interactions, *post hoc* comparisons (Fisher's LSD) were performed to further distinguish between groups.

## **5.2.2 Experiment 2: P30 Cohort**

### **5.2.2.1 Animals**

Adult male and female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC, USA) and housed in individually ventilated, microisolator polypropylene cages with specialized bedding (Alpha-Dri) and ad libitum food (PicoLab Mouse Diet 5058; Lab-Diet, Philadelphia, PA, USA) and filtered water. The colony was maintained at 22 °C on a 12:12-h light–dark cycle (lights off at 9 AM). Following acclimation to laboratory conditions for 1 week, males were paired with 2 females each for timed mating, for a maximum of 2 weeks. Females were examined twice daily for evidence of a vaginal plug [confirmation of successful mating, considered to be embryonic day (E)0], at which point they were separated from the male and housed

with another pregnant female. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

#### **5.2.2.2 DEP Exposures**

Female DEP treatment was carried out as described above. Females received 50 µg DEP suspended in 50 µl vehicle (DEP group; n=5 dams) or vehicle alone (VEH group; n = 7 dams) every 3 days E2-E17 for a total of 6 doses, as a model of intermittent exposure. Following the final exposure on E17, females were allowed to give birth normally and their offspring remained unmanipulated until P30.

#### **5.2.2.3 P30 Injections**

P30 offspring (n = 6–8/group/sex) received an i.p. injection of either sterile saline or 165 µg/kg LPS derived from *Escherichia coli* (serotype 0111:B4; Sigma, St. Louis, MO, USA) between 8 AM and 10 AM, and blood and brain tissues were collected 2 h later. This dose and time point were selected based on previous studies (Bilbo et al., 2005; Godbout et al., 2005) and initial pilot experiments from our lab demonstrating mild sickness behavior and a robust but submaximal IL-1β response under these conditions in the brains of control mice (unpublished data).

#### **5.2.2.4 Tissue Collection**

Mice were deeply anesthetized with a ketamine/xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine i.p.). Immediately afterward, mice were transcardially perfused with ice-cold saline for 2 min. Whole brains were rapidly extracted, and half

the brain was post-fixed by 4 successive changes of fresh 4% paraformaldehyde daily, and then stored in 0.1% sodium azide in PBS at 4°C until cryosectioned for histological analyses. All tissue collection occurred during the dark cycle between 10 AM and 1 PM.

#### **5.2.2.4 Microglial Immunohistochemistry**

Fixed half brains were cryoprotected in 30% sucrose plus 0.1% sodium azide for 3 d prior to slicing, then quickly frozen in -30°C isopentane and sliced coronally from the anterior hypothalamus through the ventral hippocampus in a 1:6 series at 40 µm in a -20°C cryostat. Floating sections were stored in a 0.1% sodium azide solution until immunohistochemistry was performed. All sections were stained at the same time to minimize any differences in background staining due to differences in antibody preparation and binding across days.

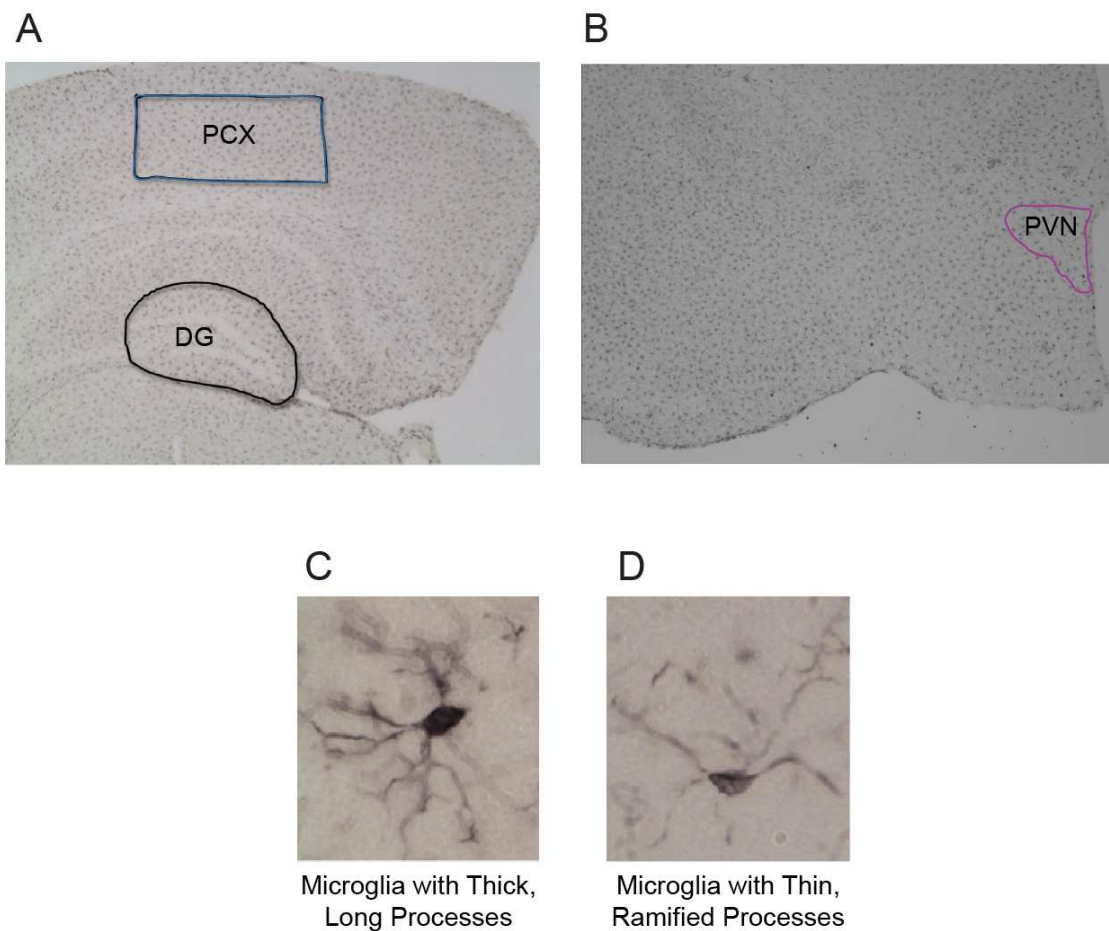
As in Experiment 1, Iba1 staining was performed. Free-floating sections were washed with PBS, incubated in 50% methanol for 30 min to quench blood vessels, and then washed in PBS again. They were then incubated for 1 h in PBS with 0.03% H<sub>2</sub>O<sub>2</sub>, 5% normal goat serum, and 0.3% Triton X in order to quench endogenous peroxidase, block, and permeabilize, respectively. Sections were incubated with primary antibody (rabbit anti-Iba1, 1:1,000; Wako Chemicals, Richmond, VA, USA) overnight at room temperature on an orbital shaker. On d 2, sections were washed and incubated with a biotinylated secondary antibody (goat anti-rabbit IgG, 1:200; Vector Laboratories, West Grove, PA, USA) for 2 h at room temperature. Sections were washed, and

immunostaining was identified by the streptavidin/HRP technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as the chromagen. Sections were mounted on gelatinized slides, dehydrated, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

#### **5.2.2.5 Stereology**

Iba1-labeled cells were quantified using the optical fractionator method within StereoInvestigator software (Microbrightfield Inc., Williston, VT, USA). Iba-1 positive cells in the P30 brain were classified as either microglia with thick, long processes or microglia with thin, ramified processes (Figure 29C-D). Unbiased stereology (Gundersen & Jensen, 1987) was used to estimate total numbers of Iba1-labeled cells and total numbers of each morphology. Microglial cell volumes were estimated using five rays of independent isotropic probes within the “Nucleator” function of StereoInvestigator software (MBF Labs, Williston, VT). Stereology was performed using a Nikon Eclipse 80i microscope at 100× (oil) and StereoInvestigator software. We analyzed 4 sections through the PVN and 5 sections through the parietal cortex and dentate gyrus for each animal. For each section examined, the area was calculated by the StereoInvestigator software based on the boundaries of the contour tracings (Figure 29A-B). Regional volume estimates were obtained by summing the areas given by the Cavalieri estimator for each section, and then multiplying this value by the product of the pre-histology thickness of each section (40 μm) and the number of sections examined. The estimated

total number of microglia per region was obtained using StereoInvestigator's Estimated Total by Mean Measured Thickness function, which bases its calculation on section thickness, series value, and number of measurements. These values were normalized by regional volume. The number of subjects analyzed per group was 5-7/group/sex for all regions analyzed.



**Figure 29: A) Example contours drawn around the P30 parietal cortex (top) and dentate gyrus (bottom) in the StereoInvestigator software. Photos were taken of Iba1-stained section at the 4X objective. C-D) The two primary microglial morphological states on P30. Representative pictures of microglia with thick, long processes (C) and thin, ramified processes in the mouse parietal cortex on P30. Photos were taken of Iba1-labeled microglia at the 100X objective.**

### 5.2.2.6 Data Analysis and Statistics

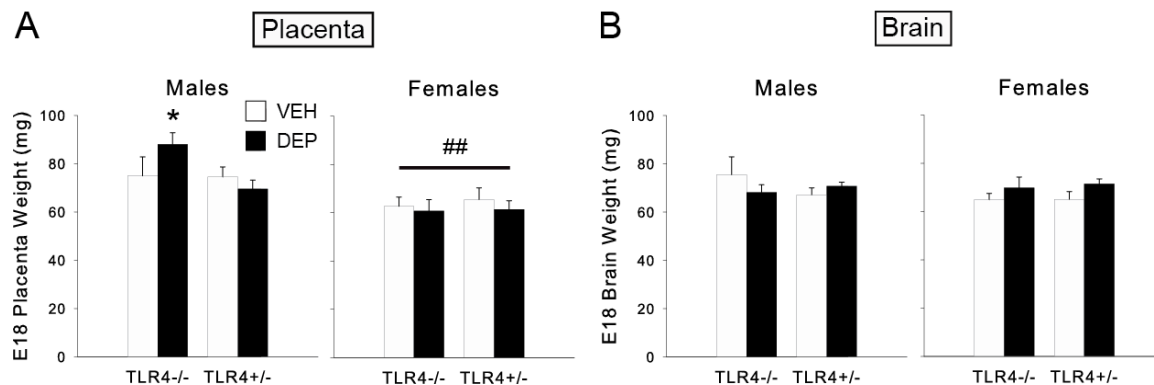
All data were analyzed with SPSS statistical software (IBM, Armonk, NY, USA). Three-way (DEP X LPS X Sex) were performed first, and following a significant or trending ( $p < 0.1$ ) interaction with Sex, two-way (DEP X LPS) ANOVAs within each sex were used to assess differences in regional volume ( $\text{mm}^3$ ), average cell volume ( $\mu\text{m}^3$ ), the total number of cells/ $\text{mm}^3$ , and the number of cells (of the 2 different morphologies)/ $\text{mm}^3$ . Following significant interactions, *post hoc* comparisons (Fisher's LSD) were performed to further distinguish between groups.

## 5.3 Results

### 5.3.1 Experiment 1: E18 Cohort

#### 5.3.1.1 Placenta and Brain Weights

We first assessed the E18 placental and brain weights to determine if there were any gross changes in fetal growth due to prenatal DEP exposure. We found a significant main effect of Sex [ $F(1,71)=17.60$ ,  $p < 0.001$ ], in that male placentas were overall heavier than female placentas, as well as a trend for a Sex X Genotype interaction [ $F(1,71)=2.72$ ,  $p=0.1$ ]. Follow-up tests revealed that male DEP TLR4<sup>-/-</sup> fetuses have an increased placental weight compared to male DEP TLR4<sup>+/-</sup> fetuses [trend for DEP X Genotype interaction,  $F(1,30)=3.26$ ,  $p=0.08$ ; *post hoc*,  $p < 0.05$ ], whereas female placental weights do not differ (Figure 30A). We failed to detect any significant differences in fetal brain weights (Figure 30B).

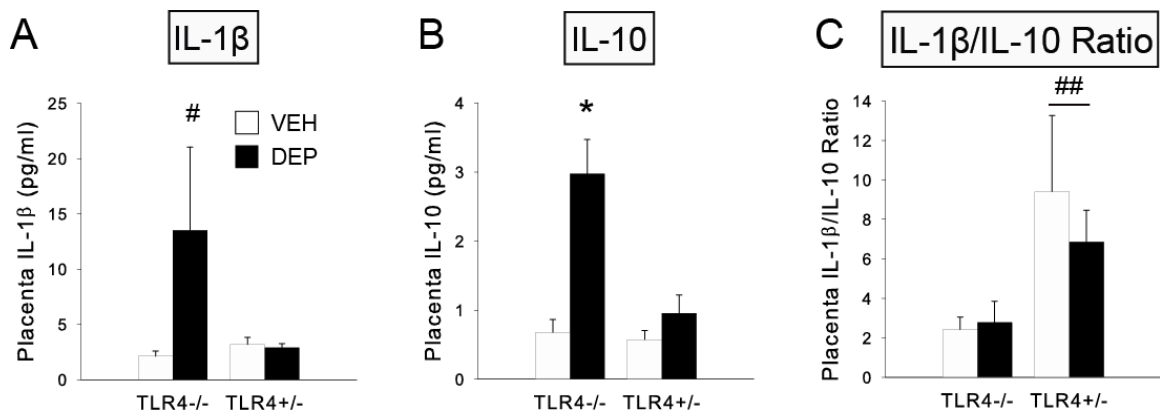


**Figure 30: A) E18 placenta weights were significantly greater in male DEP TLR4<sup>-/-</sup> fetuses than DEP TLR4<sup>+/-</sup>, whereas female placenta weights did not differ. B) No differences among E18 brain weights were detected. Data are mean  $\pm$  SEM,  $n=5-15$ /group/sex. \* $p<0.05$  vs. DEP TLR4<sup>+/-</sup>; ## $p<0.05$ , Males vs. Females.**

### 5.3.1.2 Placental Cytokines

We assessed the levels of proinflammatory IL-1 $\beta$  and anti-inflammatory IL-10 in placentas at E18 to determine whether prenatal DEP exposure induces a placental cytokine response. We failed to detect a significant difference in placental IL-1 $\beta$  at this time point, although there was a trend for a DEP X Genotype interaction [ $F(1,43)=2.83$ ,  $p=0.1$ ] due to a slight increase of IL-1 $\beta$  in DEP TLR4<sup>-/-</sup> placentas compared to VEH TLR4<sup>-/-</sup> placentas ( $p=0.09$ ; Figure 31A). Placental IL-10 did exhibit a significant DEP X Genotype interaction [ $F(1,42)=10.07$ ,  $p<0.005$ ]. Post hoc tests revealed that TLR4<sup>-/-</sup> placentas upregulate IL-10 in response to DEP exposure ( $p<0.001$ ; Figure 31B). Furthermore, there was an overall main effect of Sex, in that female placentas exhibited significantly higher levels of IL-10 than male placentas [ $F(1,42)=4.81$ ,  $p<0.05$ ]. As a measure of the relative balance between proinflammatory and anti-inflammatory cytokines in the placenta, we also analyzed the IL-1 $\beta$ /IL-10 ratio (de Wit et al., 2010).

Consistent with the above results, there was a significant main effect of Genotype [ $F(1,42)=6.90, p<0.05$ ] and a significant main effect of Sex [ $F(1,42)=4.13, p<0.05$ ], suggesting that females have a greater anti-inflammatory bias than males (data not shown), and TLR4-competent fetuses overall have a greater proinflammatory bias than TLR4-deficient fetuses (Figure 31C).



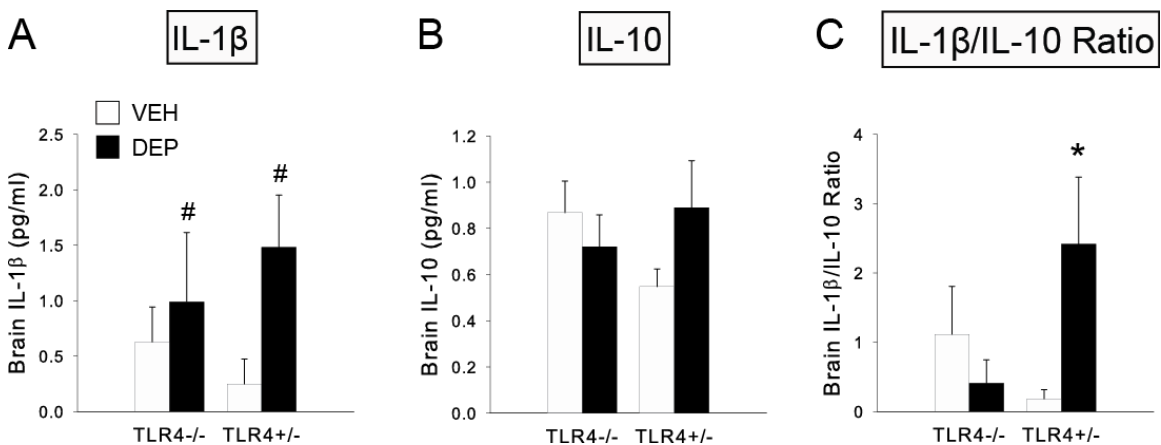
**Figure 31:** A) Levels of IL-1 $\beta$  tended to be higher in DEP TLR4 $^{-/-}$  placentas than VEH TLR4 $^{-/-}$  placentas at E18. B) Levels of IL-10 were significantly higher in DEP TLR4 $^{-/-}$  compared to all other groups. C) The proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) was significantly higher in TLR4 $^{+/-}$  placentas than TLR4 $^{-/-}$  placentas. Data are mean  $\pm$  SEM,  $n= 10-14$ /group (note that sexes are combined here). \* $p=0.09$ , DEP TLR4 $^{-/-}$  vs. VEH TLR4 $^{-/-}$ ; \* $p<0.05$  vs. all other groups; ## $p<0.05$ , TLR4 $^{+/-}$  vs. TLR4 $^{-/-}$ .

### 5.3.1.3 Placental Macrophage Counts and Morphological Analysis

As a complementary measure of the extent of the placental immune response to prenatal DEP exposure, we counted the total number of macrophages in the fetal portion of the placenta, as well as measured each macrophage's size and shape. No significant differences were found in placental macrophage cell density, cell area, or cell elongation factor due to Sex, Genotype, or DEP in the fetal portion of the placenta (data not shown).

### 5.3.1.4 Fetal Brain Cytokines

We also measured the levels of proinflammatory IL-1 $\beta$  and anti-inflammatory IL-10 in fetal brains at E18 to determine whether prenatal DEP exposure induces a fetal brain cytokine response. IL-1 $\beta$  tended to increase due to prenatal DEP exposure [trend for a main effect of DEP,  $F(1,23)=3.72$ ,  $p=0.07$ ; Figure 32A], whereas there were no significant differences in IL-10 levels in the fetal brains (Figure 32B). As in the placenta, we also analyzed the IL-1 $\beta$ /IL-10 ratio and found that DEP TLR4 $^{+/-}$  fetal brains exhibited a significantly greater proinflammatory bias than did VEH TLR4 $^{+/-}$  fetal brains [trend for DEP X Genotype interaction,  $F(1,22)=3.14$ ,  $p=0.09$ ; post hoc,  $p<0.05$ ], whereas TLR4 $^{-/-}$  brains did not differ due to prenatal treatment (Figure 32C).



**Figure 32: A) Levels of IL-1 $\beta$  tended to be higher in E18 brains due to prenatal DEP exposure. B) Levels of IL-10 did not significantly differ in the E18 brain. C) The proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) was significantly greater in DEP TLR4 $^{+/-}$  E18 brains than in VEH TLR4 $^{+/-}$  E18 brains. Data are mean  $\pm$  SEM,  $n=6-13$ /group (note that sexes are combined here). # $p=0.07$ , DEP vs. VEH; \* $p<0.05$  vs. VEH TLR4 $^{+/-}$ .**

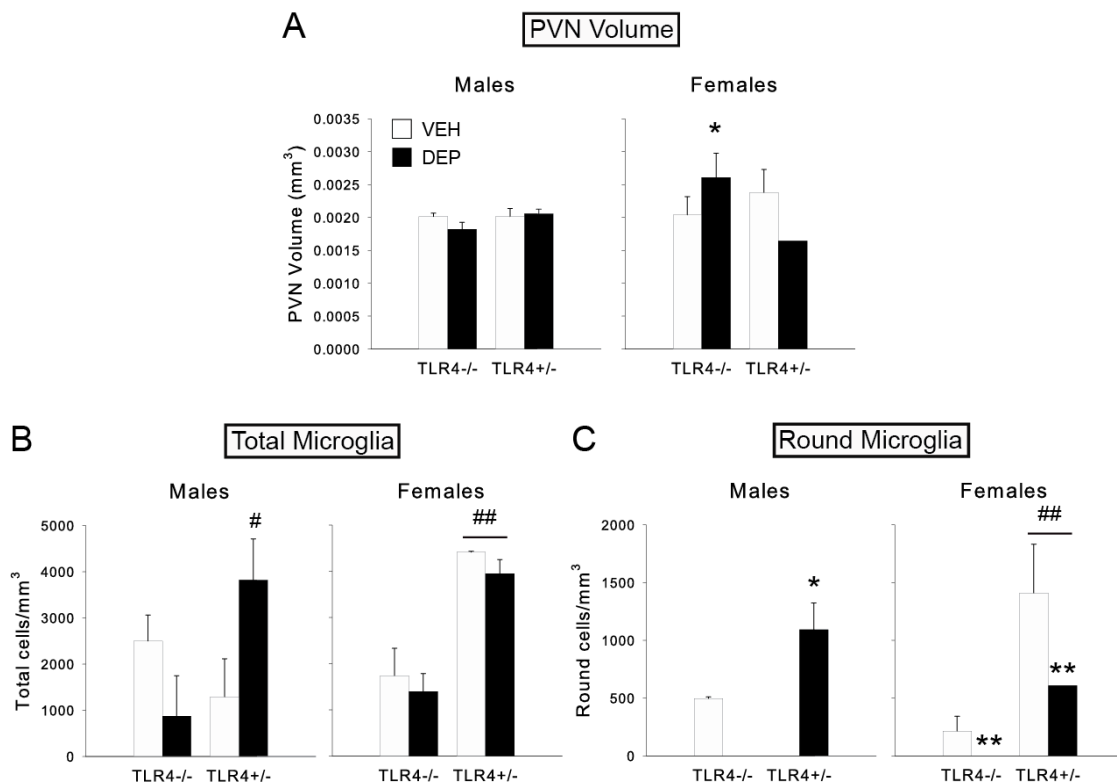
### 5.3.1.5 Fetal Brain Microglia Counts and Morphological Analysis

In order to assess whether prenatal DEP exposure altered microglial development, we exhaustively counted microglia and classified them into 4 morphological types in the PVN, DG, CA1, CA3, and PCX. We also measured the volume of each brain region in order to determine if there were any gross changes in brain structural development due to prenatal DEP exposure.

#### 5.3.1.5.1 Paraventricular Nucleus of the Hypothalamus

Analysis of the overall volume of the PVN revealed a significant Sex X DEP X Genotype interaction [ $F(1,12)=5.26, p<0.05$ ], which was determined by follow-up tests to result from DEP TLR4<sup>-/-</sup> females having an increased volume relative to DEP TLR4<sup>+/-</sup> females and DEP TLR4<sup>-/-</sup> males (post hoc,  $p<0.05$ ; Figure 33A). Furthermore, analysis of total microglial density revealed a trend for a Sex X DEP X Genotype interaction [ $F(1,12)=3.54, p=0.09$ ]. Follow-up tests revealed that males exhibited a trend for a DEP X Genotype interaction [ $F(1,6)=4.54, p=0.08$ ], whereas females showed a significant main effect of Genotype [ $F(1,6)=20.10, p<0.005$ ; Figure 33B]. Subdividing by microglial morphology, we determined that this pattern was mostly due to differences in round cells [significant Sex X DEP X Genotype interaction, [ $F(1,12)=13.59, p<0.005$ ]. Follow-up tests revealed that DEP TLR4<sup>+/-</sup> males have significantly more round microglia than VEH TLR4<sup>+/-</sup> males [significant DEP X Genotype interaction,  $F(1,6)=13.46, p<0.05$ ; post hoc,  $p<0.05$ ; Figure 33C]. In contrast, the opposite pattern was observed in females, such that DEP females had fewer round microglia than VEH females [significant main effect

of DEP,  $F(1,6)=6.40$ ,  $p<0.05$ ], and TLR4<sup>+/-</sup> females had more round microglia than TLR4<sup>-/-</sup> females [significant main effect of Genotype,  $F(1,6)=20.21$ ,  $p<0.005$ ; Figure 33C]. Within stout microglia, we detected a trend for a Sex X Genotype interaction [ $F(1,12)=3.64$ ,  $p=0.08$ ], because TLR4<sup>+/-</sup> females again possessed more stout microglia than TLR4<sup>-/-</sup> females [significant main effect of Genotype,  $F(1,6)=33.20$ ,  $p<0.005$ ], whereas males did not differ (data not shown). Analysis of thick, long microglia revealed a pattern similar to that of round cells in males, but it did not reach significance. We failed to detect any significant differences in thin, ramified microglia, likely due to their extremely low abundance in the PVN at this time point.

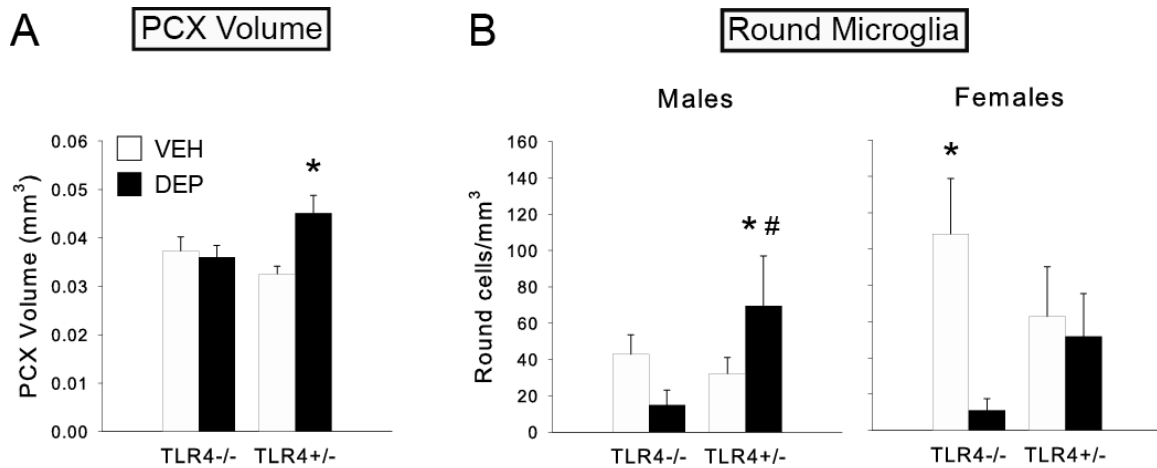


**Figure 33: A) DEP TLR4<sup>-/-</sup> females possess a significantly larger PVN volume than DEP TLR4<sup>+/-</sup> females at E18, whereas males do not differ. B) DEP TLR4<sup>+/-</sup> males tend**

to have more total microglia than VEH TLR4+/- males in the PVN, whereas TLR4+/- females have overall more total microglia than TLR4-/- females. C) DEP TLR4+/- males have more round microglia than VEH TLR4+/- males in the PVN, whereas DEP females have overall fewer round microglia than VEH females. In addition, TLR4+/- females have overall more round microglia than TLR4-/- females. Data are mean  $\pm$  SEM,  $n= 2-4/\text{group}/\text{sex}$ . \* $p<0.05$  vs. DEP TLR4+/- females for volume; # $p=0.09$ , DEP TLR4+/- vs. VEH TLR4+/-; ## $p<0.005$ , TLR4+/- vs. TLR4-/-; \*\* $p<0.05$ , DEP vs. VEH; \* $p<0.05$  vs. VEH TLR4+/- males for round cells.

#### 5.3.1.5.2 Parietal Cortex

Overall volume of the PCX was determined to be significantly larger in DEP-treated TLR4+/- fetuses compared to VEH-treated TLR4+/- fetuses, whereas TLR4-/- fetuses do not differ [significant DEP X Genotype interaction,  $F(1,26) = 5.34$ ,  $p < 0.05$ ; post hoc,  $p < 0.05$ ; Figure 34A]. No significant differences were detected in overall microglial density. However, round microglia exhibited a similar pattern to that observed in the PVN [significant DEP X Genotype interaction,  $F(1,27)=5.42$ ,  $p < 0.05$ ; trend for Sex X DEP interaction,  $F(1,27)=3.02$ ,  $p=0.09$ ]. Specifically, DEP TLR4+/- males had more round microglia than DEP TLR4-/- males [ $F(1,13)=6.07$ ,  $p < 0.05$ ; post hoc,  $p < 0.05$ ], and tended to be greater than VEH TLR4+/- males as well ( $p=0.08$ ; Figure 34B). In contrast, DEP females again exhibited the opposite pattern, showing a decrease in round microglia relative to VEH females [significant main effect of DEP,  $F(1,13)=5.03$ ,  $p < 0.05$ ; trend for DEP X Genotype interaction,  $F(1,13)=3.18$ ,  $p=0.1$ ; Figure 34B]. No further differences were detected in stout; thick, long; or thin, ramified microglia.

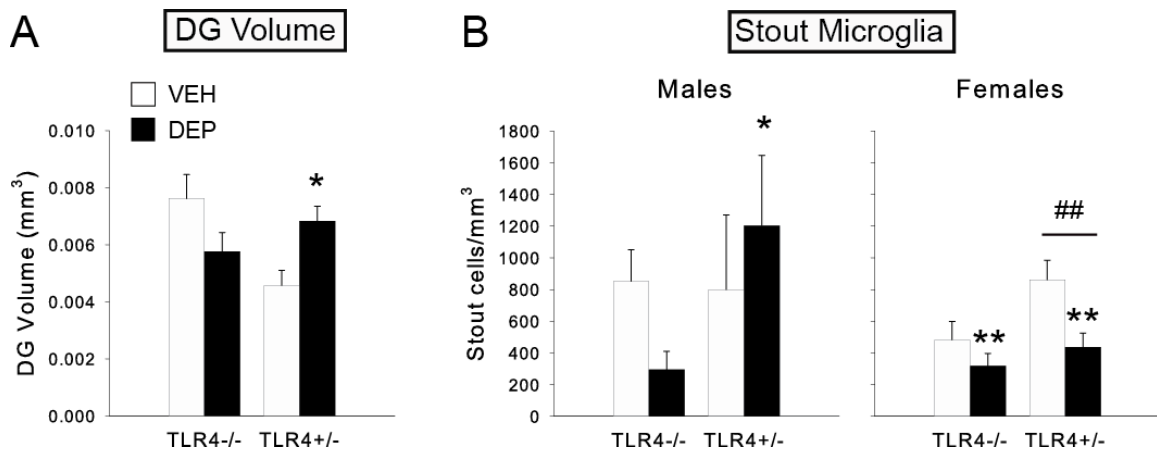


**Figure 34: A) DEP TLR4<sup>+/-</sup> animals possess a significantly larger PCX volume than VEH TLR4<sup>+/-</sup> animals and DEP TLR4<sup>-/-</sup> animals at E18. Data are mean  $\pm$  SEM,  $n= 8-9$ /group (note that sexes are combined here).  $*p<0.05$  vs. VEH TLR4<sup>+/-</sup> and DEP TLR4<sup>-/-</sup>. B) DEP TLR4<sup>+/-</sup> males have significantly more round microglia in the PCX than DEP TLR4<sup>-/-</sup> males, and tend to have more than VEH TLR4<sup>+/-</sup> males also. In contrast, VEH TLR4<sup>-/-</sup> females have significantly more round microglia than DEP TLR4<sup>-/-</sup> females in the PCX at E18. Data are mean  $\pm$  SEM,  $n= 3-5$ /group/sex.  $*p<0.05$  vs. DEP TLR4<sup>-/-</sup>;  $\#p=0.08$  vs. VEH TLR4<sup>+/-</sup>.**

#### 5.3.1.5.3 Dentate Gyrus

Overall volume of the DG followed a similar pattern to the volume change observed in the PCX, in that volumes are significantly greater in DEP-treated TLR4<sup>+/-</sup> fetuses than in VEH-treated TLR4<sup>+/-</sup> fetuses [significant DEP X Genotype interaction,  $F(1,18)=9.44$ ,  $p<0.01$ ; post hoc,  $p<0.05$ ; Figure 35A]. No significant differences were detected in total microglial density or round cell density. However, stout cells exhibited a pattern similar to that observed for round cells in the PCX and PVN [trend for Sex X DEP X Genotype interaction,  $F(1,19)=3.88$ ,  $p=0.06$ ]. Specifically, DEP TLR4<sup>+/-</sup> males had significantly more stout microglia than DEP TLR4<sup>-/-</sup> males [trend for DEP X Genotype interaction,  $F(1,9)=2.77$ ,  $p=0.1$ ; post hoc,  $p<0.05$ ; Figure 35B]. In contrast, DEP females had

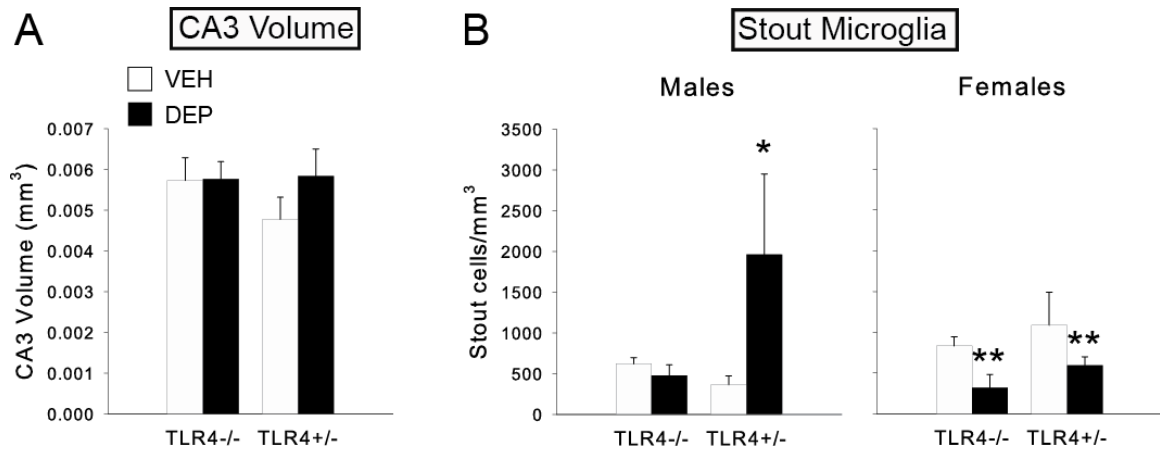
fewer stout microglia than VEH females [significant main effect of DEP,  $F(1,9)=7.49$ ,  $p<0.05$ ], and TLR4<sup>+/-</sup> females had more stout microglia than TLR4<sup>-/-</sup> females [significant main effect of Genotype,  $F(1,9)=5.37$ ,  $p<0.05$ ; Figure 35B]. Analysis of thick, long cells revealed a significant DEP X Genotype interaction [ $F(1,18)=6.89$ ,  $p<0.05$ ] that appeared to be driven by females [significant DEP X Genotype interaction,  $F(1,9)=9.60$ ,  $p<0.05$ ], due to an increased number of thick, long cells in DEP TLR4<sup>-/-</sup> females compared to VEH TLR4<sup>-/-</sup> and DEP TLR4<sup>+/-</sup> ( $p<0.05$ ; data not shown). No significant differences were detected in thin, ramified cells.



**Figure 35: A) DEP TLR4<sup>+/-</sup> animals possess a significantly greater DG volume at E18 than VEH TLR4<sup>+/-</sup> animals. Data are mean  $\pm$  SEM,  $n= 4-8$ /group (note that sexes are combined here). \* $p<0.05$  vs. VEH TLR4<sup>+/-</sup>. B) DEP TLR4<sup>+/-</sup> males have significantly more stout microglia than DEP TLR4<sup>-/-</sup> males in the E18 DG, whereas DEP females have significantly fewer stout microglia overall than VEH females. In addition, TLR4<sup>+/-</sup> females have overall more stout microglia than TLR4<sup>-/-</sup> females. Data are mean  $\pm$  SEM,  $n= 2-5$ /group/sex. \* $p<0.05$  vs. DEP TLR4<sup>-/-</sup>; ## $p<0.05$ , TLR4<sup>+/-</sup> vs. TLR4<sup>-/-</sup>; \*\* $p<0.05$ , DEP vs. VEH.**

#### 5.3.1.5.4 CA3

Overall volume of the CA3 did not differ by Sex, DEP, or Genotype (Figure 36A), nor were any differences detected in total microglia density or round cell density. However, stout microglia exhibited a similar pattern as detected in the DG [significant Sex X DEP interaction,  $F(1,16)=5.03$ ,  $p<0.05$ ; trend for Sex X DEP X Genotype interaction,  $F(1,16)=2.44$ ,  $p=0.1$ ]. Specifically, DEP TLR4<sup>+/-</sup> males possessed more stout microglia than VEH TLR4<sup>+/-</sup> and DEP TLR4<sup>-/-</sup> males [trend for DEP X Genotype interaction,  $F(1,9)=3.52$ ,  $p=0.09$ ; post hoc,  $p<0.05$ ; Figure 36B]. Once again, females exhibited the opposite pattern, as DEP females possessed fewer stout cells than VEH females [significant main effect of DEP,  $F(1,7)=6.88$ ,  $p<0.05$ ; Figure 36B]. Analysis of thick, long microglia revealed a pattern similar to that of stout cells in males, but it did not reach significance. Thin, ramified cells were not different by Sex, DEP, or Genotype.

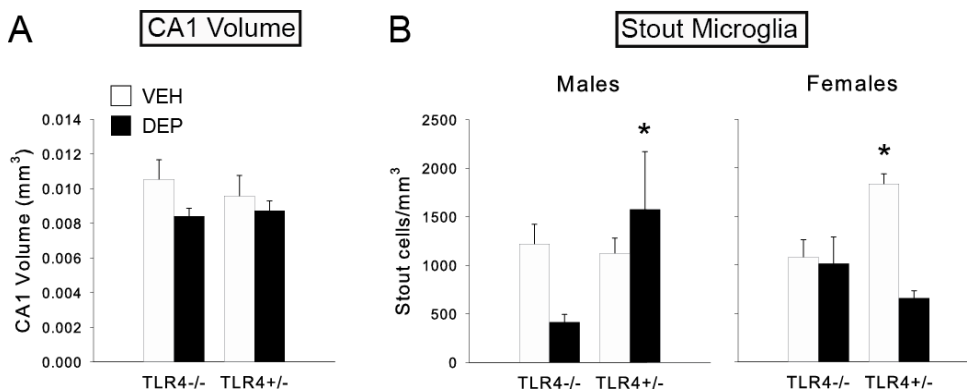


**Figure 36: A) No significant differences were detected in CA3 volume at E18. Data are mean  $\pm$  SEM,  $n= 5-7$ /group (note that sexes are combined here). B) DEP TLR4<sup>+/-</sup> males have significantly more stout microglia in the CA3 at E18 than VEH TLR4<sup>+/-</sup> males, whereas DEP females have significantly fewer stout microglia overall than VEH**

females. Data are mean  $\pm$  SEM,  $n= 2-4/\text{group}/\text{sex}$ . \* $p<0.05$  vs. VEH TLR4+/-; \*\* $p<0.05$ , DEP vs. VEH.

#### 5.3.1.5.5 CA1

Overall volume of the CA1 did not differ by Sex, DEP, or Genotype (Figure 37A), nor were any differences detected in total microglia density. Analysis of round microglia revealed a significant main effect of Sex [ $F(1,20)=6.79$ ,  $p<0.05$ ], with females possessing overall more round microglia than males (data not shown). The pattern of stout cells was similar to that found in the DG and CA3 [significant Sex X DEP X Genotype interaction,  $F(1,20)=11.29$ ,  $p<0.005$ ]. DEP TLR4+/- males possessed more stout microglia than DEP TLR4-/- males [close trend for DEP X Genotype interaction,  $F(1,10)=4.82$ ,  $p=0.05$ , post hoc,  $p<0.05$ ; Figure 37B]. On the other hand, VEH TLR4+/- females had more stout microglia than DEP TLR4+/- females [significant DEP X Genotype interaction,  $F(1,10)=7.74$ ,  $p<0.05$ , post hoc,  $p<0.05$ ; Figure 37B]. A similar pattern in females for thick, long cells was present, but it failed to reach significance. No significant differences in thin, ramified cells were detected.



**Figure 37: A) No significant differences were detected in CA1 volume at E18. Data are mean  $\pm$  SEM,  $n= 5-8/\text{group}$  (note that sexes are combined here). B) DEP TLR4+/- males**

have significantly more stout microglia in the CA1 at E18 than DEP TLR4<sup>-/-</sup> males, whereas VEH TLR4<sup>+/-</sup> females have significantly more stout microglia than DEP TLR4<sup>+/-</sup> females. Data are mean ± SEM,  $n= 2-5/\text{group}/\text{sex}$ . \* $p<0.05$  vs. DEP TLR4<sup>-/-</sup> for males; \* $p<0.05$  vs. DEP TLR4<sup>+/-</sup> for females.

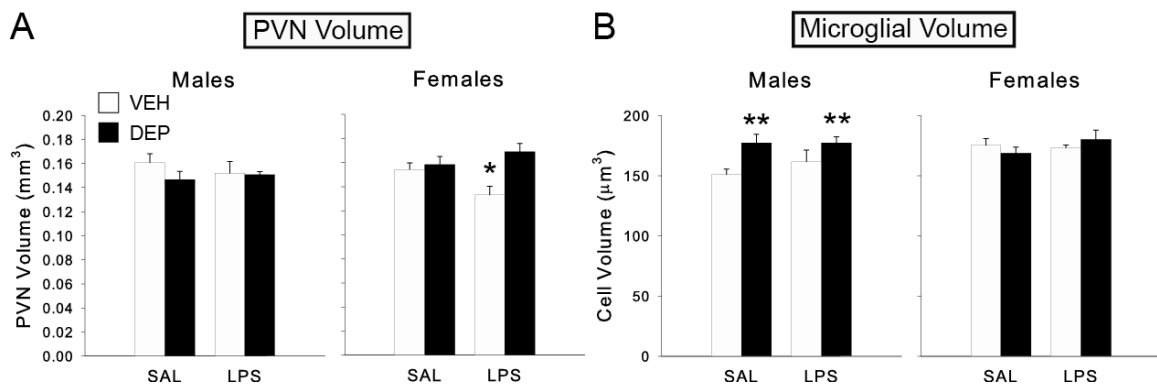
#### 5.3.1.6 P30 Brain Microglia Counts and Morphological Analysis

It is now clear that prenatal DEP exposure causes alterations in microglial development, especially in TLR4-competent males, whereas females often exhibited the opposite pattern. In order to determine if prenatal DEP exposure also results in long-term changes in microglial number and/or morphology, in conjunction with the long-term changes in behavior and microglial function that we have observed in previous studies (Bolton et al., 2013; Bolton et al., 2012; Bolton, Auten, & Bilbo, 2014), we used unbiased stereology to count microglia and classified them into one of 2 morphological types (only thick, long and thin, ramified microglia are present in the normal adult brain) in the wild-type P30 PVN, DG, and PCX. Because the number of morphological types in the adult brain are limited, we also measured microglial soma volume in order to more rigorously assess any changes in morphology. As at E18, we again measured the volume of each brain region in order to determine if there were any long-lasting gross changes in brain structure due to prenatal DEP exposure.

##### 5.3.1.6.1 *Paraventricular Nucleus of the Hypothalamus*

Overall volume of the P30 PVN exhibited a significant Sex X DEP interaction [ $F(1,39)=9.18, p<0.005$ ] and a trend for a DEP X LPS interaction [ $F(1,39)=3.20, p=0.08$ ]. Follow-up tests revealed that VEH/LPS females possessed a smaller PVN volume than

DEP/LPS females and VEH/SAL females [significant DEP X LPS interaction,  $F(1,20)=5.87$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ], whereas males do not differ (Figure 38A). Females had overall more microglia in the PVN than males [significant main effect of Sex,  $F(1,39)=6.09$ ,  $p<0.05$ ; data not shown]. We did not detect any significant differences in thick, long or thin, ramified microglia density due to Sex, DEP, or Genotype. However, analysis of average cell volume in the PVN revealed a trend for a Sex X DEP interaction [ $F(1,39)=3.44$ ,  $p=0.07$ ], which we determined to be due to an increase in microglial soma volume in males [significant main effect of DEP,  $F(1,19)=6.61$ ,  $p<0.05$ ], and no differences in females (Figure 38B). The exact same pattern for soma volume was observed in both thick, long and thin, ramified microglia when they were analyzed separately; therefore, the result applies to PVN microglia as a whole rather than just one morphological type.



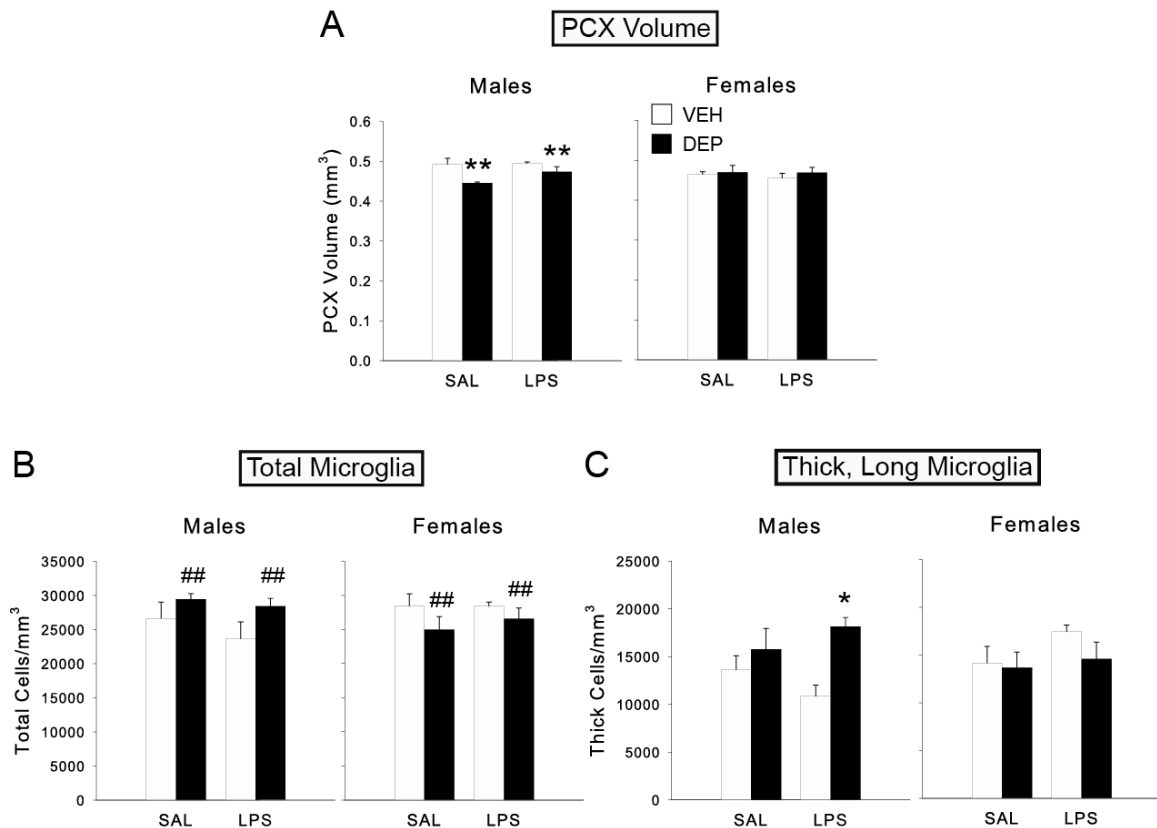
**Figure 38: A) VEH/LPS females possess a significantly smaller PVN volume than DEP/LPS and VEH/SAL females at P30, whereas males do not differ. B) DEP males have microglia with larger soma volumes in the P30 PVN than do VEH males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n= 4-6$ /group/sex. \* $p<0.05$  vs. DEP/SAL and VEH/SAL; \*\* $p<0.05$ , DEP vs. VEH.**

#### 5.3.1.6.2 Parietal Cortex

Analysis of PCX regional volume revealed a significant Sex X DEP interaction [ $F(1,43)=6.07, p<0.05$ ], which we determined to be due to a smaller volume in DEP males compared to VEH males [significant main effect of DEP,  $F(1,20)=8.55, p<0.01$ ], whereas females did not differ (Figure 39A). Analysis of total microglial cell density also revealed a significant Sex X DEP interaction [ $F(1,42)=5.88, p<0.05$ ], because DEP males tended to have more total microglia in the PCX than VEH males [trend for main effect of DEP,  $F(1,20)=3.09, p=0.09$ ], whereas females exhibited the opposite pattern [trend for main effect of DEP,  $F(1,22)=2.73, p=0.1$ ; Figure 39B]. Subdividing microglia by morphological type, we discovered that this pattern was mostly driven by thick, long cells, which also exhibited a significant Sex X DEP interaction [ $F(1,42)=8.16, p<0.01$ ], and a trend for a Sex X DEP X LPS interaction [ $F(1,42)=2.84, p=0.1$ ], whereas thin, ramified cells did not exhibit any significant differences. Specifically, DEP/LPS males have more thick, long microglia than VEH/LPS males [significant main effect of DEP,  $F(1,20)=9.00, p<0.01$ ; trend for DEP X LPS interaction,  $F(1,20)=2.65, p=0.1$ ; post hoc,  $p<0.05$ ], whereas females exhibit the opposite pattern, although this didn't reach significance (Figure 39C).

Analysis of average cell volume in the PCX revealed a significant Sex X DEP interaction [ $F(1,45)=5.34, p<0.05$ ], in which DEP males tended to exhibit a smaller cell volume than VEH males [ $F(1,22)=2.43, p=0.1$ ], and DEP females tended to exhibit the opposite pattern [ $F(1,23)=3.63, p=0.07$ ] (data not shown). However, the pattern seemed very similar to that observed with PCX regional volume, and indeed, we found a strong

positive correlation between the two measures,  $r(51)=0.50$ ,  $p<0.001$ . To account for these pre-existing group differences in regional volume, we analyzed the cell volume data again while using regional volume as a covariate (ANCOVA). In this case, the Sex X DEP interaction was eliminated entirely, suggesting that changes in PCX cell volume were dependent on changes in PCX regional volume, and not reflective of a change in activation state due to DEP exposure (data not shown).



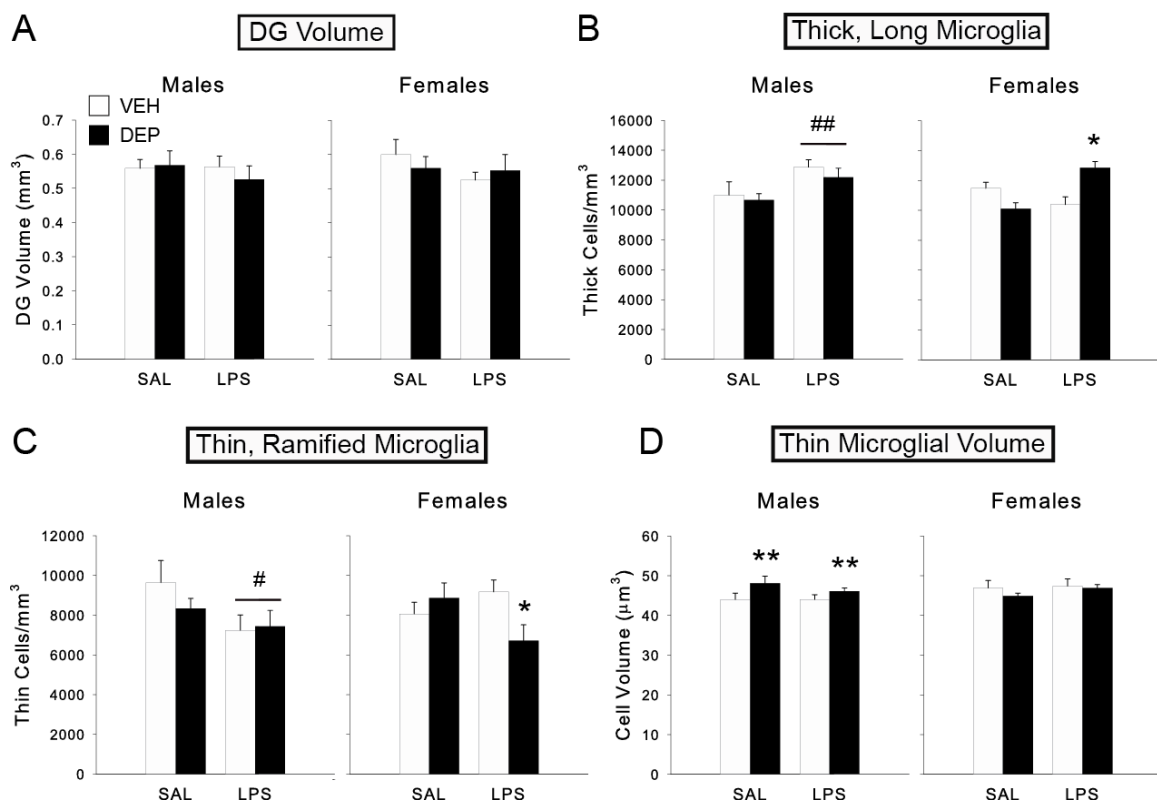
**Figure 39:** A) DEP males possess a significantly smaller PCX volume at P30 than do VEH males, whereas females do not differ. B) DEP males tend to have more total microglia in the PCX at P30 than do VEH males, whereas DEP females tend to have fewer. C) DEP/LPS males have significantly more thick, long microglia in the PCX at P30 than do VEH/LPS males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n=5-7$ /group/sex. \*\* $p<0.05$ , DEP vs. VEH; ## $p=0.1$ , DEP vs. VEH; \* $p<0.05$  vs. VEH/LPS.

#### 5.3.1.6.3 Dentate Gyrus

Neither overall volume of the DG (Figure 40A) nor total microglial cell density differed by Sex, DEP, or LPS. However, subdividing microglia by morphological type revealed a significant Sex X DEP X LPS interaction [ $F(1,40)=7.17, p<0.05$ ] within thick, long microglia. Follow-up tests that subdivided by sex revealed that LPS-injected males had significantly more thick, long microglia in the DG than SAL-injected males [significant main effect of LPS,  $F(1,21)=7.24, p<0.05$ ], whereas females exhibited a significant DEP X LPS interaction [ $F(1,19)=18.53, p<0.001$ ; Figure 40B]. Specifically, DEP/LPS females had significantly more thick, long microglia in the DG than VEH/LPS females and DEP/SAL females (post hoc,  $p<0.005$ ), much like the pattern we observed in males in the PCX. This effect was coupled with a significant Sex X DEP X LPS interaction [ $F(1,40)=4.76, p<0.05$ ] within thin, ramified microglia that represented the opposing pattern. Specifically, LPS males tended to have fewer thin, ramified microglia than SAL-injected males [trend for main effect of LPS,  $F(1,21)=3.96, p=0.06$ ], whereas DEP/LPS females had fewer thin, ramified microglia than VEH/LPS females [significant DEP X LPS interaction,  $F(1,19)=5.50, p<0.05$ ; post hoc,  $p<0.05$ ; Figure 40C]. Therefore, it appears that animals maintained the same overall amount of microglia in the DG by converting thin, ramified microglia to thick, long microglia in response to LPS.

Analysis of average cell volume in the DG revealed a trend for a Sex X DEP X LPS interaction [ $F(1,40)=3.60, p=0.06$ ]. Subdividing by morphological type, we found that thick, long cell volume overall tended to increase in LPS animals [trend for main effect

of LPS,  $F(1,40)=3.56$ ,  $p=0.06$ ; data not shown]. On the other hand, analysis of thin, ramified cell volume revealed a significant Sex X DEP interaction [ $F(1,40)=4.18$ ,  $p<0.05$ ] that is driven by DEP males having larger soma volumes than VEH males [main effect of DEP,  $F(1,20)=4.36$ ,  $p=0.05$ ], as in the PVN, whereas female cell volumes do not differ (Figure 40D).



**Figure 40:** A) No significant differences in DG volume were detected. B) LPS males had significantly more thick, long microglia in the DG at P30 than did SAL males. DEP/LPS females had significantly more thick, long microglia than did VEH/LPS and DEP/SAL females. C) LPS males tended to have fewer thin, ramified cells than SAL males in the P30 DG, whereas DEP/LPS females had significantly fewer thin, ramified cells than did VEH/LPS and DEP/SAL females. D) DEP males have thin, ramified microglia with larger soma volumes in the DG at P30 than do VEH males, whereas females do not differ. Data are mean  $\pm$  SEM,  $n=5-7$ /group/sex.  $##p<0.05$ , LPS vs. SAL;  $*p<0.05$  vs. VEH/LPS and DEP/SAL;  $\#p=0.06$ , LPS vs. SAL;  $**p=0.05$ , DEP vs. VEH.

## **5.4 Discussion**

In agreement with our previous data demonstrating long-term changes in offspring brain cytokine levels and behavior following prenatal air pollution exposure (Bolton et al., 2013; Bolton et al., 2012; Bolton et al., 2014), we have demonstrated here that DEP exposure results in altered microglial development, as well as long-lasting changes in microglial number and morphology. Furthermore, the results of the current study are also consistent with the male bias in brain and behavioral changes observed in previous studies; i.e., we observed more marked changes in microglial development at E18 and microglial number and morphology at P30 in male than female offspring. Notably, the current study is the first to directly test and confirm the critical role of TLR4, an innate pattern recognition receptor, in the effects of prenatal air pollution exposure on microglial development, which is consistent with our previous findings.

We have previously observed marked upregulation of TLR4 expression in the brains of adult male offspring in response to prenatal air pollution exposure in combination with either maternal stress during gestation or high-fat diet during adulthood (Bolton et al., 2013; Bolton et al., 2014). Those strikingly similar findings in two different models prompted us to target TLR4 in the current study to determine whether TLR4 itself was necessary for the effects of prenatal DEP exposure on brain development. We decided to focus on microglial development based on our previous findings of alterations in the brain cytokine milieu (Bolton et al., 2013) following prenatal

DEP exposure and increases in microglial antigen density with the addition of adult high-fat diet exposure (Bolton et al., 2012), as well as previous work from our laboratory that demonstrated TLR4 is expressed primarily by microglia in the brain (Schwarz & Bilbo, 2013). The current study provided the first evidence that prenatal DEP exposure alters microglial development, primarily toward a more amoeboid or “activated” morphology, in multiple regions of the fetal brain in a manner that is dependent upon TLR4 expression.

Interestingly, we observed TLR4-dependent changes not only in microglial development, but also in overall structural development (i.e., regional volume) of two of the brain regions we examined. The PCX and DG both exhibited increases in regional volume following DEP exposure only in TLR4<sup>+/-</sup> fetuses, which in conjunction with the changes in microglia, may indicate that microglia play a role in the alterations of structural development. In recent years, a rapidly growing literature has demonstrated that microglia play critical roles in normal brain development, including the promotion of neurogenesis and neuronal/glial migration, phagocytosis of surplus neural precursor cells, and pruning of superfluous synapses between neurons [reviewed in (Schwarz & Bilbo, 2011)]. The shift we observed of DEP microglia toward a more amoeboid morphology, though classically considered to represent a state of higher activation in the adult, may actually represent a more developmentally immature state in the developing brain. These immature microglia may not be as efficient at phagocytosing excess neural

cells and synapses in the developing brain, which together could contribute to the increased regional volume we observed in the PCX and DG.

The effect of prenatal DEP exposure on parietal cortex volume is reflective of the literature on autism. Many clinical studies have investigated aberrancies in brain structure and development from early childhood through adolescence, which is thought to occur in mice around P30 (Spear & Brake, 1983). It is known that in early childhood, prior to adolescence, autistic individuals have a significantly larger overall brain volume than their peers (Herbert, 2005). This suggests either excessive neurogenesis or, perhaps, aberrant synaptic pruning by microglia. Indeed, inducing deficient microglial synaptic pruning in mice by knocking out the chemokine receptor CX3CR1 results in autistic-like behaviors (Zhan et al., 2014). Moreover, parietal cortex-specific defects have been previously observed in autistic individuals, both in terms of brain structure and behavior (Courchesne, Press, & Yeung-Courchesne, 1993; Egaas, Courchesne, & Saitoh, 1995), in agreement with our data. Very recent epidemiological studies have discovered an association between the levels of maternal exposure to particulate matter air pollution during pregnancy and the risk of autism in their children (Raz et al., 2014; Volk et al., 2013). However, our study may be the first to point toward the biological underpinnings of this association.

Another characteristic of autism that is shared with the findings in our model is the striking sex difference in incidence. The prevalence of autism is four times higher in

males than females, and our study identified a male-specific alteration of microglial development following prenatal DEP exposure, in agreement with our prior findings of greater behavioral changes in male than female offspring of DEP-exposed dams. Previous data from our laboratory has revealed marked sexual dimorphisms in microglial colonization of the rat brain across development, such that males have a greater number of microglia than females shortly after birth, which has been hypothesized to render them more vulnerable to early-life immune challenges (Schwarz et al., 2012). Our data agree with this hypothesis and add to a growing literature showing sex differences in offspring outcomes following perinatal events (Clifton, 2010).

Based on our previous work and work by Clifton et al., we had previously hypothesized that a sexually dimorphic placental immune response to DEP exposure may underlie the long-term male-specific changes in brain function and behavior. However, the results of the current study fell short of directly supporting this hypothesis. We observed a marked increase in anti-inflammatory IL-10 in TLR4-null placentas in response to DEP exposure regardless of sex, in conjunction with a slight increase in IL-1 $\beta$ . In contrast, we did not observe clear evidence of an immune response to DEP in TLR4 $\pm$  placentas at this time point in either cytokine levels or macrophage number, which could suggest that the lack of TLR4 prolongs the immune response to DEP exposure beyond the normal time frame (2 days after the final DEP exposure at E16), such that the response had already equalized in TLR4-competent placentas. This

would be consistent with a previous study by de Melo and colleagues that analyzed rat placentas at E19 following maternal inhalation of fine particulate matter before and during pregnancy and out of 6 measured cytokines, found only an increase in IL-4 (de Melo et al., 2015). The authors suggested that increased IL-4, an anti-inflammatory cytokine, may be indicative of a recent placental inflammatory response, as IL-4 (like IL-10) could be responsible for the resolution of the inflammatory response. We did observe an increased proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) in TLR4 $^{+/-}$  placentas relative to TLR4 $^{-/-}$  placentas, which may be indicative of the residual effect of a previous immune response. An alternative possibility is that E18 is so close to birth that any changes due to DEP exposure are overwhelmed by the hormonal and cytokine changes required to initiate parturition, which is inflammatory in nature even during normal births (Patni et al., 2007).

Consistent with Clifton's hypothesis that male placentas prioritize growth while female placentas prioritize responding to a maternal immune challenge, we did observe an increased weight of male TLR4 $^{-/-}$  placentas in response to DEP, in conjunction with lower overall levels of IL-10 in male vs. female placentas. Female placentas also had a greater overall anti-inflammatory bias (decreased IL-1 $\beta$ /IL-10 ratio) than male placentas, which may have proved protective for the female fetus at the time of maternal exposure of DEP, despite a lack of difference due to DEP at the time of our measurement. This placental anti-inflammatory bias in females is consistent with our previous data

showing a striking anti-inflammatory bias in the adult female brain relative to the male brain following prenatal exposure to DEP and maternal stress (Bolton et al., 2013), which we hypothesized to underlie the female protection from behavioral changes observed in male offspring.

It is clear from our results that the developing brain is especially vulnerable to the effects of prenatal air pollution exposure. TLR4-competent fetuses exhibit an IL-1 $\beta$  response to DEP in the brain, when the placenta of the same fetus does not. Prenatal DEP exposure also alters the number of microglia of specific morphologies in the fetal brain at E18, whereas there is no change in the number, size, or elongation factor of placental macrophages at this time point. Furthermore, the changes in microglial maturation result in long-term changes in microglial cell number and volume that endure at least until P30. For example, the presence of more round cells in the PCX of TLR4-competent DEP-exposed males translates to more thick, long microglia (the more “activated” microglial morphology of the adult brain) at P30. The increase in PCX regional volume that was observed at E18 actually reverses to become smaller at P30, which is consistent with the autism literature as well, especially in autistic adolescent and young adults—an age in humans that roughly translates to P30 in a mouse. There is also extensive evidence linking air pollution exposure in adulthood to neurodegeneration (Block & Calderón-Garcidueñas, 2009), which would be in line with a premature loss of synapses and/or cells in the PCX due to prenatal DEP exposure.

Our previous work with prenatal DEP exposure revealed that a “second hit” (i.e., maternal stress or adult high-fat diet) was often necessary to “unmask” more profound behavioral or metabolic effects. This phenomenon is consistent with our laboratory’s previous work on the mechanisms of microglial priming, in which microglia are functionally primed to over-respond to a second immune challenge later in life, such as lipopolysaccharide injection. In the current study, although we observed extensive evidence of changes in microglial maturation and morphology in response to DEP exposure alone, we also observed some evidence of synergism between DEP and LPS in the P30 animals. For example, the combination of DEP and LPS resulted in a greater number of thick, long microglia in the male PCX than either single immune challenge alone. We have evidence supporting that microglia were primed during development because there were more round microglia in the PCX and increased IL-1 $\beta$  in the fetal brain overall in response to DEP exposure. One of the few significant changes that we observed in females was a pattern similar to the male PCX, in that there were more thick, long cells and fewer thin, long cells in the DG of DEP/LPS females. This pattern is also suggestive of microglial priming, and suggests that females were not completely unaffected after all. E18 females also exhibited the increase in IL-1 $\beta$  that males did, which could contribute to the priming environment, even though their microglia at that time were exhibiting the opposite pattern of males. Similarly, female offspring did show increased anxiety-like behavior in adulthood following prenatal DEP and maternal

stress exposure (Bolton et al., 2013), so they are not without consequence of DEP exposure. However, their consequences may occur via different mechanisms than TLR4 signaling, given that they expressed the opposite pattern of males in terms of microglial developmental changes. For example, previous work shows that LPS can induce a pro-inflammatory response by microglia independent of TLR4 activation, via the reactive oxygen species (ROS) pathway (Qin et al., 2005).

In sum, this study has demonstrated for the first time that prenatal air pollution exposure alters microglial maturation in a TLR4- and sex-dependent manner. These results are consistent with our previous observations of pronounced behavioral, metabolic, and neuroinflammatory changes primarily in the adult male offspring of air pollution-exposed dams. Notably, long-term structural changes in the parietal cortex reminiscent of those reported in the autism literature occur in conjunction with a developmentally immature microglial morphology in males. Considering the epidemiological link between prenatal air pollution exposure and autism, our model may be well-suited to further delve into the biological underpinnings of the autistic phenotype. Further research in this area will be critical for informing environmental health policy and identifying effective interventions.

## **6. Developmental Programming of Metabolism, Microglial Activation, and Behavior by “Western Diets”**

### **6.1 Introduction**

Developmental programming by maternal diet was the original phenomenon that gave birth to the field of perinatal programming. Barker and colleagues were the first in the 1980s to document a striking correlation between low birth weight (due to maternal famine, and/or fetal growth restriction) and risk for obesity/overweight and its associated health problems (e.g., heart disease, diabetes) in adulthood (Hales & Barker, 2001). A relatively more recent problem, maternal obesity and HFD consumption has begun attracting attention as another factor that can “program” offspring for lifelong obesity and associated metabolic disorders, as well as mental health dysfunction (e.g., impaired cognition and increased anxiety (de la Monte, 2009; Pasinetti & Eberstein, 2008), setting in motion a vicious cycle of propagating health problems (Grattan, 2008; McGuire et al., 2009).

The proximate brain mechanisms underlying changes in behavior as a result of diet are many, including altered serotonin receptor expression in primates (Sullivan et al., 2010), altered dopamine transmission in rats (Naef et al., 2011; Teegarden, Scott, & Bale, 2009), and increased glucocorticoid receptor expression along with increased stress-induced corticosterone release in rats (Sasaki, de Vega, St-Cyr, Pan, & McGowan, 2013). However, obesity has also been consistently associated with systemic inflammation, which is implicated in numerous disease processes, as well as in cognitive

dysfunction and mood alterations (de la Monte, 2009; Pasinetti & Eberstein, 2008).

Inflammatory molecules such as cytokines can also interact with all the aforementioned neural mechanisms. In a rodent model of maternal obesity, our lab has previously found systemic inflammation in the dams, as well as increases in both basal and LPS-induced proinflammatory IL-1 $\beta$  expression and long-term changes in microglial antigen expression within the hippocampus of their adult offspring (Bilbo & Tsang, 2010). As in our prenatal air pollution model, it is clear that prenatal inflammation has long-term consequences for both physical and mental health.

Like HFD, dietary branched-chain amino acids (BCAAs) are also enriched in a “Western diet” and implicated in human obesity and insulin resistance, and have recently been gaining recognition for their effects on mood regulation. For example, male rats fed a BCAA-supplemented diet exhibit increased anxiety-like behavior and decreased levels of serotonin in the frontal cortex (Coppola et al., 2013). This was shown to be the result of high levels of BCAAs competing with tryptophan, serotonin’s precursor, to cross the blood-brain barrier through their shared transporter, the large neutral amino acid transporter 1 (LAT1), thus resulting in diminished serotonin production within the brain. A similar mechanism appears to be at work in women with post-partum depression, as they possess very high levels of BCAAs, as well as tryptophan, in their blood, suggestive of decreased transport of tryptophan across the blood-brain barrier. Pregnant and post-partum women are especially at risk for mood

disorders related to decreased tryptophan availability, because the metabolic changes during pregnancy that are required for increased fetal nutrient transfer also result in elevated circulating levels of free amino acids, including the BCAAs (Jolly et al., 2004; S. Kalhan & Parimi, 2000; S. C. Kalhan, Rossi, Gruca, Super, & Savin, 1998). Moreover, similar to HFD, BCAAs have also been shown to interact with the immune system—BCAA supplementation results in increased expression of proinflammatory cytokines in immunosuppressed athletes (Bassit et al., 2002).

As both HFD and BCAAs have been associated with changes in mood, including increased risk of anxiety and depression, as well as inflammatory mechanisms, we propose that manipulating these dietary components both separately and in combination prior to and during pregnancy and lactation in mice may be an ideal model for postpartum depression in mothers, as well as for assessing the long-term programming effects of early-life inflammation on offspring brain and behavior. Specifically, we predict that HFDs and BCAAs may synergize to result in exacerbated brain and behavioral consequences in both dams and offspring. In order to test this hypothesis, we placed female mice on one of 4 diets: **1) high-fat diet (HFD), 2) low-fat diet (LFD), 3) HFD supplemented with BCAA (HFD/BCAA), or 4) LFD supplemented with BCAA (LFD/BCAA)**, for 6 weeks prior to breeding and continuing throughout gestation and lactation. We then assessed maternal behavioral changes postpartum, as well as long-term behavioral, metabolic, and neuroimmune outcomes in offspring.

## **6.2 Methods**

### **6.2.1 Animals**

Juvenile (P28) female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, North Carolina, USA) and housed in same-sex pairs in propylene cages with *ad libitum* access to food and filtered water. The colony was maintained at 22°C on a 12:12-h light-dark cycle (lights on at 7 AM). Sentinel animals were housed in the colony room and screened periodically for the presence of common rodent diseases; all screens were negative. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

### **6.2.2 Diets**

On P35-P40, females were placed on one of four diets: a low-fat diet [LFD; 19% of calories from protein, 71% from carbohydrate, and 10% from fat (56:44% soybean oil:lard); cat. no. D07010502, Research Diets, New Brunswick, NJ, USA;  $n=20$ ], a high-fat diet [HFD; 20% of calories from protein, 35% from carbohydrate, and 45% from fat (12:88% soybean oil:lard); cat. no. D12451, Research Diets, New Brunswick, NJ, USA;  $n=19$ ], a low-fat diet supplemented with BCAA [LFD/BCAA; 23% of calories from protein, 67% from carbohydrate, and 10% from fat (56:44% soybean oil:lard); cat. no. D07010503, Research Diets, New Brunswick, NJ, USA;  $n=19$ ], and a high-fat diet supplemented with BCAA [HFD/BCAA; 23% of calories from protein, 34% from carbohydrate, and 43% from fat (12:88% soybean oil:lard); cat. no. D06050807, Research

Diets, New Brunswick, NJ, USA;  $n=20$ ]. The HFD and HFD/BCAA diets provided 4.7 kcal/g, the LFD provided 3.84 kcal/g and the LFD/BCAA provided 3.85 kcal/g. The complete nutritional profiles of each diet are available online (<http://www.researchdiets.com>). Females were fed *ad libitum* for 6 weeks before the introduction of breeder males and remained on the diets throughout pregnancy and lactation. After weaning at postnatal day 28, offspring were fed *ad libitum* with standard laboratory chow (Rodent Diet 5001; Lab-Diet, Philadelphia, PA, USA).

### **6.2.3 Experimental Timeline**

Females were weighed prior to diet assignment, and every three days thereafter. All females were tested in elevated-zero maze (EZM) and open field test (OFT), and fasting blood glucose (FBG) was measured prior to the introduction of breeder males (6 weeks on diet; 2-3 females per male cage;  $n=26-54$ /diet). Females not pregnant after 3 weeks with males were tested after 13 weeks on diet (to mirror the time pregnant females remained on diet prior to sacrifice) in EZM, OFT, and Porsolt forced-swim test (FST), measured for FBG, and sacrificed to collect brain and tissue samples for later analysis ( $n=6-15$ /group). All litters were observed and maternal behavior scored 3 times daily from P2 to P9 ( $n=16-20$ /diet). Dams sacrificed at P8 were observed from P2 to P8, and behaviors were quantified as percent of total observations to normalize between litters. Maternal weights and food consumption were recorded on behavioral testing days.

Each litter was weighed on P1. Approximately half of the litters were then left undisturbed until P8, at which point dams were tested in EZ, OFT and FST, assessed for FBG ( $n=8-11/\text{diet}$ ), and both dams and pups were sacrificed to collect brain and blood samples for later analysis. From the remaining litters, dams were tested in EZM and OFT at P3 and P14, and at P28 in EZM, OFT and FST ( $n=9/\text{diet}$ ). On P28, dams were also tested for FBG and sacrificed for tissue analysis, and pups were weaned onto standard laboratory chow.

Offspring were maintained on standard chow until all animals reached adulthood (60-90 days old). Offspring weights and food consumption were recorded weekly. In adulthood, male and female offspring were tested separately in EZM, OFT, and FST ( $n=19-27/\text{diet}/\text{sex}$ ). Each test was performed on a different day within a four-week window, and all offspring from the same cohort and sex were tested on the same day regardless of exact age. During this time, an insulin sensitivity test was also performed on all offspring. Three days following FST testing, two-thirds of offspring were injected with saline (SAL) or lipopolysaccharide (LPS, as an immune challenge) and 2 hours later, assessed for FBG and sacrificed for tissue analysis ( $n=6-10/\text{diet}/\text{sex}$ ). The remaining third were sacrificed for flow cytometry analysis one week later.

## 6.2.4 Experimental Procedures

### 6.2.4.1 Behavioral Testing

#### 6.2.4.1.1 *Elevated-zero Maze*

Animals were assessed for anxiety using the elevated-zero maze, an adaptation of the classic elevated-plus maze used to characterize anxiety-like behaviors (Shepherd et al., 1994). The maze consisted of a circular track 4.5 cm wide, mounted 49.5 cm off the floor and divided into four quadrants. Two opposing quadrants were enclosed by black walls 15.9 cm high, forming the closed arms of the maze, while the other two were left exposed. Mice were placed at the interface of an open and closed arm and allowed to explore the maze freely for 5 min. The time spent in the closed arms of the maze and number of entries from open to closed arms were scored live by the same observer for all tests. Additional exploratory behaviors scored were body extension at the interface of closed and open arms (stretch-attend postures, SAP, an anxious risk-assessment behavior characterized by Shepherd et al.), and looking over the edge of the open arms (head dips, HD, an exploratory behavior suggesting reduced anxiety).

#### 6.2.4.1.2 *Open Field Test*

The open field test was used to assess activity levels and anxiety-like behavior. Animals were placed in a square enclosure 40 cm by 45.1 cm with walls 34.9 cm high and allowed to explore freely for 10 min. Total distance traveled, mean speed, and time mobile were used as metrics of overall activity, while time in the surround (the area closest to the walls of the maze, ~50% of total area) and percent of distance traveled in

the surround were used to quantify anxiety-like behavior. All behaviors were recorded using *ANY-maze* video tracking software (Stoelting Co., Wood Dale, IL, USA).

#### 6.2.4.1.3 Porsolt Forced-Swim Test

Animals were placed in a cylindrical container 20.3 cm in diameter x 49.5 cm high filled 2/3-full with water, and observed for 6 min. Time spent climbing (actively trying to escape from the enclosure) and time immobile (time not actively swimming or climbing) was recorded live by the same observer for all tests. Animals were then removed to an enclosure with a small towel on a heating pad to dry. Because of the high-stress nature of this test, dams were only tested immediately before they were to be sacrificed (P8 and P28) to prevent harm to pups.

#### 6.2.4.1.4 Maternal care observations

Quality of maternal care in the first ten days of life has been shown to greatly affect later-life behavior, including learning and the stress response (Liu et al., 1997). Dams were observed for one hour each three times daily from P2 to P9 (9-10 AM, 3-4 PM, and 9-10 PM; twice during the light cycle and once during the dark cycle). Observations were made at 5-min intervals without disturbing the animals, using a red flashlight during the dark cycle, and behaviors were recorded at each time point. Specific behaviors recorded included presence on or off the nest; licking and grooming (LG); arched-back nursing (ABN), blanket nursing (BN), and passive nursing (PN); building or fixing the nest; licking and grooming herself (LGH); eating; and whether any pups were scattered outside the nest (Myers et al., 1989).

#### **6.2.4.2 Insulin Sensitivity Testing**

Adult (P60-P90) offspring were assessed for blood glucose reductions after insulin challenge as a measure of insulin sensitivity. In order to assess the basal metabolic state, animals were fasted beginning at 8 AM for a period of 6 h prior to receiving an i.p. injection of insulin (0.75 U/kg; Humulin R; Eli Lilly, Indianapolis, IN, USA) (Holland et al., 2011). Whole-blood glucose was assessed using a glucometer (TRUE2go; CVS Pharmacy, Inc., Woonsocket, RI, USA) at 0, 30, 60, and 120 min. post-insulin injection, and the data are expressed as a percentage of the baseline levels (0 min) (McClung et al., 2004).

#### **6.2.4.3 Tissue Collection for Flow Cytometry Analysis**

A separate cohort of adult offspring (n=7-8/diet/sex) was utilized for flow cytometry. Mice were deeply anesthetized with ketamine-xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine i.p.) and transcardially perfused with ice-cold saline for 2 min, after which brains were rapidly extracted. Brains were dissected on ice by first removing all brainstem and cerebellar tissue, and then dissecting out the hypothalamus (HYP) to be analyzed separately from the rest of the forebrain (FB). The HYP was selected for its known role in metabolism, as well as mood regulation. Tissue collections were spread over 2 days so that both HYP and FB from each animal could be processed together (n=32 animals/day; 64 samples/day). All tissue collection occurred between 8 AM and 11 AM.

#### 6.2.4.3.1 Flow Cytometry

Immediately following tissue collection, dissected brain regions (HYP or FB) were brought to a single-cell suspension [as described in (Williamson et al., 2011)] in an enzyme mix containing 1.5 mg/ml collagenase (Roche Diagnostics, Indianapolis, IN, USA), 0.4 mg/ml DNase I (Roche Diagnostics), 5% fetal bovine serum (Hyclone, GE Healthcare, Logan, UT, USA), and 10 mM HEPES (Gibco, Carlsbad, CA, USA) in Hank's Balanced Salt Solution (without Ca<sup>2+</sup> and Mg<sup>2+</sup>; Gibco). The single-cell suspension was then demyelinated using 30% Percoll (GE Healthcare Bio-Sciences Corp., Uppsala, Sweden) by centrifuging for 10 min. at 700 × g and room temperature and discarding the fatty myelin layer. Red blood cells were then lysed using an ammonium chloride solution. Demyelinated cells were washed once in PBS and centrifuged at 350 × g for 5 min, after which the PBS was decanted.

Cells were first stained for viability with Live/Dead Aqua (1:500; Invitrogen, Carlsbad, CA, USA) for 10 min. at 4°C. Cells were then incubated with a blocking solution containing 5% normal rat serum, 5% normal mouse serum, and 1% anti-mouse Fc receptor block (CD16/CD32; BioLegend, San Diego, CA, USA) for 10 min at 4°C, followed by incubation for 30 min at 4°C in 100 µl of antibody mixture, containing PE-Cy7-conjugated CD45.2 (1:400), APC-Cy7-conjugated CD11b (1:200), PE-conjugated CD14 (1:200), APC-conjugated CCR2 (1:20), AF-700-conjugated Ly6G (1:200), V450-conjugated Ly6C (1:100), FITC-conjugated B220 (1:100), PerCP-Cy5.5-conjugated CD3e (1:100), efluor650-conjugated CD4 (1:400), and BV711-conjugated CD8 (1:200) (all

conjugated anti-mouse antibodies were purchased from eBioscience, Inc., San Diego, CA, USA except for CCR2, which was from R&D Systems, Inc., Minneapolis, MN, USA). Cells were washed in 2 ml PBS supplemented with 0.5% BSA and 2 mM EDTA, spun down at  $350 \times g$ , and fixed in 200  $\mu$ l 0.4% paraformaldehyde before analysis using an LSR II flow cytometer (4 lasers, 18 colors; BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar, Inc., Ashland, OR, USA). For each sample, at least 10,000 events were collected and doublets, debris, and dead cells were excluded from the analysis based on properties of cell size and granularity (forward scatter and side scatter) and Live/Dead staining.

#### **6.2.4.4 Tissue collection for all other analyses**

##### *6.2.4.4.1 LPS injections*

Two hours prior to sacrifice, adult offspring (i.e., those not used for flow cytometry) received an i.p. injection of either sterile saline or 165  $\mu$ g/kg lipopolysaccharide (serotype 0111:B4; Sigma, St. Louis, MO, USA), a component of bacterial cell walls known to generate a robust inflammatory response and sickness behavior in rodents. Injections were administered between 8 and 11 AM, and blood and tissue collected between 10 AM and 1 PM.

##### *6.2.4.4.2 Fasting blood glucose*

All animals (except those used for flow cytometry) were fasted for 3 h prior to sacrifice. Just prior to anesthesia, blood was drawn from the facial vein; one drop was tested with a TRUE2go portable glucometer (CVS Pharmacy, Inc., Woonsocket, RI,

USA), and the rest was collected in a 1.5-ml microcentrifuge tube containing EDTA, centrifuged at  $1,200 \times g$ , and the plasma collected and frozen at  $-80^{\circ}\text{C}$  for later analysis.

#### 6.2.4.4.3 *Tissue collection*

Mice were deeply anesthetized with a ketamine-xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine i.p.) and transcardially perfused with ice-cold saline for 2 min, after which brains were rapidly extracted. Brains were cut in half sagittally, and one half was dissected on ice into hypothalamus (HYP), prefrontal cortex (PFC), and hippocampus (HIP). We placed these regions in separate 1.5-ml microcentrifuge tubes, snap-froze in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for later analysis. We selected these regions for their known roles in the metabolic and behavioral outcomes we assessed. The other half of each brain was post-fixed in 4% paraformaldehyde for 48 h and cryoprotected in 30% sucrose plus 0.1% sodium azide for at least 2 days prior to cryosectioning. In addition, visceral fat was dissected from the abdomen and weighed prior to snap-freezing in liquid nitrogen.

#### 6.2.4.4.4 *Iba-1 immunohistochemistry*

On the day of cryosectioning, fixed half-brains were quickly frozen in  $-30^{\circ}\text{C}$  isopentane, and sliced coronally from the prefrontal cortex through the ventral hippocampus in a 1:6 series at  $40 \mu\text{m}$  in a  $-20^{\circ}\text{C}$  cryostat. Floating sections were stored at  $4^{\circ}\text{C}$  in 0.1% sodium azide until immunohistochemistry was performed. All sections were stained at the same time to minimize any differences in background staining due to differences in antibody preparation and binding across days.

Colorimetric DAB staining for ionized calcium-binding adaptor molecule-1 (Iba-1) was used to visualize microglia within the brain regions of interest. Iba-1 is a microglial-specific marker expressed constitutively throughout the cell bodies and processes, allowing observation of microglial morphology within brain slices. Free-floating sections were washed in PBS for 3 x 5 min initially, incubated for 30 min in 50% methanol to quench blood vessels, and then washed again. They were then incubated for 1 h in PBS with 0.03% hydrogen peroxide, 5% normal goat serum, and 0.3% Triton-X to quench endogenous peroxidase, block, and permeabilize, respectively. Sections were incubated overnight at room temperature in Iba-1 primary antibody (1:1,000; Wako Chemicals, Richmond, VA, USA) on an orbital shaker. On day 2, sections were washed and incubated with a biotinylated secondary antibody (goat anti-rabbit IgG, 1:200; Vector Laboratories, West Grove, PA, USA) for 2 h at room temperature. Sections were washed, and the Avidin-Biotin Complex (ABC) method was used to bind a complex of streptavidin-biotin peroxidase to the secondary antibody during a 2 h incubation. Color was then developed with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) as the chromagen for 45 min at room temperature. Sections were mounted on gelatinized slides, dehydrated, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

#### 6.2.4.4.5 *Iba-1* quantification

Microglial density was quantified with densitometry using U.S. National Institutes of Health ImageJ software (<http://rsbweb.nih.gov/ij/>) as previously described

(Bilbo & Tsang, 2010). Mounted sections were imaged at 10X using a Nikon Eclipse 80i microscope and digital camera (Nikon, Tokyo, Japan) with StereoInvestigator software (MBF Bioscience, Williston, VT, USA). Signal pixels were defined as pixels in a region of interest with gray value 3 standard deviations above the mean gray value of a cell-poor area of tissue within the region of interest. The number of signal pixels and their average gray values above the set background were multiplied to give an integrated density measurement for each section. Five consecutive slices were analyzed for each region of interest, and their values were averaged to give a single integrated density value per region per animal. A mouse brain atlas (Franklin & Paxinos, 1997) was used to identify the anatomical location of each of the 5 regions of interest for Iba1 quantification: the dentate gyrus (DG), CA1, and CA3 of the hippocampus (bregma -1.34 mm to -2.30 mm).

#### 6.2.4.4.6 *qRT-PCR*

Total RNA and native protein were isolated simultaneously from HYP and HIP using the Protein And RNA Isolation System (PARIS kit, Ambion, Waltham, MA, USA) according to manufacturer's instructions. Protein isolates were frozen at -80°C for later analysis. cDNA was synthesized from 100 ng of RNA using the QuantiTect reverse transcription kit (Qiagen, Inc., Valencia, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using a QuantiFast SYBR Green PCR kit (Qiagen) on a Mastercycler ep *realplex* (Eppendorf, Hauppauge, NY, USA). 1 µl of cDNA was added to 12 µl of master mix containing specific primers for the genes of interest. Primers for IL-1β were purchased from Qiagen, and we designed primer primers for all other genes

(GAPDH, TLR2, TLR4, Iba-1, GFAP, leptin receptor [Lepr], and insulin receptor [Insr]; see Table 5) as previously described (Williamson et al., 2011). Designed primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Optimal annealing temperatures for each primer pair were determined by running a temperature gradient, and specificity was verified by melt-curve analysis. For analysis, we determined the threshold cycle ( $C_T$ ) for each reaction and calculated relative gene expression using the  $2^{-\Delta\Delta C_T}$  method (Livak & Schmittgen, 2001; Pfaffl, 2001; Williamson et al., 2011), using GAPDH as the housekeeping gene.

**Table 5: Forward and reverse primer sequences for qRT-PCR analysis**

| <b>Gene</b>  | <b>Forward Primer (5'-3')</b> | <b>Reverse Primer (5'-3')</b> |
|--------------|-------------------------------|-------------------------------|
| <i>GAPDH</i> | ggtcaccagggctgccatt           | tgggcttcccgtgatgaca           |
| <i>TLR2</i>  | cgttcattctctggagcatcc         | gacagagactcctgagcag           |
| <i>TLR4</i>  | cagcagaggagaaagcatc           | caccaggaataaagtctctg          |
| <i>Iba-1</i> | cagacttcattctctctcttc         | ctttccactcattcccttgc          |
| <i>GFAP</i>  | tggagagaaagggtgaatcg          | gtattgagtgcgaatctctc          |
| <i>Lepr</i>  | cagtttagtctttaccag            | gtcattctgctgcttgc             |
| <i>Insr</i>  | ccaatagccatctttgtg            | ggacagaaggattagtgg            |

#### 6.2.4.4.7 IL-1 $\beta$ ELISA

Levels of the proinflammatory cytokine IL-1 $\beta$  were measured in the protein homogenates obtained from the PARIS kit procedure described above using a commercially available ELISA kit (R&D Systems, Inc.). Prior to analysis, protein homogenates were demyelinated using hexane as previously described (Bolton et al., 2013) and centrifuged at 16,000 x g to collect the supernatant, which was run in a 1:1

dilution with calibration buffer for the assay. The detection limit for the assay was 1 pg/ml and the intra-assay and inter-assay coefficients of variation were <10%.

## **6.2.5 Data Analysis and Statistics**

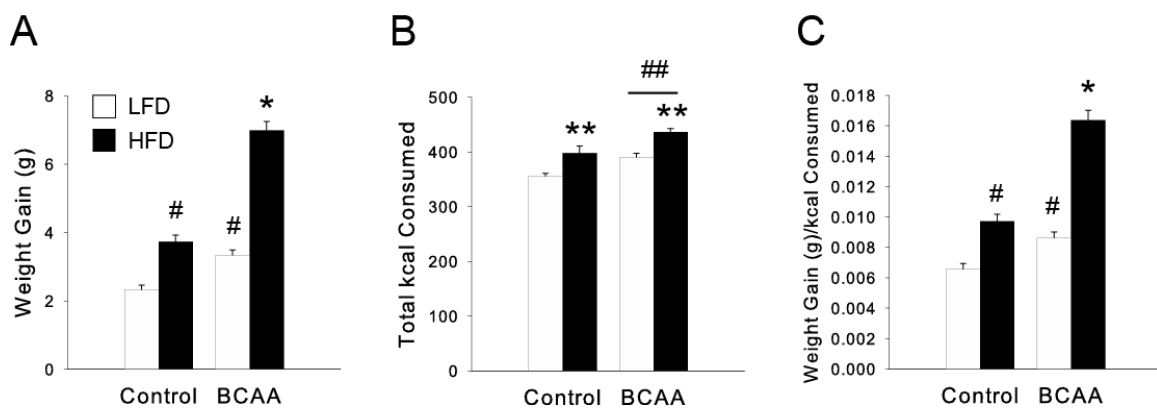
All data were analyzed using SPSS statistical software (IBM, Armonk, NY, USA). All measures were analyzed using two-way (HFD X BCAA) or three-way (HFD X BCAA X Sex) ANOVA, except densitometry, sac glucose, and ELISA measures that used four-way (HFD X BCAA X Sex X LPS) ANOVA. Following significant *F* scores for interactions in ANOVA tests, *post hoc* comparisons (Fisher's LSD) were performed to further distinguish among groups, and all differences were considered statistically significant if  $p < 0.05$ .

## **6.3 Results**

### **6.3.1 Diets high in fat and BCAA generate an obese phenotype in dams**

Both HFD and BCAA animals gained significantly more weight than controls by week 6 on the diet, with a synergistic effect in the HFD/BCAA group [significant HFD X BCAA interaction,  $F(1,185)=24.39$ ,  $p<0.001$ ; *post hoc*,  $p<0.05$ ; Figure 41A]. During the 6 weeks before male introduction, HFD and BCAA groups consumed more kilocalories of food than the control group [significant main effects of HFD,  $F(1,68)=22.75$ ,  $p<0.001$ ; and BCAA,  $F(1,68)=15.63$ ,  $p<0.001$ ; Figure 41B], probably due to the greater palatability and energy-density of these diets (Coppola et al., 2013). Intriguingly, we found that HFD/BCAA animals also gained significantly more weight per kilocalorie consumed

than did the control groups after 6 weeks on diet, suggesting an effect of diet on metabolic regulation [significant HFD X BCAA interaction,  $F(1,185)=16.84$ ,  $p<0.001$ ; post hoc,  $p<0.05$ ; Figure 41C]. No difference was observed between diets in percent weight gained during pregnancy, and HFD/BCAA animals remained heavier than all other groups (data not shown).



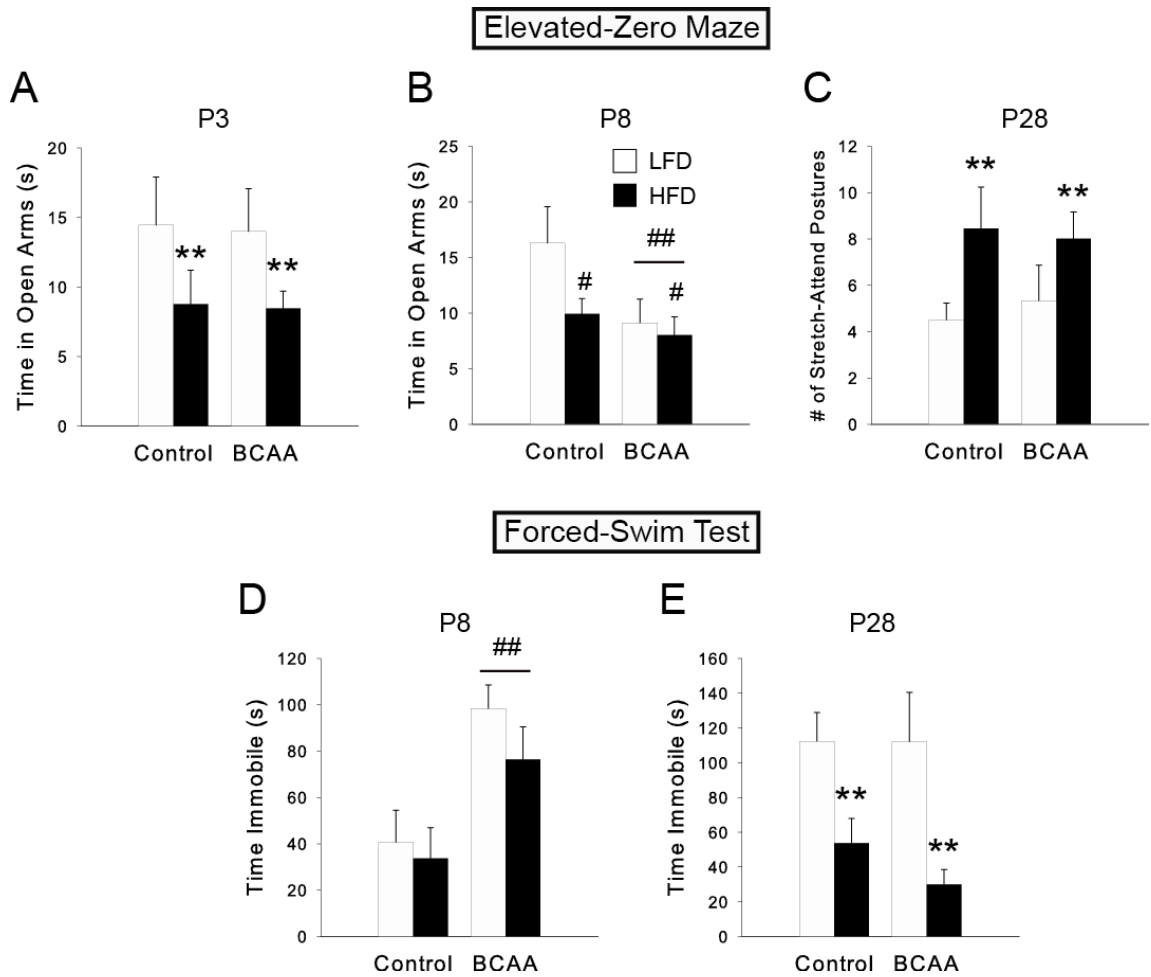
**Figure 41: HFD and BCAA generate an obese phenotype in adult females.** A) After six weeks on diet, body weight was increased in both HFD and BCAA groups, with a synergistic effect in HFD/BCAA females. B) HFD and BCAA groups consumed more kilocalories than controls. C) HFD/BCAA dams gained more weight than other groups, even after controlling for kilocalories of food consumed. Data are mean  $\pm$  SEM,  $n=35-60$  animals/group for weight gain;  $n=15-21$  cages/group for food consumption. \* $p<0.001$  vs. all other groups; # $p<0.05$  vs. LFD/Control; \*\* $p<0.001$ , HFD vs. LFD; ## $p<0.001$ , BCAA vs. Control.

### 6.3.2 HFD dams display an anxious phenotype, whereas BCAA dams exhibit postpartum depressive-like behavior

At P3, HFD dams spent less time than controls in the open arms of the EZM, which is indicative of anxiety-like behavior [significant main effect of HFD,  $F(1,30)=4.37$ ,  $p<0.05$ ; Figure 42A]. HFD dams also exhibited a tendency to spend less time in the open arms of the EZM at P8 [trend for main effect of HFD,  $F(1,29)=3.23$ ,  $p=0.08$ ; Figure 42B]. At

P28, time in the open arm was not different between groups, but HFD dams performed more stretch-attend postures, another indicator of anxiety-like behavior in the EZM [significant main effect of HFD,  $F(1,34)=5.33$ ,  $p<0.05$ ; Figure 42C]. Furthermore, P28 HFD dams spent less time immobile in the FST than controls [significant main effect of HFD,  $F(1,34)=12.12$ ,  $p<0.005$ ; Figure 42E], which may indicate hyperactivity or anxiety. Altogether, these results suggest increased anxiety-like behavior in postpartum HFD dams.

At P8, BCAA dams spent markedly more time immobile in the FST than controls [significant main effect of BCAA,  $F(1,30)=14.64$ ,  $p<0.005$ ; Figure 42D], indicating an increase in depressive-like behavior. Importantly, this effect was not present in non-pregnant animals (data not shown), and the effect disappeared by FST testing at P28 (Figure 42E). BCAA dams also exhibited more anxiety-like behavior in the EZM specifically at P8 [significant main effect of BCAA,  $F(1,29)= 4.69$ ,  $p<0.05$ ; Figure 42B]. These data indicate a profound mood disturbance in BCAA dams that is dependent on pregnancy and restricted to the early postpartum period, which is characteristic of postpartum depression in humans.



**Figure 42: HFD dams showed increased postpartum anxiety-like behavior, whereas BCAA dams exhibited a postpartum depression-like phenotype. A) At 3 days postpartum, HFD dams spent less time in the open arm of the EZM than LFD controls. B) At 8 days postpartum, BCAA dams spent less time in the open arms than controls, and HFD dams tended to show a similar pattern. C) At 28 days postpartum, HFD dams performed more stretch-attend postures in the EZM than LFD controls. D) At P8, BCAA dams were more immobile in the FST than controls. E) At P28, HFD dams were less immobile than LFD controls. Data are mean  $\pm$  SEM,  $n=7-10$ /group. \*\* $p<0.05$ , HFD vs. LFD; # $p=0.08$  HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control.**

### 6.3.3 HFD and BCAA dams show altered maternal care behaviors in the early postpartum period

Overall, HFD/BCAA dams spent more time on the nest than the LFD groups [significant HFD X BCAA interaction,  $F(1,63)=5.01$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ; Figure 43A]. However, HFD and BCAA dams also spent significantly less time eating during observations than controls, likely due to the higher caloric content of their food, which may account for this effect [significant main effect of HFD,  $F(1,63)=59.93$ ,  $p<0.001$ ; and BCAA,  $F(1,63)=13.34$ ,  $p<0.005$ ; Figure 43B]. Indeed, there was a strong negative correlation between the overall time spent on the nest and time spent eating,  $r(71)= -0.64$ ,  $p<0.001$ . Moreover, there were no significant differences in the overall time spent nursing due to maternal diet (data not shown), suggesting that the increased time spent on the nest did not necessarily translate into increased mother-pup interaction.

HFD dams overall (i.e., both HFD/Control and HFD/BCAA) spent more time licking and grooming their pups during the first postnatal week [significant main effect of HFD,  $F(1,63)=8.18$ ,  $p<0.01$ ]. This behavior is traditionally thought to be indicative of high-quality maternal care and is associated with improved offspring outcomes. However, HFD dams were especially likely to lick and groom during the dark (active) cycle [significant main effect of HFD,  $F(1,63)=22.69$ ,  $p<0.001$ ; Figure 43C], when LG is less common in normal dams (Champagne et al., 2003). Furthermore, HFD dams also spent more time during the dark cycle self-grooming [significant main effect of HFD,  $F(1,63)=9.83$ ,  $p<0.005$ ; Figure 43D] and building their nests [significant main effect of

HFD,  $F(1,63)=7.95, p<0.01$ ] than controls (data not shown). However, the increased time spent building their nests was not correlated with increased nest quality (based on a subjective score from 0 to 3; Figure 43E). Given the finding of increased anxiety-like behavior in postpartum HFD dams, it is possible that enhanced licking and grooming, together with excessive self-grooming and fidgeting with their nests, represents a repetitive, anxious phenotype.

BCAA dams, especially LFD/BCAA dams, had less well-built nests during the first postnatal week relative to control dams [close trend for main effect of BCAA,  $F(1,62)=3.86, p=0.05$ ; significant HFD X BCAA interaction,  $F(1,62)=7.05, p<0.05$ ; post hoc,  $p<0.05$ ; Figure 43E]. Moreover, during the light cycle, when dams spend most of their time nursing, LFD/BCAA moms spent a higher percentage of their time nursing their pups in passive, inefficient nursing postures (i.e., blanket and passive nursing, as opposed to arched-back nursing) compared to LFD/Control moms [significant HFD X BCAA interaction,  $F(1,63)=7.18, p<0.01$ ; post hoc,  $p<0.05$ ; Figure 43F]. These data are consistent with the postpartum depressive-like phenotype observed in the aforementioned behavioral tests.

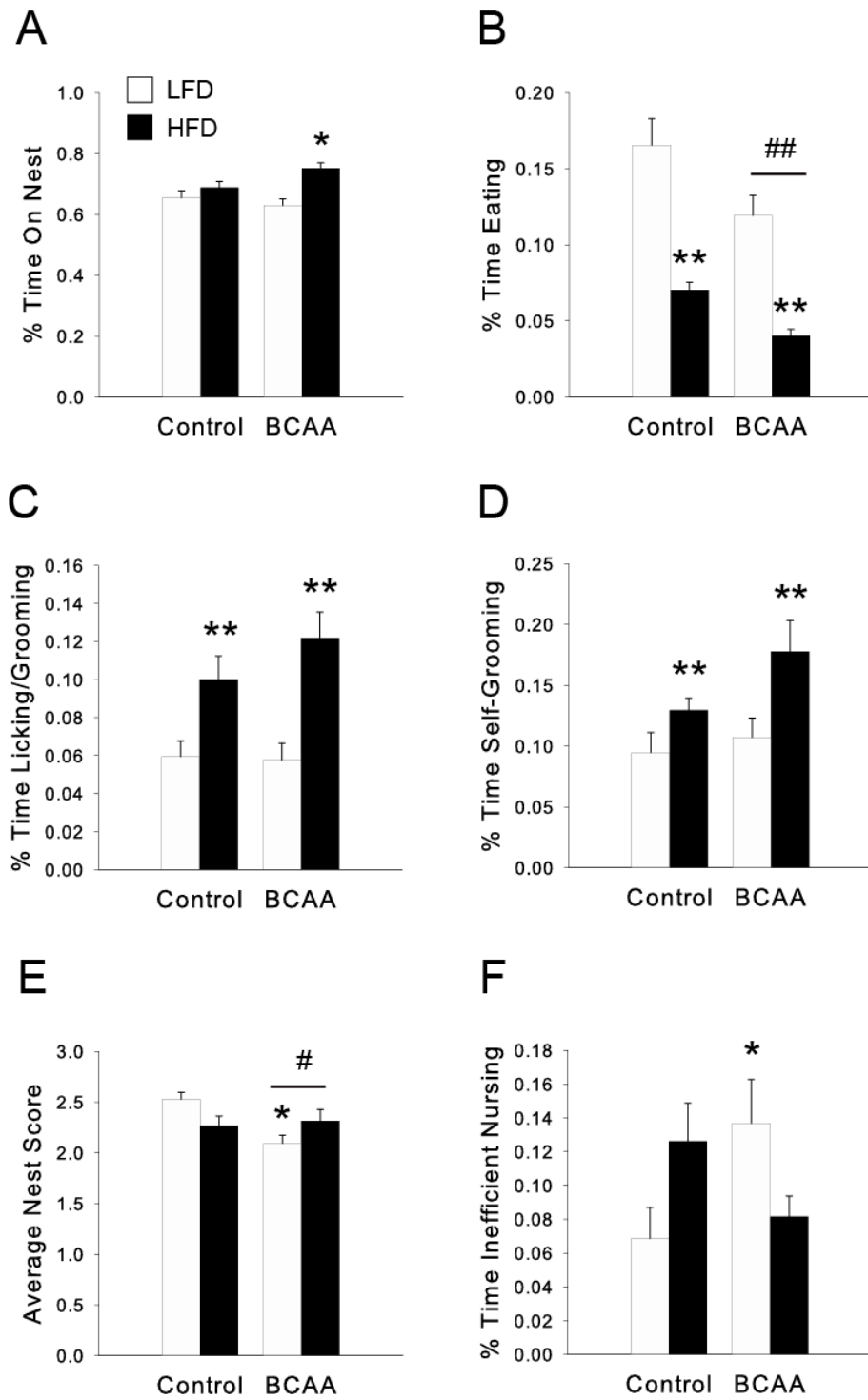


Figure 43: HFD and BCAA dams showed altered maternal care during the early postpartum period (P2-P9). A) HFD/BCAA dams spent more overall time on the nest

than LFD groups. B) However, HFD and BCAA dams both spent less time eating than controls, likely due to the higher caloric content of their diets, which may have contributed to this effect. C) HFD dams spent more time licking and grooming their pups during the dark cycle, but not the light cycle (when most LG is typically observed), than LFD controls. D) HFD dams also spent more time self-grooming during the dark cycle than LFD controls. E) BCAA dams, especially LFD/BCAA dams, had nests of lower quality than controls. F) LFD/BCAA dams spent more of their total time nursing during the light cycle (when most nursing occurs) in efficient or passive postures (i.e., blanket and passive nursing as opposed to arched-back nursing). Data are mean  $\pm$  SEM,  $n= 17-20$ /group (note that dams sacrificed at P8 and P28 are combined here). \* $p<0.05$  vs. LFD/Control and LFD/BCAA for panel A; \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control; # $p=0.05$ , HFD vs. LFD; \* $p<0.05$  vs. LFD/Control for panel F.

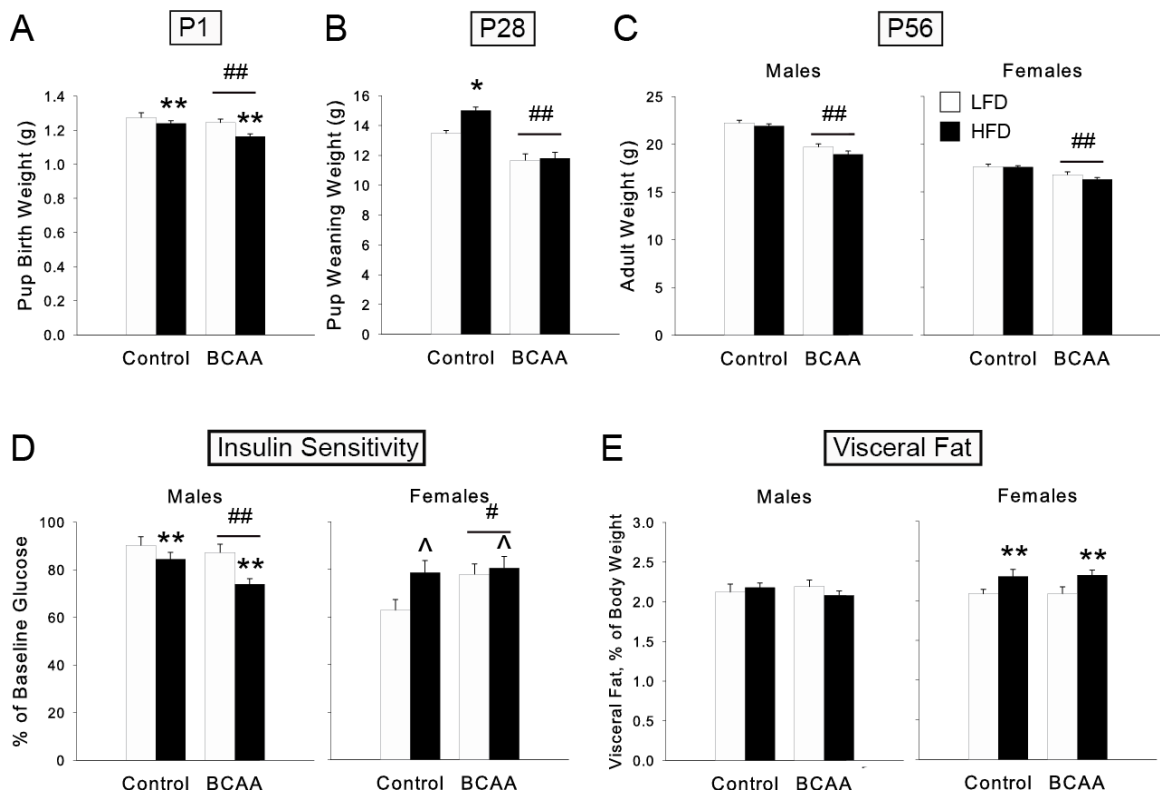
#### **6.3.4 Pups born to HFD and BCAA dams show changes in body weight and metabolism that endure into adulthood**

At P1, HFD and BCAA litters both had slightly lower average weights, an effect most pronounced in HFD/BCAA pups [significant main effect of HFD,  $F(1,63)=7.70$ ,  $p<0.01$ ; and BCAA,  $F(1,63)=6.17$ ,  $p<0.05$ ; Figure 44A]. By P28, both male and female pups born to BCAA dams weighed significantly less than controls, whereas HFD increased pup weight in control but not BCAA litters [litter average weights; significant HFD X BCAA interaction,  $F(1,32)=4.46$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ; Figure 44B]. This effect did not continue into adulthood for HFD offspring following weaning onto standard chow; in fact, HFD offspring tended to exhibit the opposite pattern and weighed slightly less than LFD offspring at 8 weeks of age [trend for main effect of HFD,  $F(1,192)=2.95$ ,  $p=0.09$ ], despite no difference in food consumption (data not shown). On the other hand, the effect of maternal BCAA on offspring weight did endure into adulthood. At 8 weeks of age, offspring of BCAA dams weighed significantly less than controls [significant main effect of BCAA,  $F(1,192)=93.73$ ,  $p<0.001$ ; Figure 44C], but this pattern was exhibited to a

greater extent in males than females [significant Sex X BCAA interaction,  $F(1,192)=18.00$ ,  $p<0.001$ ]. This decrease in weight also occurred in association with decreased consumption of standard chow [ $F(1,66)=28.90$ ,  $p<0.001$ ; data not shown]. Importantly, this effect is not the same as that seen in dams; BCAA supplementation increases body weight in adult females but decreases the weight of offspring exposed to the diet in early life. The effects of the two dietary components are also not reducible to maternal weight alone, as HFD and BCAA caused comparable weight gain in dams, but different outcomes in offspring.

In order to assess the long-term metabolic outcomes of maternal HFD and BCAA, we administered an insulin sensitivity test to adult offspring. Interestingly, male and female offspring exhibited completely opposite patterns of insulin sensitivity in response to maternal diet [significant Sex X HFD interaction,  $F(1,184)=10.58$ ,  $p<0.005$ ; significant Sex X BCAA interaction,  $F(1,184)=7.04$ ,  $p<0.01$ ]. Adult male offspring of HFD and BCAA dams exhibited an increased insulin sensitivity, such that HFD/BCAA offspring had the lowest glucose levels 30 min. post-insulin injection [the time of greatest response to insulin; significant main effect of HFD,  $F(1,97)=8.48$ ,  $p<0.005$ , and significant main effect of BCAA,  $F(1,97)=4.17$ ,  $p<0.05$ ; Figure 44D]. In contrast, maternal HFD and BCAA tended to decrease insulin sensitivity in adult female offspring, resulting in increased glucose levels 30 min. post-insulin injection [trend for main effect of HFD,  $F(1,87)=3.55$ ,  $p=0.06$ , and trend for main effect of BCAA,  $F(1,87)=3.11$ ,  $p=0.08$ ;

Figure 44D]. Female HFD offspring also had increased visceral fat deposits relative to controls [significant main effect of HFD,  $F(1,51)=6.39$ ,  $p<0.05$ ; Figure 44E], despite no overall difference in body weight, suggesting metabolic dysregulation independent of body weight. On the other hand, the size of visceral fat deposits in male offspring did not differ [significant Sex X HFD interaction,  $F(1,107)=4.82$ ,  $p<0.05$ ]. Overall, these findings suggest a long-term impact of maternal diets on body weight regulation, as well as sex-specific effects on metabolic outcomes.



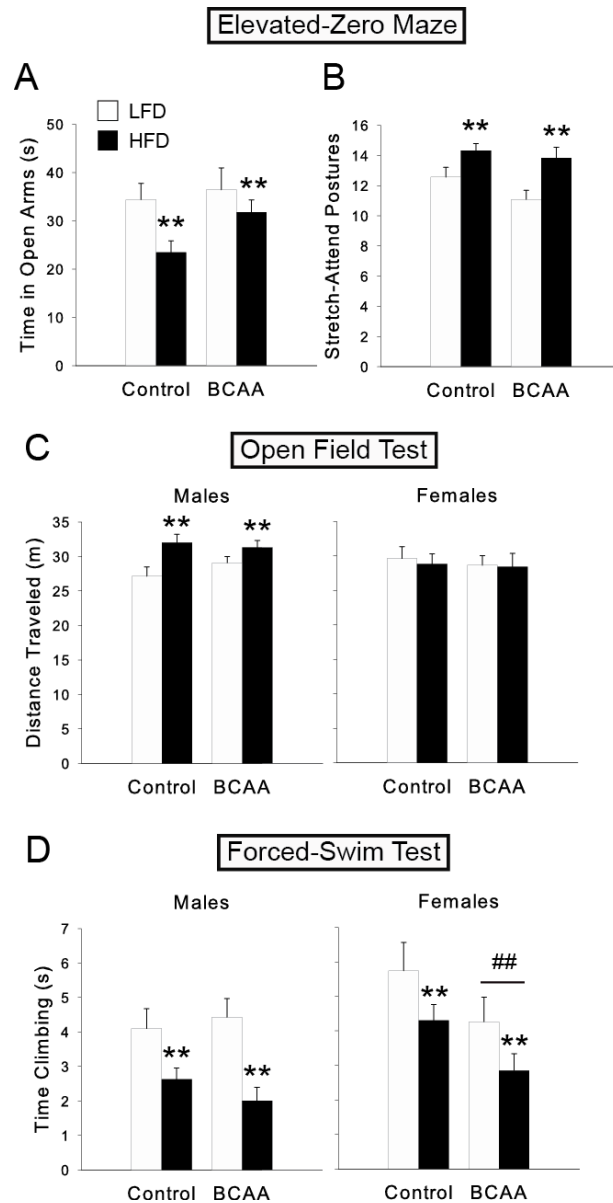
**Figure 44: Pups born to HFD and BCAA dams exhibited changes in body weight and metabolism that endured into adulthood. A) HFD and BCAA both decreased pup weight at P1 compared to controls ( $n=15-21$  litter-average weights/group). B) BCAA pups weighed less at weaning than controls at weaning, whereas HFD/Control pups weighed more than LFD/Controls ( $n=9$  litter-average weights/group). C) The decrease**

in body weight due to maternal BCAA endured into adulthood ( $n=19-30$  animals/group/sex). D) Male HFD and BCAA offspring exhibited increased insulin sensitivity (i.e., a greater glucose decrease to insulin), whereas female HFD and BCAA offspring tended to be more insulin-resistant ( $n=17-29$ /group/sex). E) Female HFD offspring had more visceral fat as a percentage of their total body weight relative to LFD controls, whereas males did not differ ( $n=7-21$ /group/sex). Data are mean  $\pm$  SEM. \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control; \* $p<0.05$  vs. LFD/Control; # $p=0.08$ , BCAA vs. Control; ^ $p=0.06$ , HFD vs. LFD.

### 6.3.5 Maternal HFD induces sex-specific behavioral changes in adult offspring

Adult HFD offspring exhibited increased anxiety-like behavior in the EZM, as measured by less time spent in the open arms [significant main effect of HFD,  $F(1,181)=4.77$ ,  $p<0.05$ ; Figure 45A] and more stretch-attend postures cautiously performed at the opening of the closed arms compared to LFD controls [significant main effect of HFD,  $F(1,181)=13.32$ ,  $p<0.001$ ; Figure 45B], regardless of sex. In the OFT, HFD males were hyperactive, as they moved more [significant main effect of HFD,  $F(1,96)=9.20$ ,  $p<0.005$ ] and at a greater mean speed than LFD controls [significant main effect of HFD,  $F(1,96)=9.31$ ,  $p<0.005$ ], whereas females did not differ [significant Sex X HFD interaction,  $F(1,184)=4.09$ ,  $p<0.05$ ; Figure 45C]. Overall time spent immobile in the FST did not differ, but the time spent actively climbing (i.e., trying to escape the water) was decreased in both male and female HFD offspring relative to LFD offspring [significant main effect of HFD,  $F(1,181)=18.75$ ,  $p<0.001$ ; Figure 45D]. Moreover, female BCAA offspring also struggled less to escape the apparatus compared to controls [significant main effect of BCAA,  $F(1,85)=5.59$ ,  $p<0.05$ ], whereas male BCAA offspring did not differ [trend for Sex X BCAA interaction,  $F(1,181)=2.86$ ,  $p=0.09$ ; Figure 45D]. This

finding may suggest the presence of increased depressive-like behavior in HFD offspring overall in conjunction with their increased anxiety-like behavior, which would be consistent with the high comorbidity of mood disorders in humans. Interestingly, the pattern of decreased struggling in BCAA female offspring may indicate depressive-like behavior similar to that observed in their mothers, although this data is difficult to interpret in the absence of a difference in time spent immobile.



**Figure 45: Adult offspring of HFD dams exhibited long-term behavioral changes. A) Both male and female offspring of HFD dams spent less time in the open arms of the EZM than LFD controls. B) HFD offspring also performed more stretch-attend postures, a cautious behavior at the interface of the closed arms in the EZM, than LFD controls. C) Male, but not female, offspring of HFD dams were hyperactive in the OFT. D) Both male and female HFD offspring spent less time actively struggling in the FST, and BCAA further exacerbated this indication of depressive-like behavior. Data are mean  $\pm$  SEM,  $n=46-49$ /group for A & B (sexes combined),  $n=18-28$ /group/sex for C & D (sexes separate). \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control.**

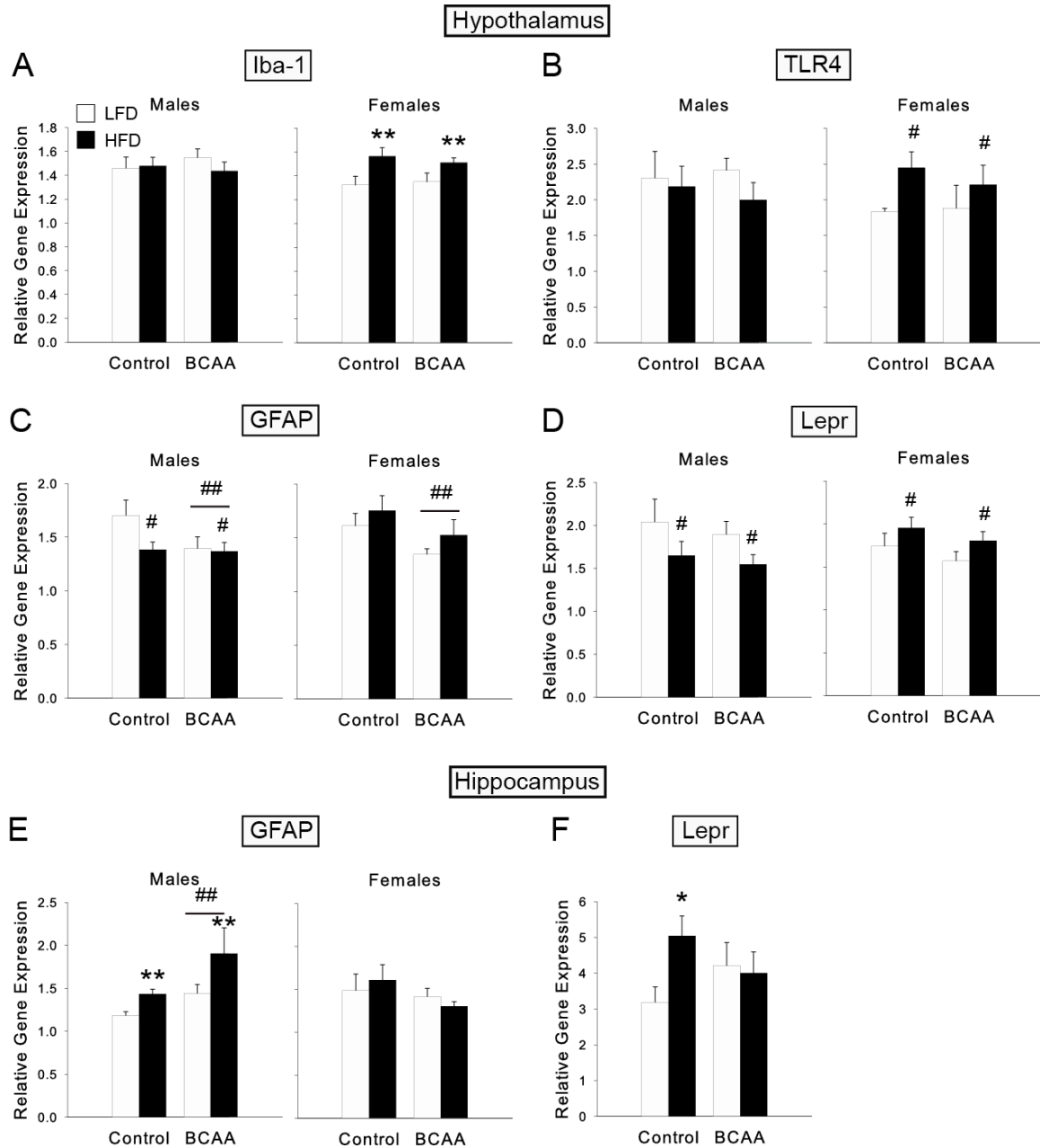
### **6.3.6 Maternal HFD induces sex- and brain region-specific changes in neuroimmune gene expression in the HYP and HIP of adult offspring**

In order to assess the neuroimmune effects of maternal diet that may underlie the observed behavioral changes, we analyzed qRT-PCR gene expression in the HYP and HIP of adult offspring. In the HYP, analysis of the microglial marker Iba-1 revealed a significant Sex X HFD interaction [ $F(1,54)=5.49, p<0.05$ ]. Follow-up tests revealed that this effect was driven by a marked increase in Iba-1 expression in HFD females relative to LFD controls [significant main effect of HFD,  $F(1,25)=8.61, p<0.01$ ], whereas males did not differ due to maternal diet (Figure 46A). Similar to Iba-1, analysis of the innate immune receptor TLR4, which is primarily expressed by microglia in the brain, revealed a trend for a significant Sex X HFD interaction [ $F(1,53)=3.58, p=0.06$ ]. Follow-up tests revealed that this effect was driven by a tendency for HFD females to exhibit increased TLR4 relative to LFD controls [trend for main effect of HFD,  $F(1,25)=2.98, p=0.09$ ], whereas males did not differ (Figure 46B). Analysis of GFAP, an astrocyte marker, in the HYP revealed a significant Sex X HFD interaction [ $F(1,52)=4.39, p<0.05$ ] and a significant main effect of BCAA [ $F(1,52)=6.64, p<0.05$ ]. Follow-up tests revealed that this effect was due to a tendency for both HFD and BCAA to decrease GFAP expression in males [trend for main effect of HFD,  $F(1,27)=2.67, p=0.1$ ; trend for main effect of BCAA,  $F(1,27)=2.36, p=0.1$ ], whereas females exhibited a significant main effect of BCAA [ $F(1,25)=4.29, p<0.05$ ; Figure 46C]. We next measured expression of the leptin receptor, which is expressed by microglia to even a greater extent than astrocytes

or neurons, and leptin has been shown to induce IL-1 $\beta$  release from microglia (Pinteaux et al., 2007). Analysis of the expression pattern revealed a significant Sex X HFD interaction [ $F(1,52)=6.96, p<0.05$ ]. Follow-up tests revealed that this effect was due to a tendency for HFD to decrease leptin receptor expression in males [trend for main effect of HFD,  $F(1,26)=3.87, p=0.06$ ], and conversely, increase leptin receptor expression in females [ $F(1,26)=3.34, p=0.08$ ; Figure 46D]. There were no significant differences detected in expression of the insulin receptor (data not shown).

In the HIP, analysis of GFAP revealed a significant Sex X BCAA interaction [ $F(1,51)=6.25, p<0.05$ ] and a trend for a Sex X HFD interaction [ $F(1,51)=2.55, p=0.1$ ]. Follow-up tests revealed that these effects were due to increased expression of GFAP as a result of both HFD and BCAA in males [significant main effect of HFD,  $F(1,28)=4.62, p<0.05$ ; significant main effect of BCAA,  $F(1,28)=4.82, p<0.05$ ], whereas females did not significantly differ by maternal diet (Figure 46E). Interestingly, this was the opposite pattern of GFAP gene expression compared to the HYP. Analysis of leptin receptor expression in the HIP revealed a trend for a HFD X BCAA interaction [ $F(1,54)=3.14, p=0.08$ ]. Post hoc tests revealed this to be caused a significant increase in the leptin receptor expression of HFD/Control animals relative to LFD/Control animals ( $p<0.05$ ), regardless of sex, whereas BCAA animals did not differ (Figure 46F). There were no significant differences detected in expression of the insulin receptor or TLR4 (data not

shown). Iba-1 gene expression was not measured in the HIPP, because we were instead able to assess its protein levels via histology.



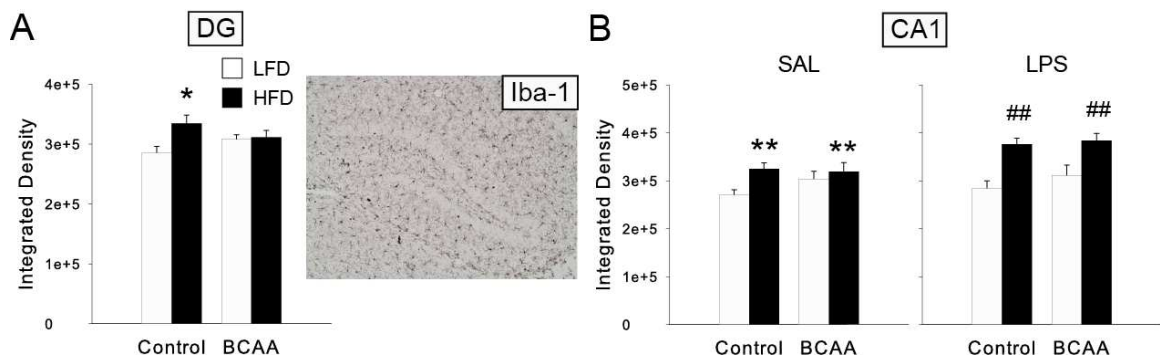
**Figure 46: Maternal HFD and BCAA induced sex- and brain region-dependent alterations in neuroimmune gene expression. A) Female HFD offspring expressed higher levels of Iba-1 in the HYP, whereas males did not differ. B) Female offspring**

of HFD dams also tended to increase TLR4 expression in the HYP relative to LFD controls. C) BCAA decreased GFAP expression in both males' and females' HYP, and HFD further decreased GFAP expression in males. D) HFD tended to decrease leptin receptor expression in the male HYP, whereas HFD tended to do the exact opposite in the female HYP. E) In the HIPP, both HFD and BCAA increased expression of GFAP in males, whereas females did not differ. F) HFD/Control offspring expressed higher levels of leptin receptor in the HIPP than LFD/Control offspring, regardless of sex. Data are mean  $\pm$  SEM,  $n=5-9$ /group/sex for A-E (sexes separate),  $n=14-16$ /group for F (sexes combined). \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control; # $p=0.1$ , HFD vs. LFD; \* $p<0.05$  vs. LFD/Control.

### **6.3.7 Maternal HFD increases microglial antigen density in the hippocampus in adulthood, and primes microglia for over-activation by an adult LPS challenge**

In order to assess the effects of maternal diet on microglial activation, we analyzed the density of Iba-1 staining in the HIPP of adult offspring treated with SAL or LPS 2 h prior to sacrifice. Male and female offspring of HFD/Control dams had increased microglial antigen staining in the dentate gyrus (DG) than LFD/Control and [significant HFD X BCAA interaction,  $F(1,108)=4.40$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ; Figure 47A] at baseline (i.e., regardless of LPS injection), whereas BCAA groups did not differ due to HFD. However within the CA1, HFD increased Iba-1 staining density at baseline [significant main effect of HFD,  $F(1,108)=26.67$ ,  $p<0.001$ ; Figure 47B], regardless of BCAA. Moreover, LPS challenge markedly increased staining density in the CA1 in HFD offspring, but not in LFD offspring [significant HFD X LPS interaction,  $F(1,108)=4.74$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ; Figure 47B]. This suggests that CA1 microglia may be “primed” to overrespond to a later-life immune challenge due to perinatal HFD exposure, consistent with previous work from our laboratory (Bilbo & Tsang, 2010). No significant

differences were detected in Iba-1 staining density within the CA3 of the hippocampus, but there was a trend for increased staining density overall due to LPS challenge [F(1,108)=2.67,  $p=0.1$ ; data not shown].



**Figure 47: Maternal HFD increased microglial antigen density in the hippocampus. A)** Adult offspring of HFD/Control dams exhibited increased Iba-1 antigen density in the DG of the hippocampus relative to offspring of LFD/Control dams, whereas BCAA groups did not differ. **B)** Maternal HFD increased Iba-1 staining density at baseline, and LPS caused an exaggerated increase in the HFD offspring only. Data are mean  $\pm$  SEM,  $n=28-32$ /group for A (injections combined),  $n=14-17$ /group for B (injections separate). Note sexes are combined here, due to a lack of significant sex differences. \* $p<0.05$  vs. LFD/Control; \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , LPS vs. SAL.

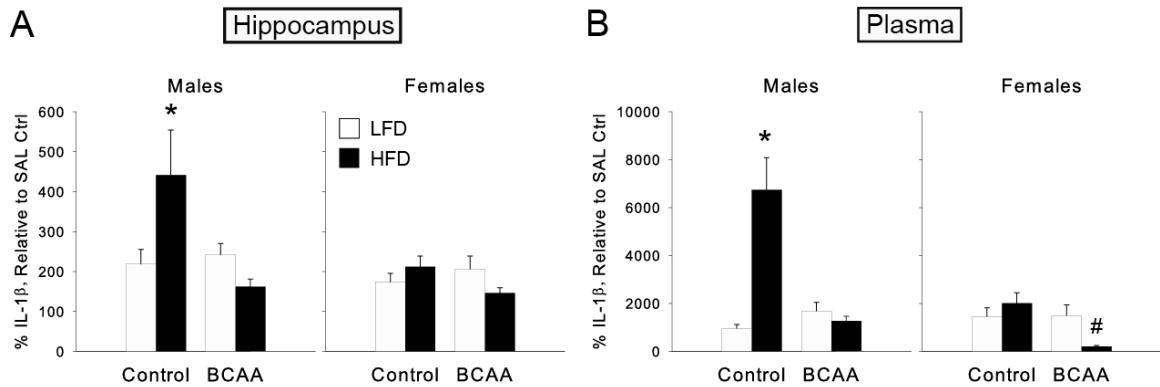
### 6.3.8 Maternal HFD causes an exaggerated IL-1 $\beta$ response following an adult LPS challenge both in the hippocampus and periphery of male offspring

In order to assess functional priming of microglia, we administered an adult immune challenge (i.e., LPS) to determine if maternal diet during the perinatal period would alter the inflammatory response of the offspring later in life. We initially analyzed the baseline IL-1 $\beta$  levels of SAL-injected offspring, and found no significant differences due to maternal diet, in either the brain or periphery. Therefore, we transformed the data to the percent increase in IL-1 $\beta$  in LPS-injected animals over their

SAL-injected controls within the same diet group, as a simpler index of the IL-1 $\beta$  response to the immune challenge. Analysis of this IL-1 $\beta$  response in the hippocampus of adult offspring revealed a significant HFD X BCAA interaction [ $F(1,51)=7.82, p<0.01$ ] and a trend for a Sex X BCAA interaction [ $F(1,51)=2.44, p=0.1$ ]. Follow-up tests revealed that male HFD/Control offspring had a significantly higher IL-1 $\beta$  response in their hippocampus than did HFD/BCAA and LFD/Control males [significant HFD X BCAA interaction,  $F(1,25)=4.95, p<0.05$ ; post hoc,  $p<0.05$ ; Figure 48A]. Female offspring exhibited a similar pattern [trend for HFD X BCAA interaction,  $F(1,26)=3.70, p=0.07$ ], but this effect did not reach significance in post hoc comparisons. Of note, both IL-1 $\beta$  and TLR2 gene expression in the HIPP and HYP of adult offspring paralleled the IL-1 $\beta$  protein levels reported here (data not shown), suggesting that the immune response in the HYP to LPS may be similar to that of the HIPP.

Analysis of the IL-1 $\beta$  response in the periphery (i.e., plasma) of the same animals revealed a similar pattern to the hippocampus [significant Sex X HFD X BCAA interaction,  $F(1,50)=7.72, p<0.01$ ]. Follow-up tests revealed that male HFD/Control offspring had a significantly higher IL-1 $\beta$  response in the periphery than did all other diet groups [significant HFD X BCAA interaction,  $F(1,24)=18.91, p<0.001$ ; post hoc,  $p<0.001$ ; Figure 48B]. Female offspring again exhibited a similar pattern [significant HFD X BCAA interaction,  $F(1,26)=6.25, p<0.05$ ], but in this case it was the HFD/BCAA females

that were most affected, showing a significantly decreased IL-1 $\beta$  response compared to all other groups ( $p<0.05$ ).



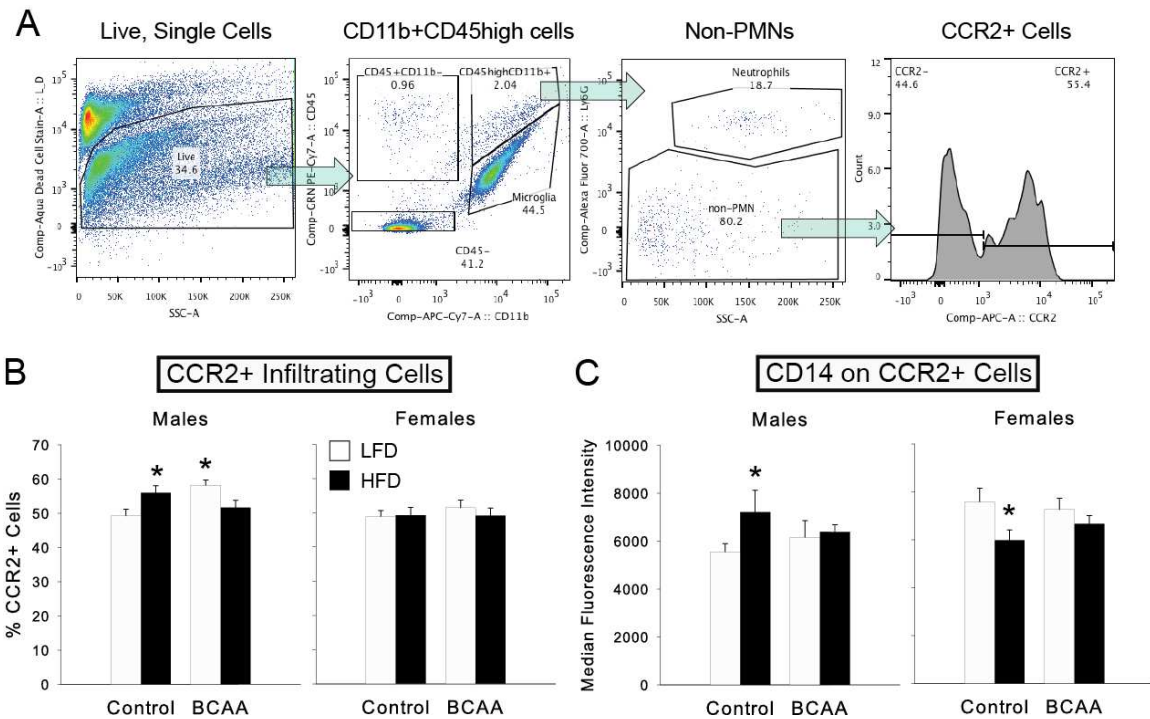
**Figure 48: HFD/Control male offspring exhibited an exaggerated IL-1 $\beta$  response to LPS in both the hippocampus (A) and plasma (B) relative to LFD groups. HFD/BCAA female offspring exhibited a significantly blunted IL-1 $\beta$  response in the periphery (B) relative to other diet groups. IL-1 $\beta$  response is calculated here as the % IL-1 $\beta$  in the LPS-injected diet groups relative to their respective SAL-injected diet group (e.g., percent increase in IL-1 $\beta$  of HFD/LPS animals over HFD/SAL animals). Data are mean  $\pm$  SEM,  $n=6-8$ /group/sex. \* $p<0.05$  vs. LFD/Control and HFD/BCAA; # $p<0.05$  vs. all other groups.**

### 6.3.9 Maternal HFD results in increased monocyte infiltration of the brains of male offspring, and these infiltrating cells are more activated in the HYP

Due to the similarities of the IL-1 $\beta$  response between the brain and periphery in the adult offspring, we next assessed whether there was any infiltration of the brain by peripheral immune cells, which can contribute to the neuroinflammatory response, as we have shown previously in adult animals consuming a HFD (Bolton et al., 2014). We analyzed the percent of CCR2 $^{+}$  monocytes (infiltrating cells of the macrophage lineage derived from the bone marrow; see Figure 49A for gating strategy) in the HYP and the FB, and found a significant Sex X HFD X BCAA interaction [ $F(1,107)=5.48$ ,  $p<0.05$ ],

regardless of brain region. Post hoc tests revealed that HFD/Control and LFD/BCAA males possessed a higher percentage of CCR2+ monocytes in their brains than LFD/Control males ( $p < 0.05$ ), whereas female offspring did not differ by maternal diet (Figure 49B).

We next analyzed this population for the per-cell surface levels of CD14 (an important co-receptor for TLR2 and TLR4 that is highly enriched in monocytes), and found a significant Sex X HFD interaction [ $F(1,52) = 7.08$ ,  $p < 0.05$ ] and a trend for a Sex X HFD X BCAA interaction [ $F(1,52) = 2.57$ ,  $p = 0.1$ ]. Post-hoc tests revealed HFD/Control males had higher levels of this activation marker on the CCR2+ monocytes in their HYP than did LFD/Control males ( $p < 0.05$ ), whereas HFD/Control females actually exhibited the opposite pattern, for decreased CD14 on the CCR2+ monocytes in their HYP relative to LFD/Control females ( $p < 0.05$ ; Figure 49C). These activation changes were specific to the HYP, as no significant changes in CD14 were detected in whole FB (data not shown).



**Figure 49: Maternal HFD altered monocytes infiltration of the brain in male offspring long-term.** A) Gating strategy for flow cytometry: cells were first gated to exclude doublets and dead cells, then gated to select the CD11b+CD45high population (infiltrating cells, not resident microglia). From this population, neutrophils were excluded, and the CCR2+ cells of the remaining non-polymorphonuclear cells were selected to end up with the population of infiltrating monocytes. B) HFD/Control and LFD/BCAA male offspring exhibited increases in the percent of CCR2+ infiltrating cells in their brains (regardless of brain region), whereas females did not differ. C) This population of CCR2+ cells in the HYP expressed higher cell-surface levels of CD14 in male HFD/Control offspring relative to LFD/Control offspring, whereas it was the opposite in female HFD/Control offspring. Data are mean  $\pm$  SEM,  $n=14-16$ /group/sex for B (brain regions HYP and FB analyzed together),  $n=7-8$ /group/sex for C (HYP only). \* $p<0.05$  vs. LFD/Control.

## 6.4 Discussion

The current study demonstrated that maternal HFD programs enduring metabolic and behavioral dysfunction in offspring independent of later diet, as well as “primes” hippocampal microglia to overreact to later-life immune challenges, consistent

with our previous findings (Bilbo & Tsang, 2010). Notably, this study was the first to demonstrate that maternal HFD also results in a long-term increase in monocyte infiltration of the brains of adult male offspring, and these cells appear more activated specifically in the hypothalamus. We have previously demonstrated that 9 weeks of HFD in adulthood increases the infiltration of peripheral immune cells in the brain, but it is surprising to find that an overall shorter period of exposure (19 days during gestation + 4 weeks during lactation) results in a long-term increase in monocyte infiltration, even when the animals are no longer consuming a HFD. It is clear from our data that maternal HFD consumption during the perinatal period has a profound ability program offspring physiology and behavior, most likely due to its inflammatory effects.

This study sought to identify the individual and synergistic effects of maternal HFD and BCAA diet on the behavior, neuroimmune profile, and metabolism of offspring exposed during gestation and lactation. Dietary supplementation with HFD and BCAA were both found to increase body weight in adult female mice, resulting in a robust model of maternal obesity. The combination of HFD and BCAA resulted in the heaviest body weight in dams, and paradoxically, the smallest birth weight in offspring, which is consistent with intrauterine growth restriction in the babies of obese humans (Ornoy, 2011). Notably, HFD resulted in increased anxiety-like behavior in postpartum dams, whereas BCAA caused a postpartum depression-like phenotype. Thus, the combination of HFD and BCAA was clearly worst for dams, but surprisingly, maternal

HFD alone was worst for offspring in terms of later-life neuroinflammation and metabolic and behavioral dysfunction. Importantly, this demonstrates that the effects on offspring outcomes were more dependent on diet composition than maternal obesity or weight alone, as HFD/Control and LFD/BCAA dams were equivalent in weight, but their offspring had very different outcomes [consistent with Sullivan et al., 2010].

Specifically, the adult offspring of HFD dams exhibited increased anxiety-like behavior, regardless of maternal BCAA supplementation, as we have previously reported (Bilbo & Tsang, 2010). In addition, male HFD offspring exhibited an additional hyperactive phenotype, whereas female offspring did not differ, suggesting a sex-specificity of behavioral programming by maternal HFD. However, the only effects we detected of maternal BCAA on adult behavioral outcomes was a decrease in escape behavior in the forced-swim test in BCAA female offspring, which may be suggestive of an increase in depressive-like behavior, similar to that observed in their mothers. In terms of metabolic outcomes, BCAA offspring were smaller at weaning and into adulthood, but did not exhibit other evidence of metabolic dysregulation. On the other hand, HFD offspring were larger at weaning, but this effect did not endure into adulthood. Nevertheless, adult male offspring of HFD dams had increased fasting blood glucose and increased insulin sensitivity compared to controls, whereas HFD females had decreased insulin sensitivity and increased visceral fat deposits. Our findings indicate lasting sex-specific effects of HFD on metabolic health as well, but this time

with females seemingly more susceptible to harmful metabolic effects. Importantly, this effect was independent of later offspring diet, as offspring were exposed to experimental diets only through maternal consumption during pregnancy and lactation.

The metabolic and behavioral changes we observed in HFD offspring were accompanied by long-term increases in microglial antigen density of the hippocampus, a region critical for metabolic and mood regulation. Specifically, maternal HFD increased baseline microglial density in the DG, but this effect was blunted in HFD/BCAA animals. Previous studies have shown that HFD acts on microglia via TLR4 to induce inflammatory signaling (Milanski et al., 2009), whereas BCAA actually decrease microglial inflammatory signaling both at baseline and in response to LPS in vitro (De Simone et al., 2013), which are consistent with our results. Furthermore, HFD increased baseline microglial antigen density in the CA1 and “primed” CA1 microglia to react more strongly to LPS challenge. This effect was in conjunction with an increased hippocampal IL-1 $\beta$  response to LPS, which is indicative of functional priming as well. Thus, maternal HFD clearly influences the development of the offspring’s central immune system, and this effect persists into adulthood even when the offspring are placed on low-fat diets at weaning.

However, programming of the immune system was not restricted to the brain, as we also observed a primed IL-1 $\beta$  response to LPS in the periphery of HFD/Control offspring. This prompted us to ask if the neuroinflammatory changes we observed may

be due to an interaction with the peripheral immune system. Indeed, we discovered that maternal HFD alone resulted in a long-term increase in monocyte infiltration in the brains of adult male offspring, which may contribute to the observed microglial reactivity. Moreover, these monocytes appeared more activated or primed (i.e., increased CD14 expression) specifically in the hypothalamus of male offspring, which may contribute to the more pronounced IL-1 $\beta$  response to LPS in both the brain and periphery in males. HFD/Control females, on the other hand, exhibited the opposite pattern, in that their monocytes expressed lower surface levels of CD14, which may have been partially protective against the exaggerated immune response observed in males. This sex-specificity is in agreement with our previous study that reported more activated infiltrating peripheral immune cells specifically in the hypothalamus of males that received prenatal air pollution exposure and adult HFD (Bolton et al., 2014). Interestingly, LFD/BCAA males also exhibited increased monocyte infiltration of their brains, similar to HFD/Control males, but they did not possess monocytes with a more activated phenotype, nor did they have an exaggerated IL-1 $\beta$  response to LPS, unlike HFD/Control males. It is possible that maternal BCAA attenuates inflammatory signaling in peripheral monocytes, as we suggested for microglia in the DG. However, the effects of BCAA in microglia in vitro in the previous study were quite complex, resulting in an intermediate M1/M2 phenotype (i.e., increased free radical generation but enhanced IL-10 production), which the authors proposed to result in a less efficient

microglial response (De Simone et al., 2013). Therefore, it is possible that the infiltrating monocytes of LFD/BCAA males also exhibit a mixed M1/M2 phenotype, in which they infiltrate the brain in greater numbers, but do not contribute significantly in terms of inflammatory signaling.

The sex differences we observed in neuroinflammatory and behavioral outcomes are consistent with previous studies reporting greater adverse outcomes in male offspring following early-life insults (Clifton, 2010). Notably, there are marked sexual dimorphisms in microglial colonization of the brain across development, such that males have a greater number of microglia than females shortly after birth, which has been hypothesized to render them more vulnerable to early-life immune challenges (Schwarz et al., 2012). This may explain why male HFD offspring exhibit greater functional priming of microglia, and could also be extended to explain the apparent priming of peripheral monocytes in males. However, female offspring in the current study did appear more vulnerable to the metabolic effects of maternal HFD than males.

Interestingly, the sex difference in microglial colonization switches later in development such that P30 and P60 females have more microglia than males (Schwarz et al., 2012).

Furthermore, a recent study demonstrated that offspring metabolic dysfunction is dependent on maternal HFD consumption during lactation, not gestation, and the resultant impairment of hypothalamic “feeding” circuit formation (Vogt et al., 2014).

Taken together, these studies suggest that females may be more sensitive later in

development than males (i.e., towards the end of lactation) to the adverse effects of maternal HFD, resulting in their more profound metabolic dysfunction.

The mechanisms by which perinatal events impact offspring behavioral, metabolic, and neuroimmune outcomes are usually complex, though poorly defined, and may involve several physiological pathways, as well as changes in maternal-offspring interactions. In the current study, we observed lower quality maternal care in BCAA dams, particularly LFD/BCAA offspring, in conjunction with their postpartum depression-like behavior. It is likely that this altered maternal behavior directly contributed to the lower body weights of BCAA offspring at weaning and into adulthood. Furthermore, our data provide suggestive evidence of increased depressive-like behavior in female BCAA offspring, which may be related to their mothers' depressive phenotype. On the other hand, HFD dams exhibited what would traditionally be called higher quality maternal care (i.e., increased licking and grooming), but it occurred atypically during the dark cycle (Champagne et al., 2003) and in conjunction with increased self-grooming and nest-building behavior, which we find to be more representative of increased anxiety-like behavior (in agreement with our EZM results). Moreover, HFD offspring suffered more adverse behavioral, metabolic, and neuroinflammatory outcomes than LFD controls, indicating that regardless, the increased maternal licking and grooming behavior was insufficient to provide protection against the long-term programming of these phenotypes.

In sum, we have demonstrated that maternal high-fat diet programs offspring for neuroimmune, metabolic, and behavioral dysregulation in a sex-specific manner. Adult male HFD offspring exhibit more pronounced behavioral changes, whereas adult female HFD offspring exhibit more severe metabolic dysregulation. Our working hypothesis is that microglia and peripheral monocytes are primed early in development by the inflammatory impact of maternal HFD, such that they overreact to later-life immune challenges. Males exhibit greater long-term neuroinflammatory changes than do females in response to maternal HFD, consistent with the male vulnerability to early-life insults reported in many other studies (Clifton, 2010). The mechanisms that allow for the greater vulnerability of males to neuroinflammation following early-life immune challenges remain to be defined, but likely involve sexual dimorphisms in microglial colonization. Future studies aimed at elucidating the sexually dimorphic mechanisms of perinatal programming by maternal diet will be critical for identifying effective interventions and treatments for the growing number of children exposed to a “Western diet” during the sensitive perinatal period.

## **7. Interaction of the Placental Immune Response with Serotonin Production and Microglial Maturation in Developmental Programming by “Western Diets”**

### ***7.1 Introduction***

Due to the key role of the placenta in nutrient transfer and serotonin production, and its immunocompetence (described in Chapter 1), we propose that our model of maternal HFD and BCAA-enriched diets (see Chapter 6) may be an ideal system to look for interactions between the placental response to maternal immune challenges and brain development, particularly serotonin production and microglial maturation.

In the current study, we placed females on one of 4 diets (LFD, LFD/BCAA, HFD, or HFD/BCAA) for 6 weeks prior to breeding, then time-mated females in order to collect placentas on E14.5. This time point was selected because it is a time of peak placental serotonin synthesis, in conjunction with forebrain neurogenesis and axon growth, which have diminished by E16.5, and at which time the dorsal raphe nucleus becomes the major source for forebrain serotonin. We predict that maternal HFD will decrease placental serotonin synthesis due to its inflammatory impact, whereas maternal BCAA will decrease placental serotonin synthesis due to its competition with maternal tryptophan in entering the placenta from the maternal bloodstream. This decrease in placental serotonin and increased inflammation (in the case of HFD) may have important implications for fetal brain development, including microglial colonization of the brain and the development of serotonergic neuronal networks, which may underlie

the microglial priming phenotype and increased anxiety-like behavior we observed in the adult offspring of HFD dams.

## **7.2 Methods**

### **7.2.1 Animals**

Juvenile (P28) female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, North Carolina, USA) and housed in same-sex pairs in propylene cages with *ad libitum* access to food and filtered water. The colony was maintained at 22°C on a 12:12-h light-dark cycle (lights on at 7 AM). Sentinel animals were housed in the colony room and screened periodically for the presence of common rodent diseases; all screens were negative. All experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

### **7.2.2 Diets**

On P40, females were placed on one of four diets: a low-fat diet [**LFD**; 19% of calories from protein, 71% from carbohydrate, and 10% from fat (56:44% soybean oil:lard); cat. no. D07010502, Research Diets, New Brunswick, NJ, USA; *n*=20], a high-fat diet [**HFD**; 20% of calories from protein, 35% from carbohydrate, and 45% from fat (12:88% soybean oil:lard); cat. no. D12451, Research Diets, New Brunswick, NJ, USA; *n*=19], a low-fat diet supplemented with BCAA [**LFD/BCAA**; 23% of calories from protein, 67% from carbohydrate, and 10% from fat (56:44% soybean oil:lard); cat. no. D07010503, Research Diets, New Brunswick, NJ, USA; *n*=19], and a high-fat diet

supplemented with BCAA [HFD/BCAA; 23% of calories from protein, 34% from carbohydrate, and 43% from fat (12:88% soybean oil:lard); cat. no. D06050807, Research Diets, New Brunswick, NJ, USA;  $n=20$ ]. The HFD and HFD/BCAA diets provided 4.7 kcal/g, the LFD provided 3.84 kcal/g and the LFD/BCAA provided 3.85 kcal/g. The complete nutritional profiles of each diet are available online (<http://www.researchdiets.com>). Females were fed *ad libitum* for 6 weeks before the introduction of breeder males and remained on the diets throughout pregnancy and lactation.

### **7.2.3 Breeding**

Following 6 weeks of diet consumption, half of the females ( $n=40$ ) were paired with adult breeder males for timed mating, for a maximum of 3 weeks. Females were examined daily for evidence of a vaginal plug [confirmation of successful mating; the morning of this day is considered to be embryonic day (E)0.5], at which point they were separated from the male and housed with other pregnant females. The other half of females ( $n=40$ ) were paired with adult breeder males for 3 weeks, but were not examined for vaginal plugs and were allowed to give birth normally.

### **7.2.4 E14.5 Tissue Collection**

In the time-mated cohort of females, we obtained fetuses on E14.5 (i.e., 14 days after finding the plug on day E0.5) by hysterotomy following lethal i.p. injection of the dam with 430 mg/kg ketamine and 65 mg/kg xylazine. Placentas were separated from

each fetus, the amniotic sac and maternal membranes were removed, and the resulting discoid placenta was cut in half, and one half placed in a 2.0-ml microcentrifuge tube and snap-frozen in liquid nitrogen. The other half was placed in a 1.5-ml tube containing 4% paraformaldehyde, where it was fixed for 48 h prior to transfer to a 30% sucrose plus 0.1% sodium azide solution for cryoprotection prior to cryosectioning. Fetal forebrains were extracted from the head of each fetus, leaving behind the cerebellum and brain stem, and snap-frozen in 2.0-ml microcentrifuge tubes. Finally, whole fetal tails were collected for later genotyping to determine the sex of each fetus. From these tails, we extracted genomic DNA and subsequently assessed SRY PCR products [as described previously in (Bolton et al., 2013)]. All tissue collection occurred between 9 AM and 5 PM.

### **7.2.5 P1 Tissue Collection**

In the cohort of females that was allowed to give birth normally, one male and one female was randomly collected from each litter at P1, rapidly decapitated, and trunk blood collected in order to obtain a measure of basal circulating CORT levels soon after birth using a commercially available CORT ELISA kit (Enzo Life Sciences, Inc., Ann Arbor, MI, USA). Blood was centrifuged at  $1,200 \times g$ , and the plasma collected and frozen at  $-80^{\circ}\text{C}$ . The whole brain was also extracted, placed in a 2.0-ml microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until later qRT-PCR analysis. All tissue collection occurred between 8 AM and 10 AM.

### **7.2.6 P8 Tissue Collection**

One week following P1 tissue collection, 1-2 males and 1-2 females from each litter were deeply anesthetized with a ketamine-xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine, i.p.) and transcardially perfused with ice-cold saline for 2 min., after which brains were rapidly extracted. Brains were cut in half sagittally, and one half was dissected on ice into hypothalamus (HYP), prefrontal cortex (PFC), and hippocampus (HIP). We placed these regions in separate 1.5-ml microcentrifuge tubes, snap-froze in liquid nitrogen, and stored at -80°C for later analysis. These were the same brain regions analyzed in adulthood in our previous study (see Chapter 6) because of their known roles in the metabolic and behavioral outcomes we assessed in the adult offspring. The other half of each brain was post-fixed in 4% paraformaldehyde for 48 h and cryoprotected in 30% sucrose plus 0.1% sodium azide for at least 2 days prior to cryosectioning. All tissue collection occurred between 10 AM and 2 PM.

### **7.2.7 Adult Tissue Collection**

All remaining pups were weaned onto standard chow (Laboratory Rodent Diet 5001; Lab-Diet, Philadelphia, PA, USA) at P28. Once animals reached adulthood (P90-P120), 1-2 males and 1-2 females from each litter were deeply anesthetized with a ketamine-xylazine cocktail (430 mg/kg ketamine; 65 mg/kg xylazine, i.p.) and transcardially perfused with ice-cold saline for 2 min., after which brains were rapidly extracted. Brains were cut in half sagittally, and one half was dissected on ice into

hypothalamus (HYP), prefrontal cortex (PFC), and hippocampus (HIP). We placed these regions in separate 1.5-ml microcentrifuge tubes, snap-froze in liquid nitrogen, and stored at -80°C for later analysis. These were the same brain regions analyzed in adulthood in our previous study (see Chapter 6). The other half of each brain was post-fixed in 4% paraformaldehyde for 48 h and cryoprotected in 30% sucrose plus 0.1% sodium azide for at least 2 days prior to cryosectioning. All tissue collection occurred between 10 AM and 2 PM.

### **7.2.7 PCR Array**

A mouse inflammatory response & autoimmunity PCR array (cat. no. PAMM-077ZA; Qiagen) was used to assess the relative gene expression of 84 different immune genes in E14.5 placentas. Total RNA was isolated from frozen E14.5 placentas using TRIzol (Chomczynski & Sacchi, 1987), and cDNA was synthesized from 500 ng of isolated RNA using the RT<sup>2</sup> First Strand Kit (Qiagen). Two samples of the same sex from the same litter were pooled to form one sample for cDNA synthesis and subsequent PCR array analysis (n=3 pooled samples from 3 different litters/diet/fetal sex); males and females from the same litters were compared to more rigorously examine sex differences. Gene expression was measured using quantitative real-time PCR with primers designed to measure 84 inflammatory cytokines, chemokines, and receptors using the RT<sup>2</sup> SYBR Green qPCR Master Mix (Qiagen) according to the manufacturer's protocol.

### 7.2.8 qRT-PCR

Total RNA and native protein were isolated simultaneously from E14.5 whole forebrain, P1 whole brain, P8 HYP and HIPP, and adult HYP and HIPP using the Protein And RNA Isolation System (PARIS kit; Ambion, Waltham, MA, USA) according to manufacturer's instructions. Protein isolates were frozen at -80°C for later analysis. cDNA was synthesized from 100 ng of RNA using the QuantiTect reverse transcription kit (Qiagen, Inc., Valencia, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using a QuantiFast SYBR Green PCR kit (Qiagen) on a Mastercycler ep *realplex* (Eppendorf, Hauppauge, NY, USA). 1 µl of cDNA was added to 12 µl of master mix containing specific primers for the genes of interest. Primers for IL-1β were purchased from Qiagen, and we designed primer primers for all other genes (GAPDH, TLR2, TLR4, Iba-1, GFAP, CD11b, 5-HT1A, 5-HT2CR, and 5-HTT; see Table 6) as previously described (Williamson et al., 2011). Designed primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Optimal annealing temperatures for each primer pair were determined by running a temperature gradient, and specificity was verified by melt-curve analysis. For analysis, we determined the threshold cycle ( $C_T$ ) for each reaction and calculated relative gene expression using the  $2^{-\Delta\Delta C_T}$  method (Livak & Schmittgen, 2001; Pfaffl, 2001; Williamson et al., 2011), using GAPDH as the housekeeping gene.

**Table 6: Forward and reverse primer sequences for qRT-PCR analysis**

| <b>Gene</b>    | <b>Forward Primer (5'-3')</b> | <b>Reverse Primer (5'-3')</b> |
|----------------|-------------------------------|-------------------------------|
| <i>GAPDH</i>   | ggtcaccagggctgccatt           | tgggcttcccgtgatgaca           |
| <i>TLR2</i>    | cgttcatctctggagcatcc          | gacagagactcctgagcag           |
| <i>TLR4</i>    | cagcagaggagaaagcatc           | caccaggaataaagtctctg          |
| <i>Iba-1</i>   | cagacttcatectctctcttc         | cttccactcattcccttgc           |
| <i>GFAP</i>    | tggagagaaagggtgaatcg          | gtattgagtgcgaatctctc          |
| <i>CD11b</i>   | ctatttggtcggctccaac           | gcatcaaagagaacaagg            |
| <i>5-HT1A</i>  | ctgccgctgatgatgatgatg         | gagtgaacaggaagggtcc           |
| <i>5-HT2CR</i> | cgatggtggacgcttgttc           | gataacgagaatgttgcccc          |
| <i>5-HTT</i>   | gctgagatgaggaacgaag           | ggcaaagaatgtggatgctg          |

### **7.2.9 High-performance liquid chromatography with electrochemical detection**

On the day of analysis, 500 µl of ice-cold standard buffer (0.5 mM sodium metabisulfite, 0.2 N perchloric acid, and 0.5mM EDTA) was added to thawed placentas, and 250 µl of ice-cold standard buffer was added to thawed adult offspring PFC. The tissue was disrupted by sonication until completely homogenized, then centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was collected and filtered through a 0.45 µm membrane via centrifugation (Durapore PVDF centrifugal filters, Millipore, Billerica, MA, USA). The filtrate was kept on ice until analysis.

Processed placenta and PFC samples were analyzed for the amino acid tryptophan (Trp), indoleamines (5-HT and 5-HIAA), and catecholamines and their respective metabolites (DA, DOPA, and HVA) using high-performance liquid chromatography with electrochemical detection (HPLC-EC). Processed samples were

separated using a 100 x 4.6 mm Kinetex (C18 5  $\mu\text{m}$  100  $\text{\AA}$ , Phenomenex) column on a reverse phase HPLC system with a BAS LC-4B electrochemical detector with dual 3 mm carbon electrode (MF-1000) and reference electrode (MF-2021) as previously described (Sánchez, Van Swearingen, Arrant, Kuhn, & Zepf, 2014). An external standard curve of all analytes was run each day. Trp quantification was performed using a mobile phase consisting of 8% acetonitrile (v/v), 0.05 M citric acid, 0.05 M  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.1 mM EDTA. No correction for pH was needed. The detector was set to 0.875 V versus Ag/AgCl reference electrode, sensitivity at 20 nA, and a flow rate of 1.0 ml/min.

Indoleamines and catecholamines were separated with a mobile phase consisting of 18% methanol (v/v), 0.1M sodium phosphate, 0.8 mM octanesulfonic acid (anhydrous), and 0.1 mM EDTA (final pH adjusted to 3.1). The detector was set to 0.70 V, sensitivity at 20 nA, and a flow rate of 1.0 ml/min.

### **7.2.10 Data Analysis and Statistics**

All data were analyzed using SPSS statistical software (IBM, Armonk, NY, USA). All measures were analyzed using two-way (HFD x BCAA) or three-way (HFD x BCAA x Sex) ANOVA. Following significant  $F$  scores for interactions in ANOVA tests, *post hoc* comparisons (Fisher's LSD) were performed to further distinguish among groups, and all differences were considered statistically significant if  $p < 0.05$ .

## **7.3 Results**

### **7.3.1 PCR array analysis reveals multiple sex-specific immune changes in the E14.5 placenta due to maternal diet**

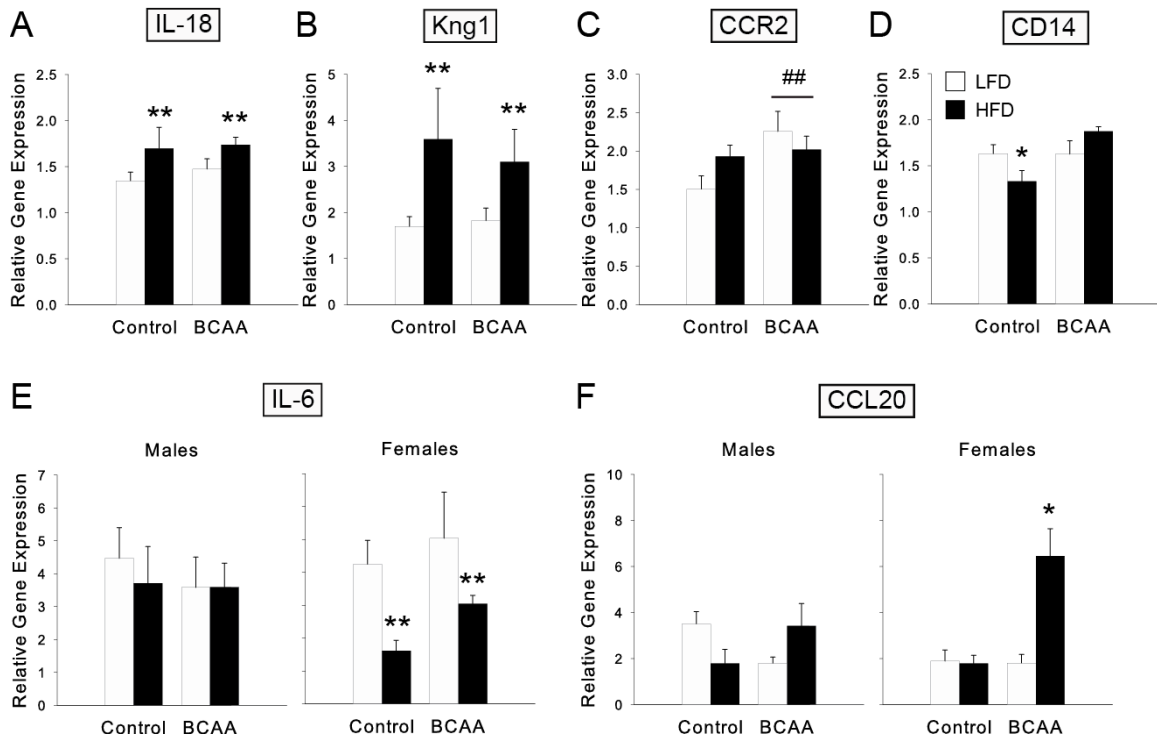
Because we were interested in sex-specific gene modulation in the placenta by maternal diet, we first examined whether there were any baseline sex differences in placental immune gene expression at E14.5. We detected 9 genes for which there was a significant difference between LFD/Control male and female placentas. Eight of these genes (CCL3, CCL4, CCR3, CXCL10, IL1rn, IL6ra, TLR2, and TLR4) were expressed at a higher level in female placentas relative to male placentas. The one gene that exhibited higher expression in male placentas than female placentas was CXCR1 (data not shown).

Ten genes were significantly modulated by maternal HFD in the placenta (i.e., we detected a main effect of HFD); 6 of these (C3ar1, CCL11, CXCL10, IL-18, Kng1, and TLR1) were increased by HFD (see Figure 50A-B for examples), whereas the other 4 (CCL8, CD14, IL-6, and COX-2) were decreased by HFD. Three of the 6 upregulated genes were primarily male-driven changes, and one was female-driven. Of the 4 downregulated genes, 3 were female-driven changes (see Figure 50E for example).

Nine genes were significantly modulated by maternal BCAA in the placenta (i.e., we detected a main effect of BCAA); 4 of these (CCL1, CCL8, CCR2, and CXCR4) were increased by BCAA (see Figure 50C for example), whereas the other 5 (CCR4, CSF-1, Fos, IL-9, and Tirap) were decreased by BCAA. One of the upregulated genes was male-

driven, and one was female-driven. Of the 5 downregulated genes, 4 were female-driven, whereas 1 was male-driven.

Nine genes were significantly modulated by the interaction of maternal HFD and BCAA in the placenta (i.e., we detected a HFD X BCAA interaction), including CCL20, C3ar1, CCL1, CD14, IL-5, Tirap, TLR4, Fos, and TNF. The directionality of these interactions is more difficult to describe in brief, but 6 of the 9 genes were modulated primarily in females (see Figure 50F for example), whereas only one gene was modulated primarily in males. In sum, it is clear that maternal diet can modulate immune gene expression in the mid-gestation placenta, and this occurs to a greater extent in female placentas than male placentas.

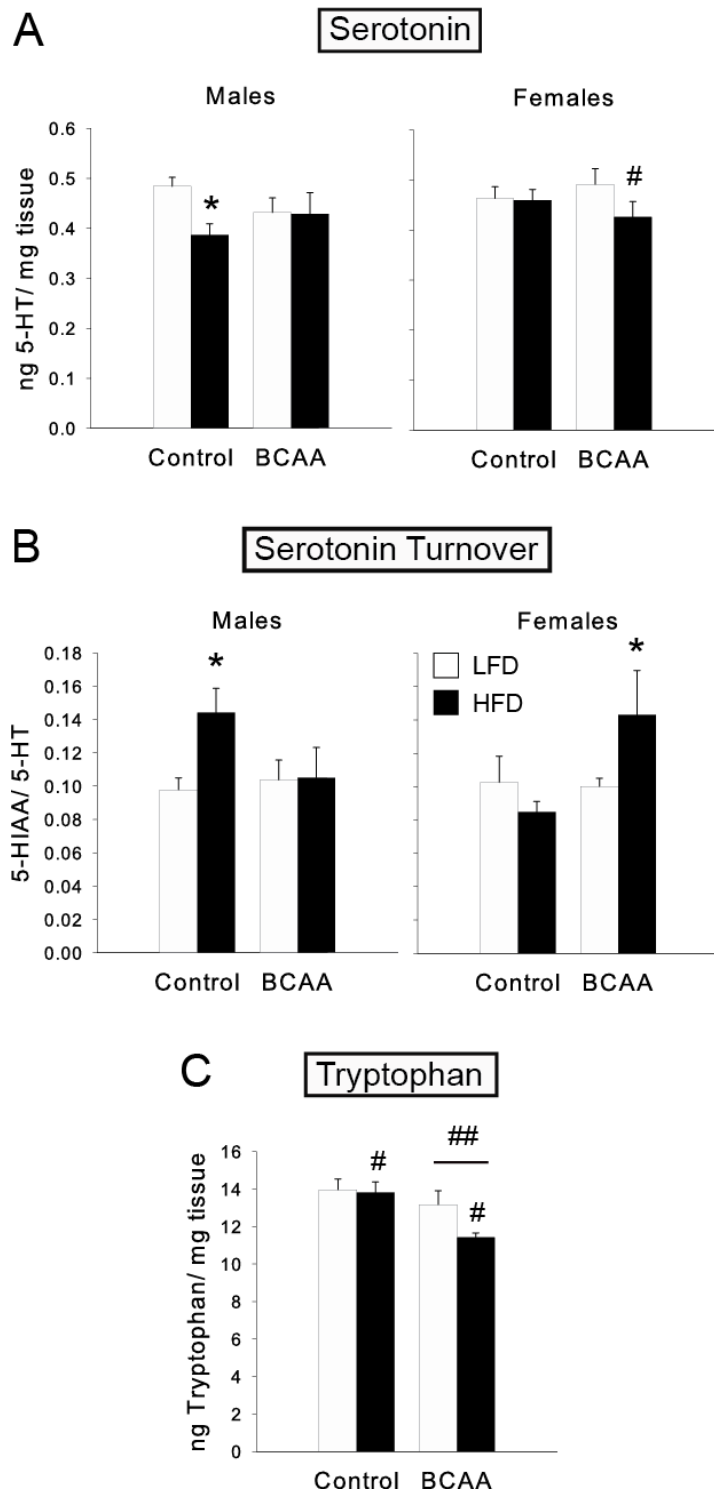


**Figure 50: Maternal diet altered immune gene expression in the E14.5 placenta. A-B) Maternal HFD caused an immune response, as shown here by an increase in IL-18, a proinflammatory cytokine, and Kng1, which is involved in coagulation and inflammation. C) Maternal BCAA also caused alterations in immune gene expression, as shown here by an increase in CCR2, a marker for infiltrating monocytes. D) There was also interaction between maternal HFD and BCAA in the regulation of immune gene expression, as shown here by decreased CD14 in HFD/Controls placentas, but not in HFD/BCAA placentas. E-F) There were sex-specific alterations in the placenta in response to maternal diet, and most were more pronounced in females than males, as shown here by decreased IL-6, an important cytokine for placental growth, in HFD female placentas, and increased CCL20 in HFD/BCAA female placentas. Data are mean  $\pm$  SEM,  $n=5-6$ /group for A-D (sexes combined),  $n=3$ /group/sex for E-F (sexes separate). \*\* $p<0.05$ , HFD vs. LFD; ## $p=0.06$ , BCAA vs. Control; \* $p<0.05$  vs. HFD/BCAA,  $p=0.07$  vs. other groups for D; \* $p<0.05$  vs. all other groups for E.**

### 7.3.2 Maternal HFD decreases serotonin production in the E14.5 placenta

Analysis of placental 5-HT content by HPLC revealed a significant main effect of HFD [ $F(1,54)=4.22, p<0.05$ ] and a trend for a Sex X HFD X BCAA interaction [ $F(1,54)=3.66, p=0.06$ ]. Post hoc tests revealed that the HFD/Control group is most decreased relative to the LFD/Control group in males ( $p<0.05$ ), whereas the HFD/BCAA is most decreased relative to the LFD/BCAA group in females ( $p=0.09$ ; Figure 51A). No significant changes were detected in the 5-HT metabolite, 5-HIAA. Consistent with these findings, the 5-HT turnover (calculated as 5-HIAA divided by 5-HT) exhibited a significant Sex X HFD X BCAA interaction [ $F(1,52)=5.78, p<0.05$ ] that was driven by an increased 5-HT turnover in HFD/Control male placentas relative to LFD/Control placentas ( $p<0.05$ ) and an increased 5-HT turnover in HFD/BCAA female placentas relative to HFD/Control female placentas ( $p<0.05$ ) and LFD/BCAA female placentas ( $p=0.06$ ; Figure 51B).

Analysis of placental tryptophan content by HPLC revealed a significant decrease due to maternal BCAA [main effect of BCAA,  $F(1,54)=7.90, p<0.01$ ], in conjunction with a tendency for a further decrease due to maternal HFD [trend for main effect of HFD,  $F(1,54)=2.94, p=0.09$ ; Figure 51C]. In this case, the effects appear to primarily derive from a pronounced decrease in placental tryptophan in the HFD/BCAA group, regardless of fetal sex.

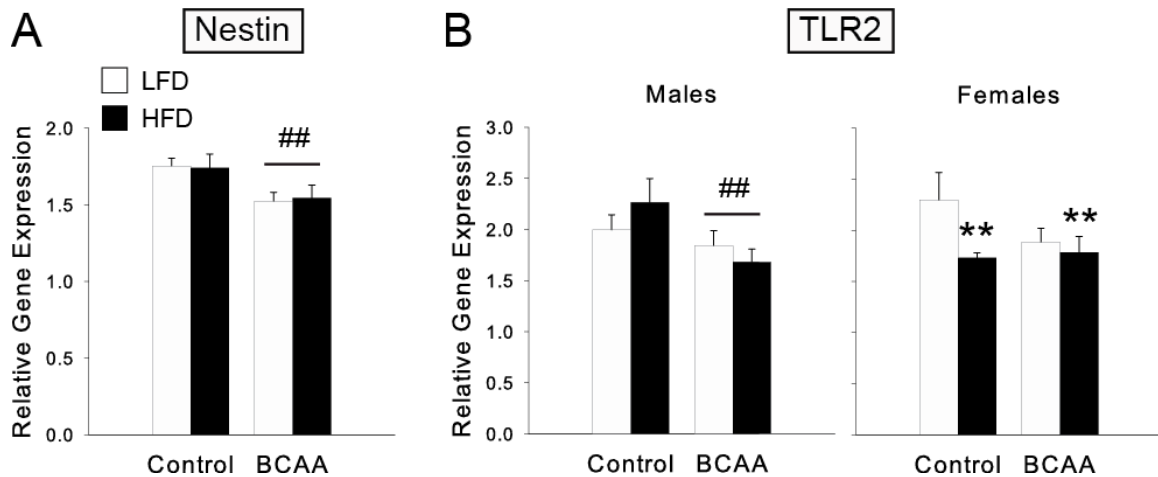


**Figure 51: Maternal HFD decreased measures of serotonin production in the E14.5 placenta. A) Maternal HFD decreased 5-HT levels in the E14.5 placenta, but especially**

for HFD/Control placentas for male fetuses, and HFD/BCAA placentas for female fetuses. B) Similarly, HFD/Control males and HFD/BCAA females exhibited increased serotonin turnover (as measured by the 5-HIAA/ 5-HT ratio). C) Placental tryptophan content was overall decreased in BCAA fetuses, and tended to be further decreased in HFD fetuses, so that HFD/BCAA fetuses had the lowest tryptophan levels. Data are mean  $\pm$  SEM,  $n=6-8$ /group/sex for A-B (sexes separate),  $n=15-16$ /group for C (sexes combined). \* $p<0.05$  vs. LFD/Control for males, and vs. LFD/BCAA and HFD/Control for females; # $p=0.09$  vs. LFD/Control in A, and HFD vs. LFD in C; ## $p<0.05$ , BCAA vs. Control.

### 7.3.3 Maternal HFD and BCAA alters gene expression in the E14.5 forebrain

qRT-PCR analysis of E14.5 whole fetal forebrains revealed a significant decrease in expression of Nestin, a marker for proliferating neuronal precursor cells, due to maternal BCAA [significant main effect of BCAA,  $F(1,52)=8.11$ ,  $p<0.01$ ], regardless of sex (Figure 52A). TLR2 gene expression exhibited a similar pattern [significant main effect of BCAA,  $F(1,54)=5.58$ ,  $p<0.05$ ], but also a close trend for a Sex X HFD X BCAA interaction [ $F(1,54)=3.73$ ,  $p=0.06$ ]. Follow-up analyses revealed a significant decrease in TLR2 expression in male forebrains due to maternal BCAA [main effect of BCAA,  $F(1,27)=5.01$ ,  $p<0.05$ ], whereas female forebrains exhibited a significant decrease in TLR2 expression due to maternal HFD [main effect of HFD,  $F(1,27)=4.26$ ,  $p<0.05$ ; Figure 52B]. Interestingly, IL-1 $\beta$  gene expression closely paralleled the pattern of TLR2 expression, although it did not reach significance (data not shown).

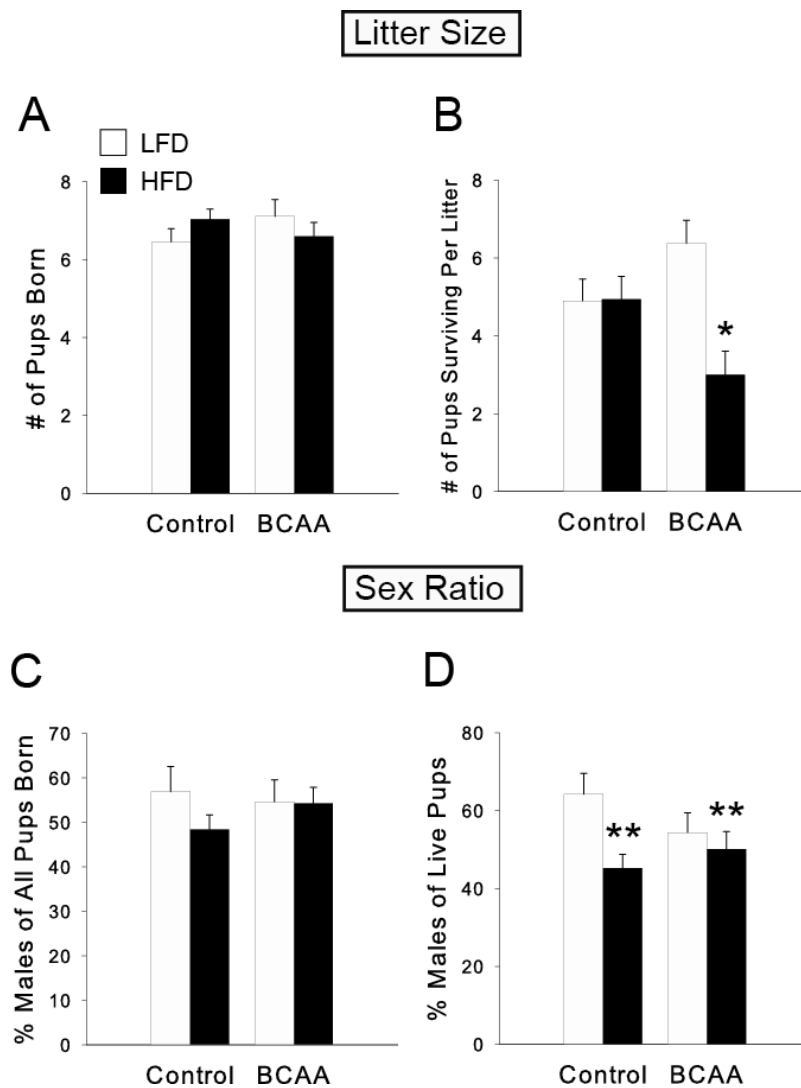


**Figure 52: Maternal HFD and BCAA altered gene expression in the E14.5 forebrain. A)** Fetuses of BCAA dams exhibited decreased Nestin expression, a marker of neural stem cells, in their forebrains at E14.5. **B)** Male fetuses of BCAA dams had decreased TLR2 expression in their forebrains, whereas female fetuses of HFD dams showed decreased TLR2 expression at E14.5. Data are mean  $\pm$  SEM,  $n=13-16$ /group for A (sexes combined),  $n=7-8$ /group/sex for B (sexes separate). ## $p<0.05$ , BCAA vs. Control; \*\* $p<0.05$ , HFD vs. LFD.

### 7.3.4 Litters of HFD/BCAA dams show increased pup mortality, especially in males

All groups had comparable litter sizes at birth (Figure 53A); however, by weaning HFD/BCAA dams had fewer surviving pups than all other groups [significant HFD X BCAA interaction,  $F(1,99)=7.34$ ,  $p<0.01$ ; post hoc,  $p<0.05$ ; Figure 53B], as HFD/BCAA dams tended to neglect or eat their pups in early life. These behaviors may be driven by either malnutrition or maternal stress, and this result indicates a strong effect of dietary fat combined with BCAA on either metabolic or mood regulation (see Chapter 6). Interestingly, HFD-fed—especially HFD/Control—dams gave birth to litters with a lower proportion of live male offspring [significant main effect of HFD,  $F(1,71)=6.47$ ,  $p<0.05$ ; trend for HFD X BCAA interaction,  $F(1,71)=2.60$ ,  $p=0.1$ ; Figure 53D],

even though the sex ratio of all identifiable pups (including stillbirths and cannibalized pups) on P1 did not differ (Figure 53C). Moreover, the sex ratio of fetuses at E14.5 did not differ significantly by maternal diet (data not shown). Taken together, these data suggest a higher mortality rate for the male offspring of HFD dams between E14.5 and P1.



**Figure 53: Litters of HFD/BCAA dams exhibited increased pup mortality, especially in males. A) The number of pups born per litter did not differ, but the number of pups**

surviving to weaning was significantly decreased in the HFD/BCAA group. B) The sex ratio does not differ for all pups born, but the % of live male pups at P1 is significantly decreased in HFD litters. Data are mean  $\pm$  SEM,  $n=17-32$  litters/group. \* $p<0.05$  vs. all other groups; \*\* $p<0.05$ , HFD vs. LFD.

### 7.3.5 Maternal HFD increases P1 pup corticosterone (CORT), whereas maternal BCAA suppresses CORT levels

HFD pups had increased CORT relative to controls shortly after birth at P1 [significant main effect of HFD,  $F(1,59)=5.96$ ,  $p<0.05$ ], whereas BCAA decreased CORT, thus suppressing the CORT increase in HFD/BCAA pups [significant main effect of BCAA,  $F(1,59)=4.12$ ,  $p<0.05$ ; Figure 54]. There were no significant differences detected due to pup sex, which suggests that this measure may be more representative of maternal CORT levels.

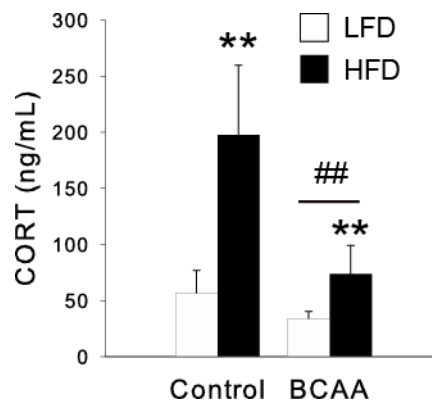
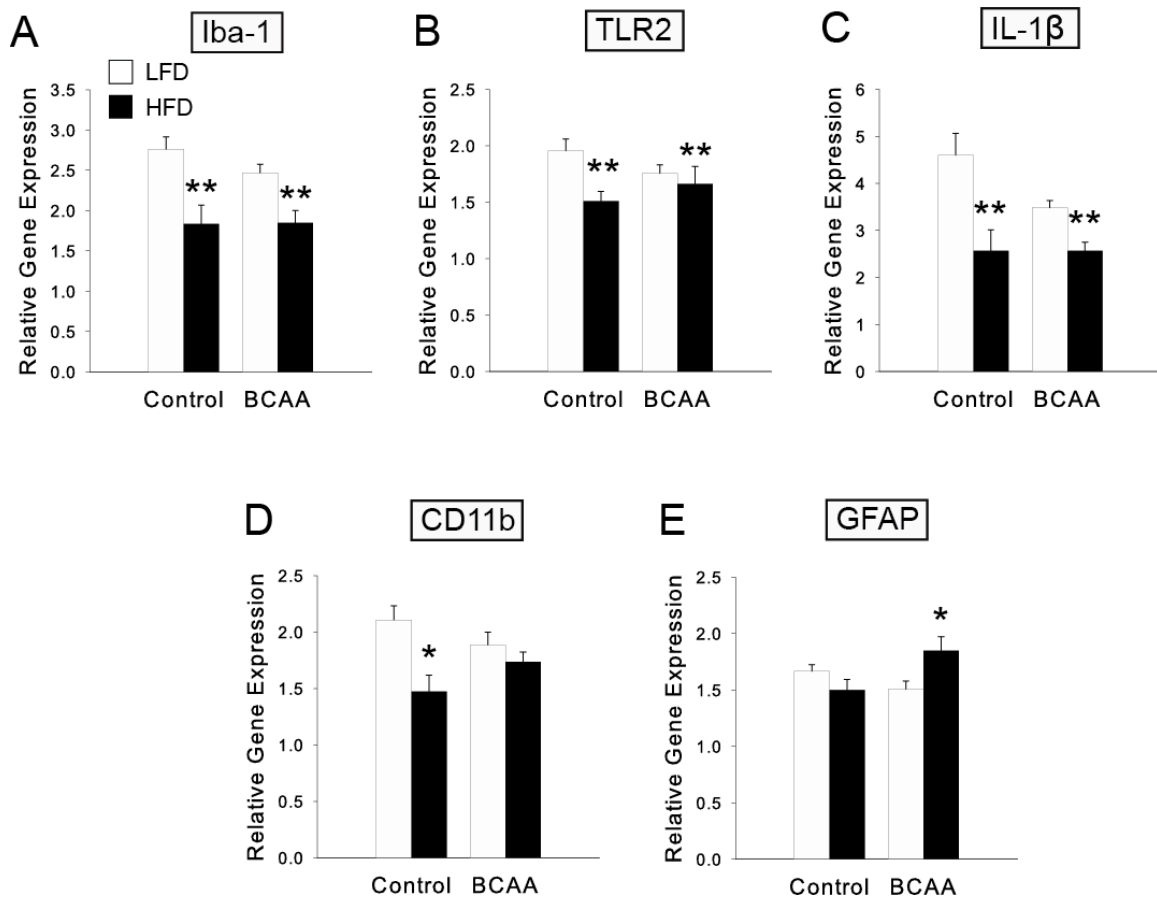


Figure 54: Pups of HFD dams exhibited increased CORT levels on P1, whereas BCAA suppressed this effect, regardless of pup sex. Data are mean  $\pm$  SEM,  $n=15-18$ /group (sexes combined). \*\* $p<0.05$ , HFD vs. LFD; ## $p<0.05$ , BCAA vs. Control.

### 7.3.6 Pups of HFD dams exhibit decreased expression of microglia markers at P1

We observed a significant main effect of HFD for expression of multiple microglial markers in the P1 brain, including decreased expression of Iba-1

[ $F(1,47)=22.68, p<0.001$ ; Figure 55A], TLR2 [ $F(1,47)=5.86, p<0.05$ ; Figure 55B], and IL-1 $\beta$  [ $F(1,46)=13.87, p<0.005$ ; Figure 55C], as well as TLR4, although this didn't reach significance (data not shown). CD11b exhibited a similar pattern [significant main effect of HFD,  $F(1,45)=9.45, p<0.005$ ], but there was also a tendency for the decrease due to HFD to be attenuated by BCAA [trend for HFD X BCAA interaction,  $F(1,45)=3.64, p=0.06$ ; Figure 55D]. In contrast, we observed a significant increase in GFAP expression due to the combination of HFD and BCAA, as the HFD/BCAA group was greater than the HFD/Control and LFD/BCAA groups [ $F(1,43)=8.75, p<0.01$ ; post hoc,  $p<0.05$ ; Figure 55E]. None of these genes exhibited significant sex differences.



**Figure 55: Maternal HFD caused decreased expression of microglial markers in P1 pup brains, including Iba-1, TLR2, and IL-1 $\beta$  (A-C). D) CD11b exhibited a similar pattern, except that BCAA blunted the effect of HFD, such that only HFD/Control brains expressed lower CD11b than LFD controls. E) GFAP, an astrocyte marker, exhibited a different pattern, in which HFD/BCAA brains expressed the highest amount of GFAP. Data are mean  $\pm$  SEM,  $n=9-18$ /group (sexes combined). \*\* $p<0.05$ , HFD vs. LFD; \* $p<0.05$  vs. LFD/Control for D, vs. HFD/Control and LFD/BCAA for E.**

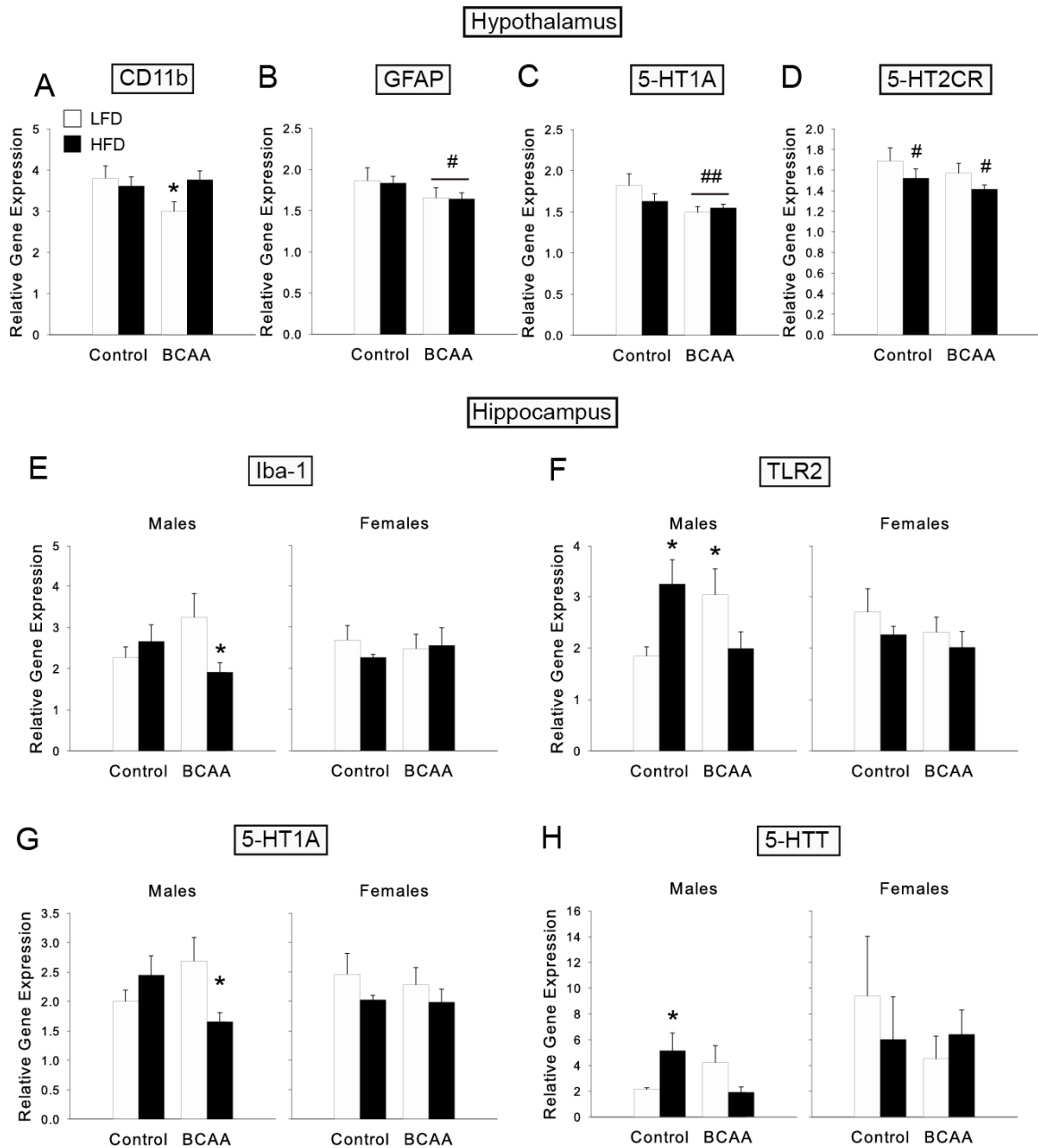
### **7.3.7 Pups of HFD and BCAA dams exhibit altered expression of immune and serotonergic genes at P8 in a brain region- and sex-specific manner**

Unlike in P1 whole brains, qRT-PCR analysis of P8 pup HYP revealed that LFD/BCAA pups expressed lower CD11b relative to HFD/BCAA and LFD/Control [trend for HFD X BCAA interaction [ $F(1,56)=3.06$ ,  $p=0.09$ ; post hoc,  $p<0.05$ ], regardless of

pup sex (Figure 56A). A very similar pattern was observed for IL-1 $\beta$  [trend for HFD X BCAA interaction,  $F(1,43)=3.06$ ,  $p=0.09$ ], because LFD/BCAA pups expressed lower IL-1 $\beta$  than LFD/Control ( $p<0.05$ ), and tended to be lower than HFD/BCAA pups also ( $p=0.1$ ; data not shown). GFAP exhibited a tendency to decrease in the HYP of BCAA pups [trend for main effect of BCAA,  $F(1,55)=3.35$ ,  $p=0.07$ ], regardless of pup sex (Figure 56B). 5-HT1A, a gene encoding a serotonergic autoreceptor, exhibited a similar pattern and was decreased by BCAA in the P8 HYP [significant main effect of BCAA,  $F(1,56)=4.43$ ,  $p<0.05$ ; Figure 56C]. 5-HT2CR, a gene encoding a different serotonergic receptor, exhibited a tendency to be decreased by HFD [trend for main effect of HFD,  $F(1,50)=3.38$ ,  $p=0.07$ ; Figure 56D]. We did not detect any significant differences in TLR2, Iba-1 or 5-HTT, a gene encoding the serotonin transporter (data not shown).

qRT-PCR analysis of CD11b expression in the P8 pup HIPP revealed a pattern that was opposite of that detected in the HYP, although it failed to reach significance (data not shown). However, Iba-1, another microglial marker, exhibited a similar pattern that did reach significance in the HIPP [significant Sex X HFD X BCAA interaction,  $F(1,52)=4.64$ ,  $p<0.05$ ]. Specifically, HFD/BCAA males expressed lower Iba-1 relative to LFD/BCAA males [significant HFD X BCAA interaction,  $F(1,26)=4.92$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ], whereas females did not differ (Figure 56E). Analysis of TLR2 revealed a similar pattern as well [significant Sex X HFD X BCAA interaction,  $F(1,50)=6.52$ ,  $p<0.05$ ]. Specifically, both HFD/Control and LFD/BCAA males exhibited higher TLR2 expression

than controls [significant HFD X BCAA interaction,  $F(1,25)=9.74$ ,  $p<0.005$ ; post hoc,  $p<0.05$ ], whereas females did not differ (Figure 56F). GFAP exhibited a similar pattern, but it failed to reach significance (data not shown). 5HT1A, unlike in the HYP, was decreased in HFD/BCAA males compared to LFD/BCAA males [significant HFD X BCAA interaction,  $F(1,26)=6.63$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ], whereas females did not differ [significant Sex X HFD X BCAA interaction,  $F(1,53)=4.48$ ,  $p<0.05$ ; Figure 56G]. 5-HT2CR exhibited a very similar pattern to 5HT1A, but it failed to reach significance (data not shown). 5-HTT exhibited a similar pattern to TLR2 in the HIPV [trend for Sex X HFD X BCAA interaction,  $F(1,44)=2.29$ ,  $p=0.1$ ], such that HFD/Control males exhibited increased 5-HTT expression compared to HFD/BCAA males and LFD/Control males [significant HFD X BCAA interaction,  $F(1,21)=7.82$ ,  $p<0.05$ ; post hoc,  $p<0.05$ ], whereas females did not differ (Figure 56H). No significant differences were detected in IL-1 $\beta$  (data not shown).



**Figure 56: Maternal HFD and BCAA altered immune and serotonergic gene expression in a sex- and brain region-dependent manner at P8. A) LFD/BCAA pups exhibited lower CD11b expression in the HYP than LFD/Control and HFD/BCAA pups, regardless of sex. B) GFAP tended to be decreased by maternal BCAA in the P8 HYP. C) BCAA pups exhibited decreased 5-HT1A expression relative to Controls in the HYP as well. D) 5-HT2CR tended to be decreased by maternal HFD in the HYP relative to LFD controls. E) HFD/BCAA male pups had decreased Iba-1 expression in**

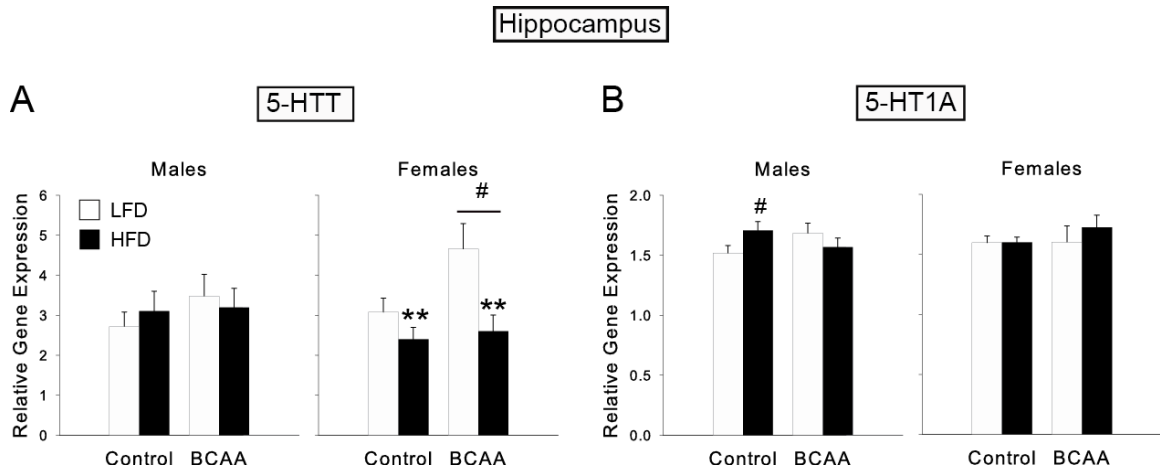
the HIPP at P8, whereas females did not differ. F) TLR2 expression was increased in HFD/Control and LFD/BCAA males in the HIPP relative to controls, whereas females did not differ. G) 5-HT1A expression was decreased in HFD/BCAA males in the HIPP, but females were not different. H) HFD/Control males exhibited increased 5-HTT expression in the HIPP relative to LFD controls, but females did not differ. Data are mean  $\pm$  SEM,  $n=12-18$ /group (sexes combined) for HYP,  $n=6-8$ /group/sex (sexes separate) for HIPP. \* $p<0.05$  vs. LFD/Control and HFD/BCAA for A, F, and H, vs. LFD/BCAA for E and G; # $p=0.07$ , BCAA vs. Control in B, HFD vs. LFD in D; ## $p<0.05$ , BCAA vs. Control.

### **7.3.8 Adult offspring of HFD and BCAA dams exhibit altered expression of serotonergic genes in a brain region- and sex-specific manner**

The immune gene expression changes in the HYP and HIPP of adult offspring due to maternal HFD and BCAA have already been described (see Chapter 6), and included altered Iba-1, TLR4, and GFAP expression, in addition to a “primed” phenotype of microglia due to maternal HFD, such that they over-respond to a second immune challenge (i.e., LPS) in adulthood. qRT-PCR analysis of the serotonin transporter, 5-HTT, in the HIPP revealed a significant Sex X HFD interaction [ $F(1,52)=4.55$ ,  $p<0.05$ ] and a close trend for a main effect of BCAA [ $F(1,52)=3.89$ ,  $p=0.05$ ]. Follow-up tests revealed that this was primarily due to a decrease in 5-HTT expression in the HIPP of HFD females relative to LFD controls, and a tendency for increased expression of 5-HTT in the HIPP of BCAA females [significant main effect of HFD,  $F(1,26)=8.93$ ,  $p<0.01$ ; trend for main effect of BCAA,  $F(1,26)=3.76$ ,  $p=0.06$ ; Figure 57A]. In contrast, we did not detect any significant differences in 5-HTT expression in the HIPP of males due to maternal diet. Analysis of the serotonin auto-receptor, 5-HT1A, in the

HIPP revealed a trend for a Sex X HFD X BCAA interaction [ $F(1,53)=2.94, p=0.09$ ].

Follow-up tests revealed that was due to a tendency for HFD/Control males to exhibit increased 5-HT1A expression in the HIPP relative to LFD/Control males [trend for HFD X BCAA interaction,  $F(1,27)=3.82, p=0.06$ ; post hoc,  $p=0.1$ ], whereas females did not differ (Figure 57B). Analysis of 5-HTT and 5-HT1A expression in the HYP did not yield any significant differences due to maternal diet (data not shown).



**Figure 57: Maternal HFD and BCAA altered serotonergic gene expression in the HIPP of adult offspring in a sex-specific manner. A) HFD females exhibited decreased expression of 5-HTT in the HIPP in adulthood relative to LFD controls, in conjunction with a tendency for BCAA to increase expression, whereas male offspring did not differ. B) Adult HFD/Control males exhibited a tendency for increased 5-HT1A expression in the HIPP relative to LFD/Control males, whereas females did not differ. Data are mean  $\pm$  SEM,  $n=6-9$ /group/sex. \*\* $p<0.05$ , HFD vs. LFD; # $p=0.06$ , BCAA vs. Control in A, # $p=0.1$  vs. LFD/Control in B.**

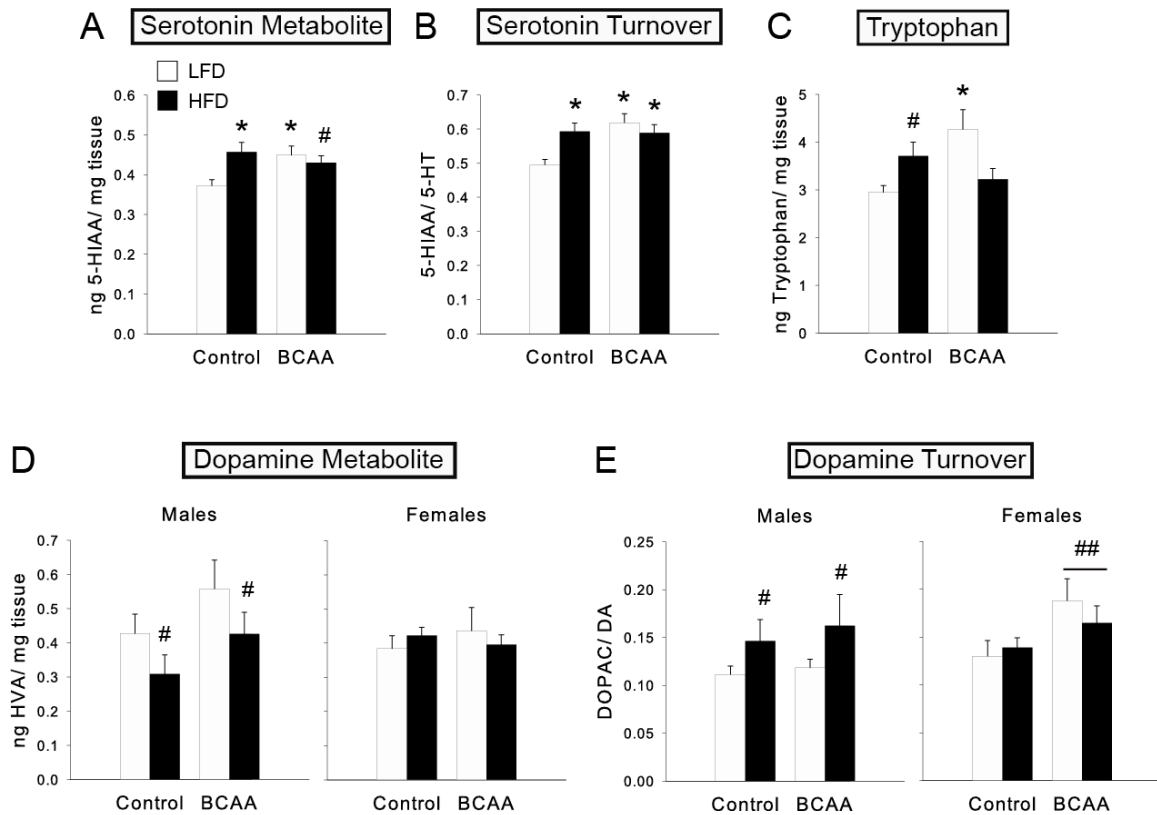
### 7.3.9 Maternal HFD and BCAA results in increased 5-HT turnover and altered dopaminergic metabolism in the PFC of adult offspring

Analysis of indoleamines in the PFC by HPLC-EC revealed a significant HFD X BCAA interaction for 5-HIAA [ $F(1,50)=5.47, p<0.05$ ], characterized by increased 5-HIAA, the major 5-HT metabolite, regardless of sex, in HFD/Control and LFD/BCAA animals compared to LFD/Control animals ( $p<0.05$ ), as well as a trend for HFD/BCAA animals to have increased 5-HIAA also ( $p=0.06$ ; Figure 58A). We did not detect any significant differences in 5-HT, but we analyzed the 5-HT turnover rate (defined as 5-HIAA divided by 5-HT) and found a similar pattern to 5-HIAA. Specifically, 5-HT turnover was increased in HFD/Control, LFD/BCAA, and HFD/BCAA animals compared to LFD/Control animals [significant HFD X BCAA interaction,  $F(1,52)=6.80, p<0.05$ ; post hoc,  $p<0.05$ ; Figure 58B]. These data are indicative of hyperserotonergic activity in the adult offspring PFC due to maternal HFD and BCAA, as 5-HIAA reflects the amount of 5-HT released in the synapse (Coppola et al., 2013).

Analysis of Trp, the 5-HT precursor, in the PFC by HPLC-EC revealed a similar pattern to 5-HIAA in the PFC [significant HFD X BCAA interaction,  $F(1,49)=9.08, p<0.005$ ], such that LFD/BCAA animals possessed significantly higher levels of Trp than LFD/Control and HFD/BCAA animals ( $p<0.05$ ), and HFD/Control animals tended to be higher than LFD/Control animals ( $p=0.09$ ; Figure 58C). We also detected an overall sex difference [significant main effect of Sex,  $F(1,49)=7.21, p<0.05$ ], because females possessed higher levels of Trp than males in the PFC overall (data not shown). Interestingly, we

observed a strong, positive correlation between Trp and 5-HIAA,  $r(57)=0.781$ ,  $p<0.001$ , but no correlation with 5-HT, which suggests that 5-HIAA may be a better measure of serotonergic activity within the brain than 5-HT itself.

Analysis of catecholamines in the PFC by HPLC-EC did not yield any significant differences in NE, DA, or DOPAC levels. However, HVA, the final-stage metabolite of dopamine, tended to be decreased in HFD males [trend for main effect of HFD,  $F(1,27)=3.47$ ,  $p=0.07$ ], whereas there were no significant differences in females' HVA levels [trend for Sex X HFD interaction,  $F(1,52)=3.11$ ,  $p=0.08$ ; Figure 58D]. There were no significant differences in the HVA/DA ratio, but the DOPAC/DA ratio did exhibit sex-specific changes [trend for Sex X HFD interaction,  $F(1,49)=2.60$ ,  $p=0.1$ ]. HFD males tended to show increased DOPAC/DA ratios [trend for main effect of HFD,  $F(1,26)=3.38$ ,  $p=0.08$ ], whereas BCAA females exhibited increased DOPAC/DA ratios in the PFC [significant main effect of BCAA,  $F(1,23)=4.96$ ,  $p<0.05$ ; Figure 58E]. These results are indicative of sex-specific alterations in dopamine metabolism.



**Figure 58: Maternal HFD and BCAA caused increased serotonin turnover and altered dopaminergic metabolism in the PFC of adult offspring.** A) HFD/Control, LFD/BCAA, and HFD/BCAA animals all exhibited an increase in 5-HIAA levels in the PFC relative to LFD/Control, regardless of sex, which is indicative of increased serotonin release at the synapse. B) The same groups also showed increased serotonin turnover (as measured by the 5-HIAA/5-HT ratio) in the PFC relative to LFD/Control, which is consistent with a hyperserotonergic phenotype. C) LFD/BCAA animals had increased tryptophan in the PFC relative to LFD/Control and HFD/BCAA animals, and HFD/Control tended to also have more tryptophan relative to LFD/Control. D) Male, but not female, HFD offspring exhibited decreased levels of HVA, a dopamine metabolite. E) Male HFD offspring also exhibited increased dopamine turnover (as measured by the DOPAC/DA ratio; DOPAC is a different dopamine metabolite) relative to LFD offspring, whereas female BCAA offspring had increased dopamine turnover relative to Controls. Data are mean  $\pm$  SEM,  $n=12-16$ /group for A-C (sexes combined),  $n=5-8$ /group/sex (sexes separate) for D-E. \* $p<0.05$  vs. LFD/Control; # $p<0.1$  vs. LFD/Control in A and C, # $p<0.09$ , HFD vs. LFD in D-E; ## $p<0.05$ , BCAA vs. Control.

## **7.4 Discussion**

This study is the first to demonstrate that maternal HFD consumption during gestation decreases placental serotonin production, which is critical for fetal brain development (Bonnin et al., 2011). Intriguingly, we also found evidence of long-term increases in serotonergic function and 5-HT turnover in the prefrontal cortex of adult HFD offspring, which corresponds well with the increased anxiety-like behavior we have observed in adult offspring in previous studies [Chapter 6; see also (Sullivan et al., 2010)]. Consistent with a multitude of studies implicating inflammatory mechanisms in the programming effects of maternal HFD [reviewed in (Bolton & Bilbo, 2014)], these changes in serotonergic function in both the mid-gestation placenta and the adult brain occurred in conjunction with widespread alterations in immune gene expression in response to maternal diet. Altered inflammatory gene expression in the brains of developing offspring, beginning as early as mid-gestation and extending into adulthood, are consistent with our previous studies demonstrating increased microglial antigen density in the adult hippocampus and an exaggerated IL-1 $\beta$  response to a later-life immune challenge (see Chapter 6).

We hypothesize that these changes in serotonergic function and immune alterations are interconnected, although this remains to be tested directly. Specifically, the inflammatory influence of maternal HFD during gestation and lactation may cause alterations in serotonergic function in the placenta and developing brain, which can then

program long-term changes in serotonergic function and behavior (Bolton & Bilbo, 2014). Serotonin and immune activation have the potential for interaction and reciprocal regulation primarily via the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is responsible for tryptophan degradation and is upregulated in response to high levels of proinflammatory cytokines (e.g., from maternal obesity), resulting in decreased serotonin synthesis and a potentially damaging by-product, quinolinic acid. Although helpful in the defense against a pathogen, quinolinic acid is also a potent NMDA receptor agonist that is capable of causing excitotoxicity and oxidative stress in the brain (Manuelpillai et al., 2005). For example, maternal endotoxin exposure during gestation results in decreased serotonin synthesis, increased cell death, and diminished serotonergic innervation of the somatosensory cortex in the brains of newborn rabbits (Kannan et al., 2010). As the placenta is the major source of serotonin for the developing forebrain prior to E15.5 (Bonnin et al., 2011), the decrease in placental serotonin output due to maternal HFD very likely resulted in a decrease in central serotonin, along with altered brain development. This idea is consistent with the observed alterations in serotonergic receptors and 5-HTT in the HYP and HIP of P8 and adult offspring, as well as the long-term increases in serotonergic activity in the PFC of adult offspring.

Although our results provide strong support for proinflammatory cytokines' interference with serotonin production in the placenta at mid-gestation, we cannot yet implicate these changes as the primary cause of the long-term changes in brain and

behavior. The developing brain may be independently affected by maternal and/or placental cytokines, such that following E16.5, when the hindbrain becomes the primary source of 5-HT for the forebrain, endogenous 5-HT production within the brain is also decreased, potentially creating an additive effect with the changes caused by the placenta. Either way, the increase in serotonergic activity in the adult PFC is indicative of a long-term programming effect, as the animals no longer have access to HFD. This is a classic example of a mismatch between the perinatal environment and the adult environment, which usually results in a compensatory mechanism and the opposite phenotype in adulthood compared to during development (e.g., the “thrifty phenotype”) (Hales & Barker, 2001). Specifically, the deficit in placental 5-HT production during mid-gestation results in the programming of the developing brain to “make due” with a limited amount of 5-HT by exaggerating its effects, perhaps via upregulation of 5-HT synthesis enzymes and downregulation of 5-HT degradation enzymes in the brain, thereby resulting in a hyperserotonergic phenotype in adulthood when a normal diet is restored.

Beyond the effect of maternal HFD on adult serotonergic activity, we also observed hyperserotonergic activity in adult offspring as a result of maternal BCAA. However, we did not detect a decrease in placental 5-HT due to BCAA alone, indicating that this may not be the cause of the observed phenotype. On the other hand, we did observe decreased placental tryptophan levels due to maternal BCAA, which may result

from increased competition with increased levels of BCAA for tryptophan to cross from the maternal bloodstream into the placenta via LAT1 (an amino acid transporter that is shared between all the large neutral amino acids) (Coppola et al., 2013). These lower levels of tryptophan in the fetal compartment may have resulted in lower levels of 5-HT production by the hindbrain after E16.5, which could result in the same compensatory hyperserotonergic phenotype in adulthood that we observed in HFD offspring. Indeed, we observed increased tryptophan levels in the adult PFC in parallel with increased 5-HIAA, particularly in LFD/BCAA offspring, which may suggest compensatory upregulation of LAT1 in the blood-brain barrier of animals that developed with decreased levels of 5-HT or Trp. Although behavioral phenotypes were not as pronounced in adult BCAA offspring as in HFD offspring, we did observe suggestive evidence of increased depressive-like behavior in the forced-swim test (see Chapter 6). It is possible that a tryptophan deficit in the absence of decreased placental 5-HT production (likely due to a compensatory mechanism) may not result in as severe of a programming phenotype. Alternatively, BCAA animals did not exhibit as widespread of changes in immune gene expression in either the placenta or brain, suggesting that the inflammatory effects of HFD may contribute significantly to the behavioral phenotype.

Our data suggests that microglial colonization of the developing brain is altered by maternal HFD, as P1 HFD pups exhibited decreased expression of microglial markers. This was initially surprising to us, as we predicted that the maternal

inflammation from HFD consumption would result in more activated microglia in the developing brain, and thus increased expression of microglial markers. However, it is possible that alteration of the cytokine milieu in the placenta by maternal HFD impacts the chemokine and growth factor signaling that is required for proper microglial colonization (Ginhoux et al., 2010; Kierdorf et al., 2013). Alternatively, microglial development may be impacted by circulating corticosterone levels (Gomez-Gonzalez & Escobar, 2010), which exhibited a very similar pattern to that observed for microglial marker expression. Either way, it is clear that microglia were altered long-term by maternal HFD, as adult HFD offspring exhibited increases in microglial antigen density in the hippocampus, as well as functional priming of the IL-1 $\beta$  response to a later-life immune challenge. In agreement with this opposite phenotype in adulthood as compared to P1, there is evidence that the pattern began to reverse later in development. For example, P8 HFD male pups exhibited increased TLR2 expression in the hippocampus, in contrast to the decreased TLR2 expression observed at P1. Furthermore, this developmental switch may again be the result of a mismatch between the perinatal environment and the adult environment, like the thrifty phenotype.

Notably, we observed sex-specific immune gene regulation in the placenta in response to maternal diet. Consistent with Clifton's hypothesis, female placentas exhibited a greater number of gene expression changes overall in response to maternal diet than did male placentas. Clifton (2010) hypothesized that male placentas prioritize

growth, whereas female placentas prioritize the immune response to a maternal immune challenge. As one example in support of this idea, we observed that female HFD placentas downregulated IL-6, a cytokine that is critical for placental growth (Hsiao & Patterson, 2011), whereas male placentas did not change. Furthermore, HFD male offspring exhibited greater mortality between E14.5 and P1, perhaps due to their lack of response in an attempt to maintain normal fetal growth. It is clear that these sex-specific changes cannot be the result of gonadal sex hormones, as E14.5 is before the gonads develop. Thus, it is more likely that the observed sex differences are the result of X-linked genes or sex-specific genomic imprinting, as has been proposed by others (Fish, 2008). We have previously described sex-specific alterations in the behavior of adult offspring; i.e., HFD male offspring exhibited hyperactivity, whereas HFD female offspring did not differ from controls (see Chapter 6). In the current study, we also detected male-specific alterations in dopaminergic metabolism in the prefrontal cortex of adult HFD offspring that may underlie the hyperactive phenotype (Rodriguez et al., 2008; Sullivan, Nousen, & Chamlou, 2014). Alternatively, the sex difference could result from an overall more pronounced neuroinflammatory phenotype in males relative to females (see Chapter 6).

In conclusion, we have demonstrated that maternal HFD causes widespread alterations in placental immune gene expression and decreased placental serotonin production in mid-gestation, which results in long-term changes in serotonergic activity

in the prefrontal cortex of HFD offspring, and may underlie their increased anxiety-like behavior (Sullivan et al., 2010). On the other hand, maternal BCAA causes decreased placental tryptophan availability, which eventually results in a similar hyperserotonergic phenotype in adult BCAA offspring. Sex-specific changes in placental immune gene expression and adult dopaminergic metabolism may contribute to the long-term sex-specific behavioral and metabolic outcomes we have previously observed. Our working hypothesis is that the inflammatory effect of HFD causes decreased placental serotonin production via IDO's conversion of available tryptophan to the kynurenine pathway, but this remains to be tested directly. Future studies aimed at elucidating these mechanisms of long-term perinatal programming will be critical for identifying effective interventions or treatments for the growing number of children impacted by maternal obesity.

## 8. Summary and Conclusions

The idea of developmental programming has gained a tremendous amount of empirical support in the nearly 30 years since David Barker's seminal work was published (Barker & Osmond, 1986). Epidemiological evidence has implicated an adverse prenatal and/or postnatal environment in the etiology of many chronic health problems and mental disorders, and more recent work with animal models is beginning to identify the mechanistic basis for the long-term consequences of early-life events. My dissertation work was based on the hypothesis that the programming of brain and behavior by an altered early-life environment is propagated by inflammatory mechanisms in the placenta and developing brain, as immune molecules are now known to have a dual role in developmental processes and host defense. Specifically, I aimed to characterize the innate immune changes, such as microglial priming and placental immune responses, that could alter normal brain and placental function during critical windows of development in order to result in the long-term behavioral, metabolic, and neuroinflammatory consequences of early-life environmental exposures.

The first goal of my dissertation research was to assess whether prenatal air pollution exposure, an extremely relevant environmental exposure in the modern world, could predispose offspring to neuroinflammation and obesity following high-fat diet consumption in adulthood (i.e., an inflammatory "second hit"). Chapter 2 (Experiment 1) presents evidence showing that prenatal air pollution exposure markedly impacts

offspring body weight, metabolic hormones, food intake, and microglial activation in ways that are adult diet- and sex-dependent. Our data thus supports the classification of air pollution as an “obesogen”, similar to tobacco smoke and pesticides (Pryor et al., 2011; Wohlfahrt-Veje et al., 2011). Although the mechanisms underlying the obesogenic properties of these environmental toxins remain unclear, we identified microglia as a potential biological substrate that may be “primed” by the prenatal air pollution exposure and then overactivated following exposure to a high-fat diet in adulthood. Previous studies had shown a key role for microglia and their proinflammatory signaling in the pathogenesis of central insulin resistance; therefore, we hypothesized that microglial overactivation in response to adult high-fat diet in our model could have accelerated the development of obesity and insulin resistance in animals exposed to air pollution prenatally.

However, given the critical role of both central and peripheral inflammation in the development of obesity (Dahlgren et al., 2001; Das, 2001; Heindel & vom Saal, 2009), we next asked if there was also an involvement of the peripheral immune system in our model. Specifically, the goal of Chapter 3 was to characterize the peripheral inflammatory response and the population of infiltrating peripheral immune cells in the brains of the “two-hit animals”. Using flow cytometry, we determined that there was a significant increase in the population of infiltrating monocytes/macrophages (CD11b+CD45<sup>high</sup> cells) in the brains of animals on a high-fat diet, which is consistent

with blood-brain barrier disruption due to the inflammatory effects of a high-fat diet. Notably, we also showed that this population of infiltrating cells exhibited higher surface levels of CD11b specifically in the hypothalamus of males that had been exposed to prenatal air pollution and later consumed a high-fat diet. This data suggests a “primed” phenotype in peripheral macrophages in addition to microglia. In order to assess functional priming of peripheral immune cells by prenatal air pollution, we administered a systemic LPS challenge in our model, and found an exaggerated peripheral, but not central, IL-1 $\beta$  response 2 hours later in air pollution-exposed males. Taken together, our results demonstrate that prenatal air pollution exposure alters the development of both the peripheral and central innate immune systems, particularly in males, which confers later vulnerability to a second immune challenge.

We now have substantial evidence that prenatal air pollution exposure can predispose offspring to adverse behavioral and neuroinflammatory outcomes following exposure to a “second hit” in adulthood, but we next asked whether prenatal air pollution exposure could also synergize with a simultaneous second hit during gestation. Thus, Chapter 4 assessed the long-term changes in adult offspring behavior and neuroimmune function when prenatal air pollution exposure was combined with maternal stress during gestation. The interaction of environmental “physical” toxins, such as air pollution, with “social toxins”, such as stress, during gestation remains relatively unexplored, despite its high relevance for expectant mothers living in areas of

low socioeconomic status. In our model, we discovered that the offspring of mothers exposed to both air pollution and stress during gestation were more anxious as adults, but only the male offspring of this group also exhibited impaired cognition. Furthermore, the brains of these male offspring exhibited increased TLR4 expression and a greater proinflammatory bias (IL-1 $\beta$ /IL-10 ratio) than their female littermates, to the extent that these changes correlated with their observed behavioral effects. As microglia are the primary cell type that express TLR4 and produce cytokines in the brain, these data are further evidence supporting the role of microglia in the programming of long-term behavioral changes due to maternal environmental exposures during gestation.

We had now observed strikingly similar long-term changes in TLR4 expression in the brains of male offspring in two distinct “2-hit” models that include prenatal air pollution exposure (one involving adult HFD and one involving simultaneous maternal stress during gestation). In both models, we had also observed greater adverse outcomes in terms of behavioral, metabolic, and neuroinflammatory changes in the male compared to the female offspring. The goal of Chapter 5 was to determine if prenatal air pollution exposure altered microglial development, such that microglia were primed long-term, and whether this was a TLR4- and sex-dependent process, as our previous data would suggest. Our data showed that prenatal air pollution did result in altered microglial development, as well as long-lasting changes in microglial number and

morphology consistent with the primed microglia phenotype we had observed in Chapters 2-4. Notably, the changes in microglial maturation were dependent on TLR4 and were much greater in males than females, as hypothesized.

Another goal of Chapter 5 was to characterize the placental inflammatory response to air pollution exposure during gestation, in order to determine if it may underlie the changes in the inflammatory milieu of the developing brain. Contrary to our hypothesis, we observed evidence of an immune response to air pollution exposure in TLR4-null placenta, but not in TLR4-competent placenta. This may suggest that the immune response is prolonged or less efficient in TLR4-null placentas, as the time point of analysis was two days after the final DEP exposure, and may have already resolved in TLR4-competent placentas. However, we did observe overall higher levels of IL-10 in female placentas, in conjunction with an increased weight in male TLR4<sup>-/-</sup> placentas in response to DEP, which is consistent with Clifton's hypothesis that male placentas prioritize growth while female placentas prioritize responding to a maternal immune challenge (Clifton, 2010). The greater anti-inflammatory bias in female placentas may have proved protective for the female fetus at the time of maternal air pollution exposure, and is consistent with our previous data in the developing and adult brain of air pollution-exposed offspring (Bolton et al., 2013).

We now had ample evidence to support sexually dimorphic fetal programming of offspring brain and behavior by prenatal air pollution exposure, as well as support for

microglial and peripheral macrophage priming by the intrauterine environment.

Another goal of my dissertation research was to determine if a different maternal environmental exposure (i.e., a “Western diet”) would result in similar long-term, sex-specific behavioral changes and macrophage priming mechanisms. Chapter 6 addressed this using a model of a “Western diet” that included a high-fat component and a high-protein component (i.e., BCAA supplementation). Consumption of this diet prior to and during gestation and lactation resulted in a robust model of maternal obesity, as well as a novel animal model of postpartum depression, which will prove useful in the future for dissecting the mechanisms of this poorly understood mood disorder in new mothers.

Although the combined HFD+BCAA supplementation resulted in the worst outcome for mothers, we determined that HFD alone resulted in the most adverse outcomes for offspring. Specifically, the offspring of HFD-fed dams were more anxious in adulthood, despite being placed on a low-fat diet at weaning. Male HFD offspring were also hyperactive, whereas female HFD offspring exhibited more severe metabolic disturbances. Furthermore, HFD offspring had greater microglial antigen density in the hippocampus, in conjunction with an exaggerated IL-1 $\beta$  response to LPS, particularly in males. These data support the idea that maternal HFD primes microglia during brain development for increased reactivity to a second immune challenge in adulthood.

However, the exaggerated immune response to a second hit was not limited to the brain, but was also present in the periphery, as in our air pollution model (Chapter 3).

Moreover, we found a long-term increase in monocyte infiltration of the brains of adult male HFD offspring, and this population of monocytes possessed increased surface levels of CD14 (an activation marker), which is consistent with the priming of peripheral macrophages in addition to microglia.

Because we found evidence of microglial priming and long-term changes in behavior, we next asked if microglial development was altered, as we had seen in Chapter 5. In Chapter 7, we presented evidence that microglial development was altered by maternal HFD, though potentially in opposite directions by prenatal vs. postnatal HFD exposure. Given the key role of the placenta in nutrient transfer and serotonin production from maternal amino acids, both of which are critical for fetal brain development, we also assessed the placental immune response to maternal HFD and its impact on placental serotonin levels. We found evidence of widespread, sex-specific immune gene regulation in response to maternal diet, in conjunction with decreased placental serotonin production due to maternal HFD. This novel finding was accompanied by a long-term alteration of serotonergic activity in the prefrontal cortex of adult HFD offspring, which is consistent with the observed increase in anxiety in these animals in Chapter 6. Moreover, consistent with the hyperactivity we observed in male HFD offspring, the prefrontal cortex of these males exhibited alterations in dopamine metabolism, whereas their female littermates did not differ.

In sum, my dissertation research has demonstrated using two different animal models and a variety of experimental techniques that developmental programming of offspring brain and behavior by maternal environmental exposures occurs in a sex-specific manner and is associated with inflammatory mechanisms in the placenta and developing brain. Specifically, macrophage priming occurs in response to maternal immune challenges in both the periphery and brain of offspring during development and may be a primary mechanism by which long-term changes in behavior and metabolism are effected. Overall, my research demonstrates that males are more vulnerable to early-life immune challenges and their long-term adverse consequences, likely due to sex differences in microglial colonization and placental immune responses. Further research aimed at establishing causation for these sexually dimorphic mechanisms in the later-life risk for mental health disorders and obesity will be critical for informing public health policy, as well as identifying effective interventions and treatments.

## References

- Abrahams, V. M. (2008). Pattern recognition at the maternal-fetal interface. *Immunological Investigations*, 37(5-6), 427-447.
- Adinolfi, M. (1985). THE DEVELOPMENT OF THE HUMAN BLOOD-CSF-BRAIN BARRIER. *Developmental Medicine & Child Neurology*, 27(4), 532-537.
- Alonso-Magdalena, P., Vieira, E., Soriano, S., Menes, L., Burks, D., Quesada, I., & Nadal, A. (2010). Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environmental Health Perspectives*, 118(9), 1243.
- Alwan, A. (2011). *Global status report on noncommunicable diseases 2010*. World Health Organization.
- Andersen, S. L. (2003). Trajectories of brain development: Point of vulnerability or window of opportunity? *Neuroscience & Biobehavioral Reviews*, 27(1), 3-18.
- Anderson, G. (2011). Neuronal-immune interactions in mediating stress effects in the etiology and course of schizophrenia: Role of the amygdala in developmental co-ordination. *Medical Hypotheses*, 76(1), 54-60.
- Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., . . . Schwartz, D. A. (2000). TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genetics*, 25(2), 187-191.
- Armitage, J. A., Khan, I. Y., Taylor, P. D., Nathanielsz, P. W., & Poston, L. (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: How strong is the evidence from experimental models in mammals? *J Physiol*, 561(Pt 2), 355-77.  
doi:10.1113/jphysiol.2004.072009 jphysiol.2004.072009 [pii]
- Auten, R. L., Potts, E. N., Mason, S. N., Fischer, B., Huang, Y., & Foster, W. M. (2009). Maternal exposure to particulate matter increases postnatal ozone-induced airway hyperreactivity in juvenile mice. *American Journal of Respiratory and Critical Care Medicine*, 180(12), 1218-1226.
- Auten, R. L., Gilmour, M. I., Krantz, Q. T., Potts, E. N., Mason, S. N., & Foster, W. M. (2012). Maternal diesel inhalation increases airway hyperreactivity in ozone-exposed offspring. *American Journal of Respiratory Cell and Molecular Biology*, 46(4), 454-460.  
doi:10.1165/rcmb.2011-0256OC
- Avishai-Eliner, S., Eghbal-Ahmadi, M., Tabachnik, E., Brunson, K. L., & Baram, T. Z. (2001). Down-regulation of hypothalamic corticotropin-releasing hormone messenger ribonucleic

- acid (mRNA) precedes early-life experience-induced changes in hippocampal glucocorticoid receptor mRNA. *Endocrinology*, 142(1), 89-97.
- Barker, D. J., & Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *The Lancet*, 327(8489), 1077-1081.
- Bartha, J. L., Romero-Carmona, R., & Comino-Delgado, R. (2003). Inflammatory cytokines in intrauterine growth retardation. *Acta Obstetrica Et Gynecologica Scandinavica*, 82(12), 1099-1102.
- Bassit, R. A., Sawada, L. A., Bacurau, R. F., Navarro, F., Martins Jr, E., Santos, R. V., . . . Costa Rosa, L. F. (2002). Branched-chain amino acid supplementation and the immune response of long-distance athletes. *Nutrition*, 18(5), 376-379.
- Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., . . . Fitzgerald, K. A. (2009). Cutting edge: NF- $\kappa$ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *The Journal of Immunology*, 183(2), 787-791.
- Bearer, C. F. (1995). Environmental health hazards: How children are different from adults. *The Future of Children*, , 11-26.
- Benirschke, K., Kaufmann, P., & Baergen, R. (2006). *Pathology of the human placenta* (5th edition ed.). New York, NY: Springer.
- Bennet, L., & Gunn, A. (2006). The fetal origins of adult mental illness. In M. Wintour-Coghlan, & J. Owens (Eds.), *Early life origins of health and disease (advances in experimental medicine and biology)* (pp. 204-211). New York: Springer.
- Bevilacqua, E., Hoshida, M., Amarante-Paffaro, A., Albieri-Borges, A., & Zago-Gomes, S. (2010). Trophoblast phagocytic program: Roles in different placental systems. *International Journal of Developmental Biology*, 54(2), 495.
- Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: All we need to know about danger. *Journal of Leukocyte Biology*, 81(1), 1-5. doi:10.1189/jlb.0306164
- Bilbo, S. D., Newsom, N. J., Sprunger, D. B., Watkins, L. R., Rudy, J. W., & Maier, S. F. (2007). Differential effects of neonatal handling on early life infection-induced alterations in cognition in adulthood. *Brain, Behavior, and Immunity*, 21(3), 332-342.
- Bilbo, S. D., & Schwarz, J. M. (2009). Early-life programming of later-life brain and behavior: A critical role for the immune system. *Frontiers in Behavioral Neuroscience*, 3, 14.

- Bilbo, S. D., & Tsang, V. (2010). Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. *The FASEB Journal*, *24*(6), 2104-2115.
- Bilbo, S. D., Biedenkapp, J. C., Der-Avakian, A., Watkins, L. R., Rudy, J. W., & Maier, S. F. (2005). Neonatal infection-induced memory impairment after lipopolysaccharide in adulthood is prevented via caspase-1 inhibition. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(35), 8000-8009. doi:10.1523/JNEUROSCI.1748-05.2005
- Black, R. A., Kronheim, S. R., Cantrell, M., Deeley, M. C., March, C. J., Prickett, K. S., . . . Hopp, T. P. (1988). Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *Journal of Biological Chemistry*, *263*(19), 9437-9442.
- Blanchard, R. J., Fukunaga, K. K., & Blanchard, D. C. (1976). Environmental control of defensive reactions to footshock. *Bulletin of the Psychonomic Society*, *8*(129), 40.
- Bland, S. T., Beckley, J. T., Young, S., Tsang, V., Watkins, L. R., Maier, S. F., & Bilbo, S. D. (2010). Enduring consequences of early-life infection on glial and neural cell genesis within cognitive regions of the brain. *Brain, Behavior, and Immunity*, *24*(3), 329-338.
- Block, M. L., & Calderón-Garcidueñas, L. (2009). Air pollution: Mechanisms of neuroinflammation and CNS disease. *Trends in Neurosciences*, *32*(9), 506-516.
- Bolton, J. L., & Bilbo, S. D. (2014). Developmental programming of brain and behavior by perinatal diet: Focus on inflammatory mechanisms. *Dialogues in Clinical Neuroscience*, *16*(3), 307.
- Bolton, J. L., Huff, N. C., Smith, S. H., Mason, S. N., Foster, W. M., Auten, R. L., & Bilbo, S. D. (2013). Maternal stress and effects of prenatal air pollution on offspring mental health outcomes in mice. *Environmental Health Perspectives*, *121*(9), 1075.
- Bolton, J. L., Auten, R. L., & Bilbo, S. D. (2014). Prenatal air pollution exposure induces sexually dimorphic fetal programming of metabolic and neuroinflammatory outcomes in adult offspring. *Brain, Behavior, and Immunity*, *37C*, 30-44. doi:10.1016/j.bbi.2013.10.029; 10.1016/j.bbi.2013.10.029
- Bolton, J. L., Smith, S. H., Huff, N. C., Gilmour, M. I., Foster, W. M., Auten, R. L., & Bilbo, S. D. (2012). Prenatal air pollution exposure induces neuroinflammation and predisposes offspring to weight gain in adulthood in a sex-specific manner. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *26*(11), 4743-4754. doi:10.1096/fj.12-210989; 10.1096/fj.12-210989
- Bonnin, A., Goeden, N., Chen, K., Wilson, M. L., King, J., Shih, J. C., . . . Levitt, P. (2011). A transient placental source of serotonin for the fetal forebrain. *Nature*, *472*(7343), 347-350.

- Boulanger, L. M. (2009a). Immune proteins in brain development and synaptic plasticity. *Neuron*, 64(1), 93-109.
- Boulanger, L. M. (2009b). Immune proteins in brain development and synaptic plasticity. *Neuron*, 64(1), 93-109. doi:DOI: 10.1016/j.neuron.2009.09.001
- Buckman, L. B., Hasty, A. H., Flaherty, D. K., Buckman, C. T., Thompson, M. M., Matlock, B. K., . . . Ellacott, K. L. (2013). Obesity induced by a high-fat diet is associated with increased immune cell entry into the central nervous system. *Brain, Behavior, and Immunity*,
- Buznikov, G. A., Lambert, W. H., & Lauder, J. M. (2001). Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis. *Cell and Tissue Research*, 305(2), 177-186.
- Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P. M., & Meaney, M. J. (1998). Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. *Proceedings of the National Academy of Sciences of the United States of America*, 95(9), 5335-5340.
- Cao, X., Laplante, D. P., Brunet, A., Ciampi, A., & King, S. (2012). Prenatal maternal stress affects motor function in 5½-year-old children: Project ice storm. *Developmental Psychobiology*,
- Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., . . . Dutta, R. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nature Neuroscience*, 9(7), 917-924.
- Caso, J. R., Pradillo, J. M., Hurtado, O., Leza, J. C., Moro, M. A., & Lizasoain, I. (2008). Toll-like receptor 4 is involved in subacute Stress-Induced neuroinflammation and in the worsening of experimental stroke. *Stroke*, 39(4), 1314-1320.
- Castagné, V., Moser, P., Roux, S., & Porsolt, R. D. (2010). Rodent models of depression: Forced swim and tail suspension behavioral despair tests in rats and mice. *Current Protocols in Pharmacology*, , 5.8.1-5.8.14.
- Castellucci, M., Celona, A., Bartels, H., Steininger, B., Benedetto, V., & Kaufmann, P. (1987). Mitosis of the hofbauer cell: Possible implications for a fetal macrophage. *Placenta*, 8(1), 65-76.
- Castellucci, M., Zaccheo, D., & Pescetto, G. (1980). A three-dimensional study of the normal human placental villous core. *Cell and Tissue Research*, 210(2), 235-247.
- Cedergren, M. I. (2004). Maternal morbid obesity and the risk of adverse pregnancy outcome. *Obstetrics and Gynecology*, 103(2), 219-224. doi:10.1097/01.AOG.0000107291.46159.00

- Challier, J., Basu, S., Bintein, T., Minium, J., Hotmire, K., Catalano, P., & Hauguel-de Mouzon, S. (2008). Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta*, 29(3), 274-281.
- Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology & Behavior*, 79(3), 359-371.
- Chen, H., Simar, D., Lambert, K., Mercier, J., & Morris, M. J. (2008). Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. *Endocrinology*, 149(11), 5348-5356. doi:10.1210/en.2008-0582
- Chomczynski, P., & Sacchi, N. (1987). Single-step RNA isolation from cultured cells or tissues. *Anal Biochem*, 162, 156-259.
- Clifton, V. L. (2010). Review: Sex and the human placenta: Mediating differential strategies of fetal growth and survival. *Placenta*, 31, S33-S39.
- Coppola, A., Wenner, B. R., Ilkayeva, O., Stevens, R. D., Maggioni, M., Slotkin, T. A., . . . Newgard, C. B. (2013). Branched-chain amino acids alter neurobehavioral function in rats. *American Journal of Physiology. Endocrinology and Metabolism*, 304(4), E405-13. doi:10.1152/ajpendo.00373.2012; 10.1152/ajpendo.00373.2012
- Courchesne, E., Press, G. A., & Yeung-Courchesne, R. (1993). Parietal lobe abnormalities detected with MR in patients with infantile autism. *AJR. American Journal of Roentgenology*, 160(2), 387-393. doi:10.2214/ajr.160.2.8424359 [doi]
- Cuadros, M. A., & Navascués, J. (1998). The origin and differentiation of microglial cells during development. *Progress in Neurobiology*, 56(2), 173-189.
- Dahlgren, J., Samuelsson, A., Jansson, T., & Holmäng, A. (2006). Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. *Pediatric Research*, 60(2), 147-151.
- Dahlgren, J., Nilsson, C., Jennische, E., Ho, H. P., Eriksson, E., Niklasson, A., . . . Holmang, A. (2001). Prenatal cytokine exposure results in obesity and gender-specific programming. *American Journal of Physiology. Endocrinology and Metabolism*, 281(2), E326-34.
- Dammann, O., & Leviton, A. (1997). Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatric Research*, 42(1), 1-8.
- Darnall, B. D., & Suarez, E. C. (2009). Sex and gender in psychoneuroimmunology research: Past, present and future. *Brain, Behavior, and Immunity*, 23(5), 595-604.

- Das, U. N. (2007). Is metabolic syndrome X a disorder of the brain with the initiation of low-grade systemic inflammatory events during the perinatal period? *The Journal of Nutritional Biochemistry*, 18(11), 701-713.
- Das, U. N. (2001). Is obesity an inflammatory condition? *Nutrition*, 17(11-12), 953-66.  
doi:S0899900701006724 [pii]
- Davidson, T. L., Kanoski, S. E., Schier, L. A., Clegg, D. J., & Benoit, S. C. (2007). A potential role for the hippocampus in energy intake and body weight regulation. *Current Opinion in Pharmacology*, 7(6), 613-616.
- de Jonge, H. J., Fehrmann, R. S., de Bont, E. S., Hofstra, R. M., Gerbens, F., Kamps, W. A., . . . ter Elst, A. (2007). Evidence based selection of housekeeping genes. *PloS One*, 2(9), e898.
- de la Monte, S. M. (2009). Insulin resistance and alzheimer's disease. *BMB Rep*, 42(8), 475-81.
- de Melo, J. O., Soto, S. F., Katayama, I. A., Wenceslau, C. F., Pires, A. G., Veras, M. M., . . . Heimann, J. C. (2015). Inhalation of fine particulate matter during pregnancy increased IL-4 cytokine levels in the fetal portion of the placenta. *Toxicology Letters*, 232(2), 475-480.
- De Simone, R., Vissicchio, F., Mingarelli, C., De Nuccio, C., Visentin, S., Ajmone-Cat, M. A., & Minghetti, L. (2013). Branched-chain amino acids influence the immune properties of microglial cells and their responsiveness to pro-inflammatory signals. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1832(5), 650-659.
- de Wit, M., Wiaterek, G. K., Gray, N. D., Goulet, K. E., Best, A. M., Clore, J. N., & Sweeney, L. B. (2010). Relationship between alcohol use disorders, cortisol concentrations, and cytokine levels in patients with sepsis. *Critical Care*, 14(6), R230.
- Demir, R., Kaufmann, P., Castellucci, M., Erben, T., & Kotowski, A. (1989). Fetal vasculogenesis and angiogenesis in human placental villi. *Cells Tissues Organs*, 136(3), 190-203.
- Deng, H., Maitra, U., Morris, M., & Li, L. (2013). Molecular mechanism responsible for the priming of macrophage activation. *Journal of Biological Chemistry*, 288(6), 3897-3906.
- Deverman, B. E., & Patterson, P. H. (2009). Cytokines and CNS development. *Neuron*, 64(1), 61-78.
- D'Mello, C., Le, T., & Swain, M. G. (2009). Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factor- $\alpha$  signaling during peripheral organ inflammation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 29(7), 2089-2102.  
doi:10.1523/JNEUROSCI.3567-08.2009 [doi]

- Dubowsky, S. D., Suh, H., Schwartz, J., Coull, B. A., & Gold, D. R. (2006). Diabetes, obesity, and hypertension may enhance associations between air pollution and markers of systemic inflammation. *Environmental Health Perspectives*, *114*(7), 992.
- Dunn, G. A., & Bale, T. L. (2009). Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice. *Endocrinology*, *150*(11), 4999-5009. doi:en.2009-0500 [pii] 10.1210/en.2009-0500
- Egaas, B., Courchesne, E., & Saitoh, O. (1995). Reduced size of corpus callosum in autism. *Archives of Neurology*, *52*(8), 794-801.
- Enders, A. C., & King, B. F. (1970). The cytology of hofbauer cells. *Anat Rec.*, *167*, 231-236.
- Esteban-Vasallo, M. D., Aragonés, N., Pollán, M., Lopez-Abente, G., & Perez-Gomez, B. (2012). Mercury, cadmium, and lead levels in human placenta: A systematic review. *Environmental Health Perspectives*, *120*(10), 1369-1377. doi:10.1289/ehp.1204952; 10.1289/ehp.1204952
- Evans, G. W., & Kantrowitz, E. (2002a). Socioeconomic status and health: The potential role of environmental risk exposure. *Annual Review of Public Health*, *23*(1), 303-331.
- Evans, G. W., & Kantrowitz, E. (2002b). Socioeconomic status and health: The potential role of environmental risk exposure. *Annual Review of Public Health*, *23*(1), 303-331.
- Fang, F., Höglund, C. O., Arck, P., Lundholm, C., Långström, N., Lichtenstein, P., . . . Almqvist, C. (2011). Maternal bereavement and childhood Asthma—Analyses in two large samples of swedish children. *PLoS One*, *6*(11), e27202.
- Fish, E. N. (2008). The X-files in immunity: Sex-based differences predispose immune responses. *Nature Reviews Immunology*, *8*(9), 737-744.
- Flannery, K. A., Liederman, J., Daly, L., & Schultz, J. (2000). Male prevalence for reading disability is found in a large sample of black and white children free from ascertainment bias. *Journal of the International Neuropsychological Society*, *6*(04), 433-442.
- Ford, A. L., Goodsall, A. L., Hickey, W. F., & Sedgwick, J. D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4 T cells compared. *The Journal of Immunology*, *154*(9), 4309-4321.
- Foster, W. M., Walters, D. M., Longphre, M., Macri, K., & Miller, L. M. (2001). Methodology for the measurement of mucociliary function in the mouse by scintigraphy. *Journal of Applied Physiology*, *90*(3), 1111-1118.

- Frank, M. G., Baratta, M. V., Sprunger, D. B., Watkins, L. R., & Maier, S. F. (2007). Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. *Brain, Behavior, and Immunity*, 21(1), 47-59.
- Frank, M. G., Watkins, L. R., & Maier, S. F. (2011). Stress-and glucocorticoid-induced priming of neuroinflammatory responses: Potential mechanisms of stress-induced vulnerability to drugs of abuse. *Brain, Behavior, and Immunity*, 25, S21-S28.
- Frank, M. G., Miguel, Z. D., Watkins, L. R., & Maier, S. F. (2010). Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to E. coli lipopolysaccharide. *Brain, Behavior, and Immunity*, 24(1), 19-30. doi:10.1016/j.bbi.2009.07.008
- Frankland, P. W., Cestari, V., Filipkowski, R. K., McDonald, R. J., & Silva, A. J. (1998). The dorsal hippocampus is essential for context discrimination but not for contextual conditioning. *Behavioral Neuroscience*, 112(4), 863.
- Franklin, K., & Paxinos, G. (1997). The mouse brain in stereotaxic coordinates (academic, san diego).
- Freeman, D. J. (2009). Effects of maternal obesity on fetal growth and body composition: Implications for programming and future health. *Semin Fetal Neonatal Med*, doi:S1744-165X(09)00087-0 [pii] 10.1016/j.siny.2009.09.001
- Garate, I., Garcia-Bueno, B., Madrigal, J. L., Caso, J. R., Alou, L., Gomez-Lus, M. L., . . . Leza, J. C. (2013). Stress-induced neuroinflammation: Role of the toll-like receptor-4 pathway. *Biological Psychiatry*, 73(1), 32-43. doi:10.1016/j.biopsych.2012.07.005; 10.1016/j.biopsych.2012.07.005
- García-Patterson, A., Aulinas, A., Sojo, L., Ginovart, G., Adelantado, J., de Leiva, A., & Corcoy, R. (2011). Poorer perinatal outcome in male newborns of women with pregestational diabetes mellitus. *Diabetic Medicine*, 28(4), 436-439.
- George Paxinos, C. W. (Ed.). (2005). *The rat brain in stereotaxic coordinates*. New York: Academic Press.
- Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., . . . Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)*, 330(6005), 841-845. doi:10.1126/science.1194637
- Giovanoli, S., Engler, H., Engler, A., Richetto, J., Voget, M., Willi, R., . . . Schedlowski, M. (2013). Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. *Science*, 339(6123), 1095-1099.
- Glaser, J., Greene, G., & Hendricks, S. (2007). *Stereology for biological research: With a focus on neuroscience* mbf Press.

- Glover, D., Brownstein, D., Burchett, S., Larsen, A., & Wilson, C. (1987). Expression of HLA class II antigens and secretion of interleukin-1 by monocytes and macrophages from adults and neonates. *Immunology*, 61(2), 195.
- Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., & Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, 19(10), 1329-1331. doi:10.1096/fj.05-3776fje
- Godfrey, K. M., & Barker, D. J. (2001). Fetal programming and adult health. *Public Health Nutrition*, 4(2B; SPI), 611-624.
- Gold, R. M. (1970). Hypothalamic hyperphagia: Males get just as fat as females. *Journal of Comparative and Physiological Psychology*, 71(3), 347.
- Gomez-Gonzalez, B., & Escobar, A. (2010). Prenatal stress alters microglial development and distribution in postnatal rat brain. *Acta Neuropathologica*, 119(3), 303-315. doi:10.1007/s00401-009-0590-4
- Gorski, J. N., Dunn-Meynell, A. A., Hartman, T. G., & Levin, B. E. (2006). Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 291(3), R768-78. doi:10.1152/ajpregu.00138.2006
- Grandgirard, A., Bourre, J., Julliard, F., Homayoun, P., Dumont, O., Piciotti, M., & Sebedio, J. (1994). Incorporation of trans long-chain n-3 polyunsaturated fatty acids in rat brain structures and retina. *Lipids*, 29(4), 251-258.
- Grattan, D. R. (2008). Fetal programming from maternal obesity: Eating too much for two? *Endocrinology*, 149(11), 5345-7. doi:149/11/5345 [pii] 10.1210/en.2008-1106
- Grün, F., & Blumberg, B. (2009). Minireview: The case for obesogens. *Molecular Endocrinology*, 23(8), 1127-1134.
- Gundersen, H., & Jensen, E. (1987). The efficiency of systematic sampling in stereology and its prediction\*. *Journal of Microscopy*, 147(3), 229-263.
- Haffner, S., Valdez, R., Hazuda, H., Mitchell, B., Morales, P., & Stern, M. (1992). Prospective analysis of the insulin-resistance syndrome (syndrome X). *Diabetes*, 41(6), 715-722.
- Hales, C. N., & Barker, D. J. (2001). The thrifty phenotype hypothesis. *British Medical Bulletin*, 60(1)

- Hancox, R. J., & Landhuis, C. E. (2011). Correlation between measures of insulin resistance in fasting and non-fasting blood. *Diabetology & Metabolic Syndrome*, 3(1), 23.
- Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry*, 90(1), 420-426.
- Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., . . . Thompson, D. A. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proceedings of the National Academy of Sciences*, 95(18), 10896-10901.
- Heindel, J. J., & vom Saal, F. S. (2009). Role of nutrition and environmental endocrine disrupting chemicals during the perinatal period on the aetiology of obesity. *Molecular and Cellular Endocrinology*, 304(1-2), 90-96.
- Herbert, M. R. (2005). Large brains in autism: The challenge of pervasive abnormality. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, 11(5), 417-440. doi:11/5/417 [pii]
- Hofbauer, J. (1903). Über das konstante vorkommen bisher unbekannter zelliger formelemente in der chorionzotte der menschlichen placenta. *Wiener Klin.Wochenschr., Bd*, 16, 1905.
- Hofer, T., Bitterle, E., Beck-Speier, I., Maier, K. L., Frankenberger, M., Heyder, J., & Ziegler-Heitbrock, L. (2004). Diesel exhaust particles increase LPS-stimulated COX-2 expression and PGE2 production in human monocytes. *Journal of Leukocyte Biology*, 75(5), 856.
- Holland, W. L., Bikman, B. T., Wang, L., Yuguang, G., Sargent, K. M., Bulchand, S., . . . Wenk, M. R. (2011). Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *The Journal of Clinical Investigation*, 121(5), 1858.
- Hsiao, E. Y., & Patterson, P. H. (2011). Activation of the maternal immune system induces endocrine changes in the placenta via IL-6. *Brain, Behavior, and Immunity*, 25(4), 604-615.
- Hsiao, E. Y., & Patterson, P. H. (2012). Placental regulation of maternal-fetal interactions and brain development. *Developmental Neurobiology*,
- Hughes, P. M., Botham, M. S., Frentzel, S., Mir, A., & Perry, V. H. (2002). Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia*, 37(4), 314-327.
- Hunt, J. S., Pace, J. L., & Gill, R. M. (2010). Immunoregulatory molecules in human placentas: Potential for diverse roles in pregnancy. *International Journal of Developmental Biology*, 54(2), 457.

- Hurvitz, P. M., Moudon, A. V., Rehm, C. D., Streichert, L. C., & Drewnowski, A. (2009). Arterial roads and area socioeconomic status are predictors of fast food restaurant density in king county, WA. *International Journal of Behavioral Nutrition and Physical Activity*, 6, 46.
- Hutchinson, M., Northcutt, A., Hiranita, T., Wang, X., Lewis, S., Thomas, J., . . . Sfregola, C. (2012). Opioid activation of toll-like receptor 4 contributes to drug reinforcement. *The Journal of Neuroscience*, 32(33), 11187-11200.
- Hwang, L. L., Wang, C. H., Li, T. L., Chang, S. D., Lin, L. C., Chen, C. P., . . . Yang, W. S. (2009). Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity*, 18(3), 463-469.
- Inoue, K., Takano, H., Yanagisawa, R., Hirano, S., Ichinose, T., Shimada, A., & Yoshikawa, T. (2006). The role of toll-like receptor 4 in airway inflammation induced by diesel exhaust particles. *Archives of Toxicology*, 80(5), 275-279.
- Ito, D., Imai, Y., Ohsawa, K., Nakajima, K., Fukuuchi, Y., & Kohsaka, S. (1998). Microglia-specific localisation of a novel calcium binding protein, Iba1. *Molecular Brain Research*, 57(1), 1-9.
- Jalleh, R., Koh, K., Choi, B., Liu, E., Maddison, J., & Hutchinson, M. R. (2012). Role of microglia and toll-like receptor 4 in the pathophysiology of delirium. *Medical Hypotheses*,
- Janeway, C., Travers, P., Walport, M., & Shlomchik, M. (2005). *Immunobiology: The immune system in health and disease* (6th ed.). New York, NY: Garland Science.
- Jansson, T., & Powell, T. (2007). Role of the placenta in fetal programming: Underlying mechanisms and potential interventional approaches. *Clinical Science*, 113, 1-13.
- Jolly, M., Bertie, J., Gray, R., Bannister, P., Venkatesan, S., Johnston, D., & Robinson, S. (2004). Increased leucine turnover in women during the third trimester of uncomplicated pregnancy. *Metabolism*, 53(5), 545-549.
- Kalhan, S., & Parimi, P. (2000). Gluconeogenesis in the fetus and neonate. Paper presented at the *Seminars in Perinatology*, , 24(2) 94-106.
- Kalhan, S. C., Rossi, K. Q., Gruca, L. L., Super, D. M., & Savin, S. M. (1998). Relation between transamination of branched-chain amino acids and urea synthesis: Evidence from human pregnancy. *The American Journal of Physiology*, 275(3 Pt 1), E423-31.
- Kannan, S., Saadani-Makki, F., Balakrishnan, B., Dai, H., Chakraborty, P. K., Janisse, J., . . . Chugani, D. C. (2010). Decreased cortical serotonin in neonatal rabbits exposed to endotoxin in utero. *Journal of Cerebral Blood Flow & Metabolism*, 31(2), 738-749.

- Kannel, W. B., Adrienne Cupples, L., Ramaswami, R., Stokes, J., Kreger, B. E., & Higgins, M. (1991). Regional obesity and risk of cardiovascular disease; the framingham study. *Journal of Clinical Epidemiology*, 44(2), 183-190.
- Keith, S. W., Redden, D. T., Katzmarzyk, P., Boggiano, M. M., Hanlon, E. C., Benca, R. M., . . . Fontaine, K. (2006). Putative contributors to the secular increase in obesity: Exploring the roads less traveled. *International Journal of Obesity*, 30(11), 1585-1594.
- Kennedy, A., Martinez, K., Chuang, C. C., LaPoint, K., & McIntosh, M. (2009). Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: Mechanisms of action and implications. *The Journal of Nutrition*, 139(1), 1-4.
- Khan, S., Katabuchi, H., Araki, M., Nishimura, R., & Okamura, H. (2000). Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. *Biology of Reproduction*, 62(4), 1075-1083.
- Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., . . . Hölscher, C. (2013). Microglia emerge from erythromyeloid precursors via pu. 1-and Irf8-dependent pathways. *Nature Neuroscience*, 16(3), 273-280.
- Kim, S. Y., Romero, R., Tarca, A. L., Bhatti, G., Kim, C. J., Lee, J., . . . Hassan, S. S. (2012). Methylation of fetal and maternal monocytes and macrophages at the Feto-Maternal interface. *American Journal of Reproductive Immunology*, 68(1), 8-27.
- Kishi, T., & Elmquist, J. (2005). Body weight is regulated by the brain: A link between feeding and emotion. *Molecular Psychiatry*, 10(2), 132-146.
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for sry. *Nature*, 351(6322), 117-121.
- Kossintseva, I., Wong, S., Johnstone, E., Guilbert, L., Olson, D. M., & Mitchell, B. F. (2006). Proinflammatory cytokines inhibit human placental 11beta-hydroxysteroid dehydrogenase type 2 activity through Ca<sup>2+</sup> and cAMP pathways. *American Journal of Physiology. Endocrinology and Metabolism*, 290(2), E282-8. doi:10.1152/ajpendo.00328.2005
- Kouduka, M., Matsuoka, A., & Nishigaki, K. (2006). Acquisition of genome information from single-celled unculturable organisms (radiolaria) by exploiting genome profiling (GP). *BMC Genomics*, 7(1), 135.
- Kreutzberg, G. W. (1996). Microglia: A sensor for pathological events in the CNS. *Trends in Neurosciences*, 19(8), 312-318.

- Krotkiewski, M., Björntorp, P., Sjöström, L., & Smith, U. (1983). Impact of obesity on metabolism in men and women. importance of regional adipose tissue distribution. *Journal of Clinical Investigation*, 72(3), 1150.
- Kumazaki, K., Nakayama, M., Yanagihara, I., Suehara, N., & Wada, Y. (2004). Immunohistochemical distribution of toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. *Human Pathology*, 35(1), 47-54.
- Langley-Evans, S., Phillips, G., Benediktsson, R., Gardner, D., Edwards, C., Jackson, A., & Seckl, J. (1996). Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. *Placenta*, 17(2), 169-172.
- Lanphear, B. P., Vorhees, C. V., & Bellinger, D. C. (2005). Protecting children from environmental toxins. *PLoS Med*, 2(3), e61. Retrieved from <http://dx.doi.org/10.1371/journal.pmed.0020061>
- Latz, E. (2010). The inflammasomes: Mechanisms of activation and function. *Current Opinion in Immunology*, 22(1), 28-33.
- Lawlor, D. A., & Chaturvedi, N. (2006). Treatment and prevention of obesity— are there critical periods for intervention? *International Journal of Epidemiology*, 35(1), 3-9.
- Levesque, S., Taetzsch, T., Lull, M. E., Kodavanti, U., Stadler, K., Wagner, A., . . . Surace, M. J. (2011a). Diesel exhaust activates and primes microglia: Air pollution, neuroinflammation, and regulation of dopaminergic neurotoxicity. *Environmental Health Perspectives*, 119(8), 1149.
- Levesque, S., Taetzsch, T., Lull, M. E., Kodavanti, U., Stadler, K., Wagner, A., . . . Surace, M. J. (2011b). Diesel exhaust activates and primes microglia: Air pollution, neuroinflammation, and regulation of dopaminergic neurotoxicity. *Environmental Health Perspectives*, 119(8), 1149.
- Ling, E. A., & Wong, W. C. (2004). The origin and nature of ramified and amoeboid microglia: A historical review and current concepts. *Glia*, 7(1), 9-18.
- Ling, Z., Chang, Q. A., Tong, C. W., Leurgans, S. E., Lipton, J. W., & Carvey, P. M. (2004). Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. *Experimental Neurology*, 190(2), 373-383.
- Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., . . . Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science*, 277(5332), 1659-1662.
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*, 25(4), 402-408.

- Lovasi, G. S., Eldred-Skemp, N., Quinn, J. W., Chang, H., Rauh, V. A., Rundle, A., . . . Perera, F. P. (2013). Neighborhood social context and individual polycyclic aromatic hydrocarbon exposures associated with child cognitive test scores. *Journal of Child and Family Studies*, , 1-15.
- Lynch, E. A., Dinarello, C. A., & Cannon, J. G. (1994). Gender differences in IL-1 alpha, IL-1 beta, and IL-1 receptor antagonist secretion from mononuclear cells and urinary excretion. *The Journal of Immunology*, 153(1), 300-306.
- Manuelpillai, U., Ligam, P., Smythe, G., Wallace, E. M., Hirst, J., & Walker, D. W. (2005). Identification of kynurenine pathway enzyme mRNAs and metabolites in human placenta: Up-regulation by inflammatory stimuli and with clinical infection. *American Journal of Obstetrics and Gynecology*, 192(1), 280-288.
- Mao, J., Zhang, X., Sieli, P. T., Falduto, M. T., Torres, K. E., & Rosenfeld, C. S. (2010). Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proceedings of the National Academy of Sciences of the United States of America*, 107(12), 5557-5562. doi:10.1073/pnas.1000440107; 10.1073/pnas.1000440107
- McAdams, R. M., & Juul, S. E. (2012). The role of cytokines and inflammatory cells in perinatal brain injury. *Neurology Research International*, 2012
- McClung, J. P., Roneker, C. A., Mu, W., Lisk, D. J., Langlais, P., Liu, F., & Lei, X. G. (2004). Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proceedings of the National Academy of Sciences of the United States of America*, 101(24), 8852-8857.
- McGuire, W., Dyson, L., & Renfrew, M. (2009). Maternal obesity: Consequences for children, challenges for clinicians and carers. *Semin Fetal Neonatal Med*, doi:S1744-165X(09)00091-2 [pii] 10.1016/j.siny.2009.09.005
- McWhorter, F. Y., Wang, T., Nguyen, P., Chung, T., & Liu, W. F. (2013). Modulation of macrophage phenotype by cell shape. *Proceedings of the National Academy of Sciences of the United States of America*, 110(43), 17253-17258. doi:10.1073/pnas.1308887110 [doi]
- Mikaelsson, M. A., Constância, M., Dent, C. L., Wilkinson, L. S., & Humby, T. (2013). Placental programming of anxiety in adulthood revealed by Igf2-null models. *Nature Communications*, 4
- Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D. E., . . . Takahashi, H. K. (2009). Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: Implications for the pathogenesis of obesity. *Science's STKE*, 29(2), 359.

- Mizutani, M., Pino, P. A., Saederup, N., Charo, I. F., Ransohoff, R. M., & Cardona, A. E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *The Journal of Immunology*, *188*(1), 29-36.
- Moore, C. L., & Morelli, G. A. (1979). Mother rats interact differently with male and female offspring. *Journal of Comparative and Physiological Psychology*, *93*(4), 677.
- Mor, G., Romero, R., Aldo, P. B., & Abrahams, V. M. (2005). Is the trophoblast an immune regulator? the role of toll-like receptors during pregnancy. *Critical Reviews in Immunology*, *25*(5), 375-388.
- Morgan, T. E., Rozovsky, I., Goldsmith, S. K., Stone, D. J., Yoshida, T., & Finch, C. E. (1997). Increased transcription of the astrocyte gene GFAP during middle-age is attenuated by food restriction: Implications for the role of oxidative stress. *Free Radical Biology and Medicine*, *23*(3), 524-528.
- Mouton, P. R., Long, J. M., Lei, D., Howard, V., Jucker, M., Calhoun, M. E., & Ingram, D. K. (2002). Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Research*, *956*(1), 30-35.
- Mueller, B. R., & Bale, T. L. (2008). Sex-specific programming of offspring emotionality after stress early in pregnancy. *The Journal of Neuroscience*, *28*(36), 9055-9065.
- Murphy, V. E., Gibson, P. G., Giles, W. B., Zakar, T., Smith, R., Bisits, A. M., . . . Clifton, V. L. (2003). Maternal asthma is associated with reduced female fetal growth. *American Journal of Respiratory and Critical Care Medicine*, *168*(11), 1317-1323.
- Murphy, V. E., Gibson, P., Talbot, P. I., & Clifton, V. L. (2005). Severe asthma exacerbations during pregnancy. *Obstetrics & Gynecology*, *106*(5, Part 1), 1046.
- Myers, M. M., Brunelli, S. A., Squire, J. M., Shindeldecker, R. D., & Hofer, M. A. (1989). Maternal behavior of SHR rats and its relationship to offspring blood pressures. *Developmental Psychobiology*, *22*(1), 29-53.
- Naef, L., Moquin, L., Dal Bo, G., Giros, B., Gratton, A., & Walker, C. (2011). Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. *Neuroscience*, *176*, 225-236.
- Nagamatsu, T., & Schust, D. J. (2010). REVIEW ARTICLE: The contribution of macrophages to normal and pathological pregnancies. *American Journal of Reproductive Immunology*, *63*(6), 460-471.

- Needleman, H. L., Schell, A., Bellinger, D., Leviton, A., & Allred, E. N. (1990). The long-term effects of exposure to low doses of lead in childhood. *N Engl J Med*, 322(2), 83-88. doi:10.1056/NEJM199001113220203
- O'Shaughnessy, P., Baker, P., Sohnius, U., Haavisto, A., Charlton, H., & Huhtaniemi, I. (1998). Fetal development of leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology*, 139(3), 1141-1146.
- Ornoy, A. (2011). Prenatal origin of obesity and their complications: Gestational diabetes, maternal overweight and the paradoxical effects of fetal growth restriction and macrosomia. *Reproductive Toxicology (Elmsford, NY)*, 32(2), 205.
- Pace, J. L., Russell, S., Torres, B., Johnson, H., & Gray, P. (1983). Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *The Journal of Immunology*, 130(5), 2011-2013.
- Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., . . . Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science*, 333(6048), 1456-1458. doi:10.1126/science.1202529
- Parney, I. F., Waldron, J. S., & Parsa, A. T. (2009). Flow cytometry and in vitro analysis of human glioma-associated macrophages. *Journal of Neurosurgery*, 110(3), 572.
- Pasinetti, G. M., & Eberstein, J. A. (2008). Metabolic syndrome and the role of dietary lifestyles in alzheimer's disease. *J Neurochem*, 106(4), 1503-14. doi:JNC5454 [pii] 10.1111/j.1471-4159.2008.05454.x
- Patni, S., Flynn, P., Wynen, L., Seager, A., Morgan, G., White, J., & Thornton, C. (2007). An introduction to toll-like receptors and their possible role in the initiation of labour. *BJOG: An International Journal of Obstetrics & Gynaecology*, 114(11), 1326-1334.
- Paylor, R., Tracy, R., Wehner, J., & Rudy, J. W. (1994). DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. *Behavioral Neuroscience; Behavioral Neuroscience*, 108(4), 810.
- Pereira, G., Nassar, N., Cook, A., & Bower, C. (2011). Traffic emissions are associated with reduced fetal growth in areas of perth, western australia: An application of the AusRoads dispersion model. *Australian and New Zealand Journal of Public Health*, 35(5), 451-458.
- Perera, F. P., Li, Z., Whyatt, R., Hoepner, L., Wang, S., Camann, D., & Rauh, V. (2009). Prenatal airborne polycyclic aromatic hydrocarbon exposure and child IQ at age 5 years. *Pediatrics*, 124(2), e195-e202.

- Perera, F. P. (2008). Children are likely to suffer most from our fossil fuel addiction. *Environmental Health Perspectives*, 116(8), 987.
- Perera, F. P., Tang, D., Wang, S., Vishnevetsky, J., Zhang, B., Diaz, D., . . . Rauh, V. (2012). Prenatal polycyclic aromatic hydrocarbon (PAH) exposure and child behavior at age 6–7 years. *Environmental Health Perspectives*, 120(6), 921.
- Pettersson, U. S., Waldén, T. B., Carlsson, P., Jansson, L., & Phillipson, M. (2012). Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PloS One*, 7(9), e46057.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9), e45-e45.
- Phillips, R., & LeDoux, J. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience*, 106(2), 274.
- Pinteaux, E., Inoue, W., Schmidt, L., Molina-Holgado, F., Rothwell, N. J., & Luheshi, G. N. (2007). Leptin induces interleukin-1 $\beta$  release from rat microglial cells through a caspase 1 independent mechanism. *Journal of Neurochemistry*, 102(3), 826-833.
- Pryor, L. E., Tremblay, R. E., Boivin, M., Touchette, E., Dubois, L., Genolini, C., . . . Cote, S. M. (2011). Developmental trajectories of body mass index in early childhood and their risk factors: An 8-year longitudinal study. *Archives of Pediatrics and Adolescent Medicine*, 165(10), 906.
- Purcell, R. H., Sun, B., Pass, L. L., Power, M. L., Moran, T. H., & Tamashiro, K. L. (2011). Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior. *Physiology & Behavior*, 104(3), 474-479.
- Qin, L., Li, G., Qian, X., Liu, Y., Wu, X., Liu, B., . . . Block, M. L. (2005). Interactive role of the toll-like receptor 4 and reactive oxygen species in LPS-induced microglia activation. *Glia*, 52(1), 78-84.
- Raz, R., Roberts, A. L., Lyall, K., Hart, J. E., Just, A. C., Laden, F., & Weiskopf, M. G. (2014). Autism spectrum disorder and particulate matter air pollution before, during, and after pregnancy: A nested Case–Control analysis within the nurses' health study II cohort. *Environ Health Perspect*,
- Rezai-Zadeh, K., Gate, D., & Town, T. (2009). CNS infiltration of peripheral immune cells: D-day for neurodegenerative disease? *Journal of Neuroimmune Pharmacology*, 4(4), 462-475.
- Rice, C. J., Sandman, C. A., Lenjavi, M. R., & Baram, T. Z. (2008). A novel mouse model for acute and long-lasting consequences of early life stress. *Endocrinology*, 149(10), 4892-4900.

- Roberts, K., Riley, S., Reynolds, R., Barr, S., Evans, M., Statham, A., . . . Denison, F. (2011). Placental structure and inflammation in pregnancies associated with obesity. *Placenta*, 32(3), 247-254.
- Robinson, N., Atkinson, D., Jones, C., & Sibley, C. (1988). Permeability of the near-term rat placenta to hydrophilic solutes. *Placenta*, 9(4), 361-372.
- Rodriguez, A., Miettunen, J., Henriksen, T. B., Olsen, J., Obel, C., Taanila, A., . . . Järvelin, M. (2008). Maternal adiposity prior to pregnancy is associated with ADHD symptoms in offspring: Evidence from three prospective pregnancy cohorts. *International Journal of Obesity*, 32(3), 550-557.
- Rundle, A., Hoepner, L., Hassoun, A., Oberfield, S., Freyer, G., Holmes, D., . . . Perera, F. (2012). Association of childhood obesity with maternal exposure to ambient air polycyclic aromatic hydrocarbons during pregnancy. *American Journal of Epidemiology*, 175(11), 1163-1172.
- Saberi, M., Woods, N. B., de Luca, C., Schenk, S., Lu, J. C., Bandyopadhyay, G., . . . Olefsky, J. M. (2009). Hematopoietic cell-specific deletion of toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metabolism*, 10(5), 419-429.
- Sánchez, C. L., Van Swearingen, A. E., Arrant, A. E., Kuhn, C. M., & Zepf, F. D. (2014). Dietary manipulation of serotonergic and dopaminergic function in C57BL/6J mice with amino acid depletion mixtures. *Journal of Neural Transmission*, 121(2), 153-162.
- Sanders, N. J. (2012). What doesn't kill you makes you weaker prenatal pollution exposure and educational outcomes. *Journal of Human Resources*, 47(3), 826-850.
- Sasaki, A., de Vega, W., St-Cyr, S., Pan, P., & McGowan, P. (2013). Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood. *Neuroscience*, 240, 1-12.
- Schwartz, M. W., & Porte Jr, D. (2005). Diabetes, obesity, and the brain. *Science*, 307(5708), 375-379.
- Schwarz, J. M., Hutchinson, M. R., & Bilbo, S. D. (2011). Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. *The Journal of Neuroscience*, 31(49), 17835-17847.
- Schwarz, J. M., Sholar, P. W., & Bilbo, S. D. (2012). Sex differences in microglial colonization of the developing rat brain. *Journal of Neurochemistry*, , 948-963.
- Schwarz, J. M., & Bilbo, S. D. (2011). The immune system and the developing brain. Paper presented at the *Colloquium Series on the Developing Brain*, , 2(3) 1-128.

- Schwarz, J. M., & Bilbo, S. D. (2013). Adolescent morphine exposure affects long-term microglial function and later-life relapse liability in a model of addiction. *The Journal of Neuroscience*, 33(3), 961-971. doi:10.1523/JNEUROSCI.2516-12.2013
- Scott, N. M., Hodyl, N. A., Murphy, V. E., Osei-Kumah, A., Wyper, H., Hodgson, D. M., . . . Clifton, V. L. (2009). Placental cytokine expression covaries with maternal asthma severity and fetal sex. *The Journal of Immunology*, 182(3), 1411-1420.
- Seckl, J. R., & Holmes, M. C. (2007). Mechanisms of disease: Glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. *Nature Clinical Practice Endocrinology & Metabolism*, 3(6), 479-488.
- Seguin, L., Potvin, L., St-Denis, M., & Loiseleur, J. (1995). Chronic stressors, social support, and depression during pregnancy. *Obstetrics & Gynecology*, 85(4), 583-589.
- Shankardass, K., McConnell, R., Jerrett, M., Milam, J., Richardson, J., & Berhane, K. (2009). Parental stress increases the effect of traffic-related air pollution on childhood asthma incidence. *Proceedings of the National Academy of Sciences*, 106(30), 12406-12411.
- Sharkhuu, T., Doerfler, D. L., Krantz, Q. T., Luebke, R. W., Linak, W. P., & Gilmour, M. I. (2010). Effects of prenatal diesel exhaust inhalation on pulmonary inflammation and development of specific immune responses. *Toxicology Letters*, 196(1), 12-20.
- Shepherd, J. K., Grewal, S. S., Fletcher, A., Bill, D. J., & Dourish, C. T. (1994). Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology*, 116(1), 56-64.
- Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. *Journal of Clinical Investigation*, 116(11), 3015-3025.
- Simmons, R. (2008). Perinatal programming of obesity. *Semin Perinatol*, 32(5), 371-4. doi:S0146-0005(08)00089-X [pii] 10.1053/j.semperi.2008.08.004
- Simon, G. E., Von Korff, M., Saunders, K., Miglioretti, D. L., Crane, P. K., van Belle, G., & Kessler, R. C. (2006). Association between obesity and psychiatric disorders in the US adult population. *Archives of General Psychiatry*, 63(7), 824.
- Simon, P., Dupuis, R., & Costentin, J. (1994). Thigmotaxis as an index of anxiety in mice. influence of dopaminergic transmissions. *Behavioural Brain Research*, 61(1), 59-64.
- Smart, J., & Preece, J. (1973). Maternal behaviour of undernourished mother rats. *Animal Behaviour*, 21(3), 613-619.

- Souza, C., Moreira, J., Siqueira, I., Pereira, A., Rieger, D., Souza, D., . . . Perry, M. (2007). Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. *Life Sciences, 81*(3), 198-203.
- Spear, L. P., & Brake, S. C. (1983). Periadolescence: Age-dependent behavior and psychopharmacological responsivity in rats. *Developmental Psychobiology, 16*(2), 83-109.
- Spector, R. (1988). Fatty acid transport through the Blood-Brain barrier. *Journal of Neurochemistry, 50*(2), 639-643.
- Stamatovic, S. M., Shakui, P., Keep, R. F., Moore, B. B., Kunkel, S. L., Van Rooijen, N., & Andjelkovic, A. V. (2005). Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. *Journal of Cerebral Blood Flow & Metabolism, 25*(5), 593-606.
- Stone, J. L., Merriman, B., Cantor, R. M., Yonan, A. L., Gilliam, T. C., Geschwind, D. H., & Nelson, S. F. (2004). Evidence for sex-specific risk alleles in autism spectrum disorder. *The American Journal of Human Genetics, 75*(6), 1117-1123.
- Strosznajder, J., Chalimoniuk, M., Strosznajder, R. P., Albanese, V., & Alberghina, M. (1996). Arachidonate transport through the blood-retina and blood-brain barrier of the rat during aging. *Neuroscience Letters, 209*(3), 145-148.
- Sullivan, E. L., Nousen, E. K., & Chamlou, K. A. (2014). Maternal high fat diet consumption during the perinatal period programs offspring behavior. *Physiology & Behavior, 123*, 236-242.
- Sullivan, E. L., Grayson, B., Takahashi, D., Robertson, N., Maier, A., Bethea, C. L., . . . Grove, K. L. (2010). Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 30*(10), 3826-3830. doi:10.1523/JNEUROSCI.5560-09.2010; 10.1523/JNEUROSCI.5560-09.2010
- Sun, Q., Yue, P., Deiuliis, J. A., Lumeng, C. N., Kampfrath, T., Mikolaj, M. B., . . . Parthasarathy, S. (2009). Ambient air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity. *Circulation, 119*(4), 538-546.
- Sutton, P., Woodruff, T. J., Perron, J., Stotland, N., Conry, J. A., Miller, M. D., & Giudice, L. C. (2012). Toxic environmental chemicals: The role of reproductive health professionals in preventing harmful exposures. *American Journal of Obstetrics and Gynecology,*
- Takahashi, K., Naito, M., Katabuchi, H., & Higashi, K. (1991). Development, differentiation, and maturation of macrophages in the chorionic villi of mouse placenta with special reference to the origin of hofbauer cells. *Journal of Leukocyte Biology, 50*(1), 57-68.

- Tamashiro, K. L. K., Terrillion, C. E., Hyun, J., Koenig, J. I., & Moran, T. H. (2009). Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. *Diabetes*, *58*(5), 1116-1125.
- Tang, X., & Sanford, L. D. (2005). Home cage activity and activity-based measures of anxiety in 129P3/J, 129X1/SvJ and C57BL/6J mice. *Physiology & Behavior*, *84*(1), 105-115.
- Tannenbaum, B. M., Brindley, D. N., Tannenbaum, G. S., Dallman, M. F., McArthur, M. D., & Meaney, M. J. (1997). High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. *American Journal of Physiology-Endocrinology and Metabolism*, *273*(6), E1168-E1177.
- Teegarden, S. L., Scott, A. N., & Bale, T. L. (2009). Early life exposure to a high fat diet promotes long-term changes in dietary preferences and central reward signaling. *Neuroscience*, *162*(4), 924-932.
- Thompson, A. L., Johnson, B. T., Sempowski, G. D., Gunn, M. D., Hou, B., DeFranco, A. L., & Staats, H. F. (2012). Maximal adjuvant activity of nasally delivered IL-1 $\alpha$  requires adjuvant-responsive CD11c cells and does not correlate with adjuvant-induced in vivo cytokine production. *The Journal of Immunology*, *188*(6), 2834-2846.
- Thompson, C., Syddall, H., Rodin, I., Osmond, C., & Barker, D. J. (2001). Birth weight and the risk of depressive disorder in late life. *The British Journal of Psychiatry : The Journal of Mental Science*, *179*, 450-455.
- Toth, F., Juhl, C., Nørskov-Lauritsen, N., Mosborg Petersen, P., & Ebbesen, P. (1990). Interferon production by cultured human trophoblast induced with double stranded polyribonucleotide. *Journal of Reproductive Immunology*, *17*(3), 217-227.
- Toth, F., Nørskov-Lauritsen, N., Juhl, C., & Ebbesen, P. (1991). Human trophoblast interferon: Pattern of response to priming and superinduction of purified term trophoblast and choriocarcinoma cells. *Journal of Reproductive Immunology*, *19*(1), 55-67.
- Tsukumo, D. M. L., Carvalho-Filho, M. A., Carvalheira, J. B. C., Prada, P. O., Hirabara, S. M., Schenka, A. A., . . . Velloso, L. A. (2007). Loss-of-function mutation in toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes*, *56*(8), 1986-1998.
- Tundidor, D., García-Patterson, A., María, M. A., Ubeda, J., Ginovart, G., Adelantado, J. M., . . . Corcoy, R. (2012). Perinatal maternal and neonatal outcomes in women with gestational diabetes mellitus according to fetal sex. *Gender Medicine*, *9*(6), 411-417.
- van den Hooven, E. H., Pierik, F. H., de Kluizenaar, Y., Willemsen, S. P., Hofman, A., van Ratingen, S. W., . . . Miedema, H. M. E. (2012). Air pollution exposure during pregnancy,

- ultrasound measures of fetal growth, and adverse birth outcomes: A prospective cohort study. *Environmental Health Perspectives*, 120(1), 150.
- Vatten, L. J., & Skjærven, R. (2004). Offspring sex and pregnancy outcome by length of gestation. *Early Human Development*, 76(1), 47-54.
- Vogt, M. C., Paeger, L., Hess, S., Steculorum, S. M., Awazawa, M., Hampel, B., . . . Hausen, A. C. (2014). Neonatal insulin action impairs hypothalamic neurocircuit formation in response to maternal high-fat feeding. *Cell*,
- Volk, H. E., Lurmann, F., Penfold, B., Hertz-Picciotto, I., & McConnell, R. (2013). Traffic-related air pollution, particulate matter, and autism. *JAMA Psychiatry*, 70(1), 71-77.
- Wahlbeck, K., Forsén, T., Osmond, C., Barker, D. J., & Eriksson, J. G. (2001). Association of schizophrenia with low maternal body mass index, small size at birth, and thinness during childhood. *Archives of General Psychiatry*, 58(1), 48-52.
- Wakselman, S., Béchade, C., Roumier, A., Bernard, D., Triller, A., & Bessis, A. (2008). Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *The Journal of Neuroscience*, 28(32), 8138-8143. doi:10.1523/JNEUROSCI.1006-08.2008
- Wang, X., Athayde, N., & Trudinger, B. (2003). A proinflammatory cytokine response is present in the fetal placental vasculature in placental insufficiency. *American Journal of Obstetrics and Gynecology*, 189(5), 1445-1451.
- Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., . . . Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience*, 7(8), 847-854.
- Wei, J., Lin, Y., Li, Y., Ying, C., Chen, J., Song, L., . . . Chen, X. (2011). Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology*, 152(8), 3049-3061.
- Weisberg, S. P., Hunter, D., Huber, R., Lemieux, J., Slaymaker, S., Vaddi, K., . . . Ferrante Jr, A. W. (2006). CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *Journal of Clinical Investigation*, 116(1), 115-124.
- Weiss, B., & Landrigan, P. J. (2000). The developing brain and the environment: An introduction. *Environmental Health Perspectives*, 108(Suppl 3), 373.
- Williams, R. L., Creasy, R. K., Cunningham, G. C., Hawes, W. E., Norris, F. D., & Tashiro, M. (1982). Fetal growth and perinatal viability in california. *Obstetrics & Gynecology*, 59(5), 624-634.

- Williamson, L. L., Sholar, P. W., Mistry, R. S., Smith, S. H., & Bilbo, S. D. (2011). Microglia and memory: Modulation by early-life infection. *The Journal of Neuroscience*, 31(43), 15511-15521.
- Wohleb, E. S., Fenn, A. M., Pacent, A. M., Powell, N. D., Sheridan, J. F., & Godbout, J. P. (2012). Peripheral innate immune challenge exaggerated microglia activation, increased the number of inflammatory CNS macrophages, and prolonged social withdrawal in socially defeated mice. *Psychoneuroendocrinology*, 37(9), 1491-1505.
- Wohleb, E. S., Hanke, M. L., Corona, A. W., Powell, N. D., La'Tonia, M. S., Bailey, M. T., . . . Sheridan, J. F. (2011). B-adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *The Journal of Neuroscience*, 31(17), 6277-6288.
- Wohleb, E. S., Powell, N. D., Godbout, J. P., & Sheridan, J. F. (2013). Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. *The Journal of Neuroscience*, 33(34), 13820-13833.
- Wohlfahrt-Veje, C., Main, K. M., Schmidt, I. M., Boas, M., Jensen, T. K., Grandjean, P., . . . Andersen, H. R. (2011). Lower birth weight and increased body fat at school age in children prenatally exposed to modern pesticides: A prospective study. *Environmental Health*, 10(1), 79.
- Wolff, R. L. (2003). Trans-18: 1 isomers in rat milk fat as effective biomarkers for the determination of individual isomeric trans-18: 1 acids in the dams' diet. *Lipids*, 38(11), 1143-1148.
- Wood, G. W. (1980). Mononuclear phagocytes in the human placenta. *Placenta*, 1(2), 113-123.
- Wright, R. J. (2009). Moving towards making social toxins mainstream in children's environmental health. *Current Opinion in Pediatrics*, 21(2), 222.
- Wu, C. H., Wen, C. Y., Shieh, J. Y., & Ling, E. A. (1992). A quantitative and morphometric study of the transformation of amoeboid microglia into ramified microglia in the developing corpus callosum in rats. *Journal of Anatomy*, 181 ( Pt 3)(Pt 3), 423-430.
- Wu, C. H., Wen, C. Y., Shieh, J. Y., & Ling, E. A. (1993). A quantitative study of the differentiation of microglial cells in the developing cerebral cortex in rats. *Journal of Anatomy*, 182 ( Pt 3)(Pt 3), 403-413.
- Wynn, R. M. (1967). Derivation and ultrastructure of the so-called hofbauer cell. *American Journal of Obstetrics and Gynecology*, 97(2), 235-248.

- Xu, X., Yavar, Z., Verdin, M., Ying, Z., Mihai, G., Kampfrath, T., . . . Chen, L. C. (2010). Effect of early particulate air pollution exposure on obesity in mice role of p47phox. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30(12), 2518-2527.
- Yagel, S., Hurwitz, A., Rosenn, B., & Keizer, N. (1987). Progesterone enhancement of prostaglandin E2 production by fetal placental macrophages. *Am.J.Reprod.Immunol.Microbiol*, 14, 45-48.
- Yeganegi, M., Watson, C. S., Martins, A., Kim, S. O., Reid, G., Challis, J. R., & Bocking, A. D. (2009). Effect of lactobacillus rhamnosus GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: Implications for treatment of bacterial vaginosis and prevention of preterm labor. *American Journal of Obstetrics and Gynecology*, 200(5), 532. e1-532. e8.
- Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity*, 25(2), 181-213.
- Zaretsky, M. V., Alexander, J. M., Byrd, W., & Bawdon, R. E. (2004). Transfer of inflammatory cytokines across the placenta. *Obstetrics & Gynecology*, 103(3), 546-550.
- Zhan, Y., Paolicelli, R. C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., . . . Ragozzino, D. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nature Neuroscience*, 17(3), 400-406.
- Zhuang, Z., Kawasaki, Y., Tan, P., Wen, Y., Huang, J., & Ji, R. (2007). Role of the CX3CR1/p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. *Brain, Behavior, and Immunity*, 21(5), 642-651.

## Biography

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