DISPARATE ENVIRONMENTAL TRIGGERS (MANGANESE, LIPOPOLYSACCHARIDE, OR HIGH-FAT DIET) OF INFLAMMATORY NATURE CAUSE NEUROLOGICAL DEFICITS AND/OR METABOLIC DYSREGULATION IN MICE

by

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(Under the Direction of Nikolay M. Filipov)

ABSTRACT

Inflammation has been implicated in the pathophysiology of metabolic syndrome and several neurological disorders; evidence is also mounting for long-term aftereffects of inflammation during early brain development. This dissertation project investigated the role of central/peripheral inflammation as a key contributor for the neurological deficits/metabolic dysregulation caused by disparate environmental triggers, including manganese (Mn; metal), lipopolysaccharide (LPS; bacterial infection mimic) or high-fat diet (HFD) in mice.

Low-level subchronic Mn drinking water exposure (0.4 g/l) led to increased brain Mn deposition (in a region- and time-independent manner), hyperactivity, and decreased anxiety in adult male mice; the latter effects were accompanied with brain region-specific glial cell activation and altered serotonin (5-HT) homeostasis. Chronic low-grade inflammation caused by repeated peripheral LPS (0.25 mg/kg) administration produced persistent depressive-like behavior and caused region-specific and time-dependent neurochemical alterations in the dopamine (DA), norepinephrine and 5-HT homeostasis in striatum, prefrontal cortex (PFC) and hippocampus of adult male mice .

HFD (60 kcal% fat) consumption for 5 weeks by female mice led to hyperactivity and anxiety, whereas mice consuming HFD for 21 weeks did not have locomotor deficits or anxiety-like behavior; after 32 weeks on HFD, the female mice were hypoactive. Mice's short-term object recognition memory remained unaffected regardless of the HFD feeding duration. Peripherally, HFD intake caused persistent glucose intolerance. Compared to age-matched low-fat diet controls, insulin sensitivity was impaired by HFD intake for 5 and 20 weeks, but not for 33 weeks. HFD consumption also caused biphasic (6 and 36 weeks) inflammation, whereas the key lipid metabolism regulatory gene, CD36, was increased at all three time points with the greatest effect at 36 weeks. Maternal HFD consumption increased anxiety in female, but not male adolescent offspring; elevated anxiety was accompanied with altered DA homeostasis in the PFC and the ventral hippocampus. Female offspring of HFD-fed dams treated with a novel anti-inflammatory glycan conjugate showed reduced anxiety.

In conclusion, in adults, Mn, LPS, or HFD exposure leads to central and/or peripheral inflammation causing neurological and/or metabolic abnormalities in mice; maternal HFD consumption produces sex-specific behavioral and monoamine alterations in adolescent offspring that are mitigated by an anti-inflammatory intervention.

INDEX WORDS: Manganese, Lipopolysaccharide, High-fat diet, Maternal high-fat diet, Inflammation, Neurological deficit, Metabolic dysregulation, Behavior, Dopamine, Serotonin

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DEDICATION

To my family for their unfailing care and support

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES x

LIST OF FIGURES xii

ABBREVIATIONS xvii

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

The impact of neurological disorders in the global population 1

Neurodegenerative diseases 1

Neurodevelopmental disorders 2

Inflammation and neurodegenerative/neurodevelopmental disorders 3

Neurodegenerative/neurodevelopmental disorders: environmental risk factors 4

Focus 1: manganese (Mn) 5

Focus 2: chronic systemic infections and ensuing inflammation 18

Focus 3: overnutrition-induced disease (obesity) 29

Overall objectives, hypothesis and specific aims 48

2 BRAIN DEPOSITION AND NEUROTOXICITY OF MANGANESE IN ADULT MICE EXPOSED VIA THE DRINKING WATER 52

Abstract 53

Introduction 54

Materials and methods 58
Results 65
Discussion 69
Acknowledgements 78
Conflict of interest statement 78
3 BEHAVIORAL AND MONOAMINE PERTURBATIONS IN ADULT MICE WITH LOW-GRADE CHRONIC INFLAMMATION INDUCED BY REPEATED PERIPHERAL LIPOPOLYSACCHARIDE ADMINISTRATION 92
Abstract 93
Introduction 95
Materials and methods 99
Results 103
Discussion 110
4 NEUROCHEMICAL AND ELECTROPHYSIOLOGICAL DEFICITS IN THE VENTRAL HIPPOCAMPUS AND SELECTIVE BEHAVIORAL ALTERATIONS CAUSED BY HIGH-FAT DIET IN FEMALE C57BL/6 MICE 137
Abstract 138
Introduction 140
Experimental procedures 142
Results 147
Discussion 151
5 HIGH-FAT DIET-INDUCED INSULIN INSENSITIVITY, GLUCOSE INTOLERANCE AND HEPATIC INFLAMMATION IN FEMALE C57BL/6 MICE. 164

Abstract 165
Introduction 166
Materials and methods 168
Results 173
Discussion 176
6 NEUROBEHAVIORAL AND METABOLIC/INFLAMMATORY RESPONSES IN FEMALE MICE FED A HIGH-FAT DIET FOR UPTO 9 MONTHS 192
Abstract 193
Introduction 195
Materials and methods 198
Results 202
Discussion 207
7 NEUROLOGICAL DEFICITS IN ADOLESCENT OFFSPRING OF MOUSE DAMS FED A HIGH-FAT DIET: INTERVENTION BY MATERNAL ADMINISTRATION OF A NOVEL ANTI-INFLAMMATORY GLYCAN 224
Abstract 225
Introduction 227
Materials and methods 230
Results 234
Discussion 238
8 OVERALL SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS 258
Overall summary 258
Conclusions 266

Unanswered questions and future directions 267

REFERENCES 271

APPENDICES

A SUPPLEMENTAL FIGURE FOR CHAPTER 2 358

B SUPPLEMENTAL TABLE FOR CHAPTER 5.....360

LIST OF TABLES

Page

Table 2.1: Region-independency of Mn deposition in the brain 79

Table 3.1: Concentrations of monoamines and their metabolites in the prefrontal cortex (PFC) of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups 123

Table 3.2: Concentrations of monoamines and their metabolites in the striatum of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups 124

Table 3.3: Concentrations of monoamines and their metabolites in the hippocampus of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups.....125

Table 4.1: Data of body weight, body weight gain, food intake, food efficiency and water intake of female C57BL/6 mice fed control (LFD) or HFD after 6 weeks (*n* = 8 per group) and 11/12 weeks (*n* = 5-6 per group).....158

Table 4.2: Concentrations of monoamines[a] or their metabolites[a] in different brain regions of female C57BL/6 mice fed control (LFD) or HFD (*n* = 8 per group) for 6 weeks.....159

Table 7.1: Concentrations of monoamines or their metabolites in different brain regions of PND 35 offspring (n = 5-6/group/sex) from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation.....249

Table 7.2: Concentrations of monoamines or their metabolites in different brain regions of PND 35 offspring (n = 5-6/group/sex) from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation.....250

LIST OF FIGURES

Page

Figure 1.1: Mechanism of Mn neurotoxicity 17

Figure 1.2: Data gaps in Mn neurotoxicity 18

Figure 1.3: Mechanism of LPS-induced microglia-dependent neurotoxicity....22

Figure 1.4: Transition from sickness behavior to a depressive-like behavior in acute immune (LPS) challenge models 27

Figure 1.5: Data gaps of chronic LPS induced peripheral inflammation 29

Figure 1.6: Inflammation and obesity pathogenesis 35

Figure 1.7: Mechanistic link between obesity and neurological disorders....37

Figure 1.8: Data gaps in adult obesity 41

Figure 1.9: Data gaps in maternal obesity 48

Figure 2.1: Effect of Mn DW (0.4 g/l) exposure on percentage (%) change in body weight (BW; a) and water intake (ml/kg BW/day; b) of adult male C57BL/6 mice during the 8 weeks of treatment duration....80

Figure 2.2: Time-independency of Mn deposition in the brain 81

Figure 2.3: T1 relaxation time (milliseconds, ms) in different brain regions of mice exposed to Mn via DW (0.4 g/l; panel a) or via a single s.c. injection (panel b; 50 mg/kg; positive control) 24 h prior to imaging....82

Figure 2.4: Representative T1 map generated using Matlab software visually displaying Mn effects on T1 relaxation time 84

Figure 2.5: Locomotor activity 85

Figure 2.6: Grip strength.....86

Figure 2.7: Forced swim test.....87

Figure 2.8: Striatal neurochemistry.....88

Figure 2.9: TH and GFAP Immunoreactivity 89

Figure 2.10: Nigral mRNA expression 91

Figure 3.1: (A) Detailed outline of the study's experimental design. Effect of saline or LPS treatment (0.25 mg/kg body weight [BW]; i.p. twice weekly) on (B) BW (g) of adult male C57BL/6 mice during the 25-weeks of treatment (*n* = 5 per group) and (C) food (g/kg BW/week) and water intake (ml/kg BW/week) (*n* = 15 per group) during the 12 week treatment duration 126

Figure 3.2: Effect of LPS (0.25 mg/kg body weight [BW]; i.p. twice weekly) on organ (liver, spleen and thymus) weights (g/kg BW) of adult male C57BL/6 mice in 13 week, 25 week and 13 week on +12 week off groups 128

Figure 3.3: Locomotor activity 129

Figure 3.4: Forced swim test.....131

Figure 3.5: Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) on spleen (A) NE and (B) 5-HT level in 13, 25 week and 13 week on +12 week off groups.....132

Figure 3.6: Plasma glucose levels.....133

Figure 3.7: Plasma cytokine levels 134

Figure 3.8: Nigral mRNA expression 136

Figure 4.1: Detailed outline of the study's experimental design 160

Figure 4.2: Effect of 5-week HFD consumption on: (a) distance traveled during each 5 min interval of the open field testing; (b) number of center crossings per 100 cm traveled in the open field arena analyzed per 5 min interval; (c) average time to turn during the pole test 161
Figure 4.3: Effect of 5-week HFD intake on: (a) total time spent swimming in a forced swim test; (b) number of marbles buried (70%) during a marble burying test; (**c**) time spent with a familiar vs. a novel object (%) in a novel object recognition test.....162
Figure 4.4: Effect of 11-12 weeks of HFD feeding on vHIP synaptic plasticity: (a) representative paired-pulse fEPSP sweeps recorded from CA1 region of vHIP slices of LFD (*n* = 6[10]) and HFD-fed mice (*n* = 5[11]); (b) quantification of paired-pulse ratio; (c) summary plots comparing the normalized fEPSP slope measurements.....163
Figure 5.1: Absolute organ weights of LFD and HFD females after 6 weeks on their respective diets 182
Figure 5.2: Glucose tolerance and insulin sensitivity in LFD and HFD-fed females after 5 weeks on their respective diets 183
Figure 5.3: pSer307 IRS1/IRS1 ratio in liver (A and B), soleus muscle (C, D) and retroperitoneal adipose tissue (E, F) of LFD/HFD female mice after 6 weeks on respective diets 185
Figure 5.4: Adipose tissue gene expression in the LFD and HFD-fed female mice after 6 weeks on their respective diets 187
Figure 5.5: Liver histology (A), total triglycerides (B), genes (C) and protein (D and E) expression in LFD and HFD-fed female mice after 6 weeks on their respective diets ...189

Figure 5.6: Gastrointestinal permeability (A), liver LPS-binding protein (LBP) (B) and liver TLR4 activation (C and D) in female mice fed LFD or HFD after 6 weeks on their respective diets.....191
Figure 6.1: Detailed outline of the study's experimental design 216
Figure 6.2: Effect of LFD or HFD consumption on mean body weights of female C57BL/6 after 22 (*n* = 8 per group) or 36 (*n* = 8 per group) weeks on respective diets.....217
Figure 6.3: Absolute (g; A) and relative (g/kg BW; B) organ weights of female C57BL/6 mice fed either a LFD or HFD for 22 or 36 weeks..... 218
Figure 6.4: Glucose and insulin sensitivity tests (GTT; 2 g/kg BW, oral; A and B) and (IST; 0.5 IU, i.p.: D and E) of female C57BL/6 mice fed either a LFD or HFD diet for 22 (A, D), or 36 (B, E) weeks 219
Figure 6.5: Effect of LFD or HFD consumption on gastrointestinal permeability (plasma FITC-dextran levels 1 h post oral gavage with FITC-labeled dextran) of female C57BL/6 mice after 22 (*n* = 8 per group) or 36 (*n* = 8 per group) weeks on respective diets 220
Figure 6.6: Effect of 22 or 36 weeks of HFD consumption on mRNA levels of tumor-necrosis factor alpha (TNFα), interleukin 6 (IL-6), haptoglobin (Hp), CD36, peroxisome proliferator-activated receptors-alpha and gamma (PPARα and PPARγ, respectively), insulin receptor substrate 1 (IRS1) and glucose transporter type 4 (GLUT4) in the liver of female mice.....221
Figure 6.7: Effect of 21 or 32 weeks of HFD consumption on: (A) distance traveled (per 5 min interval) during open field testing; (B) time spent per 5 min interval in the center of the open field arena; and (C) average time to turn during the pole test; (D) mean forelimb grip strength 222

Figure 6.8: Effect of 21 or 32 weeks of HFD consumption on: (A) time spent with a familiar vs. a novel object (%) in a novel object recognition test; (B) total time spent swimming in a forced swim test; and (C) number of marbles buried (≥ 70%) during a marble burying test 223
Figure 7.1: Mean body weight (g; A) and absolute brain (B), liver (C), kidney (D), spleen (E) and thymus (F) weights (g) of PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation 251
Figure 7.2: Distance traveled (per 5 min interval) during open field testing (A), time spent per 5 min interval in the center (B) and corner of the open field arena (C) by PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation 252
Figure 7.3: Number of approaches towards a familiar vs. a novel object (%) in a novel object recognition test (NOR; A), time spent swimming during the first 5 min (interval 1) in a forced swim test (FST; B) and number of marbles buried (≥ 70%) during a marble burying test (MBT; C) by PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation 254
Figure 7.4: Dopamine (DA; A), dihydroxyphenylacetic acid (DOPAC; B) and norepinephrine (NE; C) in the prefrontal cortex (PFC) of PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation 256
Figure 8.1: Overall summary 265

ABBREVIATIONS

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care

aCSF: artificial cerebrospinal fluid

AD: Alzheimer's disease

ADHD: attention-deficit/hyperactivity disorder

Akt2: protein kinase B

Al: aluminium

ANOVA: analysis of variance

ASD: autism spectrum disorder

AUC: area under curve

BBB: blood-brain barrier

BMI: body mass index

Cbl: cerebellum

CHD: coronary heart disease

CNS: central nervous system

Cor: cortex

COX-2: cyclo-oxygenase-2

CVO: circumventricular organs

DA: dopamine

DAB: 3, 3’ diaminobenzidine

D2DR: dopamine receptor-2

dHIP: dorsal hippocampus

DM: diabetes mellitus

DM2: type 2 diabetes mellitus

DMT1: divalent metal ion transporter 1

DOPAC: 3,4-dihydroxyphenylacetic acid

DW: drinking water

EPA: Environmental Protection Agency

EPM: elevated plus maze

fEPSPs: field excitatory post-synaptic potentials

FST: forced swim test

FXS: fragile X syndrome

GAD1: glutamate decarboxylase 1

GD: gestational day

GDM: gestational diabetes mellitus

GFAP: glial fibrillary acidic protein

GLUT4: glucose transporter type 4

Gp: globus pallidus

GTT: glucose tolerance test

GUI: graphical user interface

H & E: hematoxylin and eosin

HAPO: Hyperglycaemia and Adverse Pregnancy Outcome

HD: Huntington's disease

HDL-C: high-density lipoprotein cholesterol

HFD: high-fat diet

HFS: high frequency tetanic stimulation

5-HIAA: 5-hydroxyindoleacetic acid

Hip: hippocampus

HKG: housekeeping gene

HO-1: heme oxygenase-1

H2O2: hydrogen peroxide

Hp: haptoglobin

HRP: horseradish peroxidase

5-HT: serotonin

HVA: homovanillic acid

Hyp: hypothalamus

IACUC: Institutional Animal Care and Use Committee

IFNα: interferon alpha

IHC: immunohistochemistry

IKKβ: inhibitor of nuclear factor-κB kinase

IL-1β: interleukin-1β

IL-2: interleukin 2

IL-6: interleukin 6

iNOS: inducible nitric oxide synthase

i.p: intraperitoneal

IRS1: insulin receptor substrate 1

IST: insulin sensitivity test

i.v: intravenous

Jnk: c-Jun N-terminal kinase

LBP: LPS binding protein

LFD: low-fat diet

LI: latent inhibition

LNFPIII: lacto-N-fucopentaose III

LPS: lipopolysaccharide

LTP: long-term potentiation

MAPK: mitogen-activated protein kinases

MBT: marble burying test

MD2: myeloid-differentiation protein 2

Med: medulla

mGluR5: metabotropic glutamate receptor subtype 5

MHPG: 3-methoxy-4-hydroxyphenylglycol

MMT: methylcyclopentadienyl manganese tricarbonyl

Mn: manganese

MRI: magnetic resonance imaging

3-MT: 3-methoxytyramine

MUFA: monounsaturated fatty acids

N: Newton

NAc: nucleus accumbens

NE: norepinephrine

NFκB: nuclear factor-kappa B

NIH: National Institute of Health

NIRS: National Inorganic and Radionuclide Survey

NMDA: N-methyl-d-aspartate

NO: nitric oxide

NOR: novel object recognition test

NPIs: novelty preference indices

6-OHDA: 6-hydroxydopamine

Olf: olfactory bulb

PAMPs: pathogen-associated molecular patterns

PD: Parkinson's disease

PDD-NOS: pervasive developmental disorder-not otherwise specified

PFC: prefrontal cortex

PGs: prostaglandins

PI: pallidal index

PI3K: phosphatidylinositol 3-kinase

Pit: pituitary gland

PND: postnatal day

Poly IC: polyriboinosinic: polyribocytidylic acid

Pon: pons

PPARα: peroxisome proliferator activated receptors alpha

PPARγ: peroxisome proliferator activated receptors gamma

PPI: pre-pulse inhibition

PPR: paired-pulse ratio

PTP: protein tyrosine phosphatase

PUFA: polyunsaturated fatty acids

PVDF: polyvinylidene difluoride

qPCR: real-time quantitative polymerase chain reaction

ROIs: regions of interest

ROS: reactive oxygen species

SAT: saturated fatty acids

s.c: subcutaneous

SD: standard deviation

SEM: standard error of the mean

Sn: substantia nigra

Snpc: substantia nigra pars compacta

Snpr: substantia nigra pars reticulata

SNR: signal-to-noise ratio

SOCS3: suppressor of cytokine signaling-3

STR: striatum

Tf: transferrin

Th: tyrosine hydroxylase

TLR-4: Toll-like receptor-4

TNFα: tumor necrosis factor alpha

TPN: total parenteral nutrition

vHIP: ventral hippocampus.

WHO: World Health Organization

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The impact of neurological disorders in the global population

Neurological disorders affect people irrespective of age, gender, or socio-economic status and represent a significant share of the total global disease burden; it is estimated that 1 billion people, i.e. nearly one in six of the world's population, suffer from neurological diseases. Human neurological disorders, including neurodegenerative and neurodevelopmental diseases, are among the most devastating and complex conditions known to mankind. This is because neurological disorders not only cause morbidity and mortality, but they also impose social and economic burdens to affected patients and their families (Durnaoglu et al., 2011). According to the National Institute of Neurological Disorders and Stroke, there are more than 600 neurologic disorders, affecting approximately 50 million Americans annually. The economic cost of neurological diseases is enormous. For example, the US spends hundreds of billions of dollars each year in direct health care costs and lost opportunities (Brown et al., 2005) and in Europe, neurological diseases was estimated to cost about 798 billion euros in 2010 (Gustavsson et al., 2011). Importantly, as life expectancy increases worldwide, both the incidence and cost of age-related neurodegenerative diseases are rising dramatically.

Neurodegenerative diseases

Brain function is age-dependent: as individuals pass from childhood through adulthood to old age, their normal brain function declines after a certain age. The complexity of the nervous system and the high energy requirements for normal function can render the brain vulnerable to a

variety of insults causing neurodegeneration, characterized by the gradual and progressive loss of neuronal structure and function over and above the normal age-related loss (Cannon and Greenamyre, 2011). Neurodegenerative diseases are a diverse group of neurological disorders all having neurodegeneration as a hallmark feature. The prevalence of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) is increasing dramatically, affecting 30 million individuals worldwide (Benetti et al., 2012). Aging and certain genetic polymorphisms are considered main risk factors for the majority of the neurodegenerative disorders (Brown et al., 2005). However, the increasing evidence for certain environmental factors, including metals, infection and overnutrition-induced disease like obesity, as potential risk factors for the development of neurodegenerative diseases is increasing (Coppede et al., 2006, Cai, 2013).

Neurodevelopmental disorders

Compared to the adult brain, the developing nervous system is more susceptible to environmental neurotoxicants, which is mainly attributed to the emergence of various complex neurodevelopmental events and the lack of a mature blood-brain barrier (Rice and Barone, 2000). Any inappropriate exposure during the critical period of brain development (prenatal/fetal and early postnatal stages) can affect both the structural and functional development of the nervous system, resulting in neurodevelopmental disorders (Ehninger et al., 2008). Neurodevelopmental disorders affect approximately 1–2% of the population and include, among others, attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and genetic disorders such as fragile X syndrome (FXS) and Down syndrome (Richardson, 2006, Castren et al., 2012). Most of the neurodevelopmental disorders have their origins in abnormal brain development early in prenatal life, with behavioral abnormalities typically manifesting in

early childhood as deviation from normal development (Taber et al., 2011). Therefore it is conceivable that any adverse prenatal events, that disturb the normal maternal environment such as infection, stress and over/under nutrition can cause fetal neurodevelopmental injury (Bale et al., 2010, Marques et al., 2013), subsequently leading to neurological disorders later in the life of the offspring (Taber et al., 2011).

Inflammation and neurodegenerative/neurodevelopmental disorders

Mechanistic studies have demonstrated activation of the resident microglial populations in the CNS, causing release of inflammatory mediators and subsequent neuroinflammation by diverse external environmental stimuli (Frank-Cannon et al., 2009). Although acute self-limiting neuroinflammation is considered beneficial to the CNS, chronic neuroinflammation, characterized by sustained microglial activation and release of inflammatory mediators can cause neuronal dysfunction and subsequent death of susceptible neuronal populations (Streit et al., 2004). In addition to central inflammation, immune dysfunction outside the CNS, i.e., peripheral inflammation, is involved in the pathogenesis of nervous system dysfunctions (Trager and Tabrizi, 2013). Elevated plasma levels of pro-inflammatory cytokines and chemokines have been detected in patients with AD, PD and HD (Trager and Tabrizi, 2013). Importantly, peripheral immune system activation, evidenced by elevated plasma immune markers, showed positive correlation with AD and HD disease severity (Lucin and Wyss-Coray, 2009). On the other hand, neurodegenerative changes in the brain can also cause alterations in the peripheral immune system, indicative of an extensive bi-directional brain-immune system communication (Wrona, 2006). For example, elevated levels of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), were found in freshly isolated peripheral blood mononuclear cells from AD patients compared to their levels in cells from non-demented controls (Lucin and

Wyss-Coray, 2009). Besides neurodegenerative diseases, neurodevelopmental disorders have also been linked to peripheral immune system activation. For example, maternal infection and subsequent activation of the maternal immune system is considered a significant contributor to the etiology of disorders, such as ASD (Parker-Athill and Tan, 2010). Also, maternal infection during pregnancy has been associated with increased incidence of schizophrenia later in life of the offspring (Boksa, 2008). Thus, both central and peripheral environmentally-triggered inflammation can potentially lead to neuronal death and subsequent development of neurodegenerative and neurodevelopmental disorders (Amor et al., 2010, Theoharides et al., 2013). In summary, chronic neuroinflammation acts as a convergence point for different environmental stimuli that can promote the pathogenesis and progression of neurodegenerative and neurodevelopmental diseases (Tansey and Goldberg, 2010).

Neurodegenerative/neurodevelopmental disorders: environmental risk factors

The exact underlying cause(s) of many neurodegenerative diseases are unknown, but ageing and genetic polymorphisms are considered primary risk factors (Cannon and Greenamyre, 2011). Additionally, several lines of evidence indicate that various environmental factors can contribute significantly to the risk of developing neurodegenerative diseases (Brown et al., 2005). Emerging evidence suggests that pesticide exposure is a risk factor for neurodegenerative diseases. Several epidemiological studies have demonstrated positive association between PD and exposure to pesticides (Tanner et al., 2011), herbicides (Butterfield et al., 1993, Gorell et al., 1998) and insecticides (Butterfield et al., 1993). Neurotoxic metals, including aluminum (Al) and manganese (Mn) have long been implicated in the etiology of neurodegenerative diseases. For example, several epidemiological studies have linked Al exposure with AD (Zatta et al., 2003). Similarly, overexposure to Mn produces a neurological disorder, termed manganism that shares

many symptoms with PD (Olanow, 2004). While the involvement of Al in the etiology of neurodegenerative diseases is still somewhat controversial, both epidemiological and animal studies have pointed out the clear definitive role of Mn in the development of Parkinsonian features (Zatta et al., 2003).

In addition to the above neurotoxic metals, several studies have demonstrated a link between systemic inflammation and neurodegenerative diseases (Ferrari and Tarelli, 2011). For example, a higher risk for developing PD was demonstrated in patients suffering from Japanese encephalitis and H5N1 influenza viral infections (Shoji et al., 1993). Also, lymphocyte activation evidenced by elevated levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), IL-1β and of $CD4^+$ and $CD8^+$ T lymphocytes in the cerebrospinal fluid and in the serum of PD patients was reported (Reale et al., 2009). Multiple studies have also reported significant positive association between PD and increased body mass index (BMI) demonstrating the close association between neurodegenerative disorders and overnutrition-induced disease such as obesity (Cai, 2013). Besides neurodegenerative diseases, environmental factors also play an important role in determining the risk of neurodevelopmental disorders. For example, exposure to environmental chemicals, such as Mn, pesticides and adverse prenatal events, including maternal infection, maternal overnutrition and maternal stress. have all been implicated in the development of ASD (Krakowiak et al., 2012, Kalkbrenner et al., 2014).

Focus 1: manganese (Mn)

a) Occurrence and essentiality of Mn

Mn is a naturally occurring substance found in many types of rocks, soil and water. It is the 12^{th} most abundant element in the earth and 4^{th} most commonly used metal in the world (Santamaria, 2008). Mn is an essential metal important for protein and energy metabolism, bone

mineralization, metabolic regulation and enzymatic reactions, including amino acid, carbohydrate and lipid synthesis (Dobson et al., 2004).

b) Toxicokinetics

In humans, approximately 1-5% of ingested Mn is normally absorbed (Karki et al., 2013) and is later eliminated through the feces via hepatobiliary excretion (Aschner and Aschner, 2005). Because of this tightly controlled regulation, tissue levels of Mn are relatively stable (Aschner et al., 2005). Mn is mainly absorbed as Mn^{2+}, and is further oxidized to the more reactive Mn^{3+}, which can bind to iron binding protein called transferrin (Tf). Mn^{2+} enters the brain via Tf receptor-mediated endocytosis and also by carrier proteins, such as divalent metal ion transporter 1 (DMT1), the *N*-methyl-d-aspartate (NMDA) receptor channel and the Zip family metal transporter, Zip8 (Gunter et al., 2013). In contrast to the quick carrier-mediated Mn influx, the brain efflux of Mn is comparatively slower and occurs via diffusion mechanism, which increases the likelihood of increased brain Mn accumulation in cases of excessive Mn exposures (Zatta et al., 2003).

c) Sources of Mn and exposure levels (general population)

Diet is the primary source of Mn for the general population; Mn's average daily intake ranges from 2 to 9 mg (Santamaria, 2008). Mn is commonly found in nuts, legumes, seeds, grains, and green leafy vegetables. Particularly, grain, rice, and nuts are significant sources of Mn (30 mg/kg) (Aschner et al., 2005). Intake of Mn from drinking water (DW) is substantially less than the intake from food (U.S.EPA, 2004). Water levels of Mn are in the range of 1-100 μg/L (U.S.EPA, 2004). The National Inorganic and Radionuclide Survey (NIRS) has determined the median DW level of Mn as 10 μg/L (WHO, 2011b) and the average intake of Mn from DW for an adult is estimated to be 20 μg/day, assuming a daily water intake of 2 L (U.S.EPA, 2004).

Wind-caused erosion of dust and soil is considered an important atmospheric source of Mn (Aschner et al., 2005). Exposure to Mn from air is typically 0.04 ng/day, which is several orders of magnitude less than that from the dietary intake (U.S.EPA, 1993). The U.S. Environmental Protection Agency (EPA) has set the current inhalation reference concentration for inhalable Mn as 0.05 μg Mn/m^3, with most human exposures falling below this level (U.S.EPA, 1993).

d) Sources of Mn overexposure (occupational/non-occupational settings)

Inhalation exposure

Although, inhalation Mn exposure is considered insignificant compared to the Mn's intake via the diet, people engaged in certain occupations may be exposed to much higher levels of Mn than the general population. Production of ferroalloy, iron and steel foundries, metal fumes from welding and combustion emissions from power plants and mining contribute significantly to the concentration of Mn in air (Dobson et al., 2004). Mn level may rise to as high as 250 mg/m^3 in mining operations (Rodier, 1955); however, its level is comparatively lower in dry-cell battery and ferro-manganese plants, with the values ranging from 5-8 mg/m^3 (Saric et al., 1977). Among welders, Mn concentration has been found to vary from 0.1-4 mg/m^3 (Korczynski, 2000, Kucera et al., 2001). In addition, Mn may be released to the environment by the use of manganese tricarbonyl (MMT), a fuel additive used in unleaded gasoline (Aschner et al., 2009). Hence depending on the proximity of industries and other factors, Mn levels in the ambient air can vary widely; mean ambient air levels of Mn proximal to industrial areas have been reported to range from 220 to 300 ng/m^3, whereas average levels of Mn in ambient air in rural and remote areas are typically below 10 ng/m^3 (WHO, 2004b).

Oral exposure (drinking water; DW)

Mn occurs naturally at lower concentrations (1 to 200 ug/L) in soil, ground water and surface water (U.S.EPA, 2004). However, natural leaching processes and/or human activities can result in significant water contamination in some areas. The major anthropogenic sources of Mn contamination include municipal wastewater discharges, sewage sludge, improper land disposal of dry-cell batteries and other manganese-containing wastes (WHO, 2004b). For example, high concentration of Mn (close to 28 mg/l) in well water was reported by an epidemiological study in Japan, which was likely derived from 400 dry-cell batteries buried near the well (Kawamura, 1941). Similarly higher Mn levels (in excess of 1,000 μg/L) in well water were reported by several epidemiological studies, indicating that the anthropogenic/natural sources can easily result in Mn level, well above the range that is typically found in DW (Agusa et al., 2006, Hafeman et al., 2007, Buschmann et al., 2008). Generally, compared to food, ingestion of Mn from water is minimal; however, depending on the degree of water contamination, Mn intake from water can even rise to 20% of the total intake (Loranger and Zayed, 1995). More importantly, Mn is better absorbed from water than from food and hence the chances of developing adverse health effects, including on the nervous system, are much higher upon overexposure to Mn from contaminated DW (Bouchard et al., 2007c).

Other sources

The total parenteral nutrition (TPN) solutions are formulated to contain appropriate levels of Mn, but there are several reports of high levels of Mn contamination in the TPN solutions (Pluhator-Murton et al., 1999). Because of the parenteral administration, 100% of Mn in the TPN solution becomes systemic, largely bypassing the normal homeostatic regulatory mechanism (i.e., the gut) for Mn. Administration of TPN solutions at a dose of 0.1 mg Mn/day

have been reported to produce Mn intoxication (Nagatomo et al., 1999, Bertinet et al., 2000). Mn intoxication has also been reported in individuals who intravenously administered a designer psychostimulant drug called ephedrine (prepared using pseudoephedrine, along with potassium permanganate and acetic acid as oxidizing agents) for a chronic period (Sanotsky et al., 2007, Stepens et al., 2008). Mn neurotoxicity associated with elevated blood and brain Mn level has also been reported in humans (Spahr et al., 1996, Layrargues et al., 1998) and experimental animals (Rose et al., 1999) with cholestatic liver disorders. As biliary excretion is the primary route of Mn elimination (Ballatori et al., 1987), altered liver functions, including any interruption in the synthesis and normal flow of bile can cause impaired Mn clearance resulting in excessive Mn accumulation and associated neurotoxicity.

e) Toxic effects of Mn

Respiratory effects of Mn

Respiratory effects such as bronchitis, cough, decreased lung function, and an increased susceptibility to lung infections have been reported in chronic occupational exposures (Saric, 1992) and in populations living near ferromanganese factories (WHO, 1987). Multiple studies with experimental animals have demonstrated inflammatory response in the lungs following inhalation exposure to MnO_2 or Mn_3O_4 (Suzuki et al., 1978, Camner et al., 1985). A two fold increase in the mRNA expression of the pulmonary vascular endothelial growth factor (a potent inducer of vasculogenesis and angiogenesis) was observed in the lungs of female GVB/N mice, following nose-only inhalation exposure to $MnCl_2$ (2 mg Mn/m^3) aerosols (Bredow et al., 2007). Young male rhesus monkeys demonstrated alveolar duct inflammation and proliferation of bronchus-associated lymphoid tissue following high dose (1.5 mg Mn/m^3) subchronic inhalation exposure to $MnSO_4$ (Dorman et al., 2005c).

Reproductive effects of Mn

Human data are conflicting on the reproductive effects following occupational inhalation exposure to Mn. Several studies reported impaired sexual function, manifested by impotence, loss of libido, and reduced fertility in male workers chronically (1-19 years) exposed to excessive Mn (Lauwerys et al., 1985, ATSDR, 2008b). However, fertility of male workers in a dry alkaline battery plant chronically exposed to Mn as MnO_2 (0.71 mg manganese/m^3) was not affected (Gennart et al., 1992). There is no information regarding the reproductive effects of Mn inhalation on women (ATSDR, 2008b). In humans, there is no data regarding the reproductive effects of oral Mn exposure. In animals, oral Mn exposure is detrimental to both male and female reproductive system. For example, long-term Mn DW exposure (as $MnCl_2$ at a daily dose level of 309 mg Mn/kg/day) for 12 weeks impaired fertility in male mice (Elbetieha et al., 2001). Reproductive deficits evidenced by implantation loss and reduced number of viable fetuses were observed in female Swiss mice exposed to Mn via DW (277 mg Mn/kg/day) for a period of 12 weeks (Elbetieha et al., 2001).

Neurological effects of Mn in humans: inhalation exposure

The major neurological disorder caused by excessive Mn exposure is called manganism. Chronic inhalation exposure to excessive amounts of Mn (> 1-5 mg Mn/m^3) is the most commonly identified cause of manganism (Pal et al., 1999, ATSDR, 2008b). Several cases of Mn neurotoxicity have been reported in industrial workers, including miners, smelters and workers involved in the manufacturing of dry-cell batteries (Nelson et al., 1993, Mergler et al., 1994, Roels et al., 1999). Manganism is typically associated with increased brain Mn levels, particularly in the basal ganglia structures, including striatum, globus pallidus, substantia nigra and subthalamic nuclei (Aschner et al., 2009). The early stage of manganism is termed locura

manganica, manifested as compulsive/violent behavior, emotional instability and hallucinations. This psychiatric stage is usually succeeded by locomotor deficits, collectively called extrapyramidal motor system dysfunction that includes progressive bradykinesia, rigidity, gait disturbance and speech difficulty (Liu et al., 2006). The physical traits of manganism resemble PD and hence some of the above symptoms associated with Mn intoxication have been referred to as Parkinsonism-like disease and/or Mn-induced Parkinsonism (Olanow, 2004). However, unlike PD, patients suffering from manganism show frequent dystonia, absence of resting tremor, lack of sustained therapeutic response to levoDOPA therapy, and normal fluoroDOPA uptake in positron emission tomography studies (Santamaria, 2008). In addition to the clinical features, several pathological differences exist between manganism and PD. Specifically, diffuse degenerative lesions of striatum, globus pallidus, subthalamic nucleus, with less severe lesions of the substantia nigra have been reported by several human and animal studies with chronic Mn poisoning (Olanow, 2004). In contrast, PD is mainly characterized by the specific degenerative lesion of the substantia nigra and relative sparing of the strio-pallidal complex (Meco et al., 1994). Additionally, because of the paramagnetic nature of Mn, its accumulation in the brain can be easily visualized by T1 weighted magnetic resonance images (Olanow, 2004). However, there are relatively few studies which have used magnetic resonance imaging (MRI) to determine brain Mn deposition in rodents and have rather done post-mortem measurement of brain Mn tissue concentrations via atomic absorption spectroscopy or inductively coupled plasma atomic emission spectroscopy (Dodd et al., 2005, Avila et al., 2010). More importantly, there are no MRI reports in experimental animals, i.e. mice, where the brain deposition and distribution of Mn following its overexposure, especially via DW, were studied.

Neurological effects of Mn in experimental animals

In addition to the human data, the neurotoxic effects, including locomotor deficits and emotional disturbances following overexposure to Mn have been demonstrated in several animal studies. For example, Mn exposure (3 intraperitoneal [i.p.] injections of 4.8 mg Mn/kg/week) for 5 weeks resulted in a 3-4 fold increase of brain Mn levels and behavioral impairments, including reduction in locomotor activity and increased gait abnormalities, in female Sprague-Dawley rats (Witholt et al., 2000). Chronic Mn administration (i.p. injection of 10 mg Mn/kg BW) caused significant impairment in short-term spatial memory and anxiety-like behaviors in male Wistar rats (Hogas et al., 2011b). Pre-weaning oral Mn exposure (50 mg Mn/kg/day) resulted in hyperactivity and behavioral disinhibition in open field arena in male Sprague-Dawley neonate rats (Kern et al., 2010). Impaired locomotor deficits, characterized by akinesia and postural instability accompanied by significant reduction of striatal dopamine (DA) content was observed in mice following chronic inhalation exposure to Mn (Ordonez-Librado et al., 2010). Of note, compared to other routes of Mn exposure, limited laboratory studies have focused on the neurobehavioral consequences of Mn DW exposure. Locomotor deficits following Mn DW exposure have been demonstrated in few rat studies (Avila et al., 2010, Fordahl et al., 2012) and a single early study in mice (Chandra et al., 1979). Additionally, data regarding the non-motor impairments (i.e. anxiety or depression) in a DW exposure paradigm is limited in experimental animals (mice) as compared with other Mn exposure routes (Liu et al., 2006, Hogas et al., 2011b).

Neurological effects of Mn in humans: DW exposure

In addition to the well documented neurotoxic effects of Mn following inhalation exposure, recent evidence increasingly associates Mn exposure via contaminated DW with

adverse neurological outcomes. A wealth of epidemiological data suggests that ingestion of Mn through DW can be neurotoxic, especially to younger populations. For example, reduced intellectual performance and working memory deficits were observed in a group of children (10 year old) in Araihazar, Bangladesh, consuming high levels of Mn (0.07 mg manganese/kg BW/day) from well water (Wasserman et al., 2006, Wasserman et al., 2011). Significantly increased hyperactivity, oppositional behaviors and decreased intellectual functions were also observed in a group of children in Quebec, Canada, who were exposed to excess levels of Mn (0.02 mg Mn/kg/day) from the Mn contaminated well water (Bouchard et al., 2007c, Bouchard et al., 2011). Compared to the younger populations, relatively few studies have investigated the adverse neurological effects in adults following contaminated DW exposure. Chronic consumption of water containing high levels of Mn (1.8–2.3 mg/l) resulted in increased prevalence of neurological signs in an elderly population in Greece (Kondakis et al., 1989). In contrast, no significant differences in neurological functions, including motor coordination, was observed between adult individuals (aged ≥ 40 years) chronically (10 years) exposed to high levels of Mn (300 μg Mn/l) and the control group exposed to a lower Mn concentration (300 μg Mn/l) via well water (Vieregge et al., 1995). In light of these discrepancies in the previous reports, more experimental studies are required to evaluate the neurological consequences of Mn overexposure via consumption of contaminated DW by adults.

f) Mechanisms of Mn neurotoxicity: direct effect on neurons

Mitochondrial dysfunction

Mn produces its neurotoxic effect by targeting the CNS, particularly the basal ganglial structures, including substantia nigra, globus pallidus and striatum (Milatovic et al., 2009). Within these brain regions, Mn accumulation occurs mainly in neurons and astrocytes (Aschner

et al., 2007) (Fig.1.1). Intracellularly, mitochondria sequester most of the Mn by the calcium uniporter (Gavin et al., 1992) and the elevated mitochondrial Mn levels interfere with oxidative phosphorylation. Specifically, Mn inhibits cellular ATP synthesis by inhibiting F1/F0 ATP synthase or complex 1 (NADH dehydrogenase) of the mitochondrial electron transport chain (Gavin et al., 1992, Chen et al., 2001). Additionally, recent studies have demonstrated that Mn also interfere with ATP synthesis by inhibiting either complex II (succinate dehydrogenase) or the glutamate/aspartate exchanger (Gunter et al., 2010). This Mn-induced inhibition of ATP synthesis will cause decreased cellular ATP levels and induce oxidative stress, finally culminating in cellular toxicity (Neal and Guilarte, 2013).

Oxidative stress

Several lines of evidence point to a role for oxidative stress in Mn's neurotoxicity (Dobson et al., 2003, Erikson et al., 2007a). Mn accumulates in dopamine-rich brain regions, especially in the basal ganglia, and causes DA oxidation leading to the generation of highly reactive semiquinones, orthoquinones and free radicals, ultimately resulting in oxidative stress (Erikson et al., 2004). Another likely mechanism for Mn-induced oxidative stress is intimately related to the accumulation of Mn in mitochondria, which alters proper mitochondrial respiration, resulting in excess production of reactive oxygen species (ROS) (Burton and Guilarte, 2009). Mn-induced oxidative stress causes mitochondrial transition pore opening, increasing the permeability of the inner mitochondrial membrane to solutes, protons and ions. This increased permeability results in a loss of the mitochondrial inner membrane potential, defective oxidative phosphorylation and ATP synthesis, culminating in mitochondrial dysfunction (Karki et al., 2013). Thus, increased free radical production by interfering with the

mitochondrial function, as well as enhanced auto-oxidation of DA can both contribute significantly to the neurotoxicity induced by Mn (Neal and Guilarte, 2013) (Fig. 1.1).

g) Mechanisms of Mn neurotoxicity: indirect effects

Inflammation

In addition to the direct toxic effect of Mn on neurons, including mitochondrial dysfunction and oxidative stress, Mn also has the ability to produce neurotoxicity indirectly by causing the activation of glial cells, including microglia and astrocytes (Filipov and Dodd, 2012) (Fig.1.1). Mn-induced activation of glial cells results in the production of non-neuronal derived neurotoxic products, such as ROS, nitric oxide (NO), prostaglandins (PGs) and hydrogen peroxide (H_2O_2) (Liu et al., 2006, Liao et al., 2007, Zhang et al., 2007). Additionally, Mn enhances the release of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β from the activated glial cells. Compared to neurons, astrocytes can sequester Mn at greater than 50 fold concentration because of the high affinity transport system (Aschner et al., 2009) and are considered to be the initial target of Mn neurotoxicity (Henriksson and Tjalve, 2000). Sub-acute treatment of rats with Mn (50 mg/kg body weight, i.p/day for 1-4 days) resulted in increased Mn levels and Alzheimer type-II astrocytosis in the globus pallidus (Hazell et al., 2006). In vitro, Mn-treated astrocytes enhanced the uptake of L-arginine, the substrate for NO production (Hazell and Norenberg, 1998), and dose-dependently upregulated inducible nitric oxide synthase (iNOS) expression and NO release in TNF-α/IL-1β-stimulated astrocytes (Spranger et al., 1998) indicating that activated astrocytes contribute to Mn neurotoxicity via enhanced production of NO. Besides activating astrocytes, Mn potentiates the release of pro-inflammatory cytokines, namely TNF-α and IL-6 from microglial cells (Chang and Liu, 1999, Filipov et al., 2005) which, in turn, can activate astrocytes and cause subsequent release of inflammatory mediators such as

PGE_2 and NO (Chen et al., 2006). Mn-induced glial cell activation and subsequent gliosis was demonstrated in brains of humans exposed to Mn over a chronic period (Perl and Olanow, 2007) and in multiple animal studies following different exposure routes to Mn, namely inhalation (Antonini et al., 2009), intragastric gavage (Moreno et al., 2009), or intravenous injection (Verina et al., 2011). Among basal ganglia structures, prominent microglial activity has been shown in substantia nigra, especially pars reticulata, which is attributed to the rich iron content and DA levels of this structure (Perl and Olanow, 2007, Verina et al., 2011). Mn can displace iron from its Tf binding sites, alter iron homeostasis and can cause iron-mediated oxidative stress, thereby increasing the susceptibility of substantia nigra pars reticulata to Mn neurotoxicity (Verina et al., 2011). Collectively, the above studies suggest that glial cell activation and subsequent neuroinflammation is one of the key mechanisms underlying Mn-induced neurotoxicity. Although several animal studies have demonstrated Mn-induced activation of glial cells and subsequent neuroinflammation following oral (gavage) and inhalation exposures (Antonini et al., 2009, Moreno et al., 2009), there are no studies exploring the glial cell involvement in DW exposure to Mn.

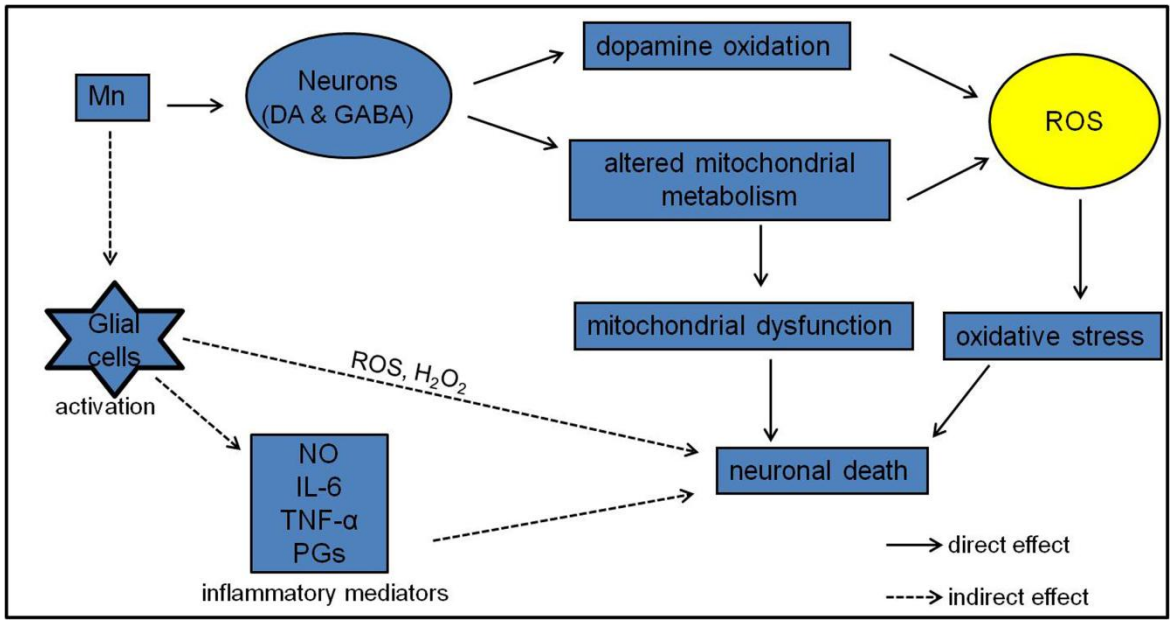

Figure 1.1. Mechanism of Mn neurotoxicity

h) Focus 1-data gaps: summary

Although occupational inhalation of Mn remains a major concern (Erikson et al., 2007a), recent epidemiological evidence has increasingly associated Mn overexposure via contaminated DW with adverse neurological outcomes, especially in younger populations (Bouchard et al., 2007c, Bouchard et al., 2011). However, limited information exists regarding the neurological consequences of Mn exposure via DW in adult humans and experimental animals, especially mice. Additionally, MRI studies investigating the brain Mn deposition/distribution following Mn overexposure via DW in experimental animals, i.e. mice, is non-existent. Furthermore, no longitudinal studies have been conducted so far to investigate how the regional distribution of Mn may vary over a period of time in the mouse brain after Mn exposure via the increasingly relevant DW exposure. The present dissertation research addresses these missing links (Fig. 1.2) by investigating the neurobehavioral consequences as well as the brain Mn deposition/distribution following Mn exposure via DW in adult mice.

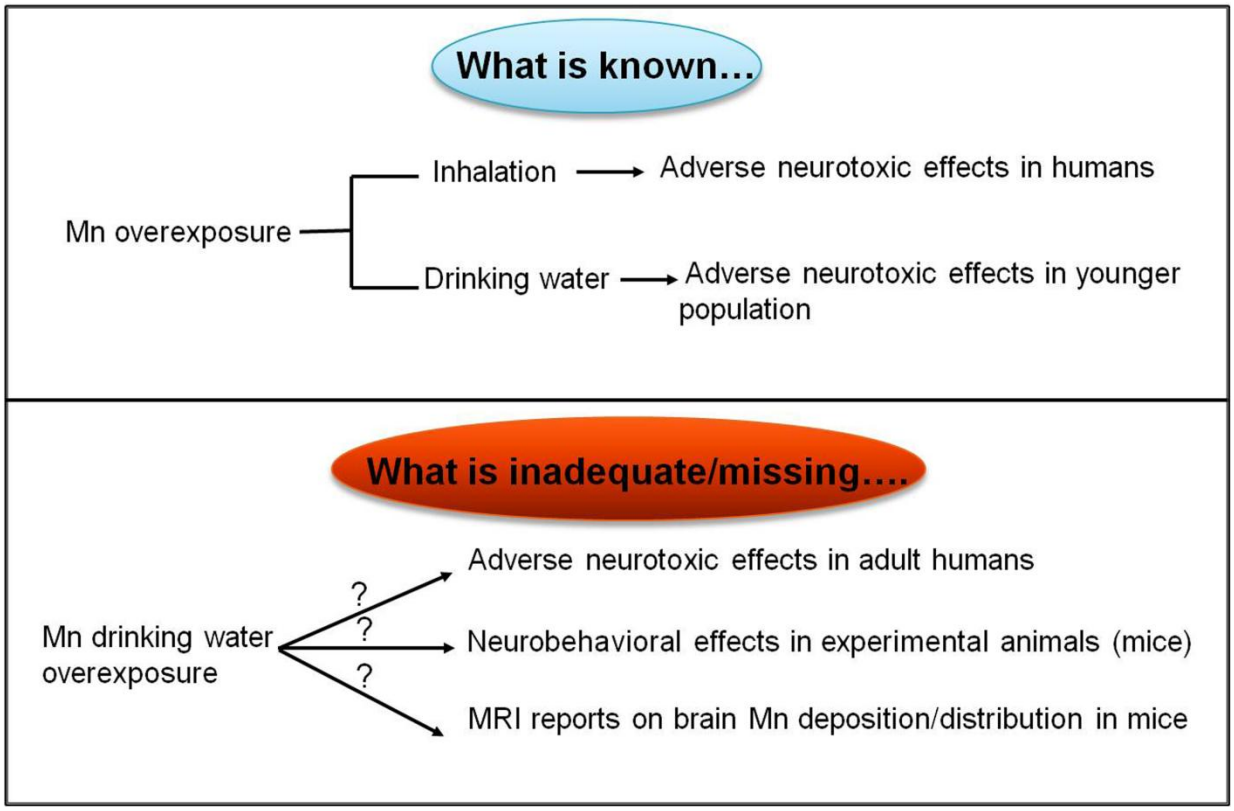

Figure 1.2. Data gaps in Mn neurotoxicity

Focus 2: chronic systemic infections and ensuing inflammation

a) Neuro-immune cross-talk

The outdated concept of the CNS as an immune-privileged site was based on early evidence from animal models which reported better persistence and smaller rejection rates of eye and brain allografts (Billingham and Boswell, 1953, Barker and Billingham, 1977). However, increasing evidence shows that the CNS can actively communicate with the immune system and that the CNS can no longer be viewed as an immune-privileged site (Shrestha et al., 2013). The blood-brain barrier (BBB) acts as a regulatory interface between CNS and immune system (Weiss et al., 2009); any disruption in the BBB integrity can lead to altered CNS microenvironment and impaired neuronal function (Stolp and Dziegielewska, 2009). BBB

damage and subsequent increase in BBB permeability to peripheral cytokines following systemic infection or inflammation has been shown dies (Erickson et al., 2012). Importantly, the infiltration of peripheral immune cells, such as CD4+ and CD8+ T lymphocytes into the midbrain of PD patients, indicates a pathogenic link between BBB dysfunction and PD (Su and Federoff, 2014). Several animal studies have also demonstrated that peripheral inflammation can enhance the degeneration of the nigrostriatal dopaminergic pathways induced by various insults (Mangano and Hayley, 2009, Pott Godoy et al., 2010, Villaran et al., 2010). Interestingly, studies have also shown that peripheral circulating cytokines can alter BBB-specific transport systems or receptors, stimulating the cells associated with the BBB to release inflammatory mediators into the CNS and cause neuroinflammation, without necessarily impairing the BBB integrity (Banks and Erickson, 2010). For example, increased cerebrospinal fluid levels of IL-1β were observed in rheumatoid arthritis patients, which is an autoimmune disease, associated with chronic peripheral immune, but not with BBB damage (Lampa et al., 2012).

b) Means of communication of peripheral inflammatory signals with the CNS

Inflammation in peripheral tissues leads to robust increase in circulating cytokines and other inflammatory molecules which can in turn communicate with the brain via several pathways (Quan and Banks, 2007). The first of these routes is the humoral pathway which involves the circumventricular organs (CVOs) that lack an intact BBB and include the median eminence, organum vasculosum of the laminae terminalis, area postrema and the suprafornical organ. In these CVOs, peripherally produced pathogen-associated molecular patterns (PAMPs) can cause the production and release of pro-inflammatory cytokines which can in turn freely diffuse from blood into the brain, further reacting with the macrophage populations within the brain, culminating in neuroinflammation (Dantzer, 2009). The second major route is via the

neural pathway wherein cytokines communicate with the brain across the intact BBB. In the neural pathway, peripheral inflammatory signals interact with the brain via the autonomic nervous system mainly via the vagal afferents and activation of the vagal afferents by peripheral inflammation results in increased level of brain cytokines (Dantzer, 2009).

c) Peripheral inflammation and Parkinson's disease (PD)

The pathogenesis and progression of PD can be significantly influenced by peripheral immunological challenges and chronic inflammatory diseases. For example, the risk for developing PD is higher in patients infected with Japanese encephalitis virus or H5N1 influenza virus (Shoji et al., 1993, Jang et al., 2009). Increased microglial activation and elevated levels of TNF-α, IL-6 and IL-1β was demonstrated in the midbrain of PD patients, indicating that pro-inflammatory cytokines act as the mediators of inflammation both in the brain and in the periphery (Hirsch and Hunot, 2009). Interestingly, reduced PD incidence was reported in users of nonsteroidal anti-inflammatory drug, such as ibuprofen, implying a role for peripheral inflammation in PD (Chen et al., 2003, Chen et al., 2005).

Besides human data, the link between peripheral infection and PD pathogenesis has been demonstrated experimentally. Bacterial infections modeled by peripheral challenge with lipopolysaccharide (LPS) in animals increase peripheral pro-inflammatory cytokine levels initially and they eventually signal to the brain to produce inflammatory mediators, resulting in progressive neurotoxicity (Perry, 2004, Puntener et al., 2012). LPS is a potent endotoxin found in the outer membrane of gram-negative bacteria and is comprised of 3 components, namely an O-specific chain, which is a surface carbohydrate polymer, a core oligosaccharide, featuring an outer and inner region, and lipid A, which is an acylated glycolipid (Rietschel et al., 1996) (Fig. 1.3). Of the 3 components, the lipid A moiety is considered to be the endotoxic or innate immune

system stimulating component of LPS (Ulevitch and Tobias, 1999). LPS mainly binds to the soluble LPS binding protein (LBP) and CD14, which are anchored on the outer leaflet of the plasma membrane (Gutsmann et al., 2001). This LPS-CD14 complex further interacts with transmembrane Toll-like receptor-4 (TLR4), activating kinases of various intracellular signaling pathways, involving mitogen-activated protein kinases (MAPK) and transcription factors such as nuclear factor-kappa B (NF-κB) and upregulating genes that are involved in inflammation and free radical generation (Janeway and Medzhitov, 2002, Takeda et al., 2003). Both peripheral immune cells, including macrophages and monocytes, as well as specific cellular populations within the brain, including microglia, can be potently stimulated by LPS, eliciting the release of TNF-α, IL-1β and free radicals (Dentener et al., 1993, Medvedev et al., 2000). Additionally, prostaglandins (PGs) and NO are biosynthesized and released by the induction of cyclo-oxygenase-2 (COX-2) and iNOS expression (Dutta et al., 2008). In contrast to the LPS-induced direct activation of microglia, neurons are not directly affected by LPS due to the absence of functional TLR4 expression (Lehnardt et al., 2003). Hence LPS models of PD are considered as an excellent tool to specifically investigate inflammation-mediated DA neurodegeneration (Liu et al., 2002).

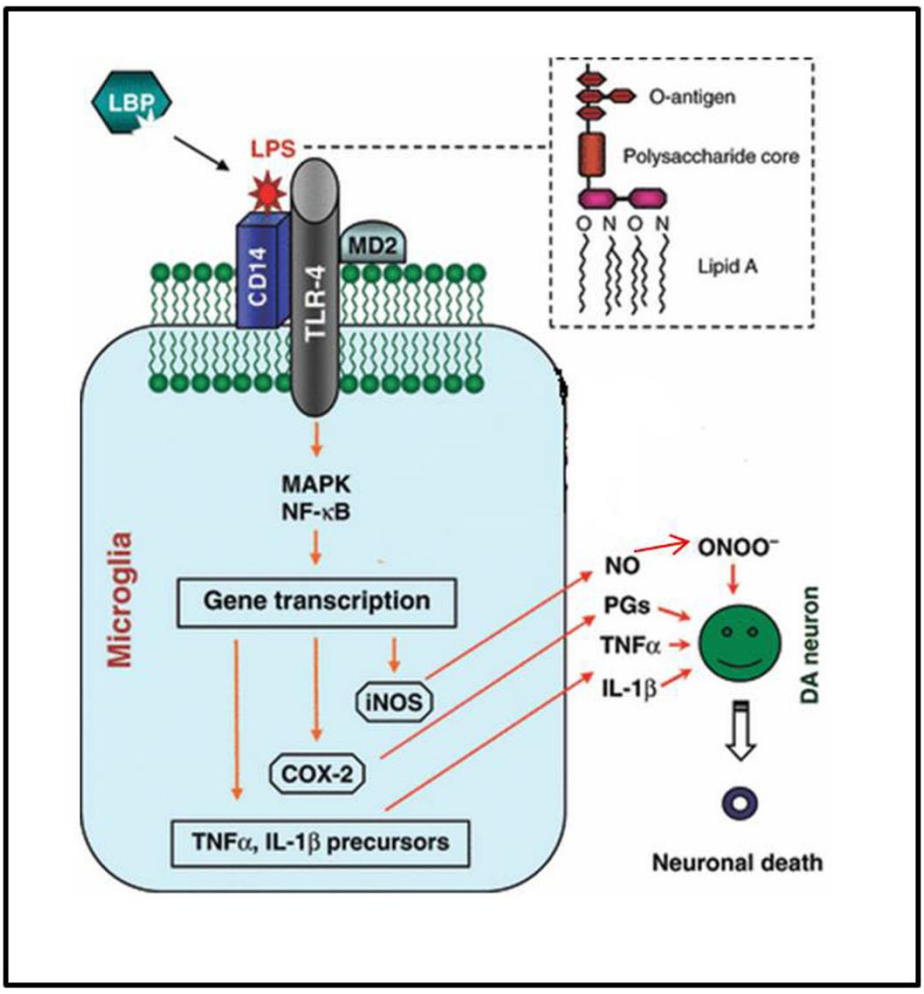

Figure 1.3. Mechanism of LPS-induced microglia-dependent neurotoxicity. Adapted from (Dutta et al., 2008)

d) LPS models of PD

Central LPS administration models

Central LPS administration models have been successful in replicating the characteristic neurodegenerative pattern seen in PD and have demonstrated significant early microglial activation followed by delayed and time-dependent nigral dopaminergic neuron degeneration. Acute intranigral injection of LPS (2 μg) in female Wistar rats resulted in selective degeneration of the dopaminergic system associated with a significant decrease in the DA levels in the striatum and substantia nigra; alterations of the dopaminergic system was evident even 3 weeks

after the LPS injection (Castano et al., 1998). Similarly, microglial activation and associated degeneration of dopaminergic substantia nigra neurons induced by single supranigral injection of LPS (5 μg) in Sprague-Dawley rats was blocked by naloxone via inhibition of cytotoxic substances from activated glial cells (Lu et al., 2000). Chronic infusion of LPS (5 ng/h for 2 weeks) into rat substantia nigra resulted in rapid microglial activation that was followed by a delayed, gradual and selective loss of dopaminergic neurons (Gao et al., 2002, McCoy et al., 2006).

Acute systemic LPS injection PD models

Compared to the central LPS administration models, relatively few studies have investigated the influence of systemic LPS-induced inflammation on the development of neurodegenerative disease in the CNS. Acute systemic LPS injection has been reported to activate microglia and consequently contribute to the chronic neurodegenerative process, similar to what is seen in PD (Tufekci et al., 2011). Systemic (i.p.) LPS administration to pregnant Sprague-Dawley rats resulted in decreased number of striatal DA and nigral dopaminergic neurons in the offspring (Carvey et al., 2003). On the other hand, central DA depletion in a rat model of PD was associated with an increased inflammatory response after systemic LPS injection, indicating the involvement of dopaminergic system in peripheral immune regulation (Engler et al., 2009). Acute systemic administration of LPS (5 mg/kg i.p.) increased peripheral TNF-α production, which activated microglia in the CNS leading to chronic neuroinflammation and subsequent progressive loss of DA neurons in the substantia nigra, similar to what is seen in PD (Qin et al., 2007).

Chronic systemic LPS injection PD models

Prolonged low-dose systemic LPS injections (0.25 mg/kg i.p. twice a week) for 3 or 6 months resulted in neuroinflammation, characterized by microgliosis, and elevated TNF-α levels in the midbrain, but the TH-immunopositive neurons in the substantia nigra of C57BL/6 mice were reduced only moderately after the longer (6-month) treatment (Frank-Cannon et al., 2008), a finding also reported by (Morrison et al., 2012). Further, the (Frank-Cannon et al., 2008) study is noteworthy that the wild type C57BL/6 mice displayed normal gross and fine locomotor performance in spite of the increased midbrain neuroinflammation triggered by the chronic systemic LPS treatment. Chronic LPS administration in *parkin*−/− and not wild type mice produced selective nigral dopaminergic neuronal loss and associated fine locomotor deficits, which is suggestive of a potentiated increase in peripheral inflammation-mediated nigral dopaminergic neurodegeneration following loss-of-function mutations in the *parkin* gene (Frank-Cannon et al., 2008).

e) Acute vs. chronic peripheral inflammation: non-PD effects

In addition to the PD-related pathological changes, peripheral LPS-induced alterations in the brain regional neurochemistry have been reported (Dunn, 2006). For example, increased turnovers of norepinephrine (NE), DA, serotonin (5-HT) and elevated tryptophan levels were observed following acute systemic LPS administration (Dunn and Welch, 1991). Similarly, single i.p. administration of LPS (250 μg) increased DA and NE turnover in the hypothalamus of male Sprague-Dawley rats (Masana et al., 1990). Also, acute peripheral LPS administration increased the in vivo release of NE and DA, as well as increased the concentration of the serotonin metabolite, 5-hydroxyindole acetic acid (5-HIAA) in the medial hypothalamus of freely moving rats (Lavicky and Dunn, 1995). CD-1 mice treated with a single i.p. dose of LPS

resulted had increased NE, DA and 5-HT turnovers in hippocampus as well as increased DA turnover in the nucleus accumbens (Lacosta et al., 1999). Acute peripheral LPS-induced neurochemical alterations have been partially attributed to the altered behavioral responses following LPS administration by several animal studies. For example, increased turnover of NE in the locus coeruleus and increased 5-HT turnover in different brain regions, namely hypothalamus, prefrontal cortex and hippocampus was partially attributed to the anxiogenic response evoked by acute systemic LPS administration in male CD-1 mice (Lacosta et al., 1999).

LPS-induced emotional alterations, specifically an increased anxiety has been demonstrated in mice (Swiergiel and Dunn, 2007, Salazar et al., 2012) and rats (Bassi et al., 2012) following acute peripheral administration. In addition to the emotional alterations, acute peripheral LPS administration causes a series of behavioral changes, collectively termed as sickness behavior. Sickness behavior is characterized by behavioural patterns, including malaise, reduction in social interactions, fatigue, behavioral inhibition, reduction of locomotor activity and exploration, anhedonia, and increased anxiety (Maes et al., 2012). Several animal studies have demonstrated sickness behavior, manifested by reduced exploratory activity, locomotion and social exploration following acute systemic LPS injection (Swiergiel and Dunn, 2007, Sah et al., 2011, Biesmans et al., 2013). For example, in male BALB/c mice, single i.p. administration of LPS (0.33 mg/kg) resulted in sickness behavior, characterized by reduced social exploration, decreased sucrose preference and food intake, and it was associated with a significant increase in the hippocampal and cortical levels of IL-6 and IL-1β mRNA (Henry et al., 2008).

f) Sickness behavior and depression

Systemic administration of a single bolus of LPS induces sickness behavior that peaks at 2-6 h later, and gradually fades over time, and further diminishes 24-48 h after LPS injection

(Dantzer et al., 2008). More importantly, studies have shown that if the peripheral immune system activation responsible for causing sickness does not subside and continues to increase, the ongoing immune signaling to the brain can transition the sickness behavior into a more depressive-like behavior in susceptible individuals (Dantzer et al., 2008, Fu et al., 2010). Symptoms of sickness behavior and depressive-like behavior are strikingly similar; both characterized by withdrawal from the social environment, malaise and decreased response to reward (Dantzer, 2009). However, recent evidence has shown that the similarities between sickness behavior and depressive-like behavior are only partial (Dantzer et al., 2008) and that the symptoms of these behaviors can be demarcated by 24 h after systemic LPS administration (Frenois et al., 2007, Henry et al., 2008, O'Connor et al., 2009) (Fig. 1.4). For example, male CD-1 mice injected systemically with LPS (830 μg/kg) showed decreased motor activity at 6 h, but not at 24 h post LPS injection, indicating the time-dependent waning of the sickness response (Frenois et al., 2007). More importantly, these LPS-treated mice exhibited depressive-like behavior evidenced by the increased immobility time in the forced-swim test 24 hours after treatment, despite normal locomotor activity at that time point (Frenois et al., 2007). This study indicates that the LPS-induced depressive-like behavior can be observed even after the acute behavioral response (reduction in locomotor activity) that is characteristic of sickness in LPS-treated mice has returned to normal (O'Connor et al., 2009). In other words, sickness behavior is a quick adaptive response to pathogenic infection in the body and is fully reversible once the pathogen is or has been cleared from the body (Dantzer et al., 2008), whereas the depressive-like behavior occurs during the exacerbation of the ongoing immune system activation and is characterized by a delayed onset and a long-lasting nature (Maes et al., 2012).

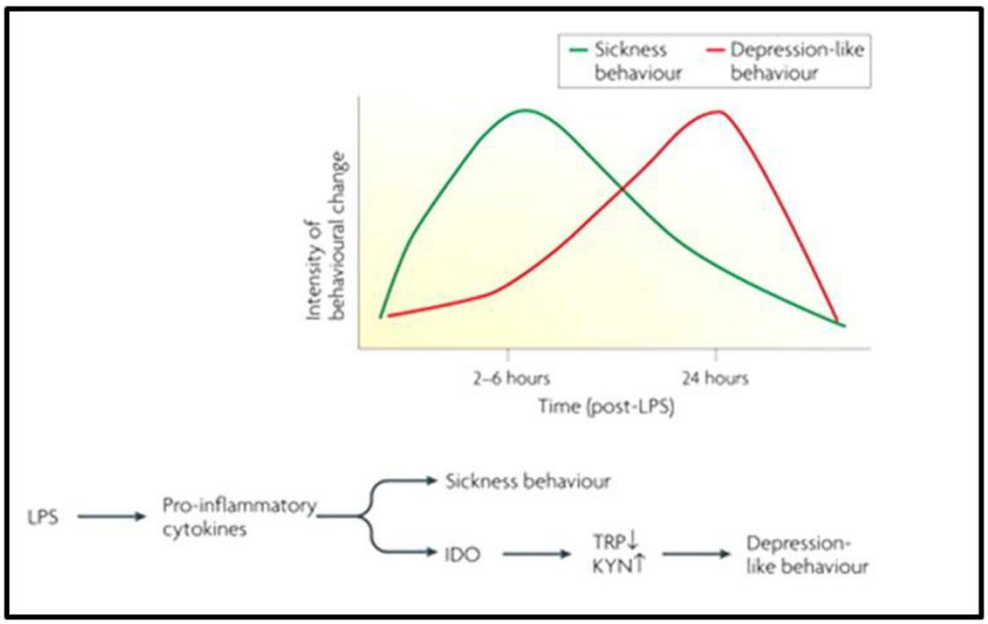

Figure 1.4. Transition from sickness behavior to a depressive-like behavior in acute immune (LPS) challenge models. Adapted and modified from (Dantzer et al., 2008)

g) Chronic peripheral inflammation and depression

Overall, animal data demonstrate that acute systemic LPS administration can cause strong activation of the peripheral immune system with accompanying neuroinflammation, neurochemical and neurobehavioral effects, including depression (Lacosta et al., 1999, Henry et al., 2008, Biesmans et al., 2013). However, inflammation-associated depression in humans is more closely related to chronic, persistent inflammation (Maes et al., 2009, Krishnadas and Cavanagh, 2012). Higher incidence rate of depressive disorders in patients suffering from chronic inflammatory conditions, as well as in those undergoing interferon-α therapy (Capuron and Miller, 2004, Steptoe et al., 2007, Williams and Steptoe, 2007) indicate close association between chronic inflammation and depression. Despite these epidemiological data, very few experimental studies have investigated the impact of chronic peripheral inflammation on

depressive-like behavior. In female C57BL/6 mice, chronic inflammation induced by repeated intermittent LPS injections produced a chronic state of depression, characterized by long-lasting anhedonic response (decreased sucrose preference) that lasted for 7 weeks (Kubera et al., 2013). Similarly, repeated i.p. administration of LPS (1 mg/kg) for 4 days resulted in depressive behavior in male Wistar rats, evidenced by the increased immobility time in forced swim test that was performed 24 h after the last LPS injection, a time point where the sickness behavior was abolished (Bay-Richter et al., 2011).

h) Focus2-data gaps: summary

Collectively, the above studies suggest that a chronic inflammatory tone is required to cause depressive-like behavior in rodents and that the chronic persistent (rather than the acute) peripheral inflammation more closely simulates the inflammation-associated depression in humans (Biesmans et al., 2013). In addition to the limited experimental data on chronic inflammation-associated depression, there are gaps of knowledge regarding the time-course of behavioral deficits as well as whether or not depression or other emotional changes will persist even after termination of chronic peripheral exposure to inflammatory stimuli. Additionally, compared to central LPS models, very few studies have investigated the influence of chronic peripheral inflammation on alterations suggestive of neurodegenerative changes in the CNS. The present dissertation addresses this missing key information (Fig. 1.5) by investigating the ability of chronic peripheral inflammation induced by chronic LPS treatment in mice to cause neurodegenerative and neurobehavioral changes reflective of neural dysfunctions.

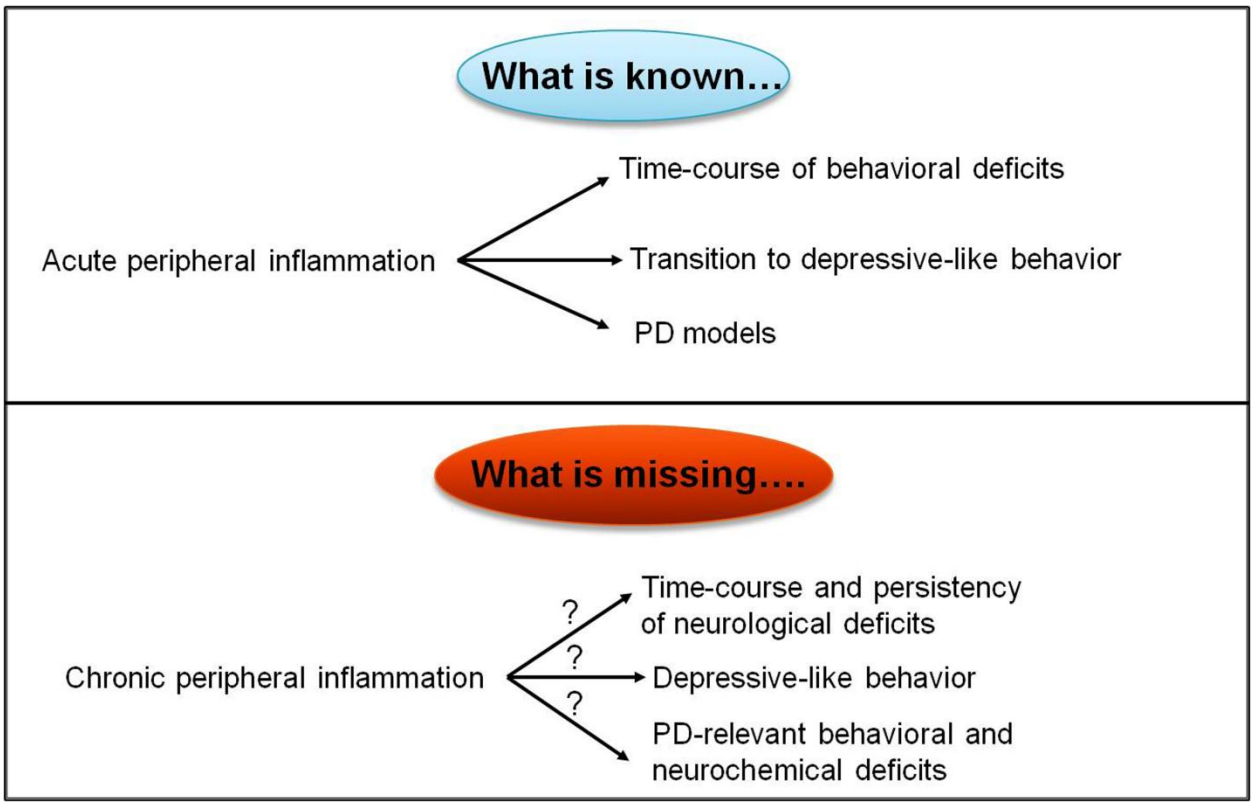

Figure 1.5. Data gaps of chronic LPS induced peripheral inflammation

Focus 3: overnutrition-induced disease (obesity)

a) Obesity

Obesity is now a worldwide epidemic, affecting all segments of the society, irrespective of age, gender and physiological status (Caballero, 2007). Obesity is a condition characterized by excess accumulation of body fat, resulting from either excessive food intake or insufficient physical activity (Despres, 2012). Energy homeostasis is maintained by three components, energy intake, energy expenditure, and energy storage; body weight changes when energy intake does not match with energy expenditure over a given period of time (Hill et al., 2012). Specifically, when energy expenditure exceeds energy intake, a state of negative energy balance occurs, resulting in loss of body mass (60%–80% from body fat) (Hill and Commerford, 1996). On the other hand, when energy intake surpasses energy expenditure, a state of positive energy

balance develops, producing an increase in body mass, of which 60% to 80% is comprised of body fat, resulting in overweight/obesity (Hill and Commerford, 1996). Although, obesity development is mostly attributed to the excessive caloric intake, recent evidence suggests that obesity is not a simple equation connecting energy intake and expenditure, rather, it is a complex neurological process including neurohormonal and neurotransmitter dysregulations (Cai, 2013).

b) Trends and prevalence of adult obesity

Obesity is most commonly measured by BMI, which is calculated by dividing body weight (in kilograms) by the square of height (in meters) (Despres, 2012). In adults, regardless of gender, a BMI score of $\geq$ 25.0 kg/m^2, $\geq$ 30.0 kg/m^2 and $\geq$ 40.0 kg/m^2 is considered overweight, obese and extreme obesity, respectively (Racette et al., 2003). The World Health Organization (WHO) estimates that globally there are more than 1 billion overweight adults, of which 300 million are obese (Greenberg and Obin, 2006). The worldwide prevalence of obesity and related metabolic disorders are increasing alarmingly, irrespective of age, gender and socioeconomic groups (James, 2004). Globally, between 1980 and 2013, the proportion of adults who were overweight or obese increased from 28.8% to 36.9% in men, and from 29.8% to 38.0% in women (Ng et al., 2014). Importantly, in 2013, the US accounted for 13% of the estimated 671 million obese individuals worldwide (Ng et al., 2014).

c) Etiology of obesity

Genetic factors

Genetics play a role in the development of obesity as it was highlighted by the twice much higher concordance rates of obesity among monozygotic than among dizygotic male twin pairs aged 20 (Stunkard et al., 1986). Obesity is a heritable trait and the heritability of the tendency to become overweight or obese was demonstrated by a study wherein the body weight

and amount of fat gain and distribution, in response to overfeeding was found to be similar within than between twin pairs (Bouchard and Tremblay, 1990). Individual's genotype mainly influences bodyweight by regulating energy expenditure and it accounts for 40% of the variability in the daily energy expenditure (excluding vigorous physical activity) (Bouchard and Tremblay, 1990). However, the alarming pace by which obesity has risen in the US and other industrialized countries in the past 2-3 decades indicate that genetic factors cannot be the sole reason for the obesity epidemic as alterations in the genetic makeup of populations occur too slowly to be accountable for this alarming increase (Pereira-Lancha et al., 2012).

Environmental factors

The rapid rise of obesity around the globe is unlikely due to genetic changes, but due to the influence of environmental factors that include the physical, social, and economic surroundings where we live. Importantly, evidence from studies comparing monozygotic, dizygotic, and virtual twins (siblings who are not biologically related, but of the same age and were raised together from infancy) suggest that environmental factors have a greater impact on BMI than was previously thought (Segal and Allison, 2002). Hence, the current consensus is that the obesity epidemic could be due to a potential interaction between several related genetic variants and environmental factors (Racette et al., 2003). Environmental and lifestyle factors, including sedentary behaviors, physical inactivity and unhealthy dietary habits are known to promote excessive caloric intake and induce a positive energy balance leading to increased weight gain (Marti et al., 2004). An inverse relationship between physical activity and adiposity has been demonstrated in multiple studies (Weinsier et al., 1998, van Rossum et al., 2002, Kyle et al., 2004). Increased consumption of food is considered to be an important determinant of the obesity phenotype. The increased food intake is attributed to easy access to food, especially fast

food that is rich in fat and calories, and increased portion sizes (Brantley et al., 2005). Although excessive caloric intake is essential for the development of obesity, it is also the quality of food consumed that determines the obesity phenotype. For example, diets rich in fat produce a greater degree of obesity than carbohydrate-rich diets (Golay and Bobbioni, 1997), due to higher caloric density, greater palatability, shorter chewing and swallowing time, as well as lower satiety level of high-fat diet (HFD) (Astrup et al., 1997). Multiple epidemiological studies have reported positive association between increased fat consumption and subsequent development of obesity (Sonne-Holm and Sorensen, 1977, Price et al., 1993, Popkin et al., 1995, Paeratakul et al., 1998). Several rodent studies have demonstrated HFD-induced obesity in various strains of mice and rats (Bray et al., 1990, West and York, 1998). Conversely, experimental animals consuming low-fat diet (LFD) do not generally develop the obesity phenotype (Bray et al., 2004). Development of obesity in animals fed a HFD is also influenced by genetics. For example, some inbred mouse strains, such as C57BL/6 and AKR/J, are more susceptible to obesity when fed HFD compared to other strains of mice, such as SWR/J (West et al., 1992, Alexander et al., 2006). Particularly, the C57BL/6 mouse strain is a good model for studying human obesity as it simulates the human metabolic abnormalities (hyperinsulinemia, hyperglycemia, and hypertension) when fed ad libitum with a HFD, while remaining lean without showing any metabolic irregularities when fed a LFD (Collins et al., 2004).

d) Obesity pathogenesis

Obesity is a condition caused by the imbalance between energy intake and energy expenditure that is reflected as stored fat and is characterized by chronic-low grade inflammation in both central and peripheral tissues (Thaler and Schwartz, 2010) (Fig. 1.6). Increased adipose tissue is associated with an enlargement of individual fat cells, called adipocytes that synthesize

and release several peptides, that drive obesity development (Bray, 1999). Leptin is one of the most important adipocyte-derived peptides that can influence the food intake through its direct effect on the hypothalamus (Kaila and Raman, 2008). Specifically, leptin acts by feedback inhibition of hypothalamic orexigenic pathways (neurons that express neuropeptide Y and agouti-related peptide) and stimulates anorexigenic neurons (neurons that express proopiomelanocortin) in response to an increase in body fat mass (Thaler et al., 2013). However, in obese states, wherein the energy intake continuously exceeds energy expenditure, the key neurocircuits in the hypothalamus fail to respond to the negative feedback control exerted by leptin, resulting in a condition called leptin resistance (Thaler et al., 2013). Emerging evidence suggest that leptin resistance is not only a causal factor for obesity, but it may be the result of obesity-associated hypothalamic inflammation (Wisse and Schwartz, 2009). Hypothalamic inflammation also contributes to the obesity-associated insulin resistance in peripheral tissues such as liver and adipose tissue.

The mechanism by which inflammatory signaling in the hypothalamus contributes to leptin and insulin resistance is by the dysregulation of signal transduction pathways downstream of both insulin and leptin receptors, mainly via the insulin receptor substrate (IRS)–phosphatidylinositol 3-kinase pathway and mitogen activated protein kinase (MAPK) pathways (Morton et al., 2006, Myers et al., 2008). Elevated levels of TNF-α associated with hypothalamic inflammation can inhibit the auto-phosphorylation of tyrosine residues of the insulin receptor (IR) and can induce serine 307 phosphorylation of IRS-1, leading to insulin resistance (Nieto-Vazquez et al., 2008). MAPKs, namely, c-Jun N-terminal kinase (Jnk) and inhibitor of nuclear factor-κB kinase (IKKβ) can inhibit insulin and leptin signaling by inducing the signal termination molecule suppressor of cytokine signaling-3 (SOCS3) and protein tyrosine

phosphatase (PTP)-1B as well as by the serine phosphorylation of the IRS (Gregor and Hotamisligil, 2011, Velloso and Schwartz, 2011). In addition to the hypothalamic inflammation, excess nutrient intake also triggers inflammation in peripheral tissues, such as liver and adipose tissue (Thaler and Schwartz, 2010). Specifically, excess visceral adiposity is characterized by increased macrophage infiltration of the adipose tissue and associated overproduction of proinflammatory cytokines such as TNF-α and IL-6, which can act in turn via intracellular pathways, including Jnk and NFκB to cause insulin resistance (Bastard et al., 2006). In conclusion, inflammatory signaling in both the hypothalamus and in peripheral tissues plays an important role in the pathogenesis of obesity via induction of insulin and leptin resistance. Importantly, the inflammation-induced insulin and leptin resistance can in turn promote obesity and associated metabolic syndrome, such as diabetes, thereby creating a vicious cycle (Thaler and Schwartz, 2010).

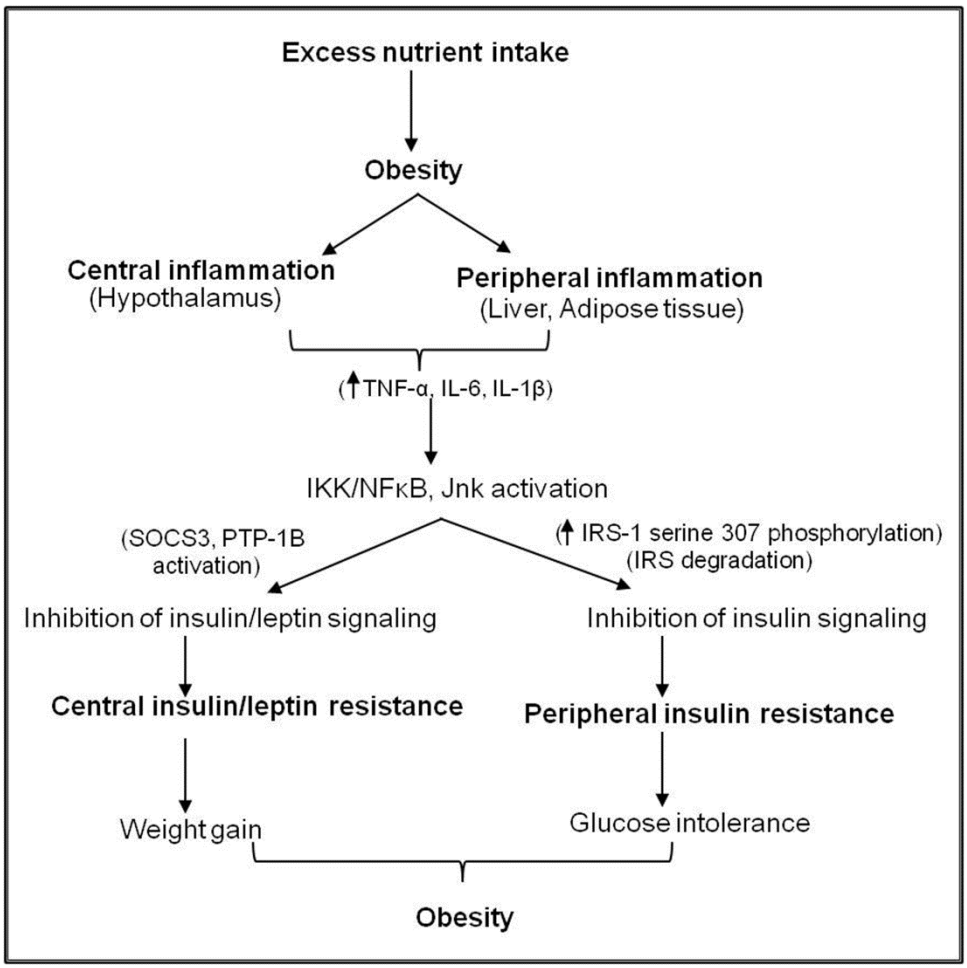

Figure 1.6. Inflammation and obesity pathogenesis. Modified and adapted from (Thaler and Schwartz, 2010)

In addition to the crucial role of hypothalamic inflammation in obesity pathogenesis, several studies have demonstrated that the chronic low-grade inflammation associated with obesity can affect other brain structures, such as the prefrontal cortex and hippocampus (Miao et al., 2013, Rojo et al., 2013). For example, hippocampal inflammation evidenced by increased protein levels of TNF-α and associated decrease in cognitive function was observed in male mice fed a HFD for 20 weeks (Jeon et al., 2012). Also, increased anxiety with associated reductions in the level of the anti-anxiety factor, neuropeptide Y, as well as altered synaptic plasticity with an associated decrease of brain derived neurotrophic factor (BDNF) was observed in the frontal

cortex and hippocampus of male rats fed a HFD for 3 weeks (Sharma et al., 2012). Studies have also reported that hippocampal and frontal lobe volume reduction was associated with increased BMI (Bruehl et al., 2011, Cazettes et al., 2011).

e) Mechanistic link between obesity and neurological disorders

As already stated, obesity is associated with an increased inflammatory signaling in the hypothalamus and other brain structures such as prefrontal cortex and hippocampus (Wisse and Schwartz, 2009). This obesity-induced neuroinflammation is dependent in large part on the cell membrane receptor TLR4 and on cytokine receptors, such as TNF-α receptors, both leading to activation of the IKKβ/NFκB intracellular signaling pathways (Romanatto et al., 2009, Konner and Bruning, 2011, Milanski et al., 2012). Besides the TLR-4 and TNF-α receptors, obesity-induced neuroinflammation is mediated by the receptor-independent mechanisms, via intracellular oxidative stress that is mainly of mitochondria and endoplasmic reticulum origin and it also activates the IKKβ/NFκB pathway (Zhang et al., 2008, Zhou et al., 2012). Additionally, dysfunction of mitochondria and ER can influence autophagy and can subsequently result in autophagic defects (Meng and Cai, 2011). Increased activation of the IKKβ/NFκB pathway can lead to an increased production of the IKKβ/NF-κB-dependent cytokines such as TNF-α and IL-1β from glial cells, mainly microglia. Because of the important role of IKKβ/NFκB pathway in the control of cell survival and apoptosis, its overactivation can disrupt the survival of the neural stem cells (NSCs) as well as inhibit their neuronal differentiation (Li et al., 2012, Zhang and Hu, 2012). Collectively, obesity-induced activation of the IKKβ/NFκB pathway and subsequent release of TNF-α, IL-1β and other neurotoxic products can hinder the survival of neural stem cells and neurogenesis (Fig.1.7), further leading to neurodegenerative

(Cai, 2013) and neurodevelopmental disorders (Fatemi et al., 2008, El-Ansary and Al-Ayadhi, 2012).

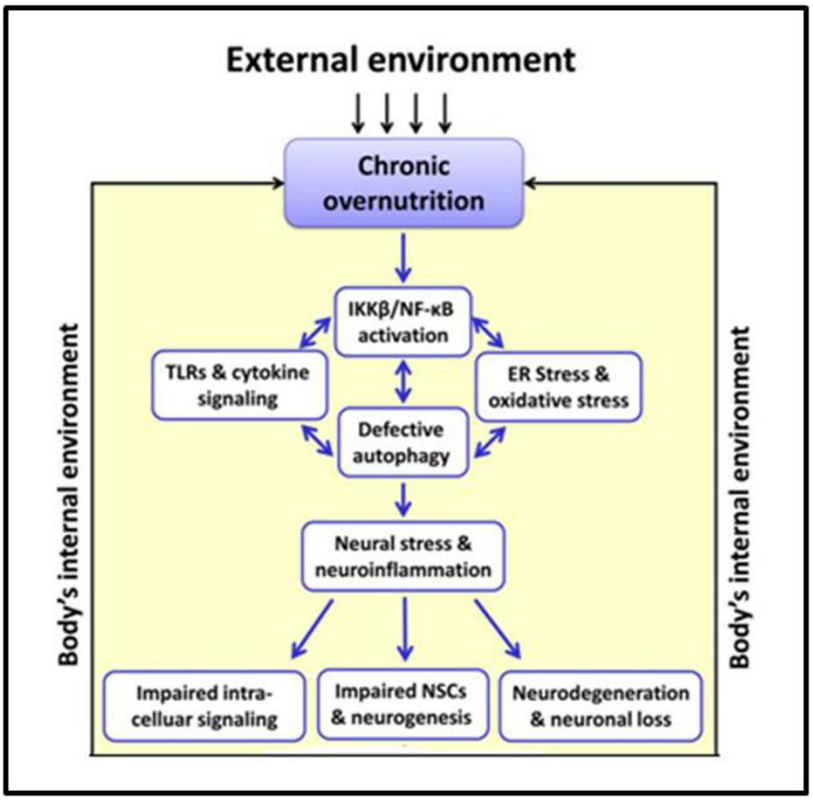

Figure 1.7. Mechanistic link between obesity and neurological disorders. Adapted and modified from (Cai, 2013)

f) Obesity and associated comorbidities

Obesity is associated with an increased risk of, among others, type 2 diabetes mellitus (DM2), hypertension, coronary heart disease (CHD), dyslipidemia, and certain cancers (endometrial, breast, colon, and prostate) (Pi-Sunyer, 1999).

Diabetes mellitus (DM)

Obesity induced insulin resistance and subsequent hyperinsulinemia plays an important role in the development of DM2 (Khaodhiar et al., 1999). In obese subjects, excess adipocytes release increased levels of free fatty acids and pro-inflammatory cytokines like TNF-α and IL-6,

which partly contribute to the insulin resistance (Bastard et al., 2006, Greenberg and Obin, 2006). Increased free fatty acid levels can inhibit muscle glucose utilization, increase hepatic glucose output and stimulate pancreatic insulin release, resulting in hyperinsulinemia (Bastard et al., 2006). Multiple cross-sectional and longitudinal studies have demonstrated the strong association between impaired glucose tolerance, insulin resistance and DM2 and overweight and obesity (Holbrook et al., 1989, Haffner et al., 1990, Pi-Sunyer, 1990, Lipton et al., 1993).

Dyslipidemia

In addition to impaired glucose homeostasis, increased circulating levels of fasting total cholesterol and triglycerides and decreased levels of high-density lipoprotein cholesterol (HDL-C) are key obesity attributes (Pi-Sunyer, 1999, Paccaud et al., 2000, Misra and Shrivastava, 2013).

Cardiovascular effects

Obesity-associated alterations in blood pressure are related to the insulin and leptin-mediated activation of the sympathetic nervous system and the activation of the renin-angiotensin system (Landsberg et al., 2013). Higher prevalence of increased blood pressure in obese individuals, suggestive of obesity being a risk factor for hypertension was reported (Landsberg et al., 2013). Obesity is an independent risk factor for CHD (Poirier et al., 2006), and it also enhances the risk of cardiovascular diseases because of its positive association with hypertension, DM2, low HDL-C, and high total cholesterol (Guh et al., 2009).

Cancer

Obesity is also considered to be one of the main preventable causes of cancer (Haslam and James, 2005). Increased mortality rates for colorectal and prostate cancer in obese men and

endometrial, cervical, ovarian, and postmenopausal breast cancer among obese women was reported in the American Cancer Society study (Pi-Sunyer, 1999).

Neurological: Cognitive and mood disorders

Besides the above mentioned obesity-associated comorbidities, increasing evidence has demonstrated the adverse effect of excessive weight on the brain. The negative association between obesity and cognition is well-documented (Elias et al., 2003, Naderali et al., 2009). Obesity is linked to an increased risk of developing AD and dementia (Kivipelto et al., 2005, Whitmer et al., 2007, Whitmer et al., 2008). Structural changes in the brain, including atrophy of the frontal and temporal lobes, white matter alterations in the frontal regions and pathophysiological alterations, characterized by central and peripheral inflammation, as well as insulin and leptin resistance have all been associated with the obesity-induced cognitive impairments (Businaro et al., 2012, Sellbom and Gunstad, 2012). In addition to cognitive impairments, multiple epidemiological and animal studies have also reported a link between obesity and emotional imbalance. For example, obesity was associated with a significant increase in the lifetime diagnosis of major depression and anxiety disorders in a nationally representative sample of US adults (Simon et al., 2006). Heightened anxiety, along with increased central (hippocampal) and peripheral (plasma) pro-inflammatory cytokines was observed in a mouse model of metabolic syndrome, characterized by obesity, hyperglycemia, insulin-resistance and hyperinsulinemia (Dinel et al., 2011).

Neurological: PD

Though relatively few, data linking obesity and PD, have also been reported (Chen et al., 2004, Morales-Briceno et al., 2012). While there are reports where no significant association between altered risk of PD and BMI, weight change, or waist circumference was found in a large

prospective cohort of US men and women (Palacios et al., 2011), several studies report a positive association. Thus, a recent study showed that overweight might be a potential risk factor for PD (Chen et al., 2014). Similarly, the risk of PD was found to be twice as high in obese men (BMI over 30) than in those with BMI under 23, and about 70% higher risk among obese women in a Finnish cohort (Hu et al., 2006). Multiple animal studies have also provided evidence in support of the idea that obesity can accelerate PD pathogenesis. A recent study showed that diet-induced obesity accelerated the age of onset of brainstem α-synucleinopathy (neuropathological hallmark of PD) in male transgenic mice expressing the human mutant A30P α-synuclein neuronally (Rotermund et al., 2014). Interestingly, exacerbation of 6-hydroxydopamine (6-OHDA)-induced nigrostriatal neurodegeneration, evidenced by significant DA depletion in both substantia nigra and striatum was observed in rats fed HFD for 5 weeks (Morris et al., 2010). Taken together, the evidence from both epidemiological and experimental studies indicates obesity as an emerging environmental risk factor for PD (Zhang and Tian, 2014).

g) Focus 3-data gaps: summary

Collectively, several animal studies have demonstrated that HFD consumption can alter neuronal function, impair neurogenesis and induce neurodegenerative changes (Lindqvist et al., 2006, de la Monte et al., 2009). However, it is worth noting that majority of these studies on the neurological consequences of HFD consumption are mostly done with male rodents; very limited information exists with females. More importantly, the reported neurological aftereffects are in advanced obese phenotypes produced by long-term HFD exposure; information on the relatively early effects of HFD consumption on the brain is limited. It is also noteworthy that experimental studies which investigated peripheral effects of HFD consumption, including metabolic and inflammatory changes, have used primarily males and have employed a single dietary exposure

duration paradigm. Considering the long-term, chronic nature of high-fat intake, as well as the occurrence of obesity epidemic in a gender-independent manner, it is imperative to conduct animal studies that can investigate the female-specific, time-dependent peripheral effects of chronic HFD consumption. This dissertation research addresses the above data gaps (Fig. 1.8) by investigating the relatively early CNS effects, as well as the time-dependent peripheral effects of HFD consumption in adult female mice.

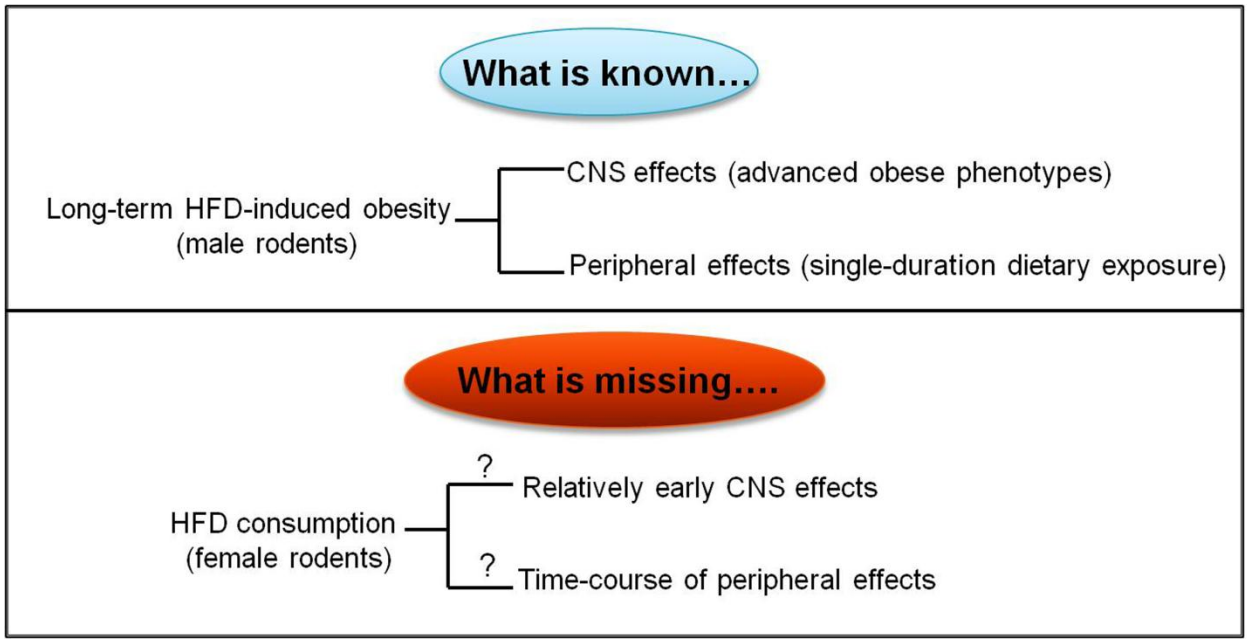

Figure 1.8. Data gaps in adult obesity

h) Maternal obesity: adverse metabolic effects on the offspring

Obesity during pregnancy increases the risk for adverse pregnancy outcomes, including preeclampsia, gestational diabetes mellitus (GDM), and cesarean delivery (Lynch et al., 2008). In the US, nearly one in two women of childbearing age is considered either overweight or obese (Vahratian, 2009). Maternal obesity is a major public health concern in the Western countries, where 28% of pregnant women are overweight and 11% are obese. In the United Kingdom, 33% of women are categorized as overweight and 23% are obese, i.e., a total of 56% are over the

recommended BMI (Bhattacharya et al., 2007). Several studies have reported an increased risk of childhood and adulthood obesity among offspring of obese mothers (Janjua et al., 2012). Also, higher maternal prepregnancy body weight gain has been linked to an increased childhood and adulthood adiposity among offspring (Rooney and Ozanne, 2011). Obesity during pregnancy is also associated with increased incidences of increased birth weight, and fetal overgrowth (Jones et al., 2009). Maternal obesity prior to pregnancy is linked to fetal macrosomia (birthweight > 4 kg), which is associated with a higher risk of overweight and obesity in childhood and adulthood (Boney et al., 2005b, Catalano and Ehrenberg, 2006).

In addition to the increased risk of offspring obesity, maternal obesity during pregnancy is also associated with adverse metabolic outcomes in offspring. For example, babies born to obese mothers were found to be insulin resistant indicating that maternal obesity can pose an increased risk for metabolic derangements in the next generation, which was apparent even at birth (Catalano et al., 2009). Similarly, a positive association between maternal BMI and neonatal hyperinsulinemia was reported by the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study (Metzger and Grp, 2010). Importantly, the higher risk of developing insulin resistance reported in 11-year-old offspring of obese mothers highlights the persistent influence of maternal obesity on insulin sensitivity (Boney et al., 2005b). In addition to the positive association between maternal obesity and altered offspring glucose/insulin homoeostasis, increased body weight gain prior to or during pregnancy is associated with high blood pressure, plasma lipid profile dysregulation, and increased heart problems such as CHD in the offspring (Forsen et al., 1997, Laor et al., 1997, Hochner et al., 2012, Moraeus et al., 2012).

i) Maternal obesity and maternal inflammation

The above evidence demonstrating the adverse effect on metabolic outcomes in offspring suggest that the negative influence of maternal obesity on the offspring most likely starts in utero and is a multi-generational risk factor (Pardo and Eberhart, 2007, Gardener et al., 2009). Pregnancy is considered to be a natural inflammatory state evidenced by the activation of leucocytes and increased plasma concentration of acute phase proteins and cytokines (Sacks et al., 2004). The basal inflammatory state of pregnancy is exacerbated by pregnancy complications such as preeclampsia and GDM (Sacks et al., 1998, Kirwan et al., 2004). Interestingly, a similar exaggeration of the basal inflammatory state of pregnancy was observed in obese women, evidenced by the increased circulating concentrations of IL-6, as well as by the 2-fold increase in the number of placental $CD14^+$ and $CD68^+$ macrophages expressing elevated levels of TNF-α and IL-6 (Challier et al., 2008). The association of maternal systemic and placental inflammation demonstrates the existence of heightened inflammatory in utero environment in obese women with increased potential for negative impact on the offspring.

j) Maternal inflammation and autism spectrum disorder (ASD)

Maternal infection and subsequent immune activation has been linked to an increased incidence of ASD in the offspring of mothers who suffer infections during pregnancy (Patterson, 2002, Shi et al., 2005, Parker-Athill and Tan, 2010). ASD is a group of developmental disorders characterized by impairments in reciprocal social interaction and communication skills compounded by increased stereotyped restrictive/repetitive behaviors and interests (Faras et al., 2010). Included in this spectrum is the classical autism or autistic disorder, which is the most severe form of ASD and usually encompasses the stereotypical social isolation, impaired verbal communication, intellectual disabilities and repetitive behaviors; Asperger syndrome, often described as a milder form of ASD, is characterized by normal language development, but with

difficulties in social interaction and less-intense repetitive behaviors; pervasive developmental disorder-not otherwise specified (PDD-NOS), or the atypical ASD, usually involves disorders that cannot otherwise be categorized (Parker-Athill and Tan, 2010).

Maternal inflammation and associated manifestation of ASD-like behavioral phenotype in offspring has also been demonstrated in animal models. Maternal immune activation by a single i.p. injection of IL-6 on gestational day (GD) 12.5 of mouse pregnancy produced behavioral deficits in adult offspring, characterized by pre-pulse inhibition (PPI) deficit and lack of significant latent inhibition (LI) (Smith et al., 2007); both PPI deficits and lack of LI are observed in mental disorders such as schizophrenia and ASD (Weiner et al., 2003, Perry et al., 2007). In the same study, another experiment conducted to induce maternal immune activation by a single i.p. injection of the viral mimic, polyriboinosinic: polyribocytidylic acid (poly IC) on GD 12.5 of mouse pregnancy also resulted in ASD-like phenotype in adult offspring, characterized by increased anxiety, decreased social interaction, PPI deficit and lack of LI (Smith et al., 2007). Significant reduction of PPI in adult offspring following prenatal treatment with poly IC was also observed in rats (Wolff and Bilkey, 2008). Overall, these studies suggest that activation of the maternal immune response and subsequent maternal inflammation are detrimental to the developing brain and can lead to long-term behavioral disorders in the offspring of ASD nature.

k) Maternal obesity and neurodevelopmental effects in the offspring

Besides the metabolic dysregulation that maternal obesity causes on the offspring, epidemiological studies also associate maternal obesity with abnormal brain development and behavior. In addition to a strong genetic component (Crawley, 2012), several environmental factors, including maternal overnutrition can pose an increased risk for ASD in the offspring.

Earlier studies identified weight gain during pregnancy as a risk factor for ASD in the offspring (Stein et al., 2006). A recent study reported that the likelihood of obese mothers to have a child with ASD was 67% more as diagnosed by standardized assessments (Krakowiak et al., 2012). Also, a significant association between maternal obesity and neurodevelopmental delay was demonstrated in this study as measured by Mullen Scales of Early Learning scores (Krakowiak et al., 2012). Furthermore, pre-pregnancy maternal weight of ≥ 90 kg as well as weight gain of 18 kg during pregnancy were reported to be independent risk factors for the development of ASD (Dodds et al., 2011). In addition to the emerging evidence of the link between maternal obesity and ASD, significant positive association of maternal obesity with another behavioral disorder, ADHD, has also been reported (Rodriguez et al., 2008). A study conducted in 5-year-old children showed that those born to obese mothers have a higher risk of developing ADHD symptoms and negative emotionality as reported by both kindergarten teachers and mothers (Rodriguez, 2010). Of note, ASD has higher rates of comorbidity with other neurodevelopmental disorders, such as ADHD and FXS (Kover and Abbeduto, 2010, Jang et al., 2013).

l) Therapeutic interventions for neurodevelopmental disorders

In general, the treatment strategies adopted for the major neurodevelopmental disorders are all symptomatic. For example, stimulants which potentiate brain dopaminergic and noradrenaline transmission, such as methylphenidate and dextroamphetamine are used to improve the focus, attention, cognition and reaction time as well as to decrease the hyperactivity and restlessness seen in ADHD patients (Kolar et al., 2008, Dias et al., 2013). Currently, the first line of therapy for ASD includes two antipsychotics, namely, aripiprazole (partial agonist of DA) and risperidone (DA and 5-HT antagonist) that can alleviate the hyperactivity and irritability symptoms in autistic patients (McPheeters et al., 2011). However, these drugs are not beneficial

in treating the core symptoms of ASD, including social and language deficits as well as repetitive behaviors (Silverman et al., 2012). A very recent report demonstrated that administration of GRN-529, a selective negative allosteric modulator of the metabotropic glutamate receptor subtype 5 (mGluR5, already implicated in FXS, a rare inherited syndrome that causes both intellectual disability and autistic behaviors) improved the repetitive behavior, stereotypic jumping, and lack of sociability in two genetic mouse models of ASD, the BTBR mouse and the C58/J mouse (Silverman et al., 2012). The pharmacological approaches used so far to treat ASD have mainly aimed at correcting the dysregulated neurotransmitter (DA and 5-HT) systems and altered glutamate receptor levels.

In spite of the proposed significant contribution of immune dysfunction in the pathogenesis of ASD (Vargas et al., 2005), none of the above-described therapeutic interventions target the immune system. However, recent studies have reported the beneficial role of anti-inflammatory agents in ASD treatment. For example, ASD children who took dietary supplement with the natural flavonoid, luteolin, that has potent anti-oxidant and anti-inflammatory activity showed improvement in their overall behaviors, such as hyperactivity, increased lethargy and inappropriate speech (Taliou et al., 2013). Intriguingly, anti-inflammatory interventions have been used to treat other neurological disorders, including multiple sclerosis. Administration of the anti-inflammatory agent, lacto-N-fucopentaose III (LNFPIII), a glycan found on schistosome parasites and in human milk successfully reduced the severity of experimental autoimmune encephalomyelitis (frequently used model for multiple sclerosis) and associated neuroinflammation in female C57BL/6 mice (Zhu et al., 2012). Interestingly, administration of LNFPIII reduced white adipose tissue inflammation and improved metabolic functions in adult male obese mice fed with a high-fat, high-carbohydrate diet for 6 weeks (Bhargava et al., 2012).

Of note, the above-mentioned studies have assessed the efficacy of anti-inflammatory therapies for neurological disorders in adults or in offspring. However, in light of the significant influence of the intrauterine environment on the normal fetal brain development, it is crucial to explore the contribution of maternal factors, such as maternal inflammation on the development of neurological disorders in offspring and initiate effective therapeutic strategies by manipulating the maternal environment. However, to date, no studies have investigated the efficacy of maternal anti-inflammatory interventions in mitigating the development of neurological deficits in the offspring. More specifically, no such approach has been tested in the context of ASD and maternal obesity.

m) Focus 3-data gaps: summary

Several lines of evidence from epidemiological studies suggest that pre-pregnancy obesity and excessive weight gain during pregnancy can lead to ASD and other neurodevelopmental disorders in the offspring. Considering the significant contribution of HFD consumption in promoting obesity, it is noteworthy that there are no studies on the precise role of diet-induced maternal obesity and ensuing maternal inflammation as a causal factor for ASD. Additionally, no studies have investigated the efficacy of maternal anti-inflammatory treatments in mitigating the development of neurological deficits in the offspring. The present dissertation research addresses this missing key information (Fig. 1.9) by investigating the contribution of HFD-induced maternal obesity in the etiology of ASD-like behavior in the offspring as well as by assessing the ability of maternal administration of anti-inflammatory glycan to alleviate the neurodevelopmental deficits in the offspring caused by maternal HFD.

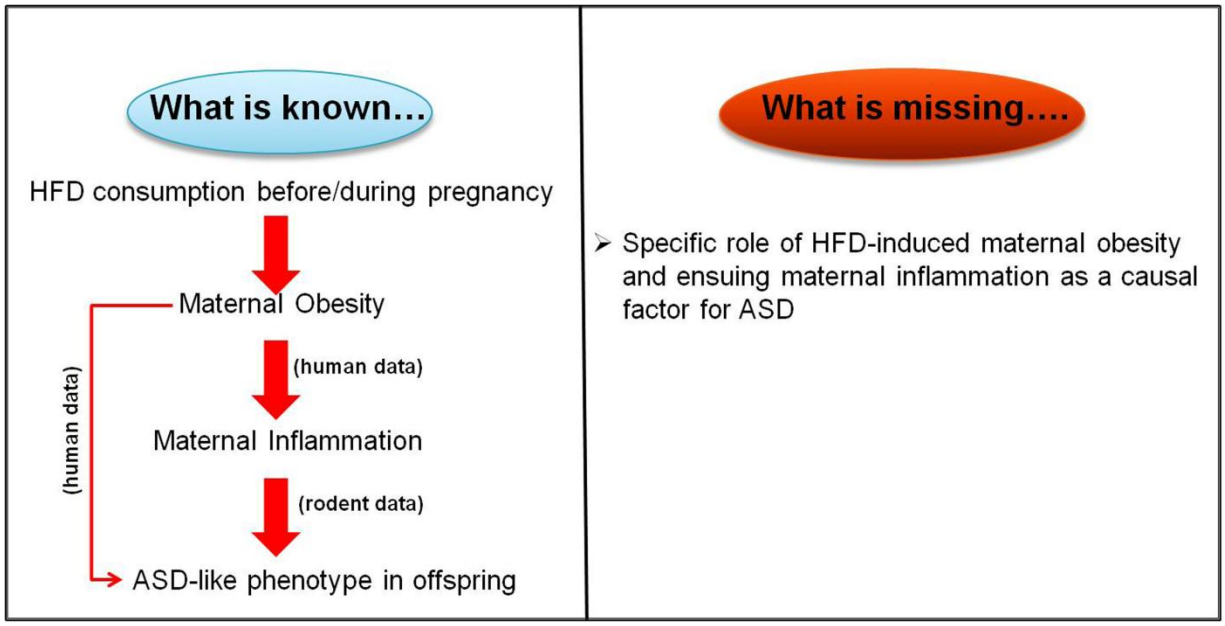

Figure 1.9. Data gaps in maternal obesity

Overall objectives, hypothesis, and specific aims

a) Overall objective

To determine the role of central or peripheral inflammation in the development of neurological deficits/metabolic dysregulation caused by disparate environmental triggers (Mn/LPS/HFD) and thereby to establish a mechanism by which these different environmental factors might contribute to the development of neuronal dysfunctions (neurochemical and/or behavioral) and/or metabolic derangements.

b) Overarching hypothesis

Overexposure to environmental triggers (Mn/LPS/HFD) for different periods, ranging from short- to long-term duration or vulnerability windows, will modulate inflammatory pathways and thereby induce heightened inflammation, both peripherally and centrally, resulting in altered neuronal function (at neurochemical/molecular & behavioral level) and/or metabolic dysregulation reminiscent of metabolic syndrome.

c) Overall hypothesis was addressed in the following Specific Aims:

Specific Aim 1: Investigate the CNS dysregulation caused by subchronic Mn exposure via drinking water (DW) in adult mice. To accomplish this aim, adult male C57BL/6 mice were exposed to low-level of Mn (0.4g Mn/l) via DW for a period of 8 weeks; behavioral, neurochemical and molecular effects of Mn DW exposure were evaluated. We hypothesized that sub-chronic Mn exposure via DW will increase the brain Mn deposition and will produce adverse effect (at neurochemical and/or molecular level) on basal ganglia structures resulting in neurobehavioral alterations in adult mice.

Specific Aim 2: Evaluate specific neurochemical responses, molecular changes and behavioral alterations in adult C57BL/6 male mice with low-grade chronic inflammation caused by repeated peripheral LPS administration. To achieve this aim, adult male C57BL/6 mice were exposed to peripheral intraperitoneal injections of low-level LPS (0.25 mg/kg BW; twice weekly) for up to 25 weeks. Additionally, to gain an insight into the persistent effects of chronic peripherally induced neuroinflammation (at neurochemical, molecular and behavioral levels), we maintained a separate set of mice exposed for 3 months to repeated low-dose LPS, followed by a 3-month wait period during which the treatment was discontinued. We hypothesized that adult mice with low-grade chronic inflammation caused by repeated peripheral LPS treatment will exhibit behavioral deficits and that these behavioral changes will correlate with increased neuroinflammation and regionalized neurochemical alterations.

Specific Aim 3: Investigate the relatively early central (neurochemical, behavioral and electrophysiological) effects of HFD consumption in adult female C57BL/6 mice. To achieve this aim, adult female C57BL/6 mice were placed on a LFD (10% kcal fat) or HFD (60% kcal fat) for a period of 11-12 weeks. Selected behavioral tests were performed after 5 weeks of LFD/HFD

intake; one week later, mice were sacrificed and brains were harvested for neurochemical analysis. Synaptic plasticity was determined in ex vivo ventral hippocampal slices from the remaining behaviorally naïve mice after an additional 5-6 weeks of LFD or HFD feeding. We hypothesized that HFD consumption even for a relatively short duration will impair certain behavioral domains and cause associated alterations in the brain monoamine homeostasis and ventral hippocampal synaptic plasticity.

Specific Aim 4: Determine the relatively early peripheral (metabolic and inflammatory) effects of HFD in adult female C57BL/6 mice. To accomplish this aim, metabolic tests such as glucose and insulin tolerance tests (GTT and ITT) were performed to assess the HFD-induced alterations in glucose homeostasis and insulin sensitivity after 5 weeks of HFD consumption. Additionally, one week later, inflammatory/insulin signaling profiles were determined in key peripheral tissues (liver, adipose tissue and muscle). We hypothesized that HFD feeding even for a relatively short duration can cause metabolic dysregulations with an associated increase in the peripheral inflammatory tone.

Specific Aim 5: Determine the time-dependent central (behavioral) and peripheral (metabolic/inflammatory) effects of HFD in adult female C57BL/6 mice. To accomplish this aim, multiple neurobehavioral tests that can effectively assess the locomotor, emotional and cognitive function of mice were employed after 21 and 32 weeks of HFD consumption. For the peripheral effects, glucose and insulin tolerance tests (GTT and ITT) were performed after 20 and 33 weeks of HFD intake to assess the HFD-induced time-dependent alterations in glucose homeostasis and insulin sensitivity. Additionally, qPCR was conducted to analyze the HFD-induced alterations in the expression of multiple metabolic and inflammatory markers in the liver after 22 and 36 weeks of HFD feeding. We hypothesized that continued HFD consumption will induce an obese

phenotype in females that will be associated with an exaggeration of the neurological deficits and/or metabolic derangements.

Specific Aim 6: Determine the neurochemical and ASD-like behavioral deficits of the offspring that are caused by maternal HFD consumption and investigate the efficacy of maternal-only administration of a potent anti-inflammatory glycan in ameliorating offspring's neuropathology caused by maternal HFD consumption. To accomplish this aim, female C57BL/6 mice were fed either a LFD or HFD for 6 weeks prior to and during mating, gestation and lactation. After 6 weeks on respective diets, females were injected (s.c.) twice per week with dextran or glycan prior to mating until weaning of the offspring. Pups were sexed and weaned on PND 21 and thereafter maintained on regular chow. After 2 weeks (PND 35), mice underwent behavioral tests followed by regional neurochemical analysis in brain. We hypothesized that maternal HFD consumption will induce ASD-like neurobehavioral deficits in the PND 35 offspring that will correlate with altered brain monoamine homeostasis and that maternal administration of the anti-inflammatory glycan will limit maternal HFD-induced neurochemical alterations/behavioral abnormalities in these offspring.

CHAPTER 2

BRAIN DEPOSITION AND NEUROTOXICITY OF MANGANESE IN ADULT MICE EXPOSED VIA THE DRINKING WATER[1]

[1]Krishna, S., Dodd, C.A., Hekmatyar, S.K., Filipov, N.M. 2014. *Archives of Toxicology*. 88:47-64. Reprinted here with the permission of the publisher.

Abstract

Natural leaching processes and/or anthropogenic contamination can result in ground water concentrations of the essential metal manganese (Mn) that far exceed the current regulatory standards. Neurological consequences of Mn drinking water (DW) overexposure to experimental animals, i.e. mice, including its brain deposition/distribution and behavioral effects are understudied. Adult male C57BL/6 mice were exposed to Mn via the DW for 8 weeks. After 5 weeks of Mn exposure, magnetic resonance imaging revealed significant Mn deposition in all examined brain regions; the degree of Mn deposition did not increase further a week later. Behaviorally, early hyperactivity and more time spent in the center of the arenas in an open field test, decreased forelimb grip strength and less time swimming in a forced swim test were observed after 6 weeks of Mn DW exposure. Eight-week Mn DW exposure did not alter striatal dopamine, its metabolites, or the expression of key dopamine homeostatic proteins, but it significantly increased striatal 5-hydroxyindoleacetic acid (a serotonin metabolite) level, without affecting the levels of serotonin itself. Increased expression (mRNA) of glial fibrillary acidic protein (GFAP, an astrocyte activation marker), heme oxygenase-1 and inducible nitric oxide synthase (oxidative and nitrosative stress markers, respectively) were observed 8 weeks post Mn DW exposure in the substantia nigra. Besides mRNA increases, GFAP protein expression was increased in the substantia nigra pars reticulata. In summary, the neurobehavioral deficits, characterized by locomotor and emotional perturbations, and nigral glial activation associated with significant brain Mn deposition are among the early signs of Mn neurotoxicity caused by DW overexposure.

Keywords: Manganese, Behavioral deficits, Serotonin imbalance, Astrocyte activation, T1 relaxation time

Introduction

Although manganese (Mn) is an essential transition metal (Talavera et al., 1999, Erikson et al., 2007b, Anderson et al., 2008), exposure to excessive Mn levels in either occupational or environmental settings may cause neurological dysfunction of the basal ganglia (Takeda, 2003, Aschner et al., 2009, Kinawy, 2009). The major neurological disorder caused by excessive Mn exposure, manganism, consists of many symptoms that are similar to Parkinson's disease (PD), i.e., postural instability, rigidity, and speech disturbances (Rajput et al., 1991, Pal et al., 1999, Cersosimo and Koller, 2006, Lucchini et al., 2009).

Occupational exposure to Mn via inhalation remains a major concern (Dorman et al., 2006b, Erikson et al., 2007a); however, exposure to Mn via contaminated drinking water (DW) is increasingly associated with adverse neurological outcomes (Ljung and Vahter, 2007). For example, exposure to Mn-contaminated well water is associated with increased infant mortality, memory deficits and lower intelligence scores in children (Woolf et al., 2002, Wasserman et al., 2006, Hafeman et al., 2007, Bouchard et al., 2011). Moreover, the Bouchard study emphasized the increased risk of overexposure to Mn through DW rather than diet as the Mn intake from water, but not diet, correlated significantly with Mn deposition in children's hair samples (Bouchard et al., 2011). Taken together, the epidemiological data suggest that ingestion of Mn through DW can be neurotoxic, especially to younger populations. However, neurological consequences of Mn overexposure via consumption of contaminated DW by adults remain unclear. Some early epidemiological studies (Kondakis et al., 1989), but not others (Vieregge et al., 1995), reported neurological impairment in elderly people after long-term exposure to Mn from DW. Even though the (Kondakis et al., 1989) study lacked a detailed exposure scenario description, it certainly underscored the potential for adverse health effects associated with

chronic consumption of Mn contaminated DW. In this regard, life-time exposure via multiple routes to moderately excessive Mn is considered a risk factor for the development of PD (Lucchini et al., 2009).

Multiple studies have reported Mn-induced locomotor deficits and emotional disturbances, such as anxiety and/or depression in humans (Bowler et al., 2003, Laohaudomchok et al., 2011) and laboratory animals (Olanow et al., 1996, Witholt et al., 2000, Dodd et al., 2005, Lazrishvili et al., 2011). Limited laboratory studies have focused on the neurobehavioral consequences of Mn DW exposure; locomotor deficits have been demonstrated in few rat studies (Avila et al., 2010, Fordahl et al., 2012) and in a single early study in mice (Chandra et al., 1979). It is also worth noting that there is very little information on the non-motor impairments (i.e., anxiety, depression) induced by Mn via DW exposure in rodents (Lazrishvili et al., 2011) as compared with other exposure routes (Liu et al., 2006, Hogas et al., 2011a). Mn-induced neurochemical changes in the basal ganglia, mainly in the striatal dopamine (DA) and serotonin (5-HT) levels have been demonstrated by several studies (Hirata et al., 2001, Tran et al., 2002, Struve et al., 2007); the DA and 5-HT alterations in the striatum caused by Mn have been implicated in the development of motor and emotional impairments, respectively (Moreno et al., 2009). Of note, the rodent studies that examined Mn-induced alterations of DA and/or 5-HT homeostasis in a DW experimental paradigm were with rats (Eriksson et al., 1987, Subhash and Padmashree, 1991); mouse data are non-existent.

In addition to the direct, oxidative stress-dependent toxic effect of Mn on neurons, (Zhang et al., 2004, Milatovic et al., 2009), recent evidence has highlighted the ability of Mn to damage neurons indirectly by causing and/or enhancing glial cell activation (Filipov et al., 2005, Zhao et al., 2009, Filipov and Dodd, 2012). In vivo, several researchers have reported Mn-

induced reactive gliosis and elevated expression of inflammatory cytokines following inhalation (Antonini et al., 2009), intragastric gavage (Moreno et al., 2009) and intravenous injection (Verina et al., 2011) of Mn; none of these studies used the increasingly relevant DW exposure route. Additionally, data regarding the basal ganglia expression of neuronal/glial-derived oxidative and nitrosative stress markers, such as heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (NOS2), which are affected by Mn in vivo (Verina et al., 2011) and in vitro (Dodd and Filipov, 2011), in a DW exposure paradigm are lacking.

Increased brain Mn deposition is considered a hallmark feature and a prerequisite for Mn neurotoxicity (Aschner et al., 2005) and is often measured via magnetic resonance imaging (Fitsanakis et al., 2006). Hyperintense signals in T1 weighted brain MRI images, indicative of increased Mn deposition, have been reported in humans (Josephs et al., 2005), non-human primates (Park et al., 2007) and rodents (Lee et al., 2005, Kim et al., 2012a). MRI studies in Mn-exposed humans and non-human primates have used the pallidal index % (PI %; the ratio of the globus pallidus to frontal white matter or neck muscle signal intensity multiplied by a factor of 100) as an indicator of brain Mn deposition (Fitsanakis et al., 2006, Guilarte et al., 2006b) because of the notable T1-weighted hyperintense signals in the globus pallidus and substantia nigra (Shinotoh et al., 1995, Dorman et al., 2006b, Uchino et al., 2007). However, PI's utility may be less than previously thought as evidence from recent human and primate studies with relatively low Mn exposure levels revealed a wide-spread pattern of Mn distribution similar to that seen in rodent brains (Chaki et al., 2000, Dorman et al., 2006b, Guilarte et al., 2006b, Fitsanakis et al., 2008, Sen et al., 2011).

Compared to humans and non-human primates, there are limited rodent studies which have exploited MRI to determine brain Mn deposition; instead, most studies have relied on post-

mortem analysis of brain Mn levels (Kontur and Fechter, 1988, Dodd et al., 2005, Avila et al., 2010). Of note, majority of the Mn neurotoxicity rodent studies that have employed MRI are in rats (Cross et al., 2004, Finkelstein et al., 2008, Kim et al., 2012a); in a single study, where high levels of Mn were tested for its ability as a contrast agent, mice were used (Lee et al., 2005). More importantly, there are no MRI reports where the Mn deposition and neurotoxic effects of Mn via DW exposure were studied in mice. It is also noteworthy that there is limited information on the brain Mn deposition over multiple time points in any rodent studies, including the C57BL/6 mouse strain, which is a model mouse strain for neurotoxicity studies (Messiha, 1990, Sedelis et al., 2000, McLaughlin et al., 2006, Jiao et al., 2012).

Hence, in the current study, the main objectives were to monitor the brain Mn deposition following subchronic DW exposure in C57BL/6 mice and to determine the neurotoxic effects of Mn DW exposure on selected behavioral, neurochemical and molecular parameters. T1 weighted MRI was used to characterize the brain deposition/regional distribution of Mn in the mouse brain following DW exposure. To assess the potential neurobehavioral consequences of Mn DW exposure, we employed a collection of behavioral tests that could effectively assess both the locomotor as well as emotional changes induced by Mn DW exposure. To investigate the neurochemical alterations induced by Mn, striatal concentrations of DA, 5-HT and their metabolites were determined. Additionally, to gain a mechanistic insight, the activation of glial cells and the expression of oxidative/nitrosative stress markers in the basal ganglia (striatum, substantia nigra) of mice exposed to Mn via the DW were examined.

Materials and methods

Reagents

Unless otherwise stated, all chemicals including manganese (II) chloride (as $MnCl_2$.4H2O) were purchased from Sigma (St. Louis, MO).

Animals

Male C57BL/6 mice (4-5 months old) weighing 28.51 ± 3.51 g (mean ± SEM) were procured from Taconic (Hudson, NY) and housed (five/cage) with food available *ad libitum* on a 12-h light/dark cycle in an AAALAC accredited facility throughout the study. All procedures involving animal handling in this study were carried out according to the latest NIH guidelines (8^{th} edition, NRC, 2010) and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Animal treatment

Control and Mn-treated groups (n = 9-10/group) were exposed to vehicle (NaCl; 0.4 g Na/l) or $MnCl_2$ (0.4 g Mn/l) in deionized water for 8 weeks. The control and Mn solutions were freshly prepared and changed weekly. Body weight (BW) and water intakes were also recorded weekly. The Mn dose and dosing route used in this study were modeled based on several reports wherein a significant increase in brain Mn, altered DA homeostasis, as well as locomotor deficits were exhibited by animals after Mn exposure for periods and different DW Mn concentrations ranging from 10 to 51 weeks and from 1 g/l to as high as 20 g/l, respectively (Chandra and Shukla, 1981, Lai et al., 1999, Calabresi et al., 2001). The concentration used in the current study was selected to be lower and it closely relates to the one used by Avila et al. that resulted in a significant, human exposure-relevant, two-fold increase in brain Mn level and subsequent

locomotor deficits in rats following chronic (4 months) exposure to Mn (as $MnCl_2$) via DW (Avila et al., 2010).

Behavioral tests were carried after 6 weeks (n = 8/group); T1 weighted MRI was conducted after 5 and 6 weeks of Mn treatment on subsets of the mice (n = 4/group). As a positive control, mice (n = 2) were given a single subcutaneous (s.c.) injection of 50 mg/kg Mn (Eschenko et al., 2010, Malheiros et al., 2012) and were subjected to MRI prior to and 24 h after Mn injection. Behavioral tests and MRI analysis are described in detail below. Mice were sacrificed and organs (brain, liver, spleen, and thymus) were harvested at the end of the exposure period (8 weeks). One-half of the brain was fixed in 4 % paraformaldehyde and flash frozen for immunohistochemistry analysis, while the other half was frozen on dry ice for neurochemistry, qPCR and western blot analyses as described below.

MRI studies

MRI was conducted using a 7 Tesla (Agilent, Santa Clara, CA) magnet, with a quadrature birdcage volume coil for excitation. Acquisition parameters of the two dimensional (2D) T1 weighted images obtained using spin echo multislice sequence were TR 500 ms, TE 17 ms, averages 8, data matrix 256 x 256, orientation coronal, slices 17, thickness 1.00 mm with no gap. T1 relaxation time measurements were performed with a fast spin-echo based inversion recovery method with the following parameters (Lee et al., 2005).

Prior to imaging, mice were anesthetized with 1.0 % isoflurane in a 30 %:70 % O_2:N_2 gas mixture with a flow rate 0.8-1.0 l/min. Anesthesia was maintained by a nose cone supplying 0.75-3.0 % isoflurane delivered in medical air at 1 l/min as necessary. Physiological parameters (respiratory rate and rectal temperature) were monitored using a small animal monitoring system (Small Animal Instruments, Inc., Stony Brook, NY) throughout the imaging period.

Defined regions of interest (ROIs) included olfactory bulb (Olf), cortex (Cor), striatum (Str), globus pallidus (Gp), hippocampus (Hip), hypothalamus (Hyp), substantia nigra (Sn), pituitary gland (Pit), cerebellum (Cbl), pons (Pon) and medulla (Med). To determine the best method for assessing Mn deposition in the mouse brain following DW exposure, signal-to-noise ratio (SNR), T1 values and PI % were the three parameters derived from the image analyses that were compared. Signal intensity from each ROIs and standard deviation (SD) of noise/background were measured using NIH Image J software (Image J 1.42). Using these values, SNR was calculated as described previously (Lee et al., 2005). T1 maps were generated using in-house graphical user interface (GUI) that runs on Matlab and T1 values across different brain regions were quantified using Matlab software (Matlab 7.9). PI % was defined as the signal intensity ratio of the Gp relative to the neck muscle multiplied by a factor of 100 in axial T1-weighted MRI images (Guilarte et al., 2006b). For the positive control group, both SNR and T1 values were collected from all slices that represented a particular ROI (refer to supplementary Fig. S1) within an animal and these data were used for statistical analysis.

Behavior

Behavioral measures were assessed at 6 weeks post Mn DW exposure (30 min open field, pole test, grip strength, and forced swim test; the 4 tests performed in succession). Behavioral tests chosen have been used extensively in rodents as measures of locomotor and emotional function (Sedelis et al., 2000, Prut and Belzung, 2003, Perona et al., 2008), alteration of which is common in Mn-exposed humans (Josephs et al., 2005, Bouchard et al., 2007). Some of these tests have also documented behavioral impairments in rodents exposed to Mn via different (non-DW) routes (Torrente et al., 2002, Dodd et al., 2005, Cordova et al., 2012). All animals were naïve to behavioral apparatuses prior to initiation of testing and all tests were performed in a

behavioral testing designated room located nearby, but separate from that in which animals were housed with the experimenter blinded to the animal treatments.

Open field. Mouse activity was monitored for a period of 30 min in an open field arena (25 cm x 25 cm x 40 cm; Coulbourn Instruments, Whitehall, PA). Total distance traveled (cm) and number of crossings in a 16 square grid area were recorded as a measure of locomotor activity (Lim et al., 2001, Takeuchi et al., 2011) using Limelight video tracking software (Actimetrics, Wilmette, IL) and analyzed per 5 min intervals. Additionally, level of anxiety was determined by measuring the time spent in defined regions, namely the center versus perimeter of the square arena (Ageta et al., 2008).

Grip strength. This test was performed using a strength gauge with an attached mouse specific square wire grid (6 cm x 6 cm; Bioseb, France), similar to (Miller et al., 2010). Mice were carefully placed in front of the wire grid and allowed to grab hold with both forepaws. Once grip was established, mice were gently lifted to induce a gripping reflex. The maximum grip force per trial was recorded in newtons (N) for a series of four trials (Dodd et al., 2005) with 1 min inter-trial interval. The average maximum grip force of the four trials was used for statistical analysis.

Pole test. Mice were placed upright on a gauze-wrapped pole (1 cm in diameter and 55 cm in height). Turning criteria included a full body turn with the head facing down the pole. The maximum time allowed for turning was 60 s and the total time per trial was 120 s (Staropoli et al., 2012). If a mouse did not turn within the first 60 s, it was gently guided and a maximum measurement (60 s) was recorded. A total of four trials were completed with a 3-5 min resting period between each trial. The average time to turn, time to descend, and total time spent on the pole from the four trials was used for statistical analysis (Royl et al., 2009).

Forced swim test. This test was performed as described previously (Petit-Demouliere et al., 2005, Perona et al., 2008), but with minor modifications. Mice were gently placed in a large cylindrical container (18 cm in diameter and 25 cm in height) filled approximately one third from the edge with tap water (27 ± 1^{o} C) and swimming behavior was recorded for a period of 15 min. Upon test completion, mice were given a 5-10 min recovery period underneath a heated lamp before returning them to their home cages. Swimming was defined by vigorous tail, forelimb, or hindlimb movement required to propel the mouse forward, climbing was defined as pawing at the sides of the container, and immobility was defined as minimal tail, forelimb, or hindlimb movement required to keep the mouse afloat but not resulting in a forward movement (Deak et al., 2005). Limelight video tracking software (Actimetrics) was utilized to score the total time spent swimming, climbing, or immobile by an experimenter blinded to treatment group and analyzed per 5 min intervals.

Neurochemistry

Neurochemical analysis to determine striatal concentration of DA, 5-HT and their metabolites (homovanillic acid, HVA; 3,4-dihydroxyphenylacetic acid, DOPAC; 5-hydroxyindoleacetic acid, 5-HIAA) was performed using HPLC with electrochemical detection as we have described it previously (Coban and Filipov, 2007). Prior to statistical analysis, all neurochemistry data were normalized on per mg of tissue basis. Tissue protein concentration was determined after sample digestion in NaOH; final protein (µg/ml) concentration was determined using the method of Bradford (Biorad, Hercules, CA, USA).

Immunohistochemistry (IHC)

Coronal sections (40-µm thick) were used for immunohistochemical analysis to examine tyrosine hydroxylase (TH; EMD Millipore, Billerica, MA, 1:2,000 dilution) immunoreactivity in

the striatum and glial fibrillary acidic protein (GFAP; EMD Millipore, 1:150 dilution) immunoreactivity in the striatum, substantia nigra pars compacta (Snpc) and substantia nigra pars reticulata (Snpr) as published previously (Coban and Filipov, 2007), but with few modifications. First, following substrate (3, 3' diaminobenzidine; DAB for TH staining; Novared for GFAP staining) addition, the respective sections were mounted on poly-L-lysine coated slides, washed in tap water for 5 min and placed on a slide warmer for 3 h. Afterwards, slides were transferred consecutively into 95 % and 100 % dehydrants (Histogene Inc, Lake Forest Park, WA) and cleared in Xylene (Decon Labs Inc, Bryn Mawr, PA). The mean integrated pixel density measured using NIH Image J software (Image J 1.42) from random medial sections (n = 2/brain; all brain sections anatomically matched) was used to analyze the intensity of TH and GFAP staining in striatum. GFAP staining intensity was also assessed from randomly selected medial sections (n = 2/brain; all brain sections anatomically matched) of the substantia nigra with staining in the Snpc and Snpr analyzed separately.

Western blotting

Effects of Mn on the expression of key striatal proteins, including TH, dopamine receptor-2 (D2DR), NOS2, GFAP, glutamate decarboxylase 1 (GAD1) and HO-1 were determined by western blot analysis according to a previously described procedure (Coban and Filipov, 2007), with minor modifications. After determining the protein concentration in the tissue lysate using the Bradford method (Bio-Rad), 10 μg of protein per sample was loaded onto10 % bis-acrylamide gels. The PVDF (polyvinylidene difluoride) membranes (EMD Millipore) on to which protein was transferred (semi-dry transfer) were blocked with 5 % milk, incubated overnight at 4 °C with primary antibodies namely anti-TH (EMD Millipore; 1:3,000 dilution), anti-D2DR, anti-HO-1, anti-GAD1, anti-NOS2 (Santa Cruz Biotechnology, Santa

Cruz, CA; all 1:500) and anti-GFAP (Biomeda; 1:1,000). Following incubation with appropriate secondary antibody conjugated with horseradish peroxidase (1: 10,000 to 1: 100,000), bands of interest were identified with chemiluminescence substrate (Supersignal West Pico substrate; Thermo Fisher Scientific, Rockford, IL); membranes were stripped using Gentle Review buffer (Amresco, Solon, OH) and were re-probed for β-actin (1:500; Santa Cruz). Quantity One software (Bio-Rad) was used to determine the pixel density of the bands of interest which was normalized to β-actin prior to statistical analysis.

Real-time quantitative PCR (qPCR)

Total RNA from substantia nigra samples was isolated using E.Z.N.A. microelute total RNA kit (Omega Bio-Tek, Inc., Norcross, GA) and quantified using Take 3 plate and Epoch microplate spectrophotometer (Biotek, Winooski, VT). One μg RNA was used to synthesize the first strand cDNA using qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD) and a peltier thermal cycler (Bio-Rad; 5 min 25 °C, 30 min 42 °C, and 5 min 85 °C). Using 10 ng of cDNA per sample, expression of TH, D2DR, HO-1, NOS2, GAD1, and GFAP were determined by qPCR using mouse-specific, certified primers and SYBR Green (Qiagen, Valencia, CA). Amplifications were performed in a Mx3005P qPCR machine (Stratagene) programmed for an initial warming (10 min, 95 °C) followed by 45 cycles (30 s, 95 °C, 1 min, 60 °C) with each sample run in triplicate. Data were analyzed using the $^{\Delta\Delta}C$t method and are presented as relative induction (fold-changes) of TH, D2DR, HO-1, NOS2, GAD1 and GFAP normalized to β-actin.

Statistical analysis

Two-way analysis of variance (ANOVA) was conducted to analyze MRI data (Mn x time) between the two imaging time points (5 and 6 weeks) and the open field (Mn treatment x interval) data. T-test was used to analyze change in BW, organ weight (g/kg BW) and water

intake (ml/kg BW), MRI parameters within a time point, immunohistochemistry, neurochemistry, western blot, qPCR and the other behavior endpoints. If ANOVA's Mn effect or interaction was found significant, treatment means were separated by Student Newman-Keuls post hoc test. All results (except for the positive control group for the MRI component of the study) are presented as mean ± SEM and are considered significant at $p \leq 0.05$. For the positive control group, paired T-test was used to compare MRI data from the different brain ROIs before and 24 h after Mn s.c. injection. The positive control MRI data are presented as mean ± SD and considered significant at $p \leq 0.05$. Following statistical analysis, all non-tabular and non-image data were presented in a graphical format with graphs created using the GraphPad Prism software (GraphPad Prism 5.01; GraphPad software Inc., San Diego, CA).

Results

Body weight, Organ weight and Water intake

BW was not affected by Mn ($p \geq 0.1$) throughout the study; both groups gained about 10 % weight by the end of the 8-week experimental period (Fig. 2.1a). Similarly, 8 weeks of Mn exposure did not affect brain, liver, spleen and thymic weights ($p \geq 0.25$; data not shown). With respect to water consumption, Mn-exposed mice showed a constant intake throughout the entire experimental period (Fig. 2.1b). Saline-treated control group exhibited lesser ($p \leq 0.05$) water intake than the Mn-treated mice for the first four weeks, which afterwards returned to the level of water intake observed in the Mn-treated group (Fig. 2.1b).

MRI

Compared to T1 weighted images acquired from control mice after 5 weeks of Mn DW exposure, images from Mn treated mice showed bilaterally widespread increased intensities, eg., in the Olf, Str, Gp, Hip, Hyp, Sn and Pit ROIs (Fig. a1, Appendix A). T1 weighted images of the

positive control brains (imaged 24 h post 50 mg/kg Mn s.c.) also displayed signal enhancement in all ROIs evaluated; the signal was more prominent than the one from DW exposed mice (Fig. a1, Appendix A).

Two-way ANOVA was conducted to compare the effect of Mn across the two imaging time points (5 and 6 weeks) and did not reveal any significant effect of time of imaging ($p \geq 0.30$). To illustrate this, representative SNR data from Olf and Pit ROIs showing no significant difference between 5 and 6 weeks data, but significantly increased SNR at both time points by DW Mn are presented in Fig. 2.2. For all subsequent analyses, 5 and 6 weeks MRI data were combined. Change in SNR (Table 2.1) and T1 values (Fig. 2.3) were calculated for each selected region of the brain. On comparison, Pit (the region deficient in blood brain barrier [BBB]) showed the highest increase (53 %), Olf and Med showed only modest increase (8 % and 9 %, respectively) in SNR following Mn DW exposure (Table 2.1). The increase in SNR in the cortex was 19%. Acute s.c. administration of Mn (50 mg/kg; used as a positive control) produced a significant increase in SNR values in all selected ROIs. Compared to the Mn DW group, short of Cor, Cbl and Pon, the increase in SNR value in the positive control group was substantially greater in all evaluated regions (Table 2.1).

Brains of Mn DW-exposed mice exhibited a significant reduction in the T1 relaxation time across all evaluated regions; the largest decrease (17 %) was observed in Pit (Fig. 2.3). The positive control group also showed a significant decrease in T1 value 24 h after Mn s.c. administration in all selected ROIs. Analogous to the findings from Mn DW group, 24 h after acute administration of a larger amount of Mn s.c., Pit showed the highest reduction (42 %) in T1 value (Fig. 2.3). T1 maps illustrate the significant effect of Mn on T1 relaxation time in Mn DW and Mn s.c. groups (Fig. 2.4). Statistical analysis of mean PI %, indicative of Mn accumulation

in the Gp, revealed a significant ($p \leq 0.05$) increase in PI in both the Mn DW and the positive control groups (9 % and 6 %, respectively; data not shown).

Behavior

After 6 weeks of Mn DW exposure, mice exhibited a significant increase in locomotor activity during the first five min (interval 1) exploration period of the open field test. Specifically, the total distance traveled (Fig. 2.5) and the total numbers of grid crossings (data not shown) were increased ($p \leq 0.05$). As expected, both control and Mn-treated mice habituated to the arena over time and their overall activity decreased (number of crossings: 185 vs 224 in first 5 min and 140 vs 156 in last 5 min of control and Mn DW groups, respectively); the difference between the two groups was not significant after the first interval ($p \geq 0.10$). Two-way ANOVA revealed an overall significant main effect of Mn with respect to the time spent in center versus periphery of the square arena over the entire 30 min of open field testing i.e., Mn-exposed mice spent more time in the center and less time in the periphery ($p \leq 0.001$; data not shown). The mean time spent per 5 min interval in the center and periphery, which was also increased (center) and decreased (periphery) significantly ($p \leq 0.001$) by Mn DW exposure, is presented in Fig. 2.5. In addition, Mn decreased the average forelimb grip strength ($p \leq 0.05$; Fig. 2.6). In the forced swim test, Mn-exposed mice exhibited a significant decrease in the mean time spent swimming and climbing per 5 min interval with a concomitant increase in the immobility time ($p \leq 0.05$; Fig. 2.7). Mn-exposed mice also exhibited a tendency toward increased time to turn and total time during the pole test, but these effects did not reach significance ($p = 0.116$ and $p = 0.085$, respectively; data not shown).

N*eurochemistry*

Mn DW exposure for 8 weeks did not affect striatal concentrations of DA and its metabolites DOPAC and HVA (DA and DOPAC data presented in Fig. 2.8; HVA data not shown). Mn treatment resulted in a small, but significant increase ($p \leq 0.01$) in the concentration of the 5-HT metabolite 5-HIAA, without affecting the parent neurotransmitter 5-HT, although a numerical trend towards an increase was observed (Fig. 2.8).

Immunohistochemistry (IHC)

Eight-week Mn DW exposure did not affect striatal expression of TH (Fig. 2.9). Similarly, GFAP expression in the striatum and Snpc was not different between the two groups (Fig. 2.9). However, compared to control mice, Mn-exposed mice showed a highly significant increase in GFAP staining in the Snpr ($p \leq 0.01$; Fig. 2.9).

Western blotting

Similar to the IHC data, Mn DW exposure for 8 weeks did not alter the protein expression of striatal TH ($p \geq 0.4$; data not shown). Western blot data for striatal protein levels of D2DR, GAD1, NOS2 and GFAP also failed to reveal any significant effect ($p \geq 0.25$) of Mn although levels of all these markers were numerically greater in the Mn-exposed mice (data not shown); protein levels of HO-1 in the striatum were undetectable.

qPCR

At the end of the 8 week exposure period, HO-1, NOS2 and GFAP mRNA in the substantia nigra of the Mn-exposed mice was significantly upregulated ($p \leq 0.05$); the nigral mRNA expression of TH, D2DR and GAD1 was not affected by Mn (Fig. 2.10).

Discussion

The current study was unique in that it utilized a repeated MRI analysis to evaluate brain Mn deposition in adult C57BL/6 mice following DW exposure. The MRI was combined with assessment of neurological effects using behavioral, neurochemical and molecular parameters. The main findings from this work include: 1) subchronic exposure to low-level Mn via DW resulted in significant brain Mn deposition in all ROIs evaluated as evidenced by a decrease in T1 relaxation time and an increase in SNR, apparently reaching a plateau; 2) mice exhibited prominent behavioral deficits following 6 weeks of Mn DW exposure without any significant alteration in striatal DA homeostasis, but in the presence of altered 5-HT homeostasis two weeks later; 3) significant activation of astrocytes and increased expression of oxidative/nitrosative stress markers, HO-1 and NOS2, respectively, was demonstrated in the substantia nigra of Mn-exposed mice with the astrocytic activation being mostly in the pars reticulata of this structure.

To our knowledge, this is the first study which have conjoined and compared all three accepted MRI image analysis parameters, namely, SNR, T1 and PI %, in order to find the best predictor of Mn deposition in the mouse brain following subchronic exposure to Mn via DW. MRI has been used to detect Mn deposition in humans, non-human primates and rats for quite some time (Dorman et al., 2006b, Bock et al., 2008, Fitsanakis et al., 2008, Selikhova et al., 2008, Sen et al., 2011), but reports assessing brain Mn deposition by MRI within the context of Mn neurotoxicity in mice are not available. There are only limited reports where acute systemic Mn administration has been used as a contrast agent for better visualization of brain neuroarchitecture (Lee et al., 2005). Ultimately, our findings suggest that MRI can be used successfully in mice to determine longitudinal brain Mn deposition following DW exposure which will be a useful tool in future analyses of brain Mn deposition dynamics in mice

chronically exposed to Mn. Consistent with the findings from other MRI Mn studies, we found that subchronic Mn exposure via DW results in hyperintense T1 weighted signals across different brain regions indicative of a widespread brain Mn deposition (Guilarte et al., 2006b, Finkelstein et al., 2008, Fitsanakis et al., 2008). Even though SNR was significantly increased in all ROIs evaluated after 5 and 6 weeks of Mn treatment, there was no significant difference across the two time points of imaging. Our findings are in accord with rodent and non-human primate studies wherein longitudinal assessment of Mn deposition showed time independence, which could be the result of a plateauing effect (Gallez et al., 1997, Guilarte et al., 2006b). Although Mn levels increased in a region independent fashion, pituitary gland showed the highest degree of Mn deposition in both the Mn DW and the high dose Mn s.c. (positive control) groups. On comparison, both SNR and T1 values changed more drastically in the positive control group than in the Mn DW group, suggesting greater brain Mn deposition after high dose acute s.c. administration of Mn.

The highest degree of Mn deposition in the pituitary gland is likely due to the absence of a BBB and is in line with a rat study wherein the pituitary gland signal intensity, compared to other brain regions, doubled following an acute s.c. administration of $MnCl_2$ (Eschenko et al., 2010). With respect to the T1 relaxation time, increasing order of the degree of T1 reduction following Mn DW exposure was cortex < medulla < globus pallidus < hippocampus < hypothalamus < striatum < pons < substantia nigra < olfactory bulb < cerebellum< pituitary gland. In this regard, another study, which investigated the dose and temporal dependence of brain contrast enhancement after intravenous (i.v.) administration of varying doses of $MnCl_2$, also found a similar trend with the largest and the smallest reduction in T1 value in the pituitary gland and cortex, respectively (Lee et al., 2005). Similarly, significant shortening of the T1 time

was observed in the pituitary gland 15 min following a single i.v. or intraperitoneal administration of $MnCl_2$ in C57BL/6 mice (Kuo et al., 2005). After 24 h of high dose Mn s.c. administration in the positive control group, the percentage (%) change (between pre and 24 h post Mn s.c.) in T1 and SNR values in different ROIs was calculated. As compared to SNR, T1 values showed a greater % change in almost all regions evaluated, which also correlated well with the hyperintense signals (indicative of brain Mn deposition) in the T1 weighted images. Taken together, these results suggest that T1 is a better predictor of Mn deposition in the brain than SNR. Consistent with our results, Dorman et al. have also demonstrated the superiority of T1 relaxation time in estimating the regional brain Mn concentration in rats exposed to Mn via inhalation over the other MRI image measurement parameters which led them to suggest that the T1 relaxation time can be used as a potential biomarker of Mn exposure (Dorman et al., 2006b). Evidence from human cases of Mn poisoning and from non-human primates exposed to Mn via inhalation or systemic administration have demonstrated significant Mn accumulation in multiple brain regions, including globus pallidus (Guilarte et al., 2006b, Struve et al., 2007, Sen et al., 2011), the basal ganglia region primarily involved in regulation of voluntary movement. In this respect, it is worth mentioning that the much lower Mn absorption route used in our study, i.e. DW exposure, also succeeded in producing measurable increase in pallidal Mn concentration in mouse brain, evidenced by the significant increase in PI %, SNR and a decrease of T1 relaxation time. Given the significant changes in the extent of brain Mn deposition assessed by SNR and T1 values across different brain ROIs, we conclude that the low level Mn DW exposure adopted in this study produced a widespread Mn deposition in mouse brain, much similar to the pattern of brain Mn distribution demonstrated by MRI studies in rats (Chaki et al., 2000), non-human

primates (Guilarte et al., 2006b), and humans (Sen et al., 2011) exposed to Mn by different routes, but at relatively low concentrations.

Contrasting results are reported in the literature regarding changes in locomotor activity following Mn exposure in rodents. For example, studies have demonstrated an increase (St-Pierre et al., 2001, Salehi et al., 2003), decrease (Witholt et al., 2000, Guilarte et al., 2006a, Avila et al., 2010) and even an absence of a change in locomotor activity after Mn exposure (Dorman et al., 2000). These variations could be due to difference in doses, exposure routes, types of tests and, especially, duration of treatment and timing of testing employed by the different studies. In the present study, Mn-exposed mice were hyperactive during the first five min of exploratory behavior in open field testing. The locomotor activity during the first five min of novel open field testing in rodents is generally considered as an indicator of emotional response to an unfamiliar environment, with an assumption that reduction in activity correlates with increased anxiety (Umezawa et al., 1999). Given the hyperactivity demonstrated in the first five min in our study, it could be inferred that Mn DW exposure produced emotional alterations, specifically an anxiolytic effect. Moreover, the increased time spent in the center versus periphery of the open field arena indicates a decreased anxiety level induced by Mn DW exposure. DW Mn-exposed mice also exhibited a significant decrease in muscle function, particularly muscle strength as demonstrated elsewhere (Bowler et al., 2006, Bagga and Patel, 2012). The significantly increased immobility time with a concomitant decrease in the total time spent swimming and climbing in the forced swim test suggest impairment in locomotion or emotional alterations, specifically depression (Deak et al., 2005), induced by Mn DW exposure. The decreased anxiety in the face of increased depressive behavior exhibited by the Mn-exposed

mice is likely related to the Mn-caused serotonergic perturbations (Mosienko et al. 2012), as we discuss in more detail later.

It is noteworthy that the neurobehavioral alterations induced by Mn occurred in the absence of a measurable effect on striatal DA homeostasis (measured two weeks post behavioral testing). A very similar result was observed by Witholt et al. who demonstrated significant impairment of neurobehavioral functions without any change in striatal DA following sub-chronic exposure to a cumulative Mn dose comparable to our study (Witholt et al., 2000). Our findings are also consistent with several other studies that reported significant neurobehavioral deficits in the absence of a measurable effect on striatal DA level or its metabolites (Pappas et al., 1997, Guilarte et al., 2006a, Kim et al., 2012a). The observed neurobehavioral alterations without overt striatal dopaminergic changes at the whole tissue level and the lack of Mn effect on both mRNA (nigral) and protein (striatal) levels of TH and D2DR (markers of DA terminal integrity) in this study further substantiate the hypothesis that at lower levels of exposure rather than altering the structural integrity of dopaminergic terminals, Mn causes functional deficits of the nigrostriatal dopaminergic system (Guilarte et al., 2006a, Guilarte et al., 2008a). In this regard, dopaminergic dysfunction was only revealed after challenging the system; marked decrease in amphetamine-induced DA release from striatal tissue slices derived from Mn-exposed animals, indicative of the adverse effect of Mn on the protein function associated with vesicular neurotransmitter release at the dopaminergic synapse was reported (Guilarte et al., 2006a). It is also worth mentioning that in our study the concentration as well as turnover of striatal DA was not altered despite significantly increased brain Mn deposition; such lack of correlation between brain Mn and change in striatal catecholamines has been reported by others (Chandra and Shukla, 1981, Shukla and Chandra, 1981, Struve et al., 2007). However, it can also

be that the Mn-induced neurochemical alterations involving dopaminergic pathways might be present in a region-dependent fashion and might have occurred in brain regions other than the striatum. For example, out of the three brain regions (prefrontal cortex, striatum and hippocampus) assessed, significant neurochemical changes characterized by depletion of DA were observed only in the hippocampus following intranasal Mn administration (0.8 mg/kg BW) in rats (Blecharz-Klin et al., 2012). Alternatively, the neurochemical changes in DA systems, if at all present, might be too subtle to be detected, especially when measuring neurotransmitter/metabolite tissue levels and not in vivo release of DA by microdialysis or other sensitive means.

The absence of alterations in DA homeostasis in the face of a significant increase in striatal 5-HT metabolite concentrations, indicative of elevated 5-HT utilization or 5-HT turnover, suggest that the serotonergic signaling appears to be more sensitive to subchronic Mn DW exposure than is the dopaminergic signaling. Generally, DA is considered the predominant neurotransmitter controlling locomotor and emotional functions and alterations in DA homeostasis are usually associated with locomotor and emotional abnormalities (Missale et al., 1998, Sotnikova et al., 2005). However, the role of other monoamines, namely 5-HT, in regulating locomotor and emotional functions has been widely studied as well. Specifically, low levels of extracellular, including synaptic levels of 5-HT, are associated with depression and reduced anxious behavior (Wurtman and Wurtman, 1995, Belmaker and Agam, 2008, Mosienko et al., 2012). Hence, the initial hyperactivity and emotional alterations demonstrated by Mn-exposed mice in the current study could be attributed to the alterations in serotonergic (increased 5-HT utilization or 5-HT turnover), but not dopaminergic neurotransmission. Previous studies have demonstrated that Mn can target both dopaminergic and serotonergic neurons in basal

ganglia and the latter has been found relatively less sensitive to the effects of Mn when relatively high level of Mn was administered via DW for a chronic period (Bonilla and Prasad, 1984), by direct unilateral intranigral injection (Parenti et al., 1986), or by intragastric gavage (Moreno et al., 2009). However, our results indicate that it is the 5-HT signaling in the striatum that is either more sensitive or is affected earlier than DA signaling following subchronic low level Mn exposure via the DW. Given the relatively low cumulative dose employed in our study in contrast to the high dose acute exposure/high cumulative dose used by the above- mentioned studies, it could be inferred that the toxic effects of Mn on the basal ganglia neurochemistry are dose-related, with impairments in 5-HT signaling appearing at lower cumulative exposures than impairments in DA signaling.

Multiple studies have demonstrated the effect of Mn overexposure on other neurotransmitter systems, namely γ-amino butyric acid (Erikson and Aschner, 2003, Burton et al., 2009). Hence, we assessed the effect of Mn DW exposure on the GABAergic neuronal marker GAD1 (key enzyme for GABA synthesis) and found no significant effect of Mn at both mRNA and protein levels in substantia nigra and striatum, respectively. These findings lend more support to the notion that the subtle serotonergic imbalance may be the major driving force for the Mn-induced behavioral impairments observed in this study. However, we cannot exclude the possibility that Mn DW exposure might have impacted GABAergic signaling independent of GAD1 expression, i.e., at the level of GABA receptors, or at the level of pre-synaptic GABA release.

Meanwhile, Mn has also been found to target and produce pathologic changes characterized by marked neuronal loss and astrocytosis in another basal ganglia structure, the globus pallidus, especially in its medial segment (Aschner, 2000, Liu et al., 2006, Burton et al.,

2009). In this regard, it is worth mentioning that the neurotoxic effects of Mn evidenced by the significant astrocytic activation in this study was observed in Snpr, the brain region which is functionally analogous to the medial segment of globus pallidus and Mn intoxication associated with neuropathological changes in Snpr has been reported earlier (Perl and Olanow, 2007, Verina et al., 2011). In order to further elucidate the effects of Mn DW exposure on the brain, we looked for molecular changes in glial cells considering their activation to be an early event, which typically precedes neuronal death (Zhao et al., 2009). Evidence has mounted in recent years that the neurotoxic effect of Mn is partly mediated by its indirect effect (activation) on glial cells (Tomas-Camardiel et al., 2002, Perl and Olanow, 2007, Filipov and Dodd, 2012), which in turn leads to neuroinflammation and associated release of a number of inflammatory mediators (Zhang et al., 2007, Liu et al., 2009, Dodd and Filipov, 2011). Previous studies have demonstrated significant increase in Mn-induced GFAP immunoreactivity in the basal ganglia (Liu et al., 2006) and hippocamus (Vezer et al., 2007) accompanied by behavioral and neurochemical changes. However, Mn-induced neurological consequences in the absence of significant astrogliosis and associated basal ganglia GFAP activation has also been demonstrated (Calabresi et al., 2001). Intriguingly, Mn DW exposure in the present study resulted in significant astrocytic activation evidenced by more than 2.5 fold nigral GFAP mRNA upregulation and increased GFAP protein expression, specifically in the Snpr. The selective effect of Mn on the Snpr, as compared to the pars compacta has been demonstrated elsewhere (Perl and Olanow, 2007, Verina et al., 2011). In comparison to other basal ganglia structures, Snpr is rich in iron content, attributed to the increased concentration of transferrin receptors in the region (Verina et al., 2011). Mn has been shown to alter iron homeostasis and produce iron-mediated oxidative stress in the brain, characterized by increase in reactive oxygen species

(ROS) and associated release of inflammatory mediators (Vezer et al., 2007, Santos et al., 2012). This enhanced ROS release either from Mn-exposed neurons or glial cells has been found to activate glial cells, successfully generating a vicious cycle (Filipov and Dodd, 2012). Hence, the significant astrocytic activation evidenced by the increased GFAP protein expression in the pars reticulata could be attributed, at least in part, to the Mn-induced iron-mediated oxidative stress and associated ROS production.

Several studies have shown increased number of reactive astrocytes and associated excess expression of the nitrosative stress marker, NOS2, following Mn exposure (Liu et al., 2006, Moreno et al., 2011). Also, Mn-potentiated expression of the oxidative stress marker, HO-1, by activated glial cells has been demonstrated in vitro (Dodd and Filipov, 2011). Consistent with these findings, Mn-induced significant upregulation of nigral NOS2 and HO-1 mRNA, indicative of nitrosative and oxidative stress, respectively, was revealed in the current study. Altogether, these research results suggest the possibility that the Mn-induced neurotoxic effects can be viewed on a continuum of neurological deficits, with Mn producing early glial activation and associated release of potent reactive nitrogen species and ROS in the basal ganglia, further leading to neuronal dysfunction as well as neurochemical imbalance in distinct signaling pathways (i.e. serotonergic) and eventually culminating in behavioral alterations of locomotor and emotional nature.

The Mn dose used in this study lies in the lowest range of fold increase in brain Mn deposition reported in humans after excessive Mn exposure. Daily intake levels of Mn in humans are based on dietary recommendations and not from DW on account of the minimal ingestion of Mn from water. However, the risk of neurotoxicity upon exposure to Mn from contaminated DW may be much higher due to the increased absorption and apparently different metabolism of

Mn from water than from food (Bouchard et al., 2011). The current study adds to the existing literature on the potential risks of Mn DW exposure by specifically addresssing the Mn-induced molecular, neurochemical and behavioral alterations in C57BL/6 mice in a DW exposure paradigm. This study provides evidence that low level Mn exposure via DW for a subchronic period causes significant increase in brain Mn deposition (in a region and time-independent manner) and region-specific glial activation in the brain. Our research results also suggest that the astrocyte activation in the Snpr as well as the serotonergic imbalance in the striatum might be associated with the locomotor and emotional perturbations observed in Mn-exposed mice. Collectively, we conclude that the neurobehavioral deficits (emotional and locomotor) and glial activation associated with significant brain Mn deposition are among the early signs of Mn neurotoxicity caused by subchronic DW exposure.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Table 2.1 Region-independency of Mn deposition in the brain. Effect on signal-to-noise ratio (SNR) in the brains of mice exposed to Mn via DW (0.4 g/l) for 5 or 6 weeks or to 50 mg/kg BW Mn s.c. (images were collected from the same mice before and 24 h post Mn). Data are represented as a mean change in SNR ± SEM in Mn DW group compared to control (n = 4/group) with percentage (%) change in parenthesis and a mean change in SNR ± SD from Mn s.c. (pre- and 24 hour post) group (n = 2) with % change in parenthesis.

Regions of Interest (ROIs)	**Mn DW Group**	**Mn s.c. Group**
	Mean Change in SNR (%)	**Mean Change in SNR (%)**
Olfactory bulb (Olf)	1.77 ± 0.38* (8)	8.88 ± 3.40* (48)
Cortex (Cor)	3.33 ± 0.88* (19)	2.14 ± 1.40* (12)
Striatum (Str)	2.50 ± 0.81*(17)	3.85 ± 2.51* (21)
Globus pallidus (Gp)	1.91 ± 0.27* (12)	3.97 ± 2.13* (23)
Hippocampus (Hip)	2.75 ± 0.61* (15)	4.11 ± 1.25* (21)
Substantia nigra (Sn)	2.88 ± 0.70* (15)	4.73 ± 0.70* (24)
Hypothalamus (Hyp)	4.01 ± 0.89* (21)	5.00 ± 0.06* (25)
Pituitary (Pit)	11.02 ± 1.68* (53)	15.39 ± 6.00* (73)
Cerebellum (Cbl)	2.38 ± 0.90* (14)	2.4 ± 0.85* (13)
Pons (Pon)	2.10 ± 0.74* (12)	2.36 ± 1.07* (13)
Medulla (Med)	2.06 ± 0.62* (9)	3.48 ± 1.09* (20)

* Indicates a significant effect of Mn ($p \leq 0.05$).

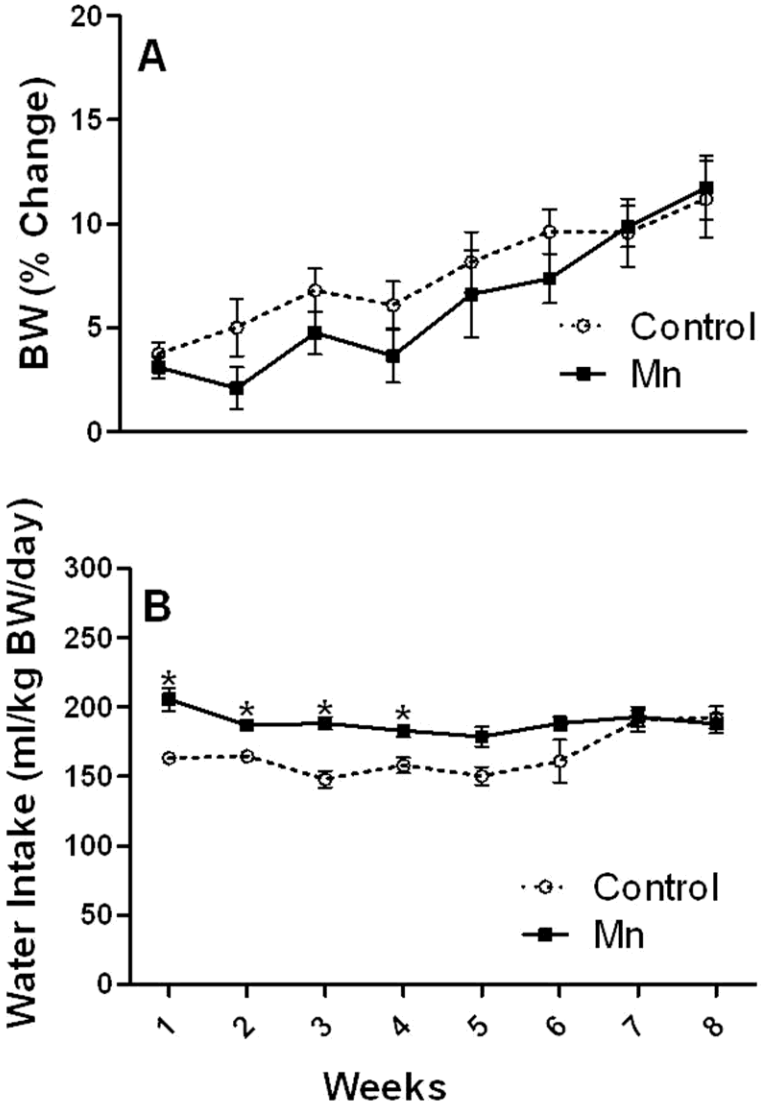


Figure 2.1. Effect of Mn DW (0.4 g/l) exposure on percentage (%) change in body weight (BW; a) and water intake (ml/kg BW/day; b) of adult male C57BL/6 mice during the 8 weeks of treatment duration. BW and water intake are presented as mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

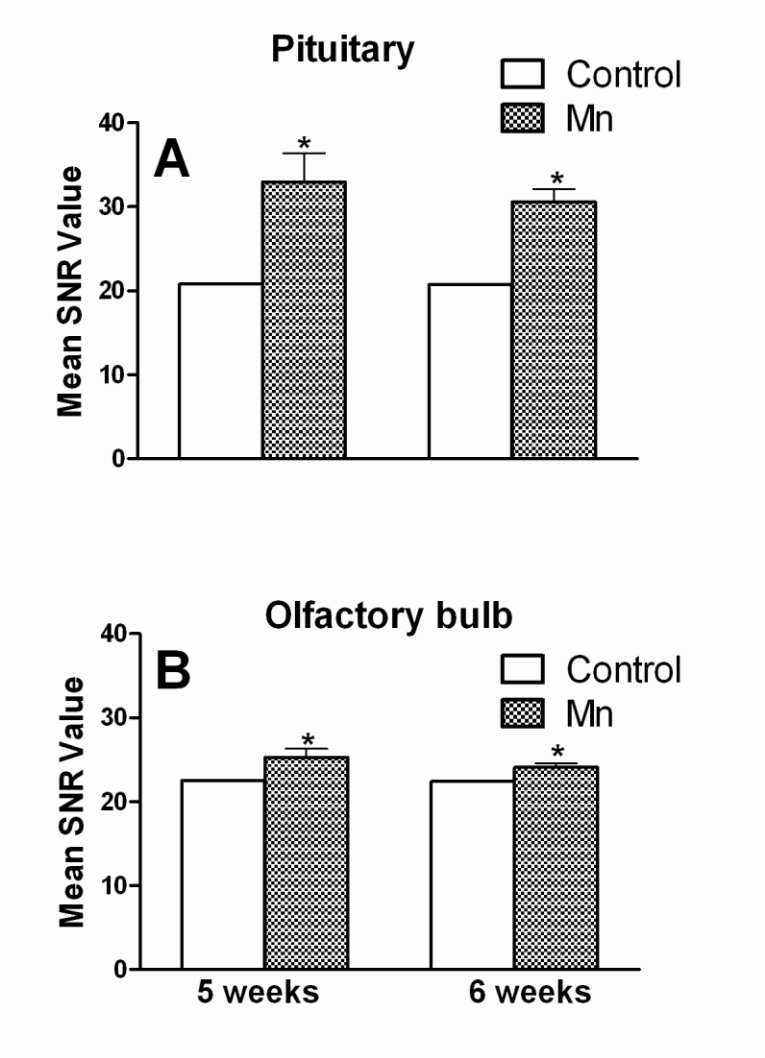


Figure 2.2. Time-independency of Mn deposition in the brain. Pituitary gland (a) and olfactory bulb (b) signal-to-noise ratio (SNR) from mice exposed to Mn DW (0.4 g/l) for 5 or 6 weeks. SNR data are presented as mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

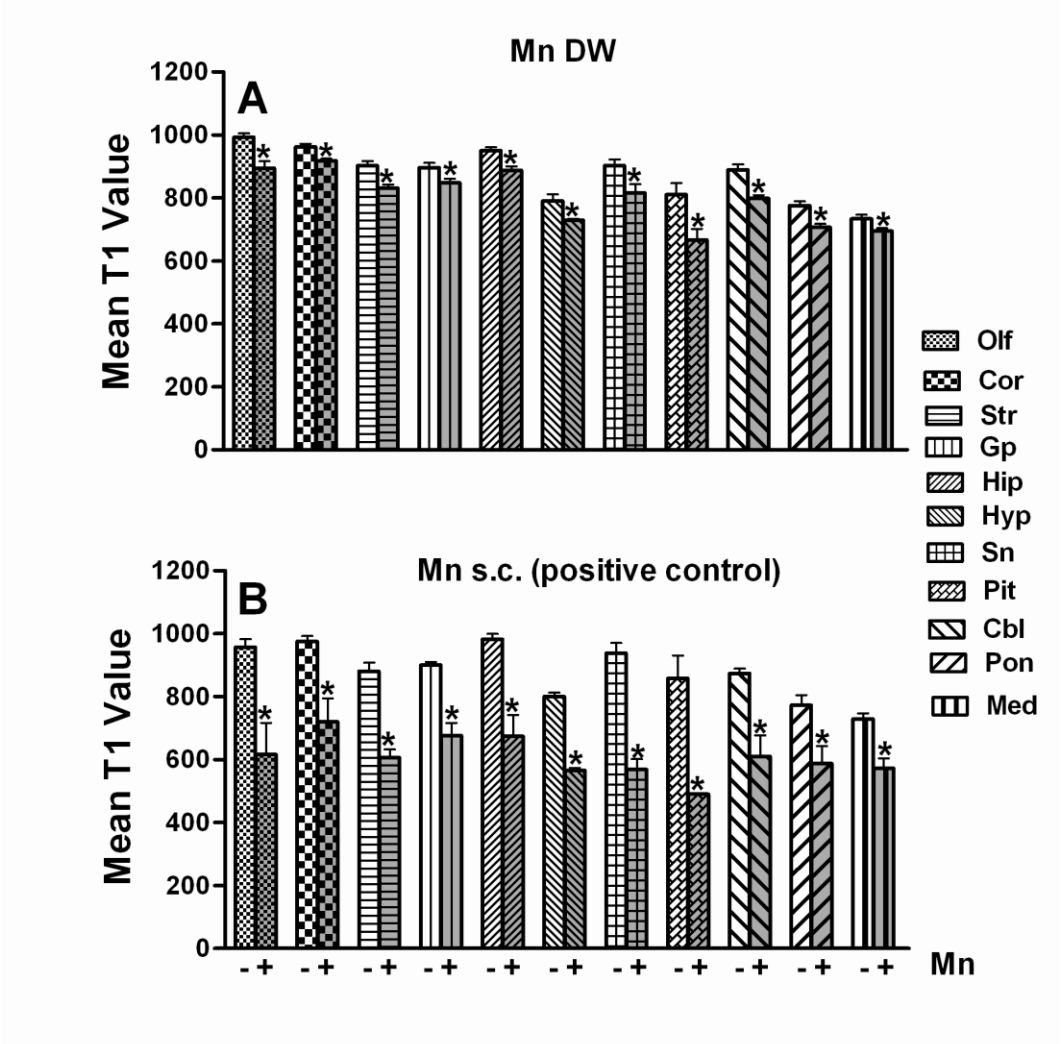


Figure 2.3. T1 relaxation time (milliseconds, ms) in different brain regions of mice exposed to Mn via DW (0.4 g/l; panel a) or via a single s.c. injection (panel b; 50 mg/kg; positive control) 24 h prior to imaging. A standard inversion-recovery, coronal T1-weighted fast spin-echo sequence with 10 inversion times (TR/TE/T1= 5000/48/10, 25, 50, 100, 200, 500, 1000, 1500, 2000 and 3000 ms, slice thickness: 1mm) was used to measure T1 relaxation time. ROIs were placed in the olfactory bulb (Olf), cortex (Cor), striatum (Str), globus pallidus (Gp), hippocampus (Hip), hypothalamus (Hyp), substantia nigra (Sn), pituitary (Pit), cerebellum (Cbl), pons (Pon) and medulla (Med). T1 values from Mn DW group (n = 4) are represented as mean ±

SEM and T1 values from Mn s.c. group (n = 2) are presented as mean ± SD.* Indicates a significant effect of Mn ($p \leq 0.05$).

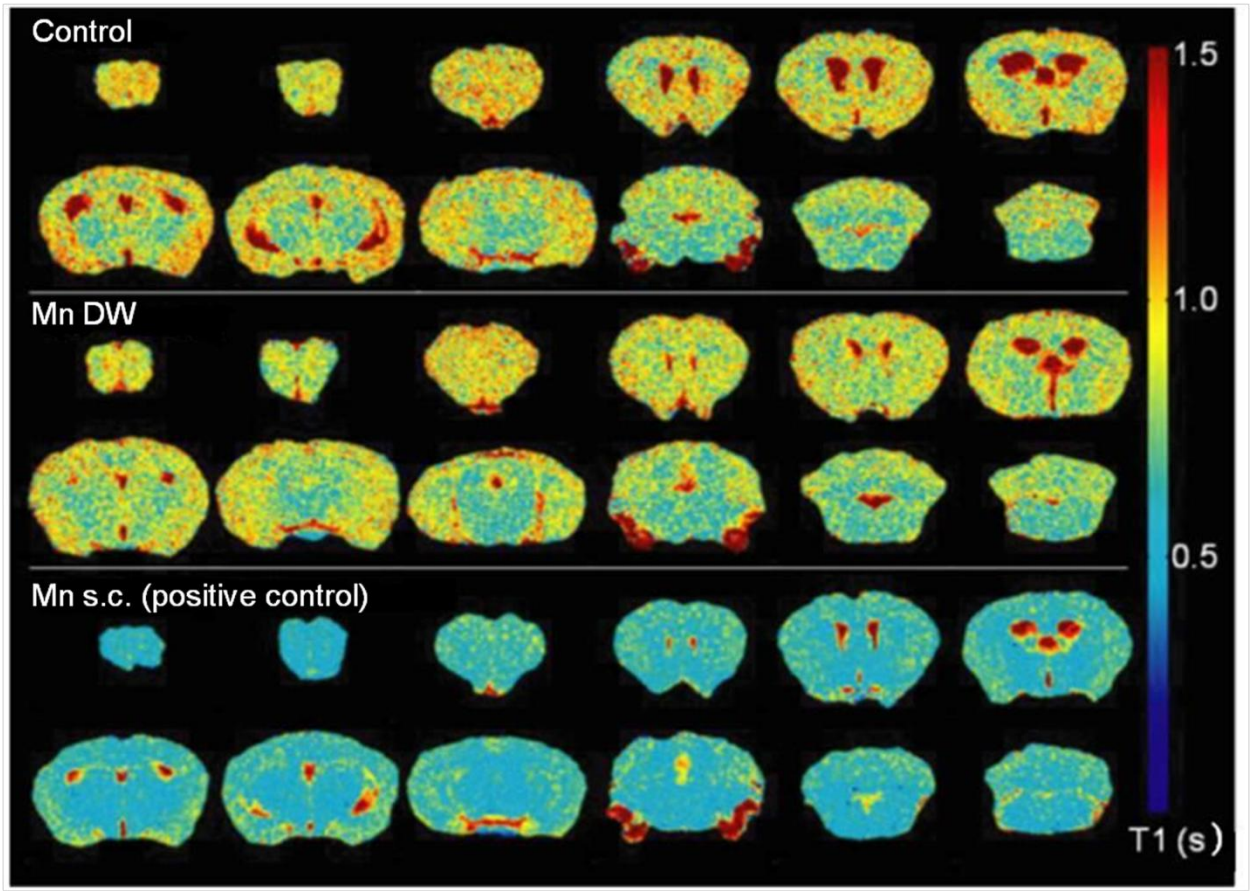


Figure 2.4. Representative T1 map generated using Matlab software visually displaying Mn effects on T1 relaxation time. Color transition from yellow to blue indicates a reduction in T1 (scale to the right). The T1 map from Mn DW group was from a mouse exposed to Mn via DW (0.4 g/l) for 5 weeks; the Mn s.c. (positive control) T1 map was from a mouse exposed to 50 mg/kg Mn s.c. 24 h prior to imaging.

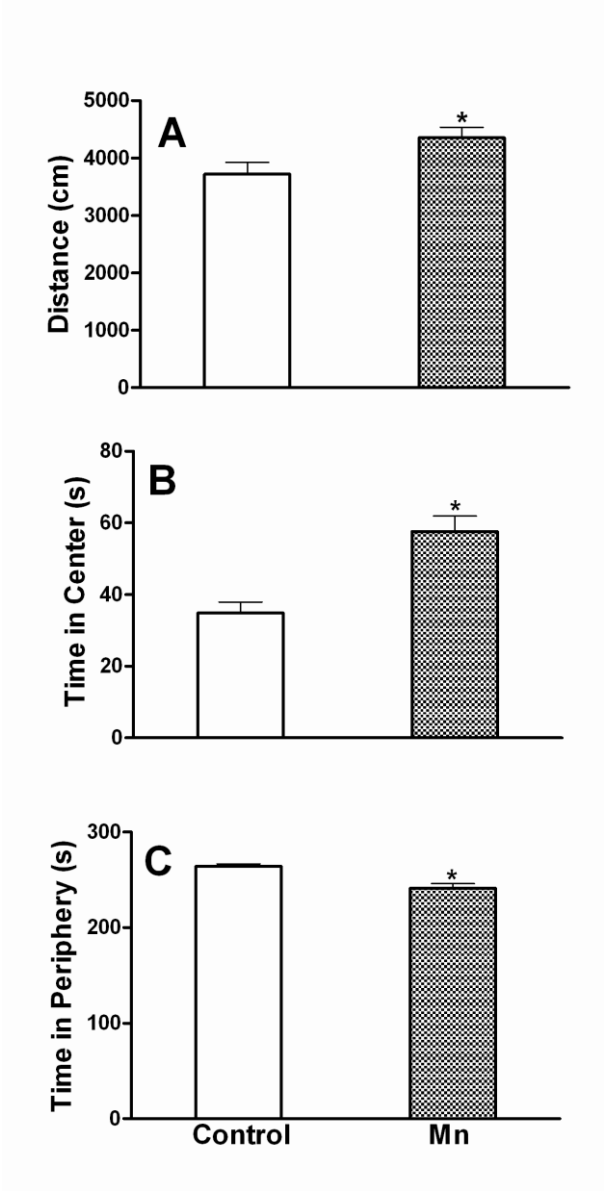


Figure 2.5. Locomotor activity. Effect of Mn after 6 weeks of DW (0.4 g/l) exposure on distance traveled (first 5 min) during open field testing (a) and on time spent per 5 min interval in the center (b) or the periphery (c) of the open field arena. Graphical representations are mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

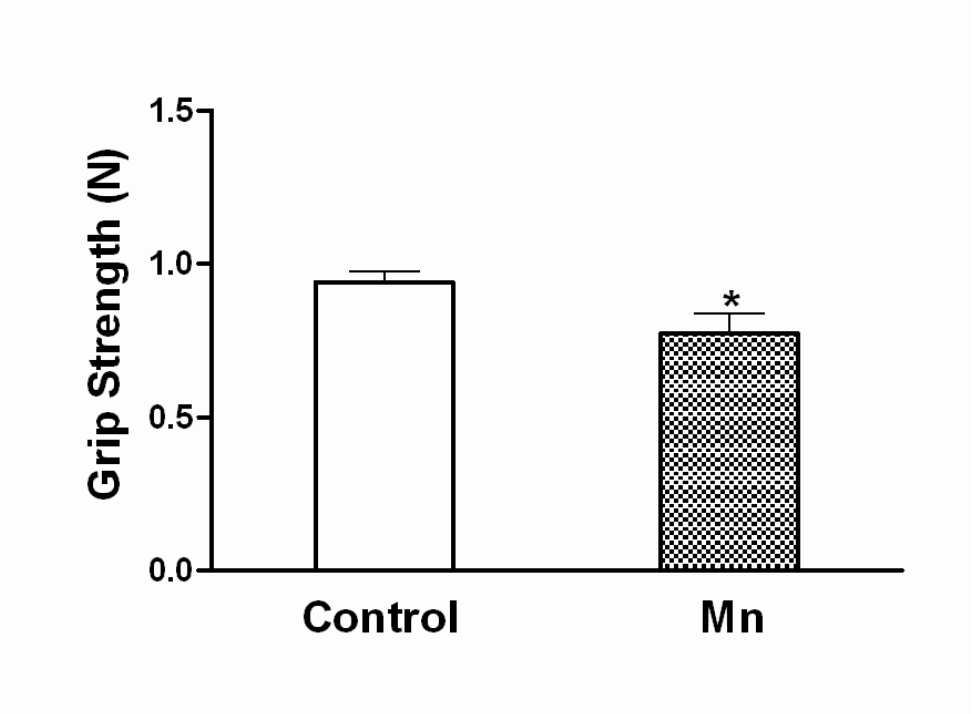


Figure 2.6. Grip strength. Effect of Mn after 6 weeks of DW (0.4 g/l) exposure on forelimb grip strength (recorded in newtons; N) and presented as mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

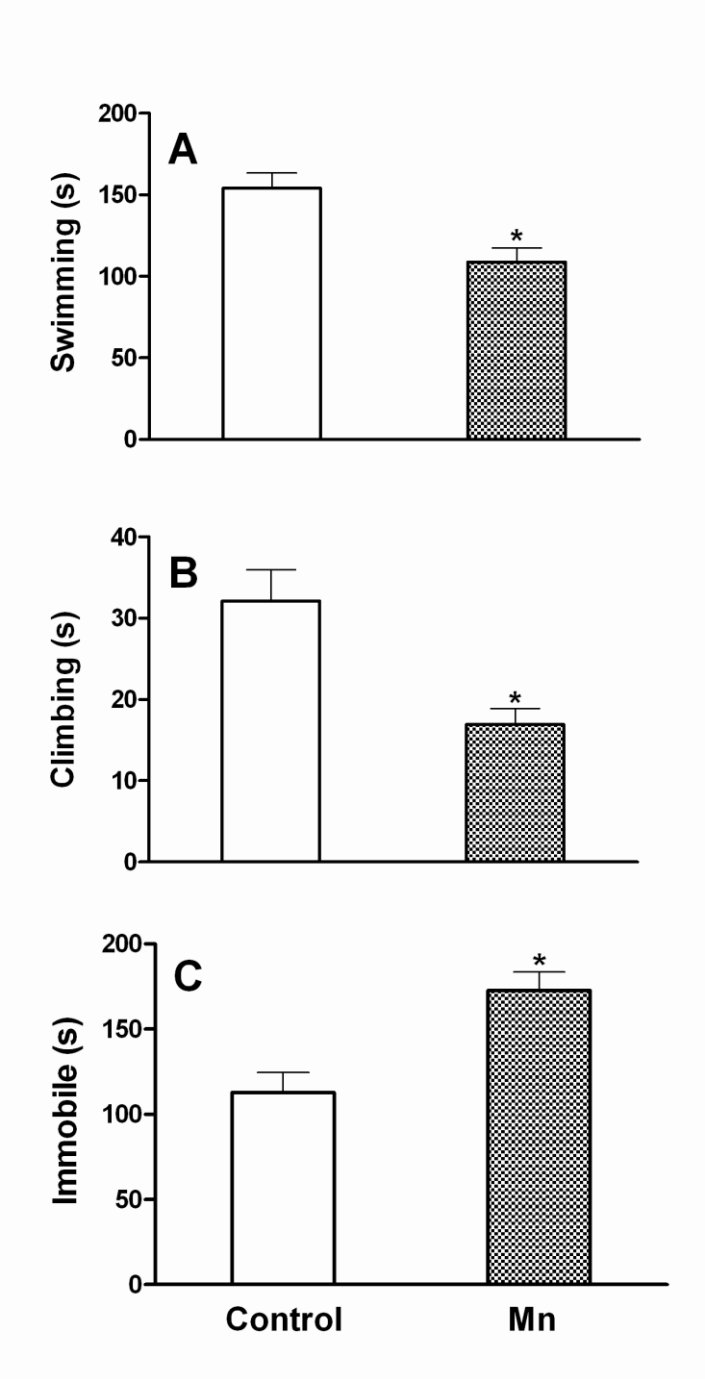


Figure 2.7. Forced swim test. Effect of Mn after 6 weeks of DW (0.4 g/l) exposure on total time spent swimming (a), climbing (b), or immobile (c) per 5 min interval in a forced swim test. Graphical representations are mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

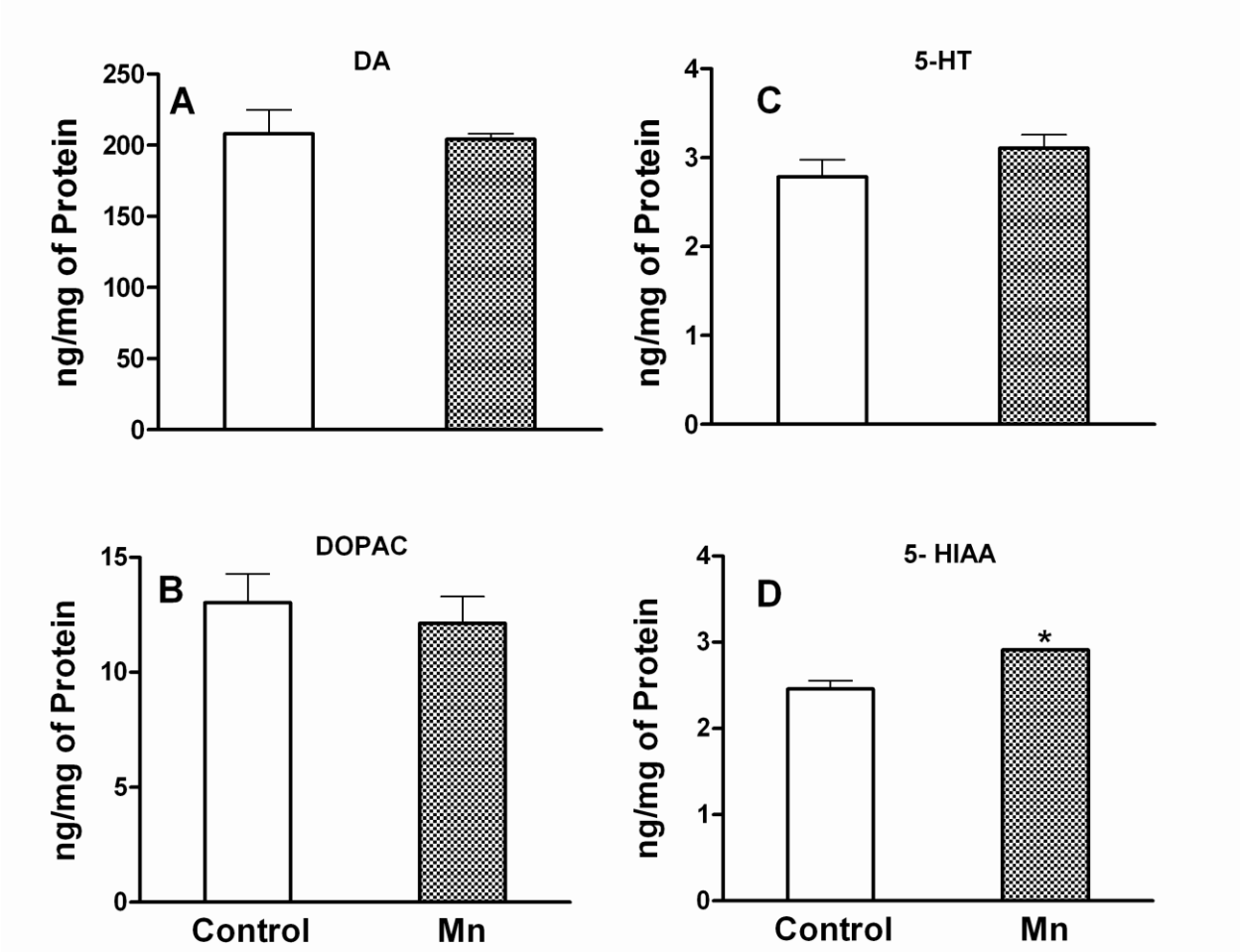


Figure 2.8. Striatal neurochemistry. Effect of Mn after 8 weeks of DW (0.4 g/l) exposure on striatal dopamine (DA; a), its metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC; b), serotonin (5-HT; c) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA; d). Neurotransmitter/metabolite concentrations are normalized on a per mg protein basis and presented as mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

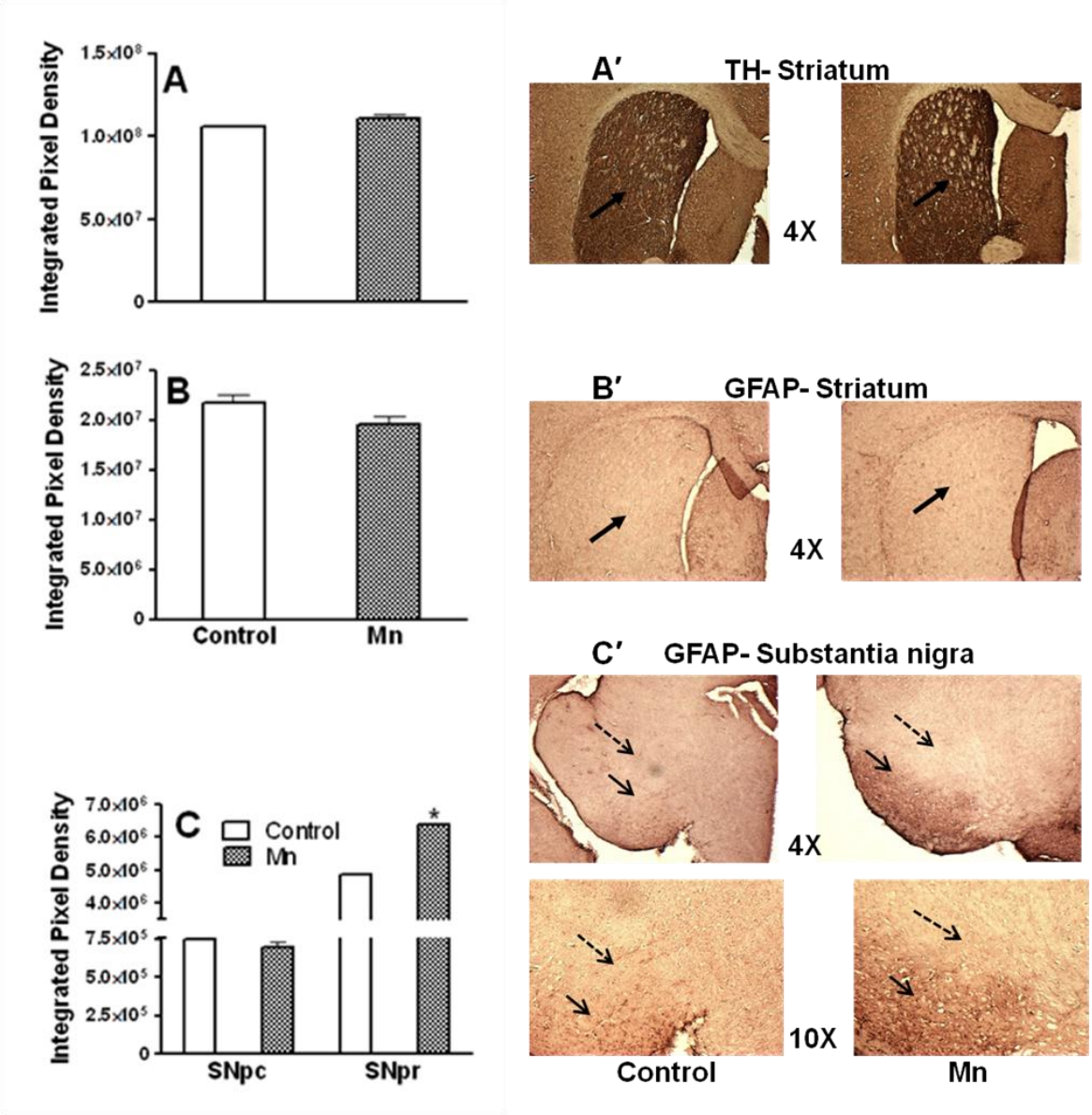


Figure 2.9. TH and GFAP Immunoreactivity. Effect of Mn after 8 weeks of DW (0.4 g/l) exposure on striatal expression of tyrosine hydroxylase (TH; a), striatal expression of glial fibrillary acidic protein (GFAP; b), GFAP expression in the substantia nigra pars compacta (Snpc) and substantia nigra pars reticulata (Snpr; c). Also shown are representative images of striatal TH (a′), striatal GFAP (b′) at 4X magnification and GFAP immunohistochemistry from substantia nigra (c′) at 4X and 10X magnifications. Solid black arrows in images a′ and b′ indicate striatum and images c′ point to Snpr; dashed black arrows in images c′ point to Snpc.

Striatal/nigral mean integrated pixel density was used to analyze the intensity of TH and GFAP staining and data are presented as mean ± SEM. * Indicates a significant effect of Mn ($p \leq 0.05$).

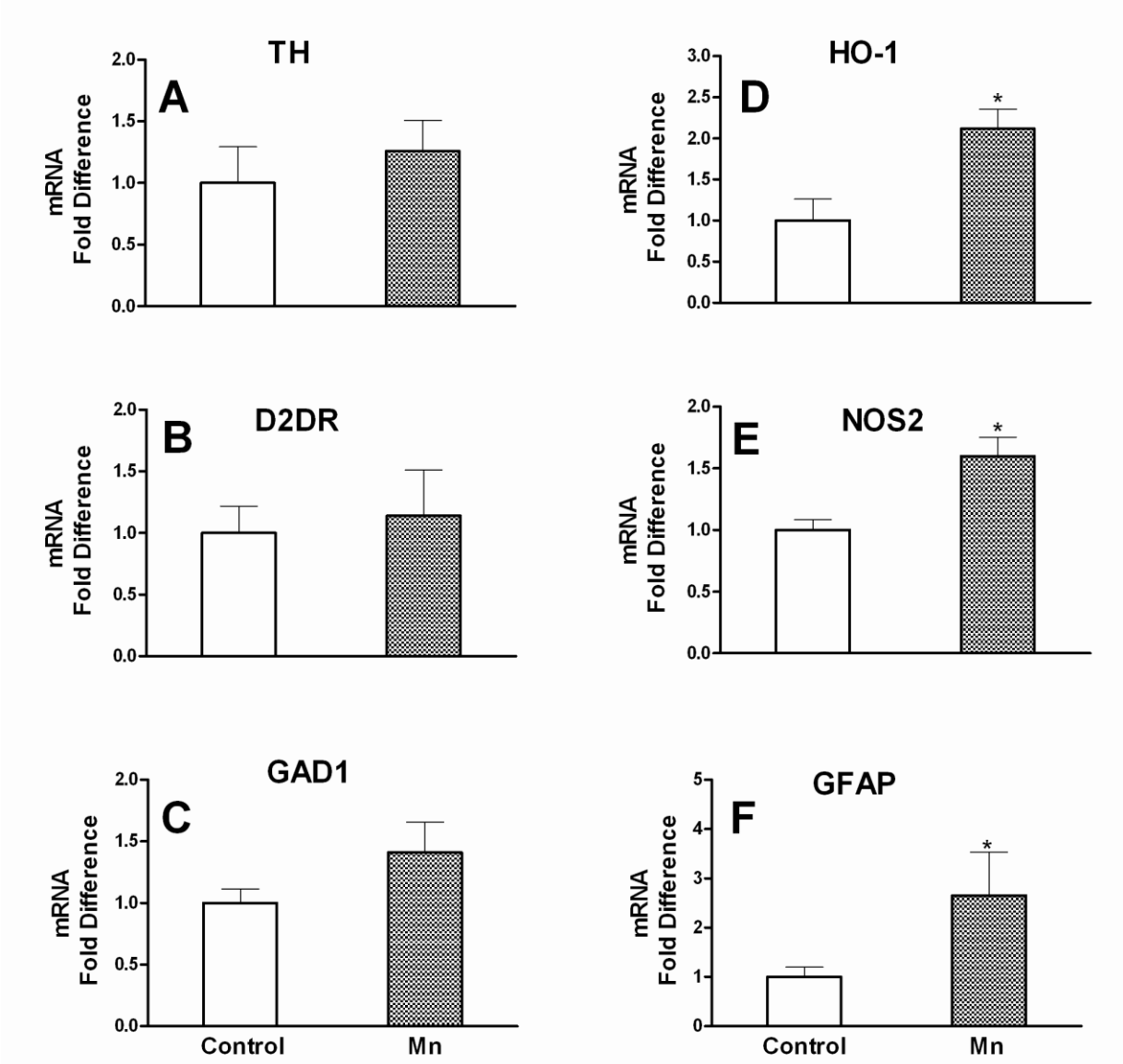


Figure 2.10. Nigral mRNA expression. Effect of Mn after 8 weeks of DW (0.4 g/l) exposure on mRNA expression levels of tyrosine hydroxylase (TH; a), dopamine receptor -2 (D2DR; b), glutamate decarboxylase 1 (GAD1; c), heme oxygenase-1 (HO-1; d), inducible nitric oxide synthase (NOS2; e) and glial fibrillary acidic protein (GFAP; f) in the substantia nigra. mRNA data are β-actin-normalized and are presented as fold change relative to control (mean ± SEM). * Indicates a significant effect of Mn ($p \leq 0.05$).

CHAPTER 3

BEHAVIORAL AND MONOAMINE PERTURBATIONS IN ADULT MICE WITH LOW-GRADE CHRONIC INFLAMMATION INDUCED BY REPEATED PERIPHERAL LIPOPOLYSACCHARIDE ADMINISTRATION[1]

[1]Krishna, S., Dodd, C.A., Filipov, N.M. To be submitted to *Brain, Behavior, and Immunity.*

Abstract

Sickness behavior following acute inflammation, modeled by a single peripheral lipopolysaccharide (LPS) administration, can later undergo transition into depressive-like behavior. Considering the limited information on the ability of chronic peripheral inflammation to induce depressive/other behavioral alterations and on the persistence of its effects after inflammatory stimuli termination, this study assessed the effects of chronic, low dose (0.25 mg/kg; twice weekly) peripheral (intraperitoneal) LPS treatment on selected behavioral, neurochemical and molecular measures at different time points in adult male C57BL/6 mice. Behavioral tests were performed 6 and 12 week post LPS/saline treatment as well as in mice exposed to 13-week LPS/saline treatment followed by an 11-week wait period without any treatment. Monoamine analysis (spleen, brain) and molecular tests (ELISA, qPCR) were conducted 13 and 25 week post LPS treatment and in the mice on 13 week on + 12 week off LPS. Behaviorally, LPS-treated mice were hypoactive in an open field after 6 week, whereas significant hyperactivity was observed in the 12 week LPS and 13 week LPS on+11 week off groups. Similar biphasic responses were observed in the center time of the open field arena between the 6 week LPS (decreased) and 13 week LPS on+11 week off groups (increased), suggestive of increased and decreased anxiety, respectively. In a forced swim test, mice exhibited significant increase in the immobility time (depressive behavior) at all times they were tested. Chronic LPS also produced a persistent increase in splenic serotonin (5-HT) and time-dependent region-specific alteration in dopamine and 5-HT homeostasis in the striatum and the prefrontal cortex. Above findings demonstrate that chronic peripheral inflammation initially causes decreased locomotion and increased anxiety, followed by persistent hyperactivity and decreased anxiety. Notably, chronic LPS-induced depressive behavior appears early, persists

long after LPS termination and it was associated with a constant increase in splenic 5-HT level. Collectively, our data emphasize the need for greater emphasis on peripheral/central monoamine alterations and lasting behavioral deficits induced by chronic peripheral inflammation as there are many pathological conditions where inflammation of a chronic nature is a hallmark feature.
Keywords: Lipopolysaccharide, Inflammation, Depressive-like behavior

Introduction

Contrary to earlier views on the central nervous system (CNS) as an immunologically privileged site, accumulating evidence highlights the existence of an extensive dynamic bidirectional interaction between the immune system and the CNS (Wrona, 2006, Ziemssen and Kern, 2007, Machado et al., 2011, Di Filippo et al., 2013). From a neuropathological perspective, peripheral inflammation can aggravate an ongoing neurological damage and can exaggerate motor and/or cognitive impairments in patients with neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Combrinck et al., 2002, Cunningham et al., 2005, Ferrari and Tarelli, 2011). Chronic peripheral inflammation induced by viral infection in humans accelerates the progression and pathogenesis of amyotrophic lateral sclerosis (Nicolson, 2008). Similarly, involvement of bacterial infections, i.e., *C.pneumoniae* in neurodegeneration has been demonstrated in patients with AD (Balin and Appelt, 2001). In animal models, peripherally generated inflammatory mediators induced by systemic challenge with lipopolysaccharide (LPS) or double-stranded RNA (poly I:C), representative of bacterial and viral infections, respectively, have been found to increase brain pro-inflammatory cytokine synthesis, resulting in progressive neurotoxicity (Perry, 2004, Puntener et al., 2012).

Peripheral LPS-induced alterations in central monoamine homeostasis have been reported (MohanKumar et al., 1999, Dunn, 2006). Specifically, single peripheral LPS administration increases the turnover of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) in different brain regions, including nucleus accumbens, hypothalamus, hippocampus and locus coeruleus (Molina-Holgado and Guaza, 1996, Lacosta et al., 1999). Behaviorally, acute peripheral LPS administration causes sickness behavior in rodents (Dantzer, 2001, Maes et al., 2012), that is characterized by disturbed (decreased) exploratory activity, locomotion and social exploration

(Swiergiel and Dunn, 2007, Henry et al., 2008, Sah et al., 2011) have been demonstrated by multitudes of animal studies. Of note, if the immune system activation persists, the heightened sickness behavior can transition into a depressive-like behavior (Dantzer et al., 2008, Fu et al., 2010).

Acute central or peripheral LPS administration leads to regionalized neuropathology, including hippocampal volume reduction (Fan et al., 2005) and increased number of apoptotic neurons in the prefrontal cortex (PFC) and basal ganglia structures (striatum, substantia nigra) (Czapski et al., 2007). These neuropathological alterations along with increased levels of pro-inflammatory cytokines are thought to be responsible for the depressive-like behavior associated with an inflammatory state (Kang et al., 2011, Shelton et al., 2011, Maes et al., 2012). There is a partial overlap in symptoms of inflammation-induced sickness behavior and depressive-like behaviors, but their course is quite different. Sickness behavior is an acute, quick, transient response to infections and it is reversible in nature once the pathogens are cleared; depressive behavior is characterized by delayed onset and long-lasting nature, which results when activation of the innate immune response is exaggerated in intensity and/or duration (Frenois et al., 2007, Dantzer et al., 2008, O'Connor et al., 2009).

Epidemiological investigations of the relationship between chronic inflammation and depression indicate increased rate of depressive disorders in patients suffering from chronic inflammatory conditions, as well as in patients undergoing interferon-α therapy (Capuron and Miller, 2004, Steptoe et al., 2007, Williams and Steptoe, 2007). In this regard, it is noteworthy that there are limited laboratory studies that have investigated the impact of chronic peripheral inflammation on the induction of depression-like behavior. Of note, only limited information (Frank-Cannon et al., 2008, Franciosi et al., 2012) exist on other motor and non-motor

behavioral domains, including anxiety and on associated neurochemical changes following long-term peripheral LPS administration. Also, data regarding the time-dependent changes in behavior, i.e. whether LPS-induced initial behavioral impairments are persistent throughout the time course of chronic inflammatory stimulation or at a later time point after treatment termination are limited.

Besides inducing emotional alterations, peripheral inflammatory events can influence the etiology and progression of many ongoing degenerative diseases, including AD and PD (Ferrari and Tarelli, 2011, Heneka et al., 2014). Chronic central LPS challenge models have successfully replicated the characteristic neurodegenerative pattern seen in PD and have demonstrated significant early microglial activation followed by delayed and time-dependent nigral dopaminergic neuron degeneration (Gao et al., 2002, McCoy et al., 2006) as well as long-term disruption of behaviorally relevant hippocampal network activity (Rosi et al., 2004, Belarbi et al., 2012). Peripheral administration of a single high dose (5 mg/kg) of LPS resulted in long-lasting neuroinflammation and nigral dopaminergic neurodegeneration (Qin et al., 2007). Of note, data that chronic peripheral LPS administration can cause both neuroinflammation and progressive dopaminergic neuron loss similar to PD is limited (Frank-Cannon et al., 2008, Morrison et al., 2012), and indicate that a genetic susceptibility might be necessary for the full pathology to occur (Frank-Cannon et al., 2008).

Besides causing neuroinflammation, acute peripheral LPS administration also induces the expression of pro-inflammatory cytokines in peripheral tissues, such as spleen and liver. Spleen, the secondary lymphoid organ, is largely innervated by the noradrenergic fibers of the sympathetic nervous system and functions as a storage site for the platelets (Walker and Codd, 1985). The neural signals transmitted by NE (major neurotransmitter of the sympathetic nervous

system) are taken by platelets that is in turn converted to immunomodulatory signals by 5-HT (Walker and Codd, 1985). Importantly, acute central administration of immune mediators such as interleukin-1 beta (IL-1β) or prostaglandins causes activation of sympathetic nerves, resulting in altered splenic tissue levels of NE or 5-HT (Vriend et al., 1993, Terao et al., 1995). Of note, information regarding the impact of chronic peripheral inflammation on splenic monoamine homeostasis is limited.

Besides triggering adverse neurological events, peripheral inflammation is also associated with metabolic dysfunctions characterized by alterations in the glucose homeostasis. For example, hypoglycemia (Roche et al., 2008, Raetzsch et al., 2009) or a biphasic response in the blood glucose level (Tsao et al., 2009, Bartolome et al., 2012) following acute inflammation has been reported. Of note, limited information exists on the time-dependent changes in glucose homeostasis following chronic peripheral inflammation.

Considering all of the aforementioned data gaps and the high prevalence of chronic inflammatory conditions of bacterial origin, the main objectives of the current study were to investigate the effect of chronic, low-dose peripheral LPS treatment in adult male C57BL/6 mice on selected monoamine profile, behavioral and molecular parameters. To assess the potential neurobehavioral consequences of repeated peripheral low-dose LPS treatment, we employed a battery of behavioral tests that could effectively assess both the locomotor (open field, grip strength and pole tests) as well as emotional (forced swim test) alterations. Additionally, to gain an insight into the persistent effects of chronic peripherally induced neuroinflammation, the above mentioned tests (behavioral, monoamine analysis and molecular) were performed on separate set of mice chronically exposed to low-dose LPS administration followed by a wait period during which the treatment was discontinued.

Materials and methods

Reagents

Unless otherwise stated, all chemicals including lipopolysaccharide *Escherichia coli*, serotype 0111:B4 (LPS) were purchased from Sigma (St. Louis, MO).

Animals

Animals were male C57BL/6 mice (4-5 months old; Taconic, Hudson, NY) weighing 30.10 ± 0.25 g (mean ± SEM) prior to treatment and were housed (n = 5/cage) with food available *ad libitum* on a 12-h light/dark cycle in an AAALAC accredited facility throughout the study. All procedures involving animal handling were in accordance with the latest NIH guidelines and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Animal treatment

Mice (n = 15 per group) were injected intraperitoneally (i.p.) with sterile normal saline (vehicle) or LPS at a dose of 0.25 mg/kg body weight (BW) twice weekly for up to 25 weeks. LPS/saline administration occurred at midmorning on treatment days. Behavioral tests (described in detail below) were carried out (24 h after the last injection) at 6 (n = 8 per group) and 12 week (n = 4 per group) post LPS/saline treatment. Randomly selected subsets (n = 5 per group) from each of the saline or LPS groups were sacrificed (24 h after the last injection) after 13 or 25 weeks of the treatment. Additionally, another subset of mice (n = 5 per group) were exposed to LPS/ saline for 13 weeks followed by a 12 week period without any treatment; these mice were sacrificed after the 12-week wait period (13 week on + 12 week off). Behavioral tests in these mice were done 1 week prior to the sacrifice, i.e., 13 week on + 11 week off. A detailed outline of the experimental design is presented in Fig. 3.1A. BW was recorded once weekly for the

entire experimental period (25 week). Food intake and water intake were measured weekly up to 12th week of the study duration. Organs (brain, liver, spleen, and thymus) were harvested from the sacrificed mice, weighed and quickly frozen at −80 °C for further analyses. Molecular tests, including ELISA, qPCR as well as plasma glucose measurements were conducted after 13 or 25 week of LPS treatment as well as 12 week after the termination of the 13 week LPS treatment. In order to assess the acute effect of LPS administration on peripheral inflammatory response, an additional experiment was performed where mice (n = 4/group/time point) were given a single i.p. injection of sterile normal saline (vehicle) or LPS (0.25 mg/kg BW) followed by sacrifice at 2, 5 and 24 h post LPS administration; plasma was collected from these mice and analyzed for pro-inflammatory cytokine (TNFα and IL-6) levels using ELISA (as described below).

Behavior

Behavioral tests, including open field, pole test, grip strength, and forced swim test were performed in succession over 2 days in a behavioral testing designated room located nearby, but separate from that in which animals were housed. All animals were naive to the behavioral apparatuses and the experimenter who conducted the behavioral tests was blinded to the animal treatments.

Open field. Each mouse was individually monitored in an open field arena (l × w × h: 25 × 25 × 40 cm, divided into 16 square grids; Coulbourn Instruments, Whitehall, PA) for 30 min with Limelight video tracking software (Actimetrics, Wilmette, IL). Total distance traveled (cm) was recorded as a measure of locomotor activity and analyzed per 5 min intervals. Additionally, the time spent in the periphery or in the center of the open field arena was measured to determine the anxiety level and analyzed per 5 min intervals (Bailey and Crawley, 2009).

Pole test. Pole test was conducted to assess the motor coordination of mice as detailed in (Krishna et al., 2014). Briefly, mice were placed upright on the top of a gauze-wrapped vertical metal pole (1 cm in diameter and 55 cm in height). The maximum turning time allowed was 60 s and the total time (complete turn + descent) per trial was 120 s (Staropoli et al., 2012). If a mouse did not turn within the first 60 s it was gently guided and a maximum measurement (60 s) was recorded. A total of 4 trials were completed for each mouse with a 3-5 min inter-trial interval and the average turn time, descent time, and total time spent on the pole from all 4 trials was used for statistical analysis (Royl et al., 2009).

Grip strength. This test was performed to assess the neuromuscular function by measuring the forelimb grip strength using mouse-specific strength gauge (Bioseb, France), as previously described (Krishna et al., 2014). The average maximum grip force (recorded in newtons [N]) of the four trials was used for statistical analysis.

Forced swim test. This test was performed as described previously (Krishna et al., 2014). Mice were gently placed in a large cylindrical container (18 cm in diameter and 25 cm in height) filled approximately two-thirds with tap water (3 L, 29±1 °C) for a period of 15 min. Limelight video tracking software (Actimetrics) was utilized to score the total time spent swimming, climbing, or immobile and analyzed per 5 min intervals.

HPLC

Monoamine analysis by HPLC was performed as described in detail in (Coban and Filipov, 2007). Briefly, brain (micropunches from PFC, striatum, and hippocampus) and spleen (20 mg) tissue samples were placed in 100 and 200 µl of 0.2 N perchloric acid, respectively, sonicated and centrifuged. A 20 µl aliquot of the supernatant was injected into HPLC with an electrochemical detector (Waters Alliance, Waters Co., Milford, MA) to determine: (1) DA and

its metabolites, namely dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT); (2) NE and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG); and (3) serotonin (5-HT) and it metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the brain tissues and 5-HT and NE in the splenic tissue. Prior to statistical analysis, brain data were normalized on a per mg protein basis (Lin et al., 2013), whereas splenic 5-HT and NE data were normalized on a per mg tissue basis.

Plasma glucose determination

Plasma glucose levels were determined with a glucometer (TRUEresult®, Nipro Diagnostics, Fort Lauderdale, FL) as per manufacturer's instructions.

ELISA Analysis for TNFα and IL-6

Plasma concentrations of TNFα and IL-6 were analyzed after acute (2, 5 and 24 h post LPS injection) and chronic LPS (13 week, 25 week and 13 week on +12 week off) administration using mouse-specific Duo set ELISA development kit (R&D Systems, Minneapolis, MN) as we have described it before. Samples and standards (TNFα: 2000-31.25 pg/ml) and (IL-6: 1000-15.625 pg/ml) were run in duplicate. Absorbance (450 nm analytical read; 570 nm background correction read) was measured using a Synergy 4 hybrid multi-mode microplate reader (BioTek Instruments, Winooski, VT) and the mean from each sample replicate was used for statistical analysis. Samples with cytokine levels below the lower limit of detection (LOD: IL-6 and TNFα were 2.5 and 5.2 pg/ml, respectively) were assigned the LODs for statistical purposes.

Real-time quantitative PCR (qPCR)

qPCR was done as we have described it before (Lin et al., 2013). Briefly, total RNA from substantia nigra samples was isolated using E.Z.N.A. microelute total RNA kit (Omega Bio-Tek,

Inc., Norcross, GA) and quantified using Take 3 plate and Epoch microplate spectrophotometer (Biotek, Winooski, VT). Using a Peltier thermal cycler (Bio-Rad; 5 min 25 °C, 30 min 42 °C, and 5 min 85 °C), 75 ng RNA was converted to cDNA with qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD). Using 0.5 ng of cDNA per sample, expression of tyrosine hydroxylase (TH), dopamine receptor-2 (D2DR), glial fibrillary acidic protein (GFAP), F4/80, interleukin-6 (IL-6) and interleukin-1β (IL-1β) were determined by qPCR using mouse-specific, certified primers and SYBR Green (Qiagen, Valencia, CA). Amplifications were performed in a Mx3005P qPCR machine (Stratagene) programmed for an initial warming (10 min, 95 °C) followed by 45 cycles (15 s, 95 °C, 1 min, 60 °C) with each sample run in triplicate. Treatment differences were calculated as a fold change using the $^{\Delta\Delta}C$t method with β-actin as the house-keeping gene.

Statistical analysis

One-way ANOVA was used to analyze the effect of LPS on organ weights (g/kg BW), behavioral endpoints (except as specified below), HPLC, molecular, and plasma glucose data within a time point. Two-way analysis of variance (ANOVA) was conducted to analyze the weekly food (g/kg BW) and water (ml/kg BW) intake and the open field (treatment x time [interval]) data. If ANOVA's overall main effect was found significant, treatment means were separated by Student-Newman–Keuls *post hoc* test. All results are presented as mean ± SEM and are considered significant at $p \leq 0.05$.

Results

Body weight (BW), food and water intakes, organ weights

Two-way ANOVA demonstrated an overall significant main effect of LPS ($p \leq 0.001$) on BW up to the 13th week that was driven mainly by the effects of LPS during the first 4 weeks of

the experiment where the BW decrease within each week was significant ($p \leq 0.05$); the largest BW drop (10%) occurred 1 week after LPS treatment initiation (Fig. 3.1B). Afterwards, BW of saline and LPS-treated mice did not significantly differ ($p \geq 0.05$) except after the 8^{th} week of LPS ($p \leq 0.05$). With respect to the BW from 13 to 25 weeks, while the saline and LPS-treated mice did not differ ($p > 0.25$; Fig. 3.1B), the BW of the mice in saline-13 week on+12 week off group increased significantly ($p \leq 0.05$) even 1 week after the discontinuation of saline injection and remained significantly elevated for the duration of the study (Fig. 3.1B). On the other hand, discontinuation of the LPS treatment (LPS-13 week on +12 week off group) did not affect the BW of mice at any time point from week 14 to week 25 ($p > 0.20$; Fig. 3.1B).

There were no significant effects of LPS ($p > 0.30$) or duration (week) of the treatment ($p > 0.80$; Fig. 3.1C) with respect to the food or water intakes, which were monitored during the first 12 weeks of the study.

Thirteen week LPS administration produced slight, but significant ($p \leq 0.05$) increase in spleen weight; liver and thymic weights were unaffected ($p > 0.25$; Fig. 3.2A). Continued LPS treatment for another 12 weeks, i.e. a total of 25 weeks moderately increased the liver weight; weights of the other organs were not changed ($p > 0.25$; Fig. 3.2B). Compared to the 25-week saline group, LPS administration for 13 weeks followed by a 12-week wait period significantly increased the liver, spleen and thymic weights ($p \leq 0.05$; Fig. 3.2B).

Behavior

Open field. There were significant main effects of LPS ($p \leq 0.01$) and interval (5 min time period; $p \leq 0.001$), but without any significant interaction between the two ($p > 0.90$) with respect to the distance traveled in the open field arena after 6 weeks of LPS treatment; LPS-treated mice were hypoactive as illustrated by the decreased distance traveled per 5 min interval

($p \leq 0.05$; Fig. 3.3A). As expected, both control and LPS-treated mice habituated to the arena over time and their overall activity decreased (e.g., distance traveled: 4054.77 ± 359.94 vs. 3637.27 ± 225.74 cm during the first 5 min and 2491.80 ± 210.94 vs. 1977.19 ± 224.36 cm during last 5 min for control and LPS groups, respectively), with LPS-treated mice being hypoactive throughout the 30 min open field session. Six weeks later, i.e. after 12 weeks of LPS treatment, mice exhibited a significant increase in locomotor activity evidenced by the significantly increased distance traveled (per 5 min interval) in the open field arena ($p \leq 0.01$; Fig. 3.3A). Compared to 6 weeks, as expected, the overall activity of both control and LPS groups was decreased (Fig. 3.3A). Control mice habituated quickly to the open field arena evidenced by 59% reduction in the locomotor activity as compared to the 6 week time point (Fig. 3.3A). However, in comparison to the 6 week, 12 week LPS-treated mice had impaired long-term habituation evidenced by only 40% reduction in their locomotor activity (Fig. 3.3A). Meanwhile, within the 30 min open field session at 12 weeks, both control and LPS-treated mice habituated to the arena over time and their overall activity decreased (e.g., distance traveled: 1541.38 ± 532.50 vs. 2337.97 ± 57.00 cm during first 5 min and 856.99 ± 232.10 vs. 1727.78 ± 483.90 cm during last 5 min for control and LPS groups, respectively. A similar trend was observed even 11 week after the termination of the 13 week chronic LPS treatment with LPS-treated mice exhibiting significantly increased locomotor activity compared to the control group ($p \leq 0.05$; Fig. 3.3A).

There was also overall significant main effect of LPS with respect to the time spent in the center versus periphery of the open field arena after 6 weeks of LPS treatment. The mean time spent per 5 min interval in the center and periphery was decreased (center; Fig. 3.3B) and increased (periphery; Fig. 3.3C) significantly ($p \leq 0.05$) at this time point. After 12 weeks of LPS

treatment, mice did not show any significant difference with respect to the time spent in center (Fig. 3.3B) versus periphery (Fig. 3.3C) of the open field arena compared to the control group. However, the mean time spent per 5 min interval in the center and periphery was increased (center; Fig. 3.3B) and decreased (periphery; (Fig. 3.3C) significantly ($p \leq 0.001$) 11 weeks after the termination of the 13-week chronic LPS administration. Thirteen week LPS administration followed by an 11-week wait period also resulted in significant decrease in the corner time ($p \leq 0.05$; data not shown).

Pole test. No significant effect of LPS was observed with respect to the mean time to turn ($p > 0.90$), time to descend ($p > 0.70$) and total time ($p > 0.70$) after 6 weeks of LPS treatment (data not shown). The pole test parameters, mentioned above were unaffected after 12 weeks of LPS administration and also 11 weeks after the termination of a 13-week LPS treatment ($p > 0.25$; data not shown).

Grip strength test. Short of an apparent trend towards an increase in the maximum grip strength after 6 weeks on LPS ($p = 0.09$), the grip strength was unaffected after 12 weeks of LPS ($p > 0.20$) or 11 weeks after the discontinuation of a 13-week LPS treatment ($p > 0.90$; data not shown).

Forced swim test. After 6 weeks on LPS, mice swam significantly less ($p \leq 0.05$; Fig. 3.4A) and had a concomitant increase in the immobility time ($p \leq 0.05$; data not shown); the mean time spent climbing per 5 min interval was unaffected ($p > 0.80$; Fig. 3.4B). Similarly, after 12 weeks on LPS, mice swam significantly less than control mice ($p \leq 0.05$; Fig. 3.4A) and had a corresponding increase in the immobility time ($p \leq 0.05$; data not shown); climbing was unaffected at this time point as well ($p > 0.10$; Fig. 3.4B). Eleven weeks after the termination of the 13-week LPS treatment, mice continued to spend significantly less time swimming ($p \leq 0.05$;

Fig. 3.4A) with a parallel increase in the immobility time ($p \leq 0.05$; data not shown); at this time point they also climbed less ($p \leq 0.05$; Fig. 3.4B).

HPLC

Spleen. Thirteen weeks of LPS treatment produced a significant reduction in the splenic NE level ($p \leq 0.05$; Fig. 3.5A); however, the levels remained unaffected after 25 weeks or 12 weeks after the termination of the 13-week LPS treatment ($p > 0.06$; Fig. 3.5A). With respect to 5-HT, LPS treatment produced a significant increase in the splenic 5-HT levels after 13 or 25 weeks of LPS treatment; the increase was also present 12 weeks after discontinuation of the 13-week LPS treatment ($p \leq 0.05$; Fig. 3.5B).

Brain. In the PFC, 13 weeks of LPS treatment did not alter the levels of DA, 5-HT or its metabolites ($p > 0.40$; Table 3.1), but it did cause non-significant trend towards a decrease in the MHPG level ($p < 0.15$; Table 3.1) as well as in the MHPG/NE ratio ($p \leq 0.10$; data not shown), without affecting the parent neurotransmitter NE levels ($p > 0.50$; Table 3.1). 25 weeks of LPS administration did not alter the levels of DA, NE or their metabolites ($p > 0.45$; Table 3.1), but significantly affected the 5-HT homeostasis. Specifically, LPS produced a significant increase in the 5-HT and 5-HIAA levels ($p \leq 0.01$; Table 3.1), without affecting the 5-HIAA/5-HT ratio ($p > 0.25$; data not shown). Thirteen weeks of LPS treatment followed by a 12 week wait period significantly altered the DA homeostasis in the PFC. Specifically, LPS resulted in an apparent trend towards a decrease in the DA level that was close to significance ($p = 0.09$; Table 3.1), along with a non-significant trend towards an increase in the concentration of the DA metabolite, DOPAC ($p < 0.20$; Table 3.1). As a result, the DOPAC/DA ratio was increased significantly ($p \leq 0.05$; data not shown). LPS did not increase the concentration of the other DA metabolite, HVA ($p > 0.50$; Table 3.1), but the HVA/DA ratio was increased to a level that was close to

significance ($p = 0.07$; data not shown). LPS treatment for 13 weeks followed by a 12-week wait period also resulted in a non-significant trend towards an increase in the MHPG level ($p = 0.13$; Table 3.1) as well as an apparent trend towards an increase in the parent neurotransmitter NE level that was close to significance ($p = 0.07$; Table 3.1); the MHPG/NE ratio was unaffected ($p > 0.70$; data not shown). No significant alteration in the 5-HT homeostasis was observed at this time point ($p > 0.50$; Table 3.1)

In the striatum, 13 week LPS treatment did not cause any alterations of DA or its metabolites ($p > 0.25$; Table 3.2); there was a non-significant trend towards an increase in the HVA/DA ratio ($p < 0.15$; data not shown). In contrast to DA, 5-HT homeostasis was significantly affected after 13 weeks of LPS administration. Specifically, LPS caused a significant increase in the 5-HT level ($p \leq 0.05$; Table 3.2) as well as an apparent trend towards an increase in the 5-HIAA level that was close to significance ($p = 0.08$; Table 3.2); there was a non-significant trend towards a decrease in the 5-HIAA/5-HT ratio ($p \leq 0.10$; data not shown). Chronic LPS administration for 25 weeks significantly increased the concentration of the DA metabolite, HVA ($p \leq 0.05$; Table 3.2), without affecting the level of DA itself ($p > 0.90$; Table 3.2). No significant alterations were observed with respect to the striatal DOPAC level ($p > 0.35$; Table 3.2) or the DOPAC/DA ratio ($p > 0.35$; data not shown). 5-HT homeostasis was not affected at this time point ($p > 0.35$; Table 3.2). LPS treatment for 13 weeks followed by a 12-week wait period did not affect striatal DOPAC ($p < 0.20$; Table 3.2) or striatal DA levels ($p > 0.50$; Table 3.2) significantly, but the DOPAC/DA ratio ($p \leq 0.05$; data not shown) was decreased significantly. No significant changes were observed with respect to the striatal HVA level ($p > 0.60$; Table 3.2) and HVA/DA ratio ($p > 0.35$; data not shown). The concentration of the DA metabolite, 3-MT, which was only detected in the striatum, was unaffected after 13 or 25

weeks of LPS administration or 12 weeks after the discontinuation of the 13-week LPS treatment ($p > 0.35$; data not shown). No significant alterations in 5-HT or 5-HIAA levels were observed after the LPS treatment termination ($p > 0.20$; Table 3.2)

Thirteen week LPS administration did not affect hippocampal monoamine homeostasis ($p > 0.25$; Table 3.3), except for a non-significant trend towards a decrease in the DA level ($p < 0.15$; Table 3.3). Similarly, LPS treatment for 25 weeks did not affect hippocampal levels of DA, NE, 5-HT or their metabolites ($p > 0.15$; Table 3.3). Similar to 13 and 25 week data, hippocampal monoamine homeostasis remained unaffected after the LPS treatment termination ($p > 0.15$; Table 3.3).

Plasma glucose measurement

Compared to saline-injected mice, 13 weeks of LPS treatment did not affect plasma glucose levels ($p > 0.70$; Fig. 3.6). However, after 25 weeks, plasma glucose in the LPS-injected mice was significantly elevated ($p \leq 0.001$; Fig. 3.6); this hyperglycemic effect of LPS was not maintained in mice that had their LPS treatment discontinued after 13 weeks ($p > 0.10$; Fig. 3.6).

ELISA

IL-6 level was undetectable in acute saline-treated mice at all time points (Fig. 3.7A). Single LPS administration increased plasma IL-6 after 2 or 5 h of LPS administration ($p \leq 0.05$; Fig. 3.7A); however, plasma IL-6 level was undetectable 24 h after LPS administration (Fig. 3.7A). Similar to IL-6, plasma TNFα in saline-treated mice was not detectable at the three time points tested (Fig. 3.7B). However, plasma TNFα was significantly elevated in mice at 2 and 5 h ($p \leq 0.05$; Fig. 3.7B) post LPS injection and, similar to IL-6, was undetectable after 24 h of LPS administration (Fig. 3.7B).

After 13 weeks, plasma TNFα level in the LPS-treated mice was minimal and was comparable to that of saline-treated group ($p > 0.30$; Fig. 3.7C). Similar to the 13-week data, plasma TNFα was detectable only in few samples from 25 weeks or 13 weeks on + 12 weeks off LPS and it was not different from age-matched controls ($p > 0.30$; Fig. 3.7C). Plasma levels of IL-6 were below the detection limit at all three time points (data not shown).

qPCR

At the mRNA level in the substantia nigra, D2DR expression was significantly upregulated after 25 weeks of LPS administration ($p \leq 0.05$; Fig. 3.8); however, its expression was unaffected after 13 weeks of LPS treatment or 12 weeks after the termination of 13 week LPS administration ($p > 0.30$; Fig. 3.8). TH mRNA expression was unaffected irrespective of the treatment duration ($p > 0.30$; data not shown). LPS treatment for 13 or 25 weeks did not alter the GFAP mRNA expression ($p > 0.25$; Fig. 3.8); however there was an apparent trend towards an increase in its expression 12 weeks after the 13-week LPS treatment termination ($p = 0.08$; Fig. 3.8). F4/80 mRNA level was significantly upregulated after 13 or 25 weeks of the LPS treatment ($p \leq 0.01$; Fig. 3.8), while no alteration was observed after the discontinuation of the LPS treatment ($p > 0.40$; Fig. 3.8). The mRNA expression of the pro-inflammatory cytokines, IL-6 or IL-1β was unaffected by LPS ($p > 0.25$; data not shown), except for a moderate, but significant decrease in the IL-6 level observed 12 weeks after the termination of the 13 week LPS treatment ($p \leq 0.05$; data not shown).

Discussion

Chronic peripheral inflammation has long been implicated in the etiology of immune-mediated diseases like rheumatoid arthritis and lupus erythematosus (Ku et al., 2009, Ohl and Tenbrock, 2011). Besides peripheral immune dysregulation, inflammation-related events in the

periphery can communicate with the CNS, producing an exacerbation of the CNS immune response and subsequent alterations in cognition, mood, and behavior (Maier, 2003). Clinical evidence for a clear association between chronic, low-grade inflammatory response and depression, which is the most common mood disorder, is accumulating (Berk et al., 2013), but previous animal studies have largely focused on the impact of acute peripheral inflammation on emotional alterations. However, inflammation-associated emotional disorders, including depression in humans, are typically lifelong progressive conditions that are more likely to be related to chronic, low-grade inflammatory response (Dantzer et al., 2008). In light of the limited experimental data on the impact of chronic peripheral inflammation on the induction of depressive behavior and other neurological behavioral alterations and on their persistence after the termination of inflammatory stimuli exposure, we conducted this study. The current study is unique in that it assessed the time-dependent changes in the LPS-induced behavior and associated neurochemical and molecular substrates not only during the treatment but also after the treatment termination in a chronic inflammatory model paradigm. The main findings of this study include: 1) Repeated low-dose peripheral LPS administration for 6 weeks resulted in depression-like emotional alterations, that persisted after 12 weeks of treatment and even long after the termination of LPS administration; 2) chronic low-dose LPS caused brain region-specific and time dependent neurochemical alterations in the DA, NE and 5-HT homeostasis; 3) prolonged LPS treatment increased splenic 5-HT that persisted even long after the LPS termination.

Behavioral effects of acute systemic LPS administration, collectively termed as sickness behavior and characterized by reduced exploratory activity and locomotion are well documented (Bluthe et al., 1992, Swiergiel and Dunn, 2007, Sah et al., 2011, Biesmans et al., 2013). Previous

rodent studies have shown that sickness behavior peaks 2-6 h after a single LPS challenge with an associated increase in peripheral pro-inflammatory cytokines, and gradually fades over time, diminishing at 24-48 h after the LPS injection (Dantzer et al., 2008, Henry et al., 2008, MacDonald et al., 2014). Consistent with these earlier findings, we found that the plasma TNFα and IL-6 levels were significantly increased after 2 and 5 h, but not 24 h after a single LPS challenge. In light of this result, for the chronic experimental paradigm adopted in this study, mice were given a 24 h rest period after the previous LPS administration and thereafter underwent behavioral tests or sacrifice in order to minimize the possible acute effects of LPS.

Acute LPS-induced sickness behavior can transform to a depressive-like behavior over time, which can persist even after the immediate behavioral response (reduction in locomotor activity) to LPS administration has been normalized (Frenois et al., 2007, Henry et al., 2008, O'Connor et al., 2009). For example, 24 h after the LPS treatment, mice exhibited increased immobility time in the tail-suspension test and in the forced-swim test, indicative of a depressive-like behavior, while showing normal locomotor activity at that time point (Frenois et al., 2007).

It has to be noted that the above studies have adopted an acute inflammatory model paradigm to dissociate the LPS-induced sickness and depressive-like behaviors, but the association between depression and chronic, low-grade inflammation is understudied, but mounting. Higher rates of depressive disorders in patients suffering from chronic inflammatory conditions as well as in patients undergoing interferon (IFN)-α therapy are evidence for the close association between chronic inflammation and depression in humans (Hauser et al., 2011, Krishnadas and Cavanagh, 2012, Hoyo-Becerra et al., 2014). Experimentally, repeated i.p. administration of LPS (1 mg/kg) for 4 days resulted in depressive behavior in male Wistar rats,

evidenced by the increased immobility time in forced swim test that was performed 24 h after the last LPS injection, a time point where the sickness behavior was abolished (Bay-Richter et al., 2011). Repeated intermittent LPS injections induced long-lasting anhedonic response (decreased sucrose preference) that lasted for 7 weeks (Kubera et al., 2013).

Taken together, the above studies suggest that a chronic inflammatory tone is required to cause long lasting depressive-like behavior in rodents and that chronic, persistent (rather than the acute) inflammation is more closely associated with depression in humans (Biesmans et al., 2013). In this study, we demonstrate that chronic low-dose LPS administration resulted in depression-like emotional alterations (evidenced by the significantly decreased swimming time and concomitant increase in the immobility time in the forced swim test) which was apparent early on (6 weeks) and persisted after 12 week of LPS treatment. Most intriguingly, we found that the depressive-like behavior persisted even long (11 weeks) after the termination of chronic LPS administration, indicating the ability of prolonged LPS treatment to have a long-lasting depressogenic effect. Interestingly, the long-lasting depressogenic effect observed in our study has been demonstrated by several case studies in humans where depressive symptoms are present even after termination of IFN-α therapy, which causes enhanced production of pro-inflammatory cytokines and downregulates 5-HT synthesis during the treatment (Janssen et al., 1994, Rifflet et al., 1998, Koseki et al., 2000, Hoyo-Becerra et al., 2014).

A recent study reported chronic depression in female, but not male C57BL/6 mice, characterized by long-lasting anhedonic response (decreased sucrose preference) following repeated, intermittent LPS injection for 4 months (Kubera et al., 2013). However, it is worth noting that studies in rats and mice have reported a clear sex-specific difference in the sucrose consumption, with females showing a greater sucrose preference than males (Sclafani et al.,

1987, Pelloux et al., 2005). More importantly, male, but not female rats, showed complete absence of sucrose preference after an extended access to sucrose (Sclafani et al., 1987). In this regard, it could be that in (Kubera et al., 2013) study, the females were more responsive to the sucrose preference test or the long-term availability of sucrose solution might have resulted in a lack of sucrose preference in control male mice, which in turn have masked the effect of chronic LPS treatment.

Alterations in glucose homeostasis in response to stress-induced hypercortisolemia or peripheral inflammation have been increasingly associated with depression (Gans, 2006, Bartolome et al., 2012, Hannestad et al., 2012). To investigate the possibility that the persistent depressive behavior displayed by mice in this study may have resulted from the stress associated with chronic low-dose LPS injection, we measured the plasma glucose of mice at different time points of the LPS treatment. Interestingly, the plasma glucose level was unaffected after 13 weeks of LPS administration and 11 weeks after the LPS treatment termination, but was only increased after 25 weeks of LPS treatment. In light of the persistent depressive behavior we observed, our finding implies that the chronic LPS-induced depression is independent of the stress-induced alterations in glucose homeostasis; however circulating cortisol needs to be measured to confirm this assumption. Peripheral inflammation induced alterations in glucose homeostasis has been previously reported. For example, some studies have demonstrated decrease in glucose production and glucose utilization (Raetzsch et al., 2009), while others have reported a biphasic response in blood glucose level (Tsao et al., 2009, Bartolome et al., 2012) following acute LPS administration. Taken together, the hyperglycemia exhibited by 25-week LPS treated mice could be due to either an increased cortisol level or an aftereffect of the chronic

peripheral inflammation induced by LPS treatment; however, the exact mechanism is unclear at this stage.

In order to investigate the potential peripheral monoamine alterations underlying the observed persistent depressive behavior, we assessed NE and 5-HT levels in the splenic tissue. In this study, we found significant reduction in the splenic NE after 13 weeks, but not after 25 weeks of LPS treatment or after LPS treatment termination. Interestingly, we observed a significant increase in splenic weight after 13 week of LPS treatment. Changes in the tissue volume of lymphoid organs has been reported to affect the availability of NE as this neurotransmitter released from sympathetic nerves may have to travel larger distances to reach target cells in the lymphoid organs; hence with the increase in the splenic size, the target cells that are distant from the nerve terminal is less likely to receive NE (Lorton et al., 1997). In this regard, given the lack of NE alterations after 25 week and long after the termination of the LPS treatment, it is likely that the decreased splenic NE observed after 13 week is merely a consequence of the increased splenic size, rather than an altered metabolism of NE. In contrast to NE, we observed consistent and lasting increase in splenic 5-HT. Although platelets cannot synthesize 5-HT, they can sequester large amount of 5-HT by an active uptake system mediated by NE (Walker and Codd, 1985). Hence the chronic LPS-induced elevation in splenic 5-HT level observed in this study is suggestive of an inflammation-dependent redistribution of platelets from blood to spleen and subsequent reduction in the circulating platelets. Low levels of circulating platelet 5-HT have been implicated in depression (Mann et al., 1992, Cleare, 1997); hence the chronic persistent depressive behavior exhibited by mice in this study could be a result of the LPS-induced net reduction in the whole blood 5-HT. More importantly, in light of the proposed LPS-induced platelet redistribution from blood to spleen as well as considering the strong

association between the splenic 5-HT levels and the depressive behavior observed in our study, it seems that, at least in chronic inflammation-dependent depression, the levels of serotonin in the blood platelets may not be a good marker to assess the depressive state of the patients.

Besides splenic 5-HT dyshomeostasis, chronic LPS treatment produced time-dependent brain region-specific impairment in 5-HT homeostasis. Specifically, the LPS-induced significant elevation in striatal (13-week) and cortical (25-week) 5-HT levels is indicative of an increased tissue 5-HT reserve so as to compensate for the augmented 5-HT demand (increased 5-HIAA levels) observed after 13 and 25 weeks of LPS treatment. Hence it is likely that the depressive behavior exhibited by mice at these time points could be a consequence of the LPS-induced augmentation of the 5-HT tissue reserve and subsequent reductions in the synaptic 5-HT levels. However, it is worth noting that although mice showed depressive-like behavior long after the discontinuation of LPS administration, 5-HT homeostasis was not significantly altered in the brain regions (PFC, striatum and hippocampus) at this stage. It appears therefore that although chronic LPS treatment impairs both central and peripheral 5-HT homeostasis, it is mainly the peripheral splenic 5-HT alteration that drives the persistent depressive behavior observed in this study.

Besides depression-like emotional alterations, peripheral LPS-induced behavioral changes, including increased anxiety, have been demonstrated in mice (Lacosta et al., 1999, Swiergiel and Dunn, 2007, Salazar et al., 2012) and rats (Bassi et al., 2012) after acute LPS treatment. Data regarding the time-dependent changes in the LPS-induced anxiety level in a chronic peripheral inflammatory model is lacking. Increased anxiety has been reported to be highly comorbid with depression in patients suffering from chronic inflammatory conditions as well as in patients undergoing IFN-α-based immunotherapy (Bonaccorso et al., 2001, Hauser et

al., 2011). In our study, mice exhibited increased anxiety coupled with depressive-like behavior after 6 weeks of chronic LPS administration. Importantly, prolonged LPS administration for another 6 weeks, i.e. a total of 12 week only had a significant impact on depressive behavior and not on anxiety, indicative of a time-dependent dissociation of the behavioral pathologies in the continuum of neurological dysfunctions induced by chronic peripheral inflammation. Interestingly, mice exhibited a decreased anxiety level long after the termination of chronic low-dose LPS treatment. Taken together, our findings demonstrate a biphasic effect of chronic LPS administration on anxiety response with the initial increased anxiety-like behavior dissipating over time and further following an opposite trend (decreased anxiety) in the absence of an active peripheral inflammatory stimulus. Regardless, additional behavioral tests that are specific for anxiety, such as elevated plus maze and marble burying tests (Bailey and Crawley, 2009), may be employed in the future to further characterize the anxiety response induced by chronic peripheral inflammation in male mice.

Reduction in locomotor activity following a single LPS administration is a key response demonstrated in multiple studies (Swiergiel and Dunn, 2007, Sah et al., 2011). Here we found that repeated low-dose LPS administration for 6 weeks reduced the locomotor activity of mice. However, with continued LPS treatment for another 6 weeks, i.e. a total of 12 weeks, mice displayed a significant increase in locomotion that persisted even long after the termination of LPS treatment. Previous studies reported an absence of gross locomotor deficit in mice following chronic (12 week) low-dose LPS administration and also at a time point long after the termination of the long-term LPS exposure (Frank-Cannon et al., 2008, Nguyen et al., 2013). Of note, the locomotor performance of mice was evaluated based on a 5 min open field testing in the above studies and data regarding the open field parameters (distance traveled, time spent in

center, corner or periphery of the open field arena) that we measured during the open field testing, were not reported in the above studies. While, the first 5 min of open field testing is typically used to assess novel environment exploration (Prut and Belzung, 2003), an extended session length (usually for 30 min), is often required to assess the habituation of animals to the open field environment (Bailey and Crawley, 2009). We found that within the 30-min session, both saline and LPS-treated mice habituated to the arena over time at all behavioral time points (6 weeks, 12 weeks and 13-week on +11-week off) tested.

We also assessed the intersession habituation in mice by repeated exposure of mice to the open field apparatus between 6, 12 and 24 weeks (13 week on+ 11 week off group). Generally, with repeated open field testing, rodents, especially C57BL/6 mice, will display intersession habituation, characterized by a general decrease in activity over time across sessions (Bolivar, 2009). Interestingly, we found that compared to saline group, LPS-treated mice showed a 2-fold lesser decrease in long-term habituation at 12 and 24 week (13 week on +11 week off group). Increased familiarity of the open field test environment over repeated test sessions may reflect a form of recognition memory (Crawley, 2007). Significant reduction in recognition memory following acute peripheral LPS administration has been demonstrated in several animal studies (Jacewicz et al., 2009, Bossu et al., 2012, Song et al., 2013). In this regard, the decreased long-term habituation over repeated open field testing exhibited by LPS-treated mice in this study could be due to the LPS-induced deficits in recognition memory; but this needs to be verified by further testing.

In light of the significant locomotor deficits exhibited by 13 week LPS-exposed mice and the lack of significant effect on DA homeostasis in the multiple brain regions (PFC, striatum and hippocampus) assessed in this study, it is likely that the neurochemical changes in dopaminergic

pathways, if at all present, might be too low to be detected in the regions we assessed, especially when measuring the tissue level of neurotransmitter/metabolites and not in vivo DA release by sensitive methods, such as microdialysis (Chefer et al., 2009). Alternatively, it is conceivable that unlike the acute effects, chronic low-dose LPS-induced neurochemical alterations involving dopaminergic pathways might be present in a highly region-dependent fashion and might have occurred in brain regions other than the ones we focused on. Interestingly, continued LPS administration for another 12 week, i.e. a total of 25 week impaired DA homeostasis in the striatum indicative of a time-dependent region-specific neurochemical changes induced by chronic LPS treatment. Thirteen week of LPS treatment followed by 12 week wait period significantly decreased DA level in PFC, which is indicative of increased DA utilization or increased synaptic DA release. In this regard, the increased locomotor activity exhibited by mice in the open field test at this time point could be attributed to the altered DA homeostasis in PFC.

In order to further elucidate the mechanism of LPS-induced locomotor impairments, we looked for the molecular changes in the dopaminergic neuronal markers, such as TH and D2DR in the substantia nigra. The present study shows that the nigral mRNA expression of selected dopaminergic markers, such as TH and D2DR was not affected after 13 weeks and 12 weeks after the termination of 13 week chronic LPS treatment. However, by 25 week of continuous LPS administration, the nigral mRNA expression of D2DR was significantly upregulated, which is indicative of an impaired DA synthesis and release (Liu et al., 2011). Overall, our findings suggest that the LPS-induced neurotoxicity may be mediated by a time-dependent perturbation in the nigral dopaminergic system.

To further investigate the possibility that the LPS-induced behavioral and neurochemical deficits may have resulted from an increased neuroinflammatory response, we analyzed the

nigral mRNA expression of microglia and astrocyte activation marker, F4/80 and GFAP, respectively, and pro-inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-1β (IL-1β). Interestingly, we found that chronic low-dose LPS administration produced microglial activation, evidenced by the significant upregulation of nigral F4/80 at 13 and 25 week post LPS treatment. Interestingly, in contrast to the LPS-induced microglial activation, the nigral GFAP mRNA expression remained unaltered, indicating that the neurotoxic effects of chronic low-dose LPS administration is mainly mediated by microglia, but not astrocytes. However, it is worth mentioning that the significant enhancement in microglial activation observed at above two time points (13 and 25 week) in this study was not accompanied by an increase in the nigral expression of pro-inflammatory cytokines (IL-6 or IL-1β). Immune activation, with an associated increase of pro-inflammatory cytokines, including IL-6, IL-1β, IL-2 and IFN-α has been implicated in the etiology and progression of depression (Schiepers et al., 2005). Anhedonia, a core symptom of depression (Moreau, 2002), has been demonstrated in cancer patients undergoing treatment with pro-inflammatory and antiviral cytokines, namely IL-2 and IFN-α (Meyers, 1999). Interestingly, systemic administration of IL-2, but not IL-6 or IL-1β induced anhedonic response, evidenced by the decreased responding for rewarding hypothalamic self-stimulation in rats (Anisman et al., 1998). In this regard, the possibility of microglial-derived cytokines (other than what we assessed) such as IL-2 and IFN-α in mediating the LPS-induced depressive-like behavior observed in this study, cannot be ruled out.

Previous reports have shown an enhanced neuroinflammatory response in different brain regions, including PFC, hippocampus, striatum and midbrain following acute LPS administration in adult mice (Chung et al., 2010, Cazareth et al., 2014, Noh et al., 2014). Importantly, abnormalities in the normal PFC or hippocampal functioning have been demonstrated in patients

with mood disturbances, including major depressive disorders (Drevets, 2000, Koenigs and Grafman, 2009, Savitz et al., 2013) and hence it is possible that the depressive behavior observed in this study could be mediated by the neuroinflammatory changes in the PFC or hippocampus; however LPS-induced neuroinflammatory responses in these brain regions has to be analyzed to verify this assumption.

Besides looking at the chronic LPS-induced neuroinflammation, we evaluated the effects of prolonged LPS administration on peripheral (plasma) pro-inflammatory cytokine expression. Higher levels of peripheral pro-inflammatory cytokine levels have been increasingly associated with depression (Kwant and Sakic, 2004, Leonard and Maes, 2012). Surprisingly, compared to saline-injected group, we did not find any significant difference with respect to the plasma TNFα and IL-6 levels, suggesting that the persistent depressive behavior exhibited by mice in this study is not associated with an increased peripheral inflammatory response. Similar result has been reported by a recent study wherein mice exhibited depressive behavior following chronic (4 months) LPS administration, while showing no significant changes in the peripheral (spleen) levels of pro-inflammatory cytokines; this finding was attributed to a phenomenon called LPS tolerance (Kubera et al., 2013). LPS tolerance is characterized by an attenuated response to repeated LPS administration with an associated reduction in the peripheral pro-inflammatory cytokine expression (Draisma et al., 2009). This being said, it is conceivable that mice may have developed LPS tolerance over the course of prolonged LPS administration, which in turn may have led to the diminished peripheral inflammatory response observed in this study. Meanwhile, it is also likely that the inflammatory mediators (other than what we assessed), such as IL-1β or IL-12 might be mediating the depressive behavior, as reported previously (Owen et al., 2001, Kim et al., 2002).

In summary, we have shown that chronic peripheral LPS treatment induces a chronic state of depression in male C57BL/6 mice lasting even long after the termination of LPS treatment. Prolonged LPS treatment also affects monoamine pathways in a brain-region specific manner. The long-lasting depressogenic effect of chronic LPS treatment could be attributed to the persistently increased 5-HT in the spleen and a subsequent decrease in the circulating 5-HT levels. Collectively, the findings from this study suggest that repeated low-dose peripheral LPS administration can be used successfully to model chronic depression and also emphasize the need to pay more attention on the peripheral/central monoamine alterations and lasting behavioral deficits induced by chronic peripheral inflammation as there are many pathological conditions where are characterized by chronic inflammation.

Table 3.1.Concentrations of monoamines and their metabolites in the prefrontal cortex (PFC) of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups. Data are presented as mean ± SEM; unit: ng/mg protein.

Table 3.1. Brain neurotransmitters and neurotransmitter metabolites							
Brain region	**PFC**						
Treatment	**13 wk**						
	DA	DOPAC	HVA	NE	MHPG	5-HT	5-HIAA
Saline	0.56 ± 0.23	0.20 ± 0.12	0.47 ± 0.08	6.52 ± 0.28	6.73 ± 0.28	1.04 ± 0.06	0.53 ± 0.07
LPS	0.51 ±0.15	0.19 ± 0.06	0.39 ± 0.09	6.71 ± 0.16	5.90 ± 0.38	0.97 ± 0.08	0.48 ± 0.05
	25 wk						
Saline	1.06 ± 0.21	1.70 ± 0.30	0.23 ± 0.02	9.32 ± 0.40	15.76 ± 2.17	1.01 ± 0.13	0.49 ± 0.06
LPS	0.97 ± 0.19	1.45 ± 0.09	0.25 ± 0.03	8.94 ± 0.39	14.74 ± 0.62	**2.37 ± 0.34***	**0.87 ± 0.07***
	13 wk on+12 wk off						
Saline	1.24 ± 0.45	1.69 ± 0.06	0.47 ± 0.12	8.34 ± 0.25	14.77 ± 0.89	1.07 ± 0.11	0.47 ± 0.04
LPS	**0.34 ± 0.09***	2.17 ± 0.29	0.59 ± 0.13	10.07 ± 0.81	16.76 ± 0.79	1.19 ± 0.15	0.47 ± 0.07

Abbreviations: PFC: prefrontal cortex; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid. *$p < 0.05$ compared to 13 wk saline-injected control group.

Table 3.2. Concentrations of monoamines and their metabolites in the striatum of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups. Data are presented as mean ± SEM; unit: ng/mg protein.

Table 3.2. Brain neurotransmitters and neurotransmitter metabolites							
Brain region	**Striatum**						
Treatment	**13 wk**						
	DA	DOPAC	HVA	NE	MHPG	5-HT	5-HIAA
Saline	252.72 ± 10.73	17.10 ± 0.75	17.19 ± 1.09	ND	ND	1.56 ± 0.38	2.10 ± 0.09
LPS	241.07 ± 11.16	17.17 ± 2.14	18.60 ± 0.76	ND	ND	**4.43 ± 1.05***	2.51 ± 0.18
	25 wk						
Saline	201.82 ± 9.03	19.61 ± 0.90	12.64 ± 0.52	ND	ND	1.83 ± 0.22	2.02 ± 0.40
LPS	201.62 ± 10.68	18.46 ± 0.82	**15.71 ± 0.95***	ND	ND	1.49 ± 0.20	1.56 ± 0.15
	13 wk on + 12 wk off						
Saline	199.25 ± 6.72	20.58 ± 1.18	11.30 ± 0.96	ND	ND	1.91± 0.15	2.08 ± 0.37
LPS	206.05 ± 8.02	18.32 ± 0.85	10.78 ± 0.57	ND	ND	2.39 ± 0.35	1.53 ± 0.26

Abbreviations: DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; ND: not detected; *$p < 0.05$ compared to 13 wk saline-injected control group.

Table 3.3. Concentrations of monoamines and their metabolites in the hippocampus of male C57BL/6 mice exposed to control (saline) or LPS (0.25 mg/kg BW; i.p. twice weekly; *n* = 5 per group) in 13, 25 and 13 wk on +12 wk off groups. Data are presented as mean ± SEM; unit: ng/mg protein.

Table 3.3. Brain neurotransmitters and neurotransmitter metabolites							
Brain region	**Hippocampus**						
Treatment	**13 wk**						
	DA	DOPAC	HVA	NE	MHPG	5-HT	5-HIAA
Saline	0.46 ± 0.09	0.48 ± 0.28	0.23 ± 0.10	7.48 ± 0.54	6.06 ± 0.42	3.22 ± 0.20	2.84 ± 0.21
LPS	0.28 ± 0.05	0.19 ± 0.02	0.22 ± 0.10	7.42 ± 0.76	6.47 ± 0.50	2.77 ± 0.16	2.48 ± 0.13
	25 wk						
Saline	0.93 ± 0.35	2.77 ± 0.13	0.18 ± 0.03	10.69 ± 1.13	11.81 ± 0.58	3.63 ± 0.78	3.25 ± 0.28
LPS	1.13 ± 0.19	2.67 ± 0.19	0.37 ± 0.12	10.48 ± 0.48	11.33 ± 0.53	3.70 ± 0.73	3.31 ± 0.28
	13 wk on + 12 wk off						
Saline	0.87 ± 0.13	2.72 ± 0.22	0.32 ± 0.07	10.34 ± 0.53	11.74 ± 0.92	3.43 ± 0.48	3.01 ± 0.37
LPS	1.07 ± 0.06	3.15 ± 0.38	0.25 ± 0.10	9.90 ± 0.74	13.67 ± 1.88	2.41 ± 0.60	2.36 ± 0.64

Abbreviations: DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid. *$p < 0.05$ compared to 13 wk saline-injected control group.

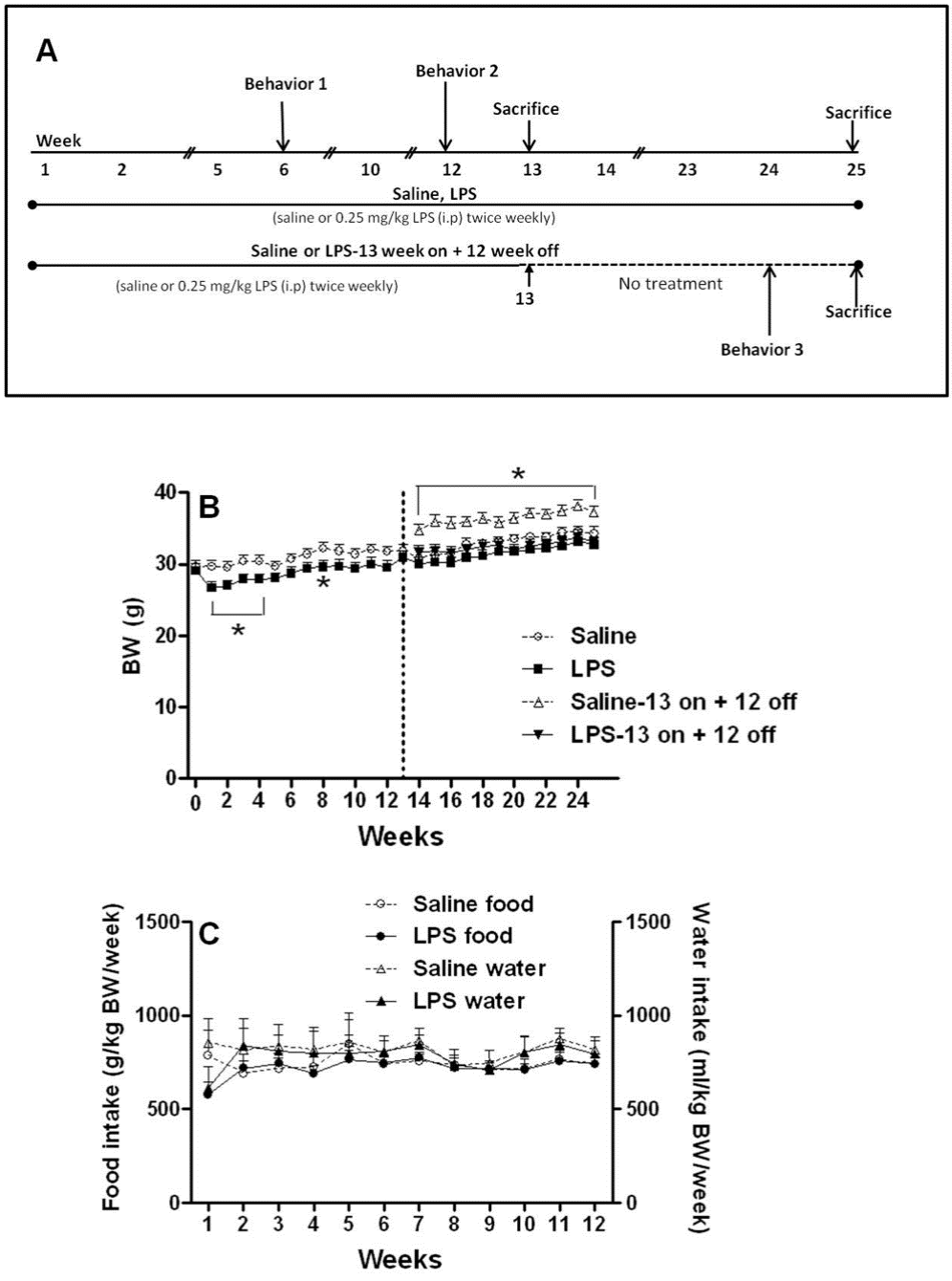


Figure 3.1. (A) Detailed outline of the study's experimental design. Effect of saline or LPS treatment (0.25 mg/kg body weight [BW]; i.p. twice weekly) on (B) BW (g) of adult male C57BL/6 mice during the 25-weeks of treatment (n = 5 per group) and (C) food (g/kg BW/week)

and water intake (ml/kg BW/week) (n = 15 per group) during the 12 week treatment duration. Graphical representations are mean ± SEM. *$p \leq 0.05$ when compared to the saline group.

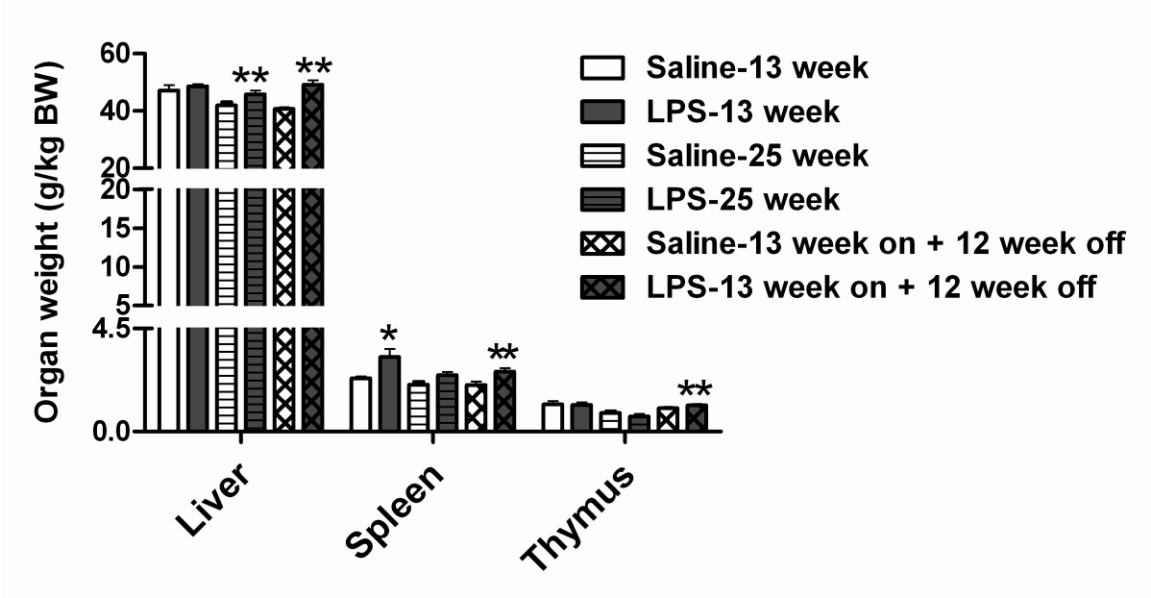


Figure 3.2. Effect of LPS (0.25 mg/kg body weight [BW]; i.p. twice weekly) on organ (liver, spleen and thymus) weights (g/kg BW) of adult male C57BL/6 mice in 13-week, 25-week and 13 week on +12 week off groups. Graphical representations are mean ± SEM (n = 5/group).

*$p \leq 0.05$ when compared to the saline group within a time point.

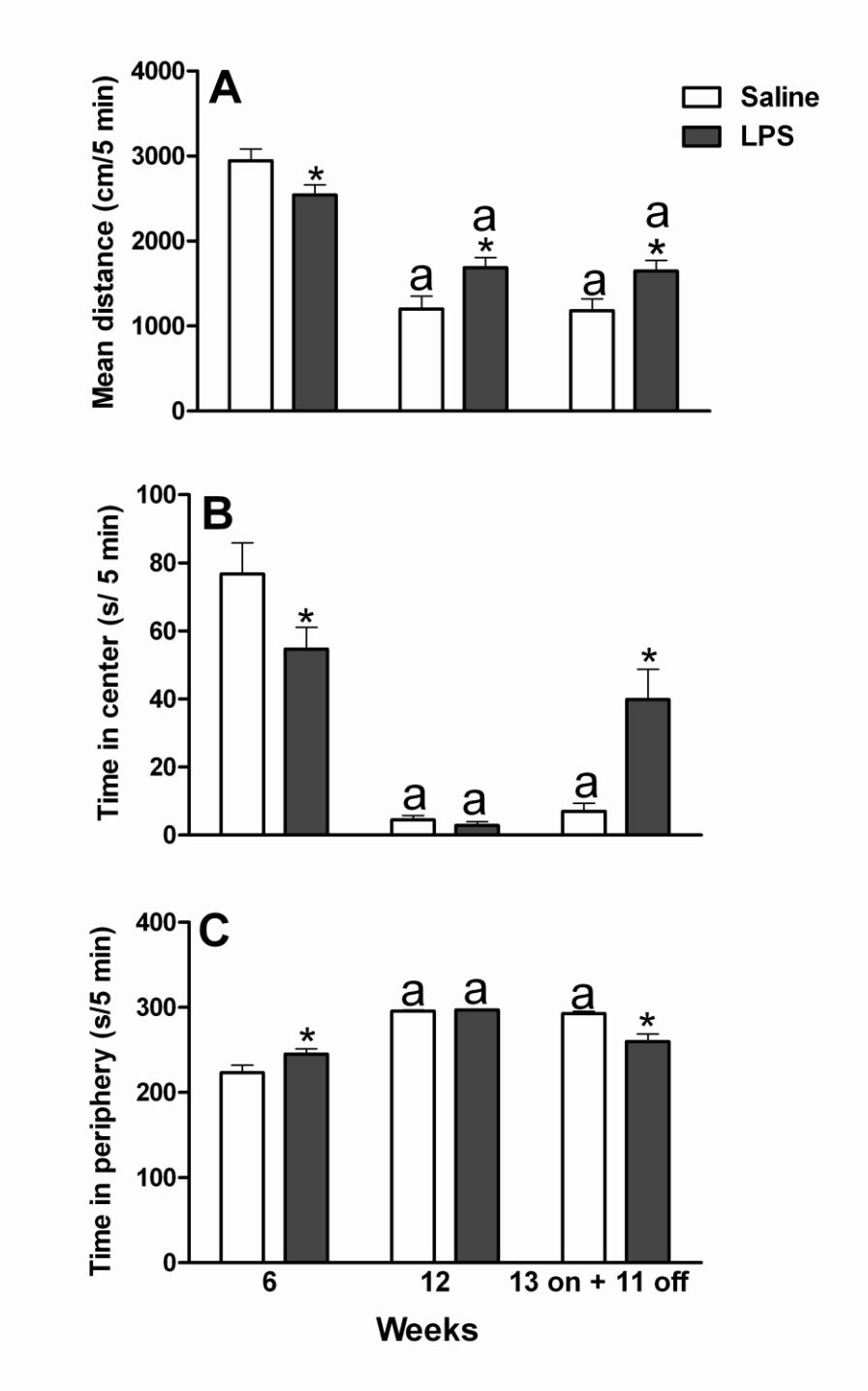


Figure 3.3. Locomotor activity. Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) after 6, 12 or 13-week on +11-week off LPS treatment on (A) distance traveled per 5 min interval, on (B) time spent in the center per 5 min interval or (C) the periphery of the open field arena in open field

testing. Graphical representations are mean ± SEM (n = 5/group). * indicates significant effect of LPS within a time point ($p \leq 0.05$). “a” indicates significant differences across different durations within a group ($p \leq 0.05$).

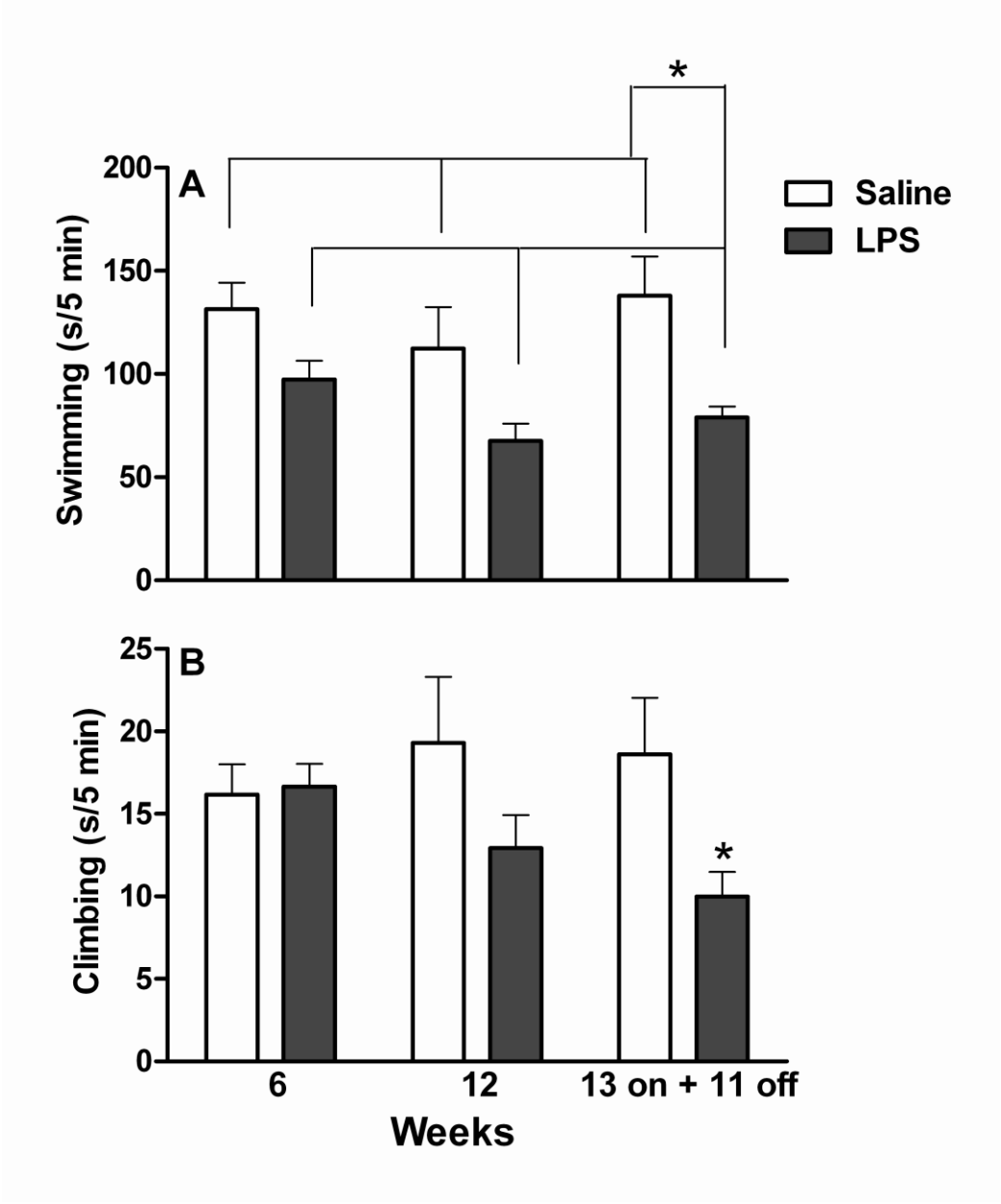


Figure 3.4. Forced swim test. Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) after 6, 12 or 13-week on +11-week off LPS treatment on (A) time spent swimming or (B) climbing per 5 min interval in the forced swim test. Graphical representations are mean ± SEM (n = 5/group). *$p \leq 0.05$ when compared to the saline group within a time point.

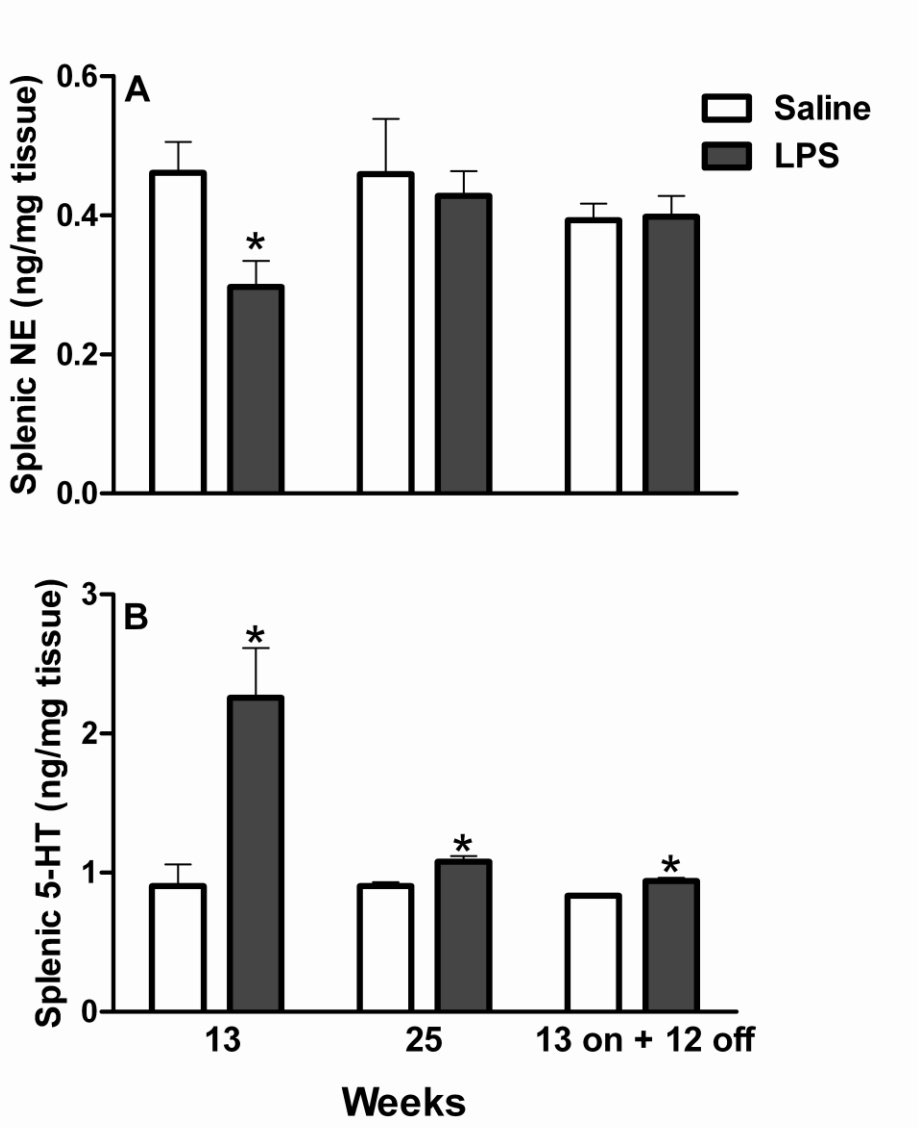


Figure 3.5. Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) on spleen (A) NE and (B) 5-HT level in 13-week, 25-week and 13-week on +12-week off groups. Graphical representations are mean ± SEM (n = 5/group). * indicates significant effect of LPS within a time point ($p \leq 0.05$).

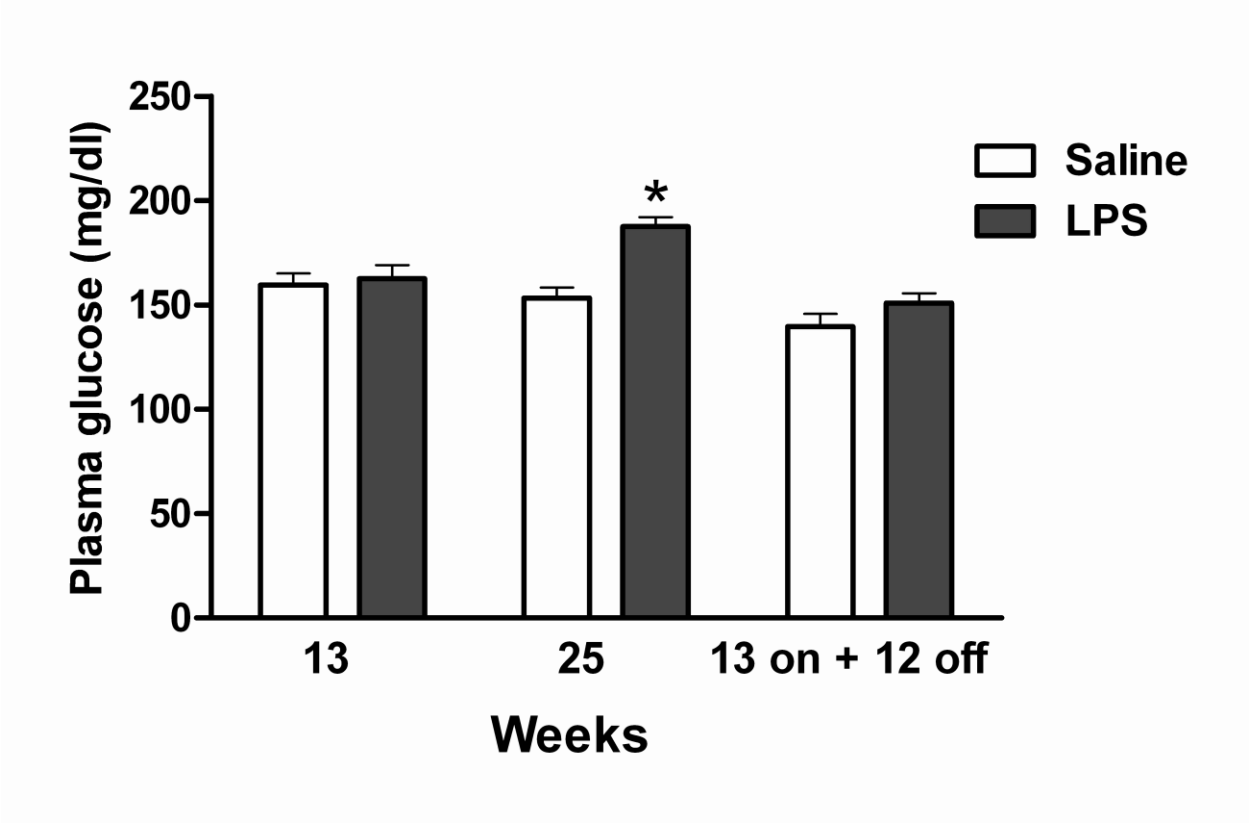
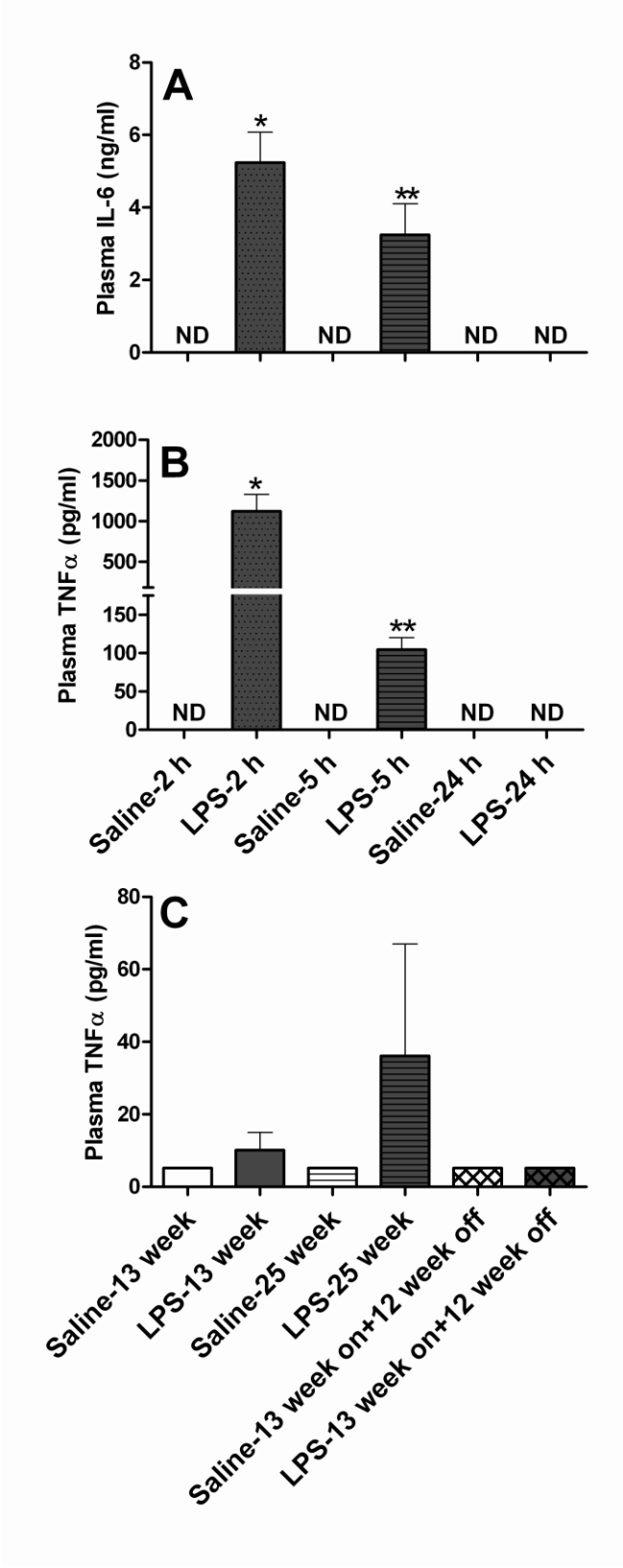


Figure 3.6. Plasma glucose levels. Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) on plasma glucose levels after 13, 25 or 13-week on +12-week off LPS treatment. Graphical representations are mean ± SEM (n = 5/group). * indicates significant effect of LPS within a time point ($p \leq 0.05$).


A
Plasma IL-6 (ng/ml)
8
6
4
2
0
*
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ND
ND
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ND
B
Plasma TNFα (pg/ml)
2000
1500
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500
150
100
50
0
*
**
ND
ND
ND
ND
Saline-2 h
LPS-2 h
Saline-5 h
LPS-5 h
Saline-24 h
LPS-24 h
C
Plasma TNFα (pg/ml)
80
60
40
20
0
Saline-13 week
LPS-13 week
Saline-25 week
LPS-25 week
Saline-13 week on+12 week off
LPS-13 week on+12 week off

Figure 3.7. Plasma cytokine levels. Effect of acute LPS (0.25 mg/kg BW; i.p.) administration on plasma (A) IL-6 and (B) TNFα levels 2, 5 and 24 h post LPS injection as well as chronic LPS (0.25 mg/kg BW; i.p. twice weekly) administration on plasma (C) TNFα level in 13-week, 25-week and 13-week on +12-week off groups. Graphical representations are mean ± SEM (n = 4-5/group). * indicates significant effect of LPS within a time point ($p \leq 0.05$).

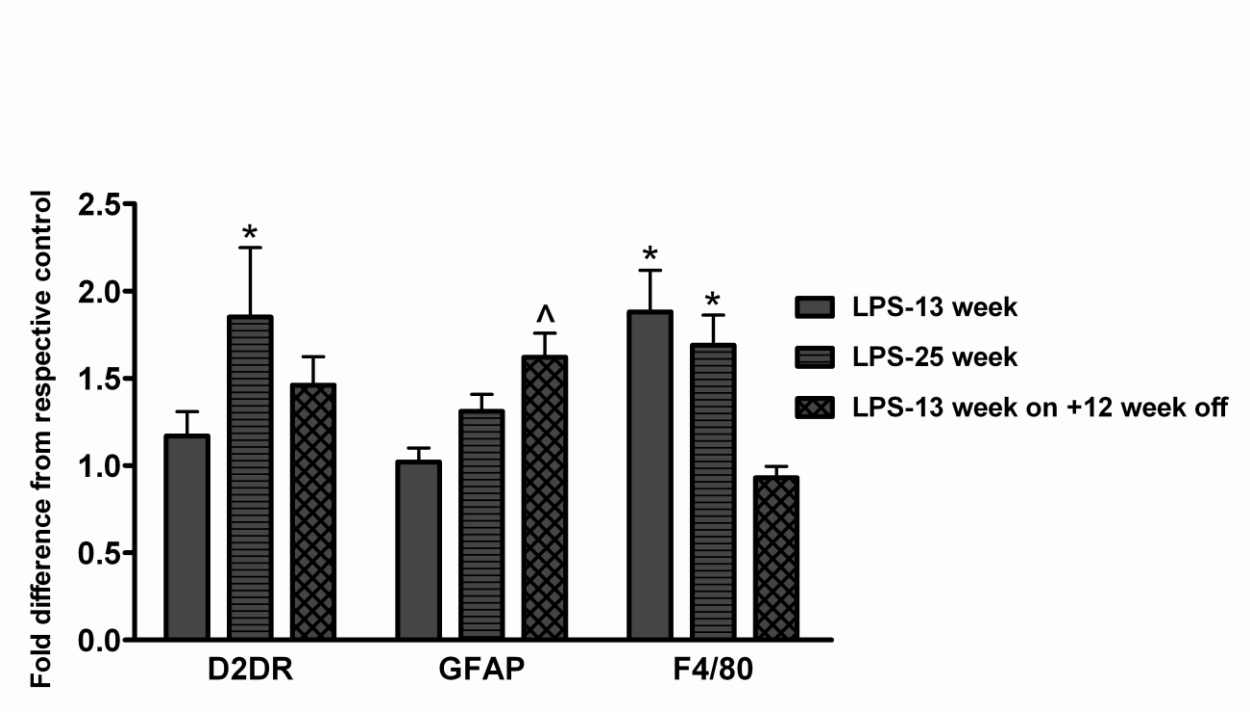

Figure 3.8. Nigral mRNA expression. Effect of LPS (0.25 mg/kg BW; i.p. twice weekly) after 13, 25 or 13-week on + 12 week off LPS treatment on mRNA expression levels of dopamine receptor-2 (D2DR), glial fibrillary acidic protein (GFAP) and F4/80 in the substantia nigra of adult male C57BL/6 mice (n = 5 per group). mRNA data are β-actin-normalized and are presented as fold change relative to respective control at each time point (mean ± SEM). * indicates significant effect of LPS ($p \leq 0.05$). ^ indicates significant trend ($p \leq 0.08$).

CHAPTER 4

NEUROCHEMICAL AND ELECTROPHYSIOLOGICAL DEFICITS IN THE VENTRAL HIPPOCAMPUS AND SELECTIVE BEHAVIORAL ALTERATIONS CAUSED BY HIGH-FAT DIET IN FEMALE C57BL/6 MICE[1]

[1]Krishna, S., Keralapurath, M.M., Lin, Z., Wagner, J.J., de La Serre, C.B., Harn, D.A., Filipov, N.M. Submitted to *Neuroscience.* 10/07/2014.

Abstract

Mounting experimental evidence, predominantly from male rodents, demonstrates that high-fat diet (HFD) consumption and ensuing obesity are detrimental to the brain. To shed additional light on the neurological consequences of HFD consumption in female rodents and to determine the relatively early impact of HFD in the likely continuum of neurological dysfunction in the context of chronic HFD intake, this study investigated effects of HFD feeding for up to 12 weeks on selected behavioral, neurochemical, and electrophysiological parameters in adult female C57BL/6 mice; particular focus was placed on the ventral hippocampus (vHIP). Selected locomotor, emotional and cognitive functions were evaluated using behavioral tests after 5 weeks on HFD or control (low-fat diet) diets. One week later, mice were sacrificed and brain regional neurochemical (monoamine) analysis was performed. Behaviorally naïve mice were maintained on their respective diets for additional 5-6 weeks; synaptic plasticity was determined in ex vivo slices from the vHIP. HFD-fed female mice exhibited increased: (i) locomotor activity in the open field testing, (ii) mean turn time on the pole test, (iii) swimming time in the forced swim test, and (iv) number of marbles buried in the marble burying test. In contrast, the novel object recognition memory was unaffected. Mice on HFD also had decreased norepinephrine and dopamine turnover in the prefrontal cortex and vHIP, respectively. HFD consumption for a total of 11-12 weeks altered vHIP synaptic plasticity, evidenced by significant reductions in the paired-pulse ratio and long-term potentiation (LTP) magnitude. In summary, in female mice, HFD intake for several weeks induced multiple behavioral alterations of mainly anxiety-like nature and impaired monoamine pathways in a brain-region specific manner, suggesting that in the female, certain behavioral domains (anxiety) and associated brain regions, i.e., the vHIP, are preferentially targeted by HFD.

Keywords: High-fat diet (HFD), Anxiety, Dopamine, Ventral hippocampus (vHIP), Long-term potentiation (LTP)

Introduction

Overweight and obesity affect all societal segments, irrespective of age, gender and ethnicity (WHO, 2000, Wang and Beydoun, 2007). Besides genetics, which plays important, but a minor role, several environmental factors, with high-fat consumption being a major one, drive the obesity epidemic (Swinburn et al., 2004). Worldwide prevalence of overweight and obesity in adults is increasing in both genders (Stevens et al., 2012). Importantly, obesity prevalence in adult American females from certain ethnic groups, i.e., African and Mexican Americans, is higher than in their male counterparts (Terrell, 2002, Kanter and Caballero, 2012).

Epidemiological and animal data have demonstrated emotional disturbances, such as anxiety and/or depression, associated with high-fat consumption and subsequent weight gain/obesity (Petry et al., 2008, Gadalla, 2009, Mizunoya et al., 2013). As it is the case with the research in the biomedical field in general, including in neuroscience (Beery and Zucker, 2011), male subjects in obesity-related laboratory animal research are over-represented i.e. (Del Rosario et al., 2012, Cone et al., 2013); information on the adverse central effects of high-fat diet (HFD) consumption and HFD-induced obesity in females is available, i.e. (Sato et al., 2010, Balasubramanian et al., 2012), but, compared to available data in males, is rather limited. Besides using mostly males, the majority of animal studies investigating the neurological effects of HFD have done so in advanced obese phenotypes induced by long-term (6-12 months) HFD consumption (Hwang et al., 2010, Heyward et al., 2012, Karimi et al., 2013); data regarding the relatively early impact of HFD in the likely continuum of neurological deficits/dysfunctions in the face of continued HFD feeding are scarce.

HFD consumption alters brain neurochemistry in a region-specific manner (Molteni et al., 2004, Sharma et al., 2013) and this alteration might be partially responsible for the HFD-

induced behavioral impairments. For example, chronic (3 months) HFD intake alters striatal and mesolimbic dopamine (DA) signaling in rodents (Davis et al., 2008, Sharma and Fulton, 2013). Besides DA, the use of selective serotonin (5-HT) and norepinephrine (NE) reuptake inhibitors as anti-obese/anti-depressant agents demonstrates their important roles in regulating obesity and associated behavioral deficits (Chudasama and Bhatt, 2009).

HFD and other palatable foods also affect the hypothalamus, the prefrontal cortex (PFC) and the hippocampus (Heyward et al., 2012, Rojo et al., 2013). Hypothalamic DA and 5-HT plays a key role in regulating food intake (Meguid et al., 2000); several animal studies have reported DA dysfunction in hypothalamus following chronic HFD consumption, characterized by decreased DA turnover (Levin et al., 1986), increased D4 receptor mRNA expression (Huang et al., 2005) and upregulation of genes involved in the synthesis and release of DA (Lee et al., 2010a). In male rodents chronically fed HFD, compromised hippocampal synaptic plasticity and cognitive deficits are observed (Stranahan et al., 2008, Valladolid-Acebes et al., 2012, Karimi et al., 2013); however, female data are limited. Besides cognition, the hippocampus also modulates anxiety and preferential roles for the dorsal hippocampus (dHIP) in spatial memory and the ventral hippocampus (vHIP) in anxiety-related behaviors have been suggested (Bannerman et al., 2004, Fanselow and Dong, 2010).

In this study, we aimed to investigate the early neurological effects caused by relatively short-term HFD intake in female C57BL/6 mice using behavioral, neurochemical and electrophysiological measures. The C57BL/6 mouse strain is the most widely used strain in neuroscience research (Kalueff and Nguyen, 2014). Importantly, compared to other strains of mice, such as SWR/J, C57BL/6 is a good model for studying human obesity as it simulates the human metabolic abnormalities (hyperinsulinemia, hyperglycemia, and hypertension) when fed

ad libitum with a HFD, while not showing metabolic irregularities when fed a low-fat diet (Collins et al., 2004). Behavioral tests were conducted to evaluate the effects of HFD on: (i) motor function (open field, pole and grip strength), (ii) emotional disturbances, such as anxiety or depression (marble burying and forced swim) and (iii) cognitive changes (novel object recognition). To investigate the HFD-induced neurochemical alterations underlying the behavioral changes (if any), levels of DA, 5-HT, NE and their metabolites were determined in multiple brain regions, namely PFC, nucleus accumbens [NAc], striatum, dHIP and vHIP that are implicated in the regulation of locomotor, emotional and/or cognitive functions. The two hippocampal zones (dorsal and ventral) were analyzed separately due to their distinct roles in memory and anxiety (Bannerman et al., 2004, Fanselow and Dong, 2010). Additionally, based on the neurochemical and behavioral data gathered after 5-6 weeks on HFD, synaptic function was evaluated in the vHIP after additional 5-6 weeks of HFD consumption.

Experimental procedures

Animals

Young adult female C57BL/6 mice (6-7 weeks old, Harlan, Indianapolis, IN) were housed (4-5 per cage) in an environmentally controlled room (22–24°C) with food and water available *ad libitum* on a 12h light/dark cycle in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility throughout the study. All procedures were in accord with the latest National Institutes of Health (NIH) guidelines and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Animal treatment

Mice weighing 16.0 ± 0.20 g (mean ± SEM) were randomly divided into two groups (n = 13-14 per group) and placed on either a low-fat diet (LFD; 10% kcal from fat, D12450J, Research Diets, Inc., New Brunswick, NJ) or a high-fat diet (HFD; 60% kcal from fat, D12492, Research Diets) that are micronutrients and simple sugar (sucrose) balanced for the entire duration of the study. Body weight was recorded weekly; food and water intakes were recorded twice weekly. Subset of the mice (n = 8 per treatment group) were subjected to behavioral tests after 5 weeks on LFD/HFD; a week later (6 weeks on diets, 48 h after the last behavioral test), these mice were sacrificed, brains were harvested, weighed, and quickly frozen at -80 °C for neurochemical analysis. The remaining behaviorally naïve mice (n = 5-6 per treatment group) were maintained on their respective diets for 11-12 weeks; at that time, brains were harvested and ex vivo hippocampal slices prepared for electrophysiology. The experimental design is depicted in Fig. 4.1.

Behavioral tests

Behavioral tests were performed in succession over 3 days (Figure 1a). Animals were naïve to the testing ambience prior to testing initiation and all tests were performed by a treatment-blinded experimenter in a specially equipped behavioral testing room separate from the one where the mice were housed.

Open field. Mouse activity was monitored for a period of 30 min in an open field arena (25 cm x 25 cm x 40 cm; Coulbourn Instruments, Whitehall, PA) as we have described it previously (Krishna et al., 2014). Parameters evaluated using the Limelight software (Actimetrics, Wilmette, IL) included: (1) distance traveled (cm/5 min intervals); (2) number of crossings into the center or periphery of the arena per 100 cm distance analyzed for the 30 min

and per 5 min intervals (Thomas et al., 2009); (3) number of rearings during the first 5 min, counted

Pole test. After 5 min resting period (following the open field test), mice were placed upright on a gauze-wrapped pole (1 × 55 cm; d × h) as in (Lin et al., 2013). A total of four trials were completed with a 3-5 min inter-trial interval. The average times to turn, to descend, and the total time spent on the pole from the four trials were used for statistical analysis. The maximum turning time allowed was 60 sec and the total time per trial was 120 sec (Krishna et al., 2014).

Grip strength. A strength gauge (Bioseb, France) with attached mouse-specific square wire grid (6 × 6 cm) was used to measure forelimb grip strength (10 min rest after the pole test) as previously described (Lin et al., 2013, Krishna et al., 2014). The average maximum grip force (recorded in newtons [N]) of the four trials was used for statistical analysis.

Novel object recognition (NOR). The NOR was conducted at the beginning of day 2 of behavioral testing with the previous day open field test used as a habituation phase, as detailed in (Lin et al., 2013). Briefly, mice were allowed to explore two identical objects for 5 min. After 1 h rest in their home cages, they were reintroduced into the arenas with one familiar and one novel object for 5 min. The number of approaches towards the familiar (N_f) or the novel object (N_n), as well as the time spent exploring the familiar (T_f) or the novel object (T_n), were extracted using the Limelight software. To determine novelty preference, the percent of time exploring the familiar vs. the novel object and the novelty preference indices (NPIs) based on the number of approaches (NPI_A= [Nn – Nf]/[Nn + Nf]) or time (NPI_T = [Tn – Tf]/[Tn + Tf]) were calculated and analyzed (Cyrenne and Brown, 2011).

Forced swim test (FST). Following the NOR test and a 1.5-h home cage rest period, FST was performed as in (Krishna et al., 2014). Mice were gently placed in a large cylindrical

container (18 × 25 cm; d × h) filled approximately two-thirds with tap water (3 L, 29±1 °C) for 15 min. The total times spent swimming, climbing, or immobile, as well as the total distance swam, were scored using the Limelight video tracking software (Actimetrics).

Marble burying test (MBT). This test was performed on day 3 as described previously, but with some modifications (Gaikwad et al., 2010). Mice were individually placed in cages containing 4-5 cm-thick pine bedding (American Wood Fibers, Columbia, MD) for 10 min (habituation phase). After a 40-min home cage resting period, mice were reintroduced into the cages which now contained twenty glass marbles (diameter ~10 mm, Panacea Products Corp., Columbus, OH), overlaid on the bedding arranged in a 4 x 5 matrix for a 10 min testing phase. The number of marbles buried (≥ 70%) was counted based on images collected at times 0 and 10 min of the testing phase (Gaikwad et al., 2010).

Identification of stage of estrus cycle

After 6 or 11-12 weeks on respective diets, vaginal smears were obtained from mice in the morning (between 08.00 and 09.00 h) of the day prior to their sacrifice and were assessed for the estrus cycle stage as detailed in (Caligioni, 2009).

Neurochemistry

Whole brain tissue concentration of monoamines and their metabolites was determined as previously described (Coban and Filipov, 2007). Briefly, micropunches (1.5-mm diameter) from PFC, NAc, striatum, dHIP and vHIP were collected from 500-μm thick sections and placed in 100 μl of 0.2 N perchloric acid, sonicated and then centrifuged. An aliquot (20 μl) of the supernatant was injected into HPLC with an electrochemical detector (Waters Alliance, Waters Co., Milford, MA) for determination of: (i) DA and its metabolites dihydroxy-phenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT); (ii) NE and its metabolite

3-methoxy-4-hydroxyphenylglycol (MHPG); (iii) 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). Prior to statistical analysis, all neurochemistry data were normalized on per mg of protein basis as previously described (Coban and Filipov, 2007, Krishna et al., 2014).

Hippocampal electrophysiology

Beginning after 11 weeks and terminating before the end of 12 weeks on LFD or HFD diets, hippocampal electrophysiology was performed on vHIP slices as in (Thompson et al., 2004). The brain was removed quickly from CO_2 anesthetized mice and submerged in ice-cold, oxygenated (95% O_2/ 5% CO_2) dissection artificial cerebrospinal fluid (aCSF) containing: 120 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose; 400-µm thick horizontal brain slices were harvested; slices were sub-dissected, the CA3 region removed, leaving the CA1 region intact (Keralapurath et al., 2014), and then incubated in a submersion chamber for 45 min at room temperature and an additional 45 min at 30°C continuously perfused with oxygenated (95% O_2 / 5% CO_2) standard aCSF containing: 120 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. Using a bipolar stimulating electrode (Kopf Instruments, CA) Schaffer collaterals were stimulated at the CA3-end of the CA1 region, and the field excitatory post-synaptic potentials (fEPSPs) generated at the stratum radiatum layer were recorded using a 1.0 MΩ tungsten recording electrode (WPI, Sarasota, FL). The initial slope of the fEPSPs was measured by fitting two straight lines to a 1 ms window immediately following the fiber volley. Paired-pulse responses with a 50 ms inter-stimulus interval were obtained, and paired-pulse ratio (PPR) was calculated as previously described in (Thompson et al., 2004) using the formula, PPR = (initial slope of pulse 2)/(initial slope of pulse 1).

For the long term potentiation (LTP) experiments, stable fEPSPs were recorded for at least 30 min at baseline frequency before stimulating the slices with a high frequency tetanic stimulation (HFS) consisting of 3 trains of 100 pulses at 100 Hz, with 20s inter-train interval (Keralapurath et al., 2014). fEPSPs were recorded at baseline frequency for additional 60 min post-tetanus. fEPSP slopes were normalized to the slopes of the last five responses prior to the HFS and the LTP magnitude was assessed by comparing the average of normalized slopes from the last 5 responses of pre-tetanus and 55-60 min post-tetanus phases. Data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunnyvale, CA). *n*-values (x[y]) indicate the number of mice (x) and the number of slices (y) assessed.

Statistical analysis

Student's T-test was used to analyze the effect of HFD on body weight within a time point, behavioral endpoints (except as specified below), neurochemistry data, and for planned comparisons between the two groups for the hippocampal electrophysiology data. Two-way analysis of variance (ANOVA) was conducted to analyze the effect of estrus cycle stage on behavioral, neurochemical and hippocampal electrophysiology data as well as to analyze the food and water intake and the open field (treatment x time [interval]) data. If ANOVA's overall main effect was found significant, treatment means were separated by Student-Newman-Keuls *post hoc* test. All results are presented as mean ± SEM and are considered significant at $P < 0.05$.

Results

Body weight, body weight gain, food intake, food efficiency and water intake

There was no significant difference in initial body weights between the LFD and HFD groups ($P = 0.63$; Table 4.1). After 6 or 11-12 weeks on respective diets, mice in HFD group had

a significantly greater body weight and body weight gain compared to the respective LFD group ($P < 0.05$; Table 4.1). The food intake (g/mouse/day) was not significantly different between the LFD and HFD groups during the first 6 weeks or the last 5-6 weeks of the study duration ($P \geq 0.20$; Table 4.1). However, on a weekly basis, the daily caloric (kcal/mouse/day) intake of HFD-fed mice was consistently and significantly higher than that of the LFD-fed mice; however the caloric intake of HFD-fed mice did not increase over time (Table 4.1). The food efficiency % (g body weight gain/100 g or 100 kcal, expressed as percentage) was significantly ($P < 0.05$) higher in HFD mice compared to the respective LFD group during the first 6 weeks and the last 5-6 weeks of the experimental period (Table 4.1). With respect to water intake (ml/mouse/day), no significant difference was observed between LFD and HFD mice during the first 6 weeks of the study ($P > 0.20$; Table 4.1); however, during the last 5-6 weeks, HFD-fed mice showed a moderate, but significant increase in water intake than the LFD group ($P < 0.01$; Table 4.1).

Behavior

Open field. Two-way ANOVA revealed overall significant main effects of diet ($P < 0.01$) and interval (5 min time period; $P < 0.001$), but without any significant interaction between the two ($P = 0.74$) with respect to the distance traveled in the open field arena. HFD-fed mice had significantly ($P < 0.05$) greater locomotor activity as evidenced by the increased distance traveled per 5 min interval (LFD vs. HFD: 3491.50 ± 110.18 vs. 3914.86 ± 103.06 cm). Mice on HFD traveled more distance than LFD-fed mice during each 5-min bin of the 30 min open field test (Fig. 4.2a). Both LFD and HFD-fed mice, as expected, habituated to the arenas over time; their locomotor activity during the 5^{th} and 6^{th} 5-min interval compared to the activity during the 1^{st} interval (first 5 min) of open field testing of open field testing was significantly lower (Fig. 4.2a). Vertical activity (number of rearings during the first 5 min of open field exploration) was

unaffected (P = 0.18; data not shown). Similarly, number of (i) crossings into the center (LFD vs. HFD: 2.24 ± 0.14 vs. 2.09 ± 0.09; Fig. 4.2b) and (ii) peripheral crossings (LFD vs. HFD: 6.96 ± 0.22 vs. 7.23 ± 0.32; data not shown) per 5-min interval was decreased and increased, respectively, only numerically, but not significantly (P > 0.40).

Pole test. Five weeks on HFD resulted in robust, significant (2-fold) increase in the mean turn time during the pole test (P < 0.05; Fig. 4.2c). The other two pole test parameters, time to descend (P = 0.28) and total time (P = 0.08), were increased by HFD only numerically (data not shown).

Grip strength test. After 5 weeks of HFD intake, mice exhibited an apparent trend towards an increase in the mean grip strength that was almost significant (LFD vs. HFD: 0.70 ± 0.02 vs. 0.79 ± 0.04; P = 0.06).

FST. During the entire 15-min FST session, HFD-fed mice swam 75% more (time) than the LFD-fed mice (P < 0.05; Fig. 4.3a). Also, HFD feeding resulted in increased climbing time (P < 0.05; data not shown), and decreased time spent immobile (P < 0.05; data not shown). Additionally, the HFD-fed mice swam longer distance during the FST (LFD vs. HFD: 5914.99 ± 367.41 vs. 8182.70 ± 838.52 cm; P < 0.05).

MBT. Compared to LFD-fed mice, 5 weeks on HFD was associated with a robust, significant (2.2-fold) increase (P < 0.05) in the number of buried marbles (Fig. 4.3b).

NOR. Performance in the NOR test was not affected by diet as mice from both LFD and HFD groups exhibited greater novel object preference during the novel object testing phase (P < 0.05; Fig. 4.3c) and spent equal (~ 50%) time with each of the two identical objects during the acquisition phase (data not shown). Similarly, the number of approaches towards the novel object vs. the familiar one was increased in both LFD and HFD groups to a similar extent (P <

0.01; data not shown). If these data are expressed in terms of novelty preference indices (NPIs), 5 weeks on HFD did not affect either the NPI_T ($P = 0.68$; data not shown) or the NPI_A ($P = 0.59$; data not shown) for time or approaches, respectively.

Estrus cycle stage

After 6 weeks, out of 8 LFD mice, 7 were in diestrus and 1 was in estrus, while out of 8 HFD mice, 5 were in diestrus and 3 were in estrus. There was no main effect of estrus stage ($P >$ 0.30) on the behavioral or neurochemical endpoints measured after 5-6 weeks of LFD/HFD feeding. After 11-12 weeks, out of 6 LFD mice, 4 were in diestrus and 2 were in estrus, whereas, out of 5 HFD mice, 3 were in diestrus and 2 were in estrus. Similar to 5-6 week time point, estrus stage did not have a main effect ($P >$ 0.80) on the hippocampal electrophysiology measure that was evaluated after 11-12 weeks of LFD/HFD intake.

Neurochemistry

In the PFC, 6 weeks of HFD consumption did not alter the levels of DA, 5-HT or their metabolites ($P >$ 0.23; Table 4.2). However, HFD significantly decreased ($P <$ 0.05) the MHPG concentration, without affecting the parent (NE) neurotransmitter levels ($P = 0.83$; Table 4.2). As a result, the MHPG/NE ratio also decreased ($P <$ 0.01; Table 4.2). In the NAc, 6-week HFD intake did not cause any alterations of DA, 5-HT or their metabolites ($P >$ 0.28; Table 4.2). Similarly, no neurochemical alterations caused by HFD feeding were observed in the striatum ($P >$ 0.25; Table 4.2). The DA metabolite 3-MT, which was only detected in the NAc and striatum, was unaffected by HFD ($P >$ 0.25; data not shown) as it was dHIP neurochemistry ($P >$ 0.10; Table 4.2).

Contrary to the lack of HFD effects on the dHIP, vHIP monoamine homeostasis was substantially affected by HFD. Specifically, HFD intake significantly decreased the

concentration of the DA metabolite, HVA ($P < 0.05$; Table 4.2), without affecting the level of DA itself ($P > 0.35$). Hence, vHIP HVA/DA ratio decreased significantly ($P < 0.05$; Table 4.2). HFD feeding decreased the level of the other DA metabolite, DOPAC ($P = 0.29$) only numerically, but it decreased the DOPAC/DA ratio significantly ($P < 0.05$; Table 4.2). The vHIP concentration of NE, 5-HT and their metabolites was unaffected by HFD ($P > 0.10$; Table 4.2).

Hippocampal electrophysiology

Paired pulse fEPSP responses (Fig. 4.4a) were recorded in the stratum radiatum layer to evaluate short-term synaptic plasticity in the CA1 region of vHIP slices. Compared to LFD, 11-12 weeks on HFD was associated with a significant reduction in the PPR ratio (LFD vs. HFD: 1.41 ± 0.10, $n = 6[10]$ vs. 1.17 ± 0.03, $n = 5[11]$); mice [slices]; $P < 0.05$, Fig. 4.4b). In addition, LTP was induced via HFS (100 Hz/300 pulses) to assess synaptic plasticity in the LFD and HFD-fed mice (Fig. 4.4c). LTP measured 60 min following induction was significantly decreased in the HFD group (LFD vs. HFD: 1.82 ± 0.13, $n = 6[10]$ vs. 1.46 ± 0.08; $n = 5[11]$; $P < 0.05$, Fig. 4.4d).

Discussion

Although multiple factors are implicated in obesity's etiology, a clear role for the environment in its rapid rise has been established (Sallis and Glanz, 2009). Among the environmental factors, increased dietary fat consumption is considered a major contributor (Swinburn et al., 2004). As previous, largely done with males, rodent studies have focused mainly on the neurological consequences of chronic HFD intake, we evaluated the effects of HFD consumption at a relatively early time point in the likely continuum of neurological dysfunctions and did so in female mice. Our main findings include: (i) 5-weeks of HFD consumption leads to increased locomotor activity and anxiety, but it does not affect short-term

object recognition memory; (ii) HFD intake selectively alters monoamines in the vHIP and to a smaller extent in the PFC; (iii) continued (11-12 weeks) HFD feeding reduces synaptic plasticity in the vHIP.

In this study, we found no significant difference in food intake between LFD and HFD-fed mice as reported recently (Liu et al., 2014). However, mice on HFD showed a sustained significant increase in caloric intake than LFD mice throughout the study duration which correlated with their significantly increased body weight observed after 6 or 11-12 weeks of the study.

In male rodents, HFD induces anxiety-like (Heyward et al., 2012, Sharma et al., 2012), depressive-like (Chudasama and Bhatt, 2009, Yamada et al., 2011) as well as anxiety coupled with depressive-like behaviors (Sharma and Fulton, 2013). The significantly increased swimming time in the FST and the number of marbles buried in the MBT observed after 5 weeks of HFD consumption is typically indicative of less depressive behavior and increased anxiety, respectively. Although commonly used to assess animal's depressive state (Castagne et al., 2011, Slattery and Cryan, 2012), the behavior of animals in the FST could also be related to their anxiety level; increased swimming in FST may be also be reflective of an increased anxiety level (Nishikawa et al., 2004, Pechnick et al., 2004). Hence, putting the FST and MBT results together, it seems that 5 weeks of HFD consumption increases anxiety-like behavior in female mice. The absence of an anxiety-like nature in the open field test (there was only a numerical decrease in the number of central crossings) suggests that this behavioral measure may be less applicable to female mice in part due to the greater general anxiety level in females compared to males in an open field arena (Anchan et al., 2014). Additional behavioral tests, such as elevated

plus maze (EPM), may be employed in the future for further characterization of the anxiogenic properties of HFD to female mice.

Five weeks of HFD intake also caused locomotor alterations, evidenced by significant increases in locomotor activity and turning time in the open field and pole tests, respectively. Pole test is typically used to assess motor coordination in mice (Fleming et al., 2004, Brooks and Dunnett, 2009) and the significantly increased turn time exhibited by HFD fed mice in this study is primarily indicative of an impaired motor coordination. However, the pole test performance can also be affected by the animal's anxiety level. For example, greater anxiety can lead to increased hesitance to descend during the pole test, thereby lengthening the total time the mouse spends on the pole (Balkaya et al., 2013). Thus, the increased turning time exhibited by HFD-fed mice in pole test of the current study could be anxiety-related.

With respect to FST, taking into account the relatively short period (5 weeks) of HFD consumption and the BW (LFD vs. HFD: 20.0 vs. 25.0 g) of the mice, it is unlikely that body fat and buoyancy have influenced the outcome. In light of the previous studies with male rodents demonstrating either an absence of a gross locomotor deficit (Hwang et al., 2010, Sharma and Fulton, 2013) or a HFD-induced reduction in locomotor activity (Sharma et al., 2012), the significant hyperactivity demonstrated by HFD-fed female mice in this study indicates a sex-dependent effect of HFD on the locomotor outcomes, which is likely due to increased anxiety (Vaanholt et al., 2008, Hwang et al., 2010). Besides sex, species, mouse strain and duration of feeding are some other factors (Bray et al., 2004, Murphy and Mercer, 2013, Lai et al., 2014) that may impact behavior and should be considered.

Majority of the existing rodent data that investigated HFD-induced neurochemical changes have centered on the mesolimbic reward pathways and associated reward-related

behaviors in response to palatable foods (Davis et al., 2008, Sharma et al., 2013). Few studies have investigated the molecular changes underlying HFD-induced emotional states, such as anxiety or depression (Yamada et al., 2011, Sharma and Fulton, 2013). Acute (1 week) HFD feeding increased anxiety-like behavior and it caused cognitive deficits that were associated with increased HVA concentration in the cortex and hippocampus of male mice (Kaczmarczyk et al., 2013), suggesting that hippocampal DA homeostasis is under distress even at this early time point. Information on the monoamine correlates underlying HFD-induced behavioral impairments in female rodents is lacking. Here, we demonstrate that HFD consumption region-specifically altered the brain neurochemistry by reducing NE and DA turnover in the PFC and vHIP, respectively.

HFD-induced neurochemical changes in the dorsal and ventral hippocampal zones were analyzed separately due to the increasing evidence of their regional dissociation. Interestingly, dHIP monoamine homeostasis was unaffected by HFD. Previous rodent studies, which investigated the adverse effect of HFD consumption on dHIP, have shown impairments in spatial memory (Molteni et al., 2004, Valladolid-Acebes et al., 2012), but information about HFD's effect on nonspatial object memory is limited. Significant NOR memory impairment has been reported in either long-term (2-4 months) HFD-fed older male mice (Carey et al., 2014) or in sucrose-induced obese adult male rats (Jurdak and Kanarek, 2009, Carey et al., 2014b) NOR is a widely used task to investigate short- or long-term object recognition memory through modification of the retention interval between the acquisition and testing phases (Antunes and Biala, 2012). Specifically, retention intervals varying from 2-90 min (Jurdak and Kanarek, 2009, Stefanko et al., 2009, Moore et al., 2013) are used to investigate short-term memory and a retention time of 24 h (Shi et al., 2012) is typically used to assess long-term object recognition

memory. In line with our study with female mice, intact short-term NOR memory was observed in male Long-Evans rats following HFD consumption for 2 months (Jurdak and Kanarek, 2009). Also, adult male mice tested for long-term object memory showed normal memory performance after chronic HFD exposure for 22 weeks (Heyward et al., 2012). Overall, it appears that, except for older male mice (Carey et al., 2014), HFD does not affect nonspatial object recognition memory in both adult females and males. However, the HFD's effect on female mice's spatial memory was not assessed in our study; given the evidence for adverse effects in males (Valladolid-Acebes et al., 2011, Heyward et al., 2012), spatial memory needs to be evaluated in females fed HFD in future studies with Morris water maze, radial arm maze, or other appropriate tests.

Intriguingly, in contrast to the dorsal sector, the vHIP monoamines were significantly altered by HFD. Involvement of vHIP DA and DA receptors in anxiety modulation was demonstrated by a rat study where rats exhibited increased anxiety in the EPM test following apomorphine (a DA agonist) administration directly into the vHIP (Zarrindast et al., 2010). Thus, in light of the significantly decreased HVA concentration and DA turnover, we presume that HFD consumption caused higher synaptic DA levels in the vHIP and an associated increase in anxiety; however, synaptic DA has to be measured to verify this assumption. PFC is thought to act as the downstream target in the modulation of anxiety-like behaviors because of direct efferent projections to it from the vHIP (Adhikari et al., 2010). As both PFC and vHIP can functionally interact during anxiety (Adhikari et al., 2010), our findings suggest that the anxiogenic effect of HFD in the MBT and FST could be due to altered monoamine homeostasis in both brain regions. Collectively, our results indicate that the vHIP/prefrontocortical circuit

may be more sensitive to diet-induced monoamine alterations than the dHIP, at least in female C57BL/6 mice after shorter feeding duration.

Considering the significantly altered neurochemistry in the ventral, but not dHIP, HFD's effect on synaptic plasticity was assessed in vHIP slices. Earlier studies reported HFD-induced significant impairments of hippocampal LTP in male rodents (Stranahan et al., 2008, Karimi et al., 2013); female data are limited. (Hwang et al., 2010) demonstrated hippocampal (primarily dorsal) LTP impairment only in obese male, but not female mice fed HFD for longer duration (9-12 months). Interestingly, we found a significant reduction in the ventral hippocampal LTP in female mice at a relatively early time point (after 11-12 weeks) of HFD exposure. The varied electrophysiological outcome between (Hwang et al., 2010) group and our study could be due to the differences in the hippocampal sectors (dHIP vs. vHIP) chosen for the measurement of synaptic plasticity changes, duration of HFD feeding (9-12 months vs. 11-12 weeks), selection of control diets (regular rodent chow vs. micronutrients and simple sugar-matched LFD) and/or the quantity (45% kcal% vs. 60% kcal%) of dietary fat in the HFD diets used.

In light of the HFD's targeted effect on ventral hippocampal monoamine homeostasis and related behavioral deficit (anxiety) observed after 5-6 weeks of the experimental period, we performed ventral hippocampal electrophysiology after an additional 6 weeks to determine whether the HFD's initial negative influence on vHIP will continue to persist with prolonged HFD consumption. We found that continued HFD intake produced a persistent adverse effect on the normal ventral hippocampal function evidenced by significant reduction in the vHIP LTP magnitude. Importantly, our results indicate that the neurological deficits induced by HFD intake can be viewed along a continuum, with some of the initially targeted brain regions still being impaired at a later stage of HFD consumption. In light of the intact short-term object recognition

memory and unaltered dHIP monoamine homeostasis observed at 6 weeks, we did not assess HFD's effect on dHIP synaptic plasticity at 11-12 week time point; however it is possible that unlike the early effects, continued HFD consumption may alter the functioning of dHIP (with respect to synaptic plasticity, neurochemistry, and cognition); this needs to be determined in future studies.

In summary, HFD intake by adult female mice for relatively short duration induces multiple behavioral abnormalities and affects monoamine pathways in a brain-region specific manner. The fact that HFD consumption selectively impairs emotional function, but spares object recognition memory, indicates that, in adult female mice, certain behavioral domains and associated brain regions are impaired by HFD preferentially. Importantly, considering the significantly decreased vHIP LTP with continued HFD feeding, it is apparent that HFD compromises the normal functioning of this hippocampal sub-structure. One likely implication of the present results is that, potentially, HFD is among the contributing factors for the recognized increased female vulnerability to mood disorders (An et al., 2011).

Table 4.1.Data of body weight, body weight gain, food intake, food efficiency and water intake of female C57BL/6 mice fed control (LFD) or HFD after 6 weeks (*n* = 8 per group) and 11/12 weeks (*n* = 5-6 per group).

Variable	LFD	HFD
Body weight (g)		
Week 0	15.76 ± 0.31	16.28 ± 0.18
Week 6	20.08 ± 0.38	**25.00 ± 0.99***
Week 11/12	20.62 ± 0.63	**29.58 ± 1.80***
Body weight gain (g; 0-6 weeks)	4.05 ± 0.59	**8.73 ± 1.21***
Body weight gain (g; 0-11/12 weeks)	5.22 ± 0.98	**13.30 ± 1.78***
Food intake (g/day; 0-6 weeks)	2.13 ± 0.05	2.27 ± 0.15
Food intake (g/day; 6-11/12 weeks)	2.24 ± 0.05	2.07 ± 0.12
Food intake (kcal/day; 0-6 weeks)	8.21 ± 0.25	**11.31 ± 0.87***
Food intake (kcal/day; 6-11/12 weeks)	8.62 ± 0.20	**10.83 ± 0.63***
Food efficiency % (g; 0-6 weeks)	452.7	**915.1***
Food efficiency % (g; 6-11/12 weeks)	665.4	**1835.7***
Food efficiency % (kcal; 0-6 weeks)	117.5	**183.7***
Food efficiency % (kcal; 6-11/12 weeks)	167.9	**350.9***
Water intake (ml/day; 0-6 weeks)	2.93 ± 0.18	3.20 ± 0.10
Water intake (ml/day; 6-11/12 weeks)	2.77 ± 0.06	**3.01 ± 0.03***

Data are presented as mean ± SEM; Food efficiency % calculated as g body weight gain/100 g or 100 kcal, expressed as percentage;
* Indicates significant effect of HFD ($P < 0.05$).

Table 4.2.Concentrations of monoamines[a] or their metabolites[a] in different brain regions of female C57BL/6 mice fed control (LFD) or HFD (n = 8 per group) for 6 weeks.

Table 4.2. Brain neurotransmitters, neurotransmitter metabolites and metabolite/neurotransmitter ratios

Diet	Brain region										
						PFC					
	DA	DOPAC	HVA	DOPAC/DA	HVA/DA	NE	MHPG	MHPG/NE	5-HT	5-HIAA	5-HIAA/5-HT
LFD	0.44 ± 0.07	0.23 ± 0.08	1.30 ± 0.52	0.56 ± 0.15	2.90 ± 0.82	4.88 ± 0.50	30.26 ± 4.27	6.18 ± 0.48	2.54 ± 0.51	2.79 ± 0.42	1.39 ± 0.30
HFD	0.32 ± 0.07	0.42 ± 0.27	1.04 ± 0.31	0.51 ± 0.12	3.20 ± 0.99	4.99 ± 0.15	**23.41 ± 0.69***	**4.71 ± 0.18***	2.62 ± 0.60	2.21 ± 0.19	1.04 ± 0.15
						NAc					
LFD	111.92 ± 9.65	12.88 ± 1.79	9.19 ± 0.97	0.11 ± .01	0.08 ± 0.01	ND	ND	ND	3.94 ± 0.28	3.32 ± 0.32	0.88 ± 0.13
HFD	106.38 ± 5.80	14.57 ± 1.85	9.31 ± 0.74	0.14 ± 0.02	0.09 ± 0.01	ND	ND	ND	3.48 ± 0.32	3.20 ± 0.27	0.95 ± .08
						Striatum					
LFD	146.75 ± 8.60	12.77 ± 1.07	10.82 ± 0.68	0.09 ± 0.01	0.08 ± .01	ND	ND	ND	4.26 ± 0.51	3.29 ± 0.23	0.83 ± 0.13
HFD	143.25 ±13.67	13.90 ± 1.42	10.78 ± 1.54	0.10 ± 0.01	0.07 ± 0.01	ND	ND	ND	3.72 ± 0.70	3.59 ± 0.62	1.01 ± 0.08
						dHIP					
LFD	0.38 ± 0.07	0.04 ± 0.01	0.83 ± 0.23	0.16 ± 0.05	2.45 ± 0.75	7.81 ± 0.48	49.24 ± 7.45	6.41 ± 0.87	4.64 ± 0.49	4.93 ± 0.63	1.16 ± 0.21
HFD	0.39 ± 0.05	0.06 ± 0.02	0.58 ± 0.15	0.14 ± 0.03	1.81 ± 0.51	7.61 ± 0.51	36.61 ± 1.97	4.88 ± 0.24	3.70 ± 0.30	4.31 ± 0.32	1.23 ± 0.17
						vHIP					
LFD	1.22 ± 0.21	0.16 ± 0.02	1.00 ± 0.13	0.20 ± 0.09	0.89 ± 0.08	8.27 ± 0.77	32.60 ± 2.83	4.06 ± 0.42	7.31 ± 1.51	5.95 ± 0.49	1.16 ± 0.21
HFD	1.58 ± 0.34	0.12 ± 0.03	**0.64 ± 0.06***	**0.09 ± 0.02***	**0.54 ± 0.12***	7.95 ± 0.26	27.76 ± 0.99	3.52 ± 0.17	6.16 ± 0.69	5.02 ± 0.38	1.23 ± 0.17

PFC: prefrontal cortex; NAc: nucleus accumbens; dHIP: dorsal hippocampus; vHIP: ventral hippocampus; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; ND: not detected/determined.

[a] Data are presented as mean ± SEM; unit: ng/mg protein (ratios are unitless). * Indicates significant effect of HFD ($P < 0.05$).

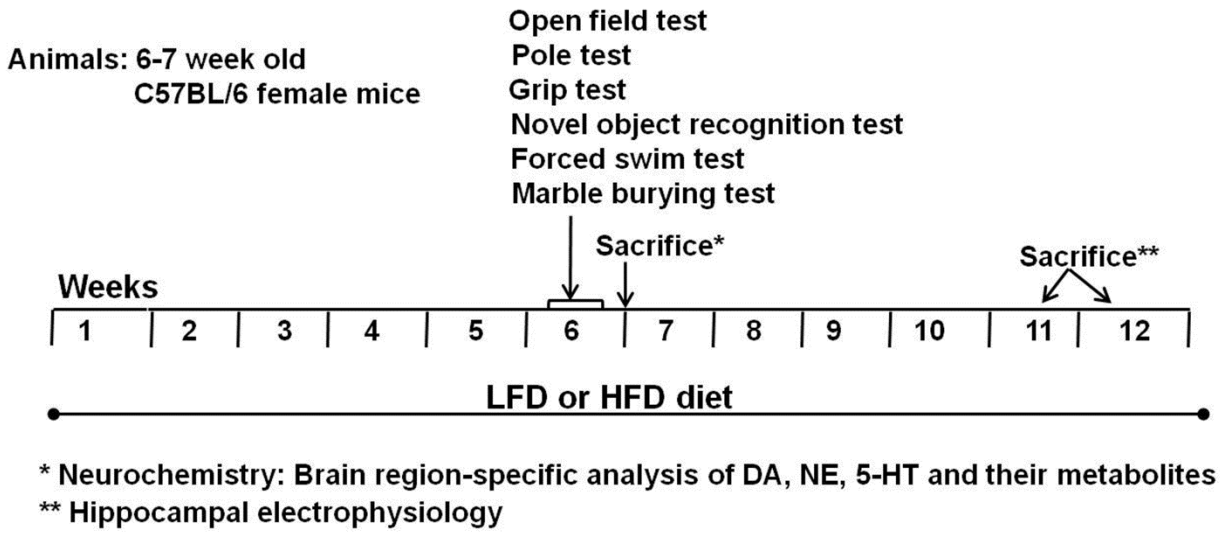

Figure 4.1.Detailed outline of the study's experimental design.

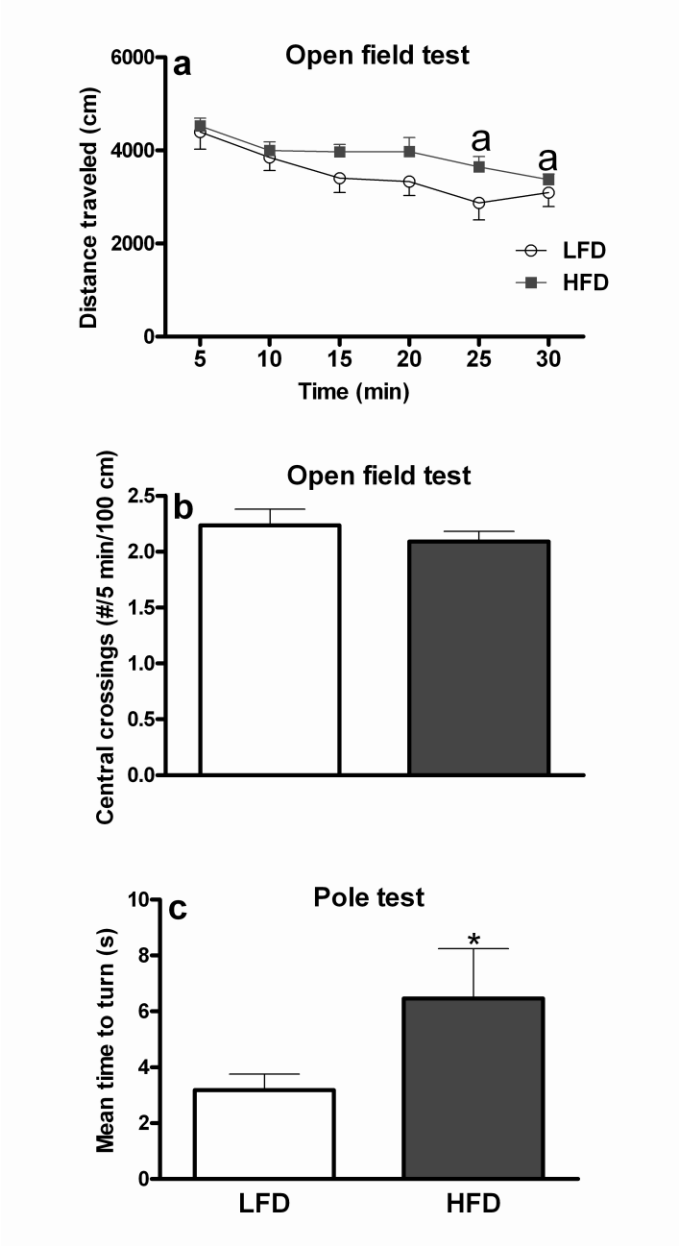

Figure 4.2. Effect of 5-week HFD consumption on: (**a**) distance traveled during each 5 min interval of the open field testing; (**b**) number of center crossings per 100 cm traveled in the open field arena analyzed per 5 min interval; (**c**) average time to turn during the pole test. Graphical representations are mean ± SEM (n = 8 per group). * indicates significant effect of HFD (P < 0.05). "a" indicates significant difference from 1st interval (first 5 min) in LFD/HFD groups (P < 0.05).

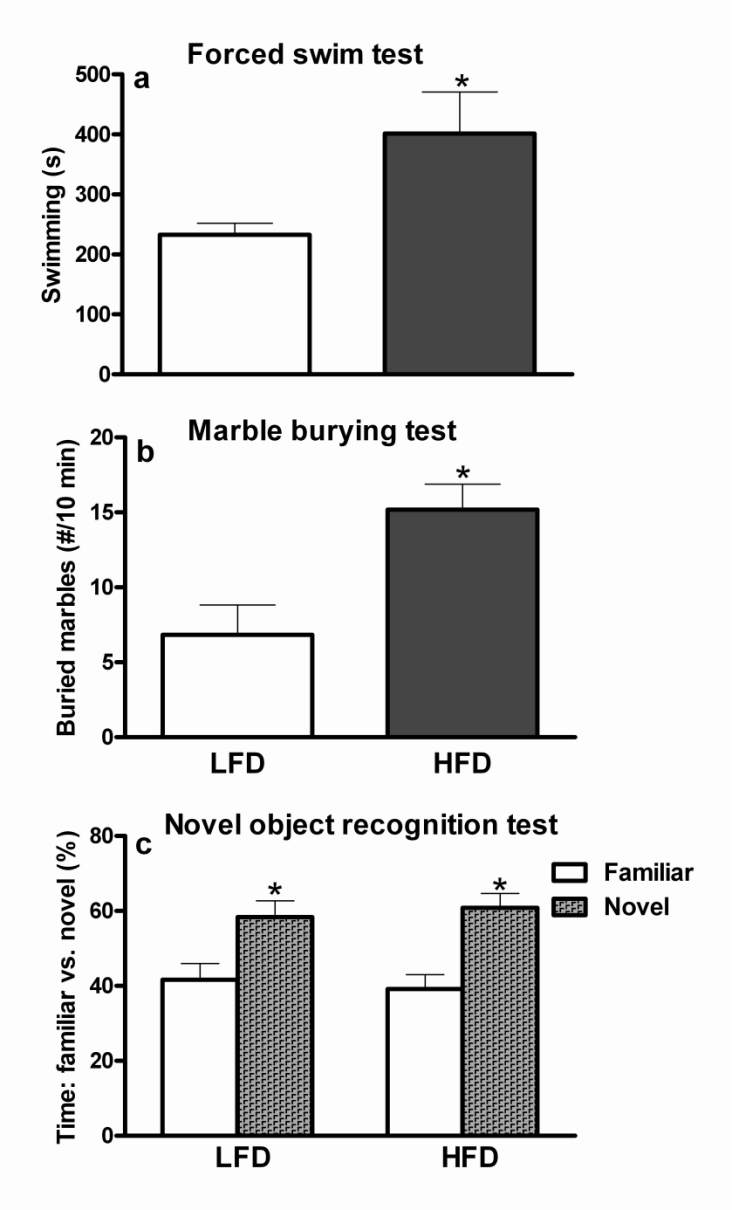

Figure 4.3. Effect of 5-week HFD intake on: (a) total time spent swimming in a forced swim test; (b) number of marbles buried (70%) during a marble burying test; (c) time spent with a familiar vs. a novel object (%) in a novel object recognition test. Graphical representations are mean ± SEM (n = 8 per group). * indicates significant effect of HFD ($P < 0.05$).

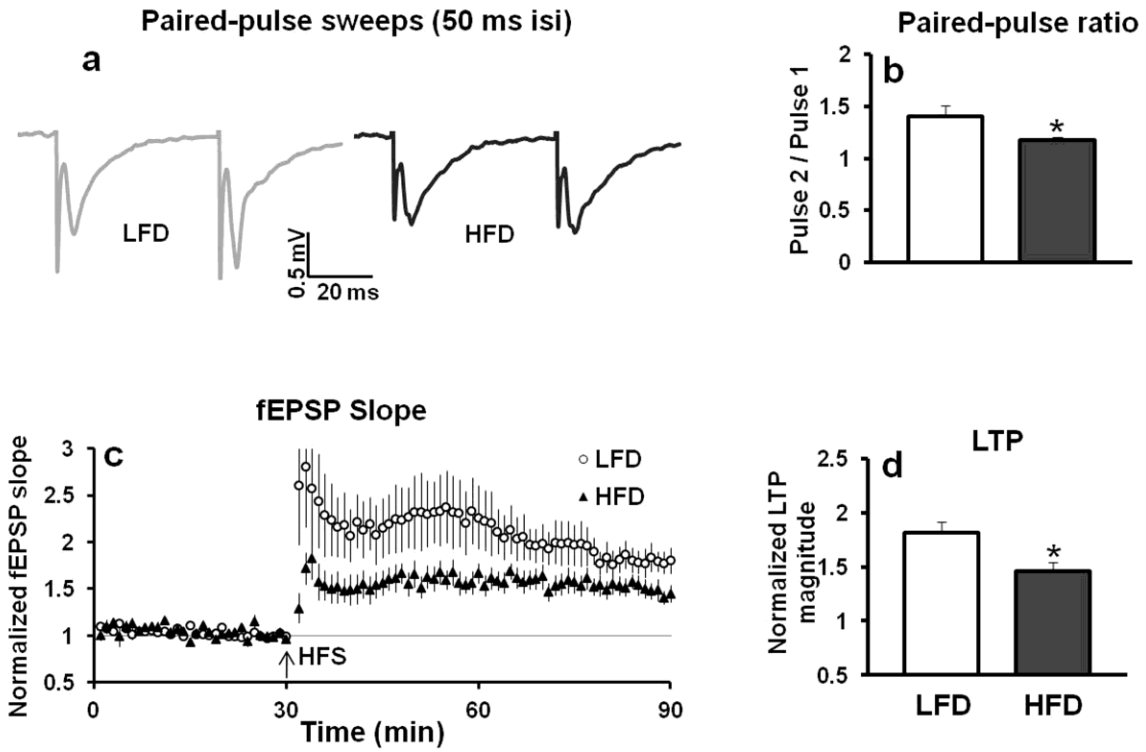

Figure 4.4. Effect of 11-12 weeks of HFD feeding on vHIP synaptic plasticity: (a) representative paired-pulse fEPSP sweeps recorded from CA1 region of vHIP slices of LFD (n = 6[10]) and HFD-fed mice (n = 5[11]); (b) quantification of paired-pulse ratio; (c) summary plots comparing the normalized fEPSP slope measurements. The x- axis indicates time for which the fEPSP slopes were recorded pre-and post- high frequency stimulation (HFS). Inset highlights superimposed 50 ms representative fEPSP sweeps from the last 5 min of pre-tetanus baseline and from the 55-60 min post-tetanus; (d) summary quantification of the LTP magnitudes. * indicates significant effect of HFD ($P < 0.05$). n-values (x[y]) indicate the number of mice (x) and the number of slices (y) assessed.

CHAPTER 5

HIGH-FAT DIET-INDUCED INSULIN INSENSITIVITY, GLUCOSE INTOLERANCE AND HEPATIC INFLAMMATION IN FEMALE C57BL/6 MICE[1]

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[1]Krishna, S., de La Serre, C.B., Srivastava, L., Norberg, T., Kirkland, R.A., Lee, S.H., Harn, D.A., Filipov, N.M. Submitted to *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 11/03/2014.

Abstract

Obesity plays a central role in the progression of metabolic syndrome that includes impaired glucose tolerance, dyslipidemia and insulin resistance. Increased intake of high-fat diets (HFD) is a key player in the development of obesity and associated metabolic syndrome. While the obesity epidemic and related metabolic dysfunctions occur in a gender-independent manner, most of the experimental studies with HFD focus on males. Considering the limited female-specific HFD data on metabolic dysregulation and associated inflammatory changes, this study characterized the female mice's response to 5-6 weeks of HFD feeding, a duration long enough to induce insulin resistance in male peripheral tissues. Glucose tolerance and insulin sensitivity tests were performed after 5 weeks on HFD. One week later, mice were sacrificed and inflammatory/insulin signaling markers were determined in key peripheral tissues (liver, adipose tissue and muscle). Female mice fed HFD were mildly hyperglycemic (baseline), substantially glucose intolerant and insulin resistant. Six-weeks of HFD intake did not affect total insulin receptor substrate 1 (IRS1) expression, but it specifically increased pSer307 IRS1 in the liver; IRS1 and pSer307 IRS1 levels were unaffected by HFD in adipose or muscle tissues. HFD consumption also increased hepatic mRNA markers of inflammation (interleukin-6, haptoglobin and CD36) and lipid metabolism (peroxisome proliferator-activated receptors alpha and gamma), accompanied with *toll-like receptor 4* activation; adipose tissue gene expression was unaffected. Collectively, our data suggest that in female mice, compared to adipose or muscle tissues, hepatic tissue is relatively more sensitive to the metabolic/inflammatory effects of HFD and that HFD-induced insulin insensitivity at this time point is likely driven by increased hepatic inflammation likely via increased IRS1 phosphorylation.

Key words: High-fat diet, Insulin resistance, Glucose tolerance, Hepatic inflammation

Introduction

Obesity and its associated comorbidities, such as type-II diabetes, are worldwide public health concern (Hu, 2011). In the United States alone, 25.8 million people, half of which are women, are affected by diabetes (CDC, 2014). Yet, obesity-related laboratory research has relied predominantly on male rodent models (Anai et al., 1999, Cano et al., 2013, Gao et al., 2014). Fat-enriched, so-called high-fat diets (HFD) have long been used by researchers to generate obese rodent models that can mimic the metabolic disorders characteristic of human obesity (Lai et al., 2014). For example, previous studies with male rodents fed a HFD have demonstrated visceral obesity and associated metabolic dysfunctions, including glucose intolerance, impaired peripheral insulin sensitivity and hyperlipidemia (Touati et al., 2011, Cano et al., 2013, Gao et al., 2014); information regarding the metabolic consequences of HFD feeding in females is limited but suggests metabolic dysregulation (Chalkiadaki and Guarente, 2012).

Compared to men with similar adiposity, women have less visceral fat and are less prone to insulin-resistance (Krotkiewski et al., 1983), especially free fatty acids-induced insulin resistance (Frias et al., 2001). A recent study which investigated the consequences of short-term (4 days) HFD feeding found that female mice, unlike their male counterparts, maintained normal insulin sensitivity in liver tissue (Senthil Kumar et al., 2014). However, clinical evidence shows that diabetic women are notably at higher risk for developing cardiovascular diseases (Logue et al., 2011, Kwon, 2014) and have a lower quality of life than diabetic men (Franzini et al., 2013, CDC, 2014). To shed more light into the nature of the metabolic dysregulation caused by HFD in the females, this study, characterized effects of 5-6 weeks of HFD feeding, a time of exposure long enough to induce insulin resistance in males (Kim et al., 2008b, Turner et al., 2013), in female C57BL/6 mice.

Obesity is associated with increased endotoxemia, due to changes in microflora composition and/or a compromised intestinal epithelial barrier (Cani et al., 2007, de La Serre et al., 2010a), facilitating the passage of the bacterial endotoxin, lipopolysaccharide (LPS) from the gut in to the systemic circulation. Increased circulating LPS, activates the transmembrane Toll-like receptor 4 (TLR4) signaling cascade, to cause increased synthesis of pro-inflammatory cytokines, such as tumor-necrosis factor alpha (TNFα) and interleukin 6 (IL-6) (Backhed et al., 2003); these cytokines impair insulin signaling via downregulation of signaling proteins, such as IRS1 and GLUT4 (Lumeng et al., 2007a, Qatanani and Lazar, 2007), and increase serine phosphorylation of IRS1 (Hotamisligil et al., 1996, Andreozzi et al., 2007, D'Alessandris et al., 2007).

In insulin-sensitive tissues, inflammatory signaling can be downregulated by peroxisome proliferator-activated receptors (PPARs). PPARs are fatty acid-activated transcription factors involved in lipid and carbohydrate metabolism. PPARα is notably involved in lipid catabolism (Patsouris et al., 2006), while specific activation of PPARγ stimulates lipid storage and causes weight gain (Wagener et al., 2010). PPARs may exert anti-inflammatory effects by modulating the activity of nuclear factor (NF)-κB, leading to a decreased pro-inflammatory cytokine production (Wahli and Michalik, 2012, Magliano et al., 2013).

Inflammation associated with obesity is believed to play a causal role in the development of insulin resistance (Bastard et al., 2006). Hyperinsulinemia, indicative of systemic insulin resistance, has been demonstrated in female mice (Winzell and Ahren, 2004) and rats (Flanagan et al., 2008) following short-term HFD consumption. In addition to causing systemic metabolic dysregulations, HFD intake affects the insulin sensitivity of key peripheral tissues, such as liver, adipose tissues and muscle. For example, 4 weeks of HFD feeding impaired insulin signaling,

evidenced by decreased levels of insulin signaling pathway regulating genes, such as insulin receptor substrate 2 (IRS2), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt2) was observed in the livers of male C57BL/6 mice following short-term (4 weeks) HFD feeding (Gao et al., 2014). Insulin resistance associated with impairment in PI3K activation was observed in the muscle and adipose tissue of male Sprague-Dawley rats after 2 weeks of HFD consumption (Anai et al., 1999). Of note, majority of the rodent studies that investigated the short-term effect of HFD feeding on peripheral tissue insulin sensitivity have used male subjects; female-specific information on HFD-induced metabolic and inflammatory dysregulation in specific peripheral tissues is limited and there is evidence that females may maintain liver insulin sensitivity better than males (Senthil Kumar et al., 2014). This is why we placed female C57BL/6 mice on HFD for 5-6 weeks and determined HFD's effects on glucose tolerance and insulin sensitivity, as well as on IRS1 Ser307 phosphorylation and inflammatory/lipid markers in liver and adipose tissues.

Materials and methods

Animals

Female C57BL/6 mice (6-7 weeks old) were purchased from Harlan (Indianapolis, IN) and housed (4-5 per cage) with water and food available *ad libitum* under constant temperature (22 °C) on a 12 h:12 h (light:dark) cycle in an AAALAC accredited facility throughout the study. Mice were given one-week acclimatization period before initiation of the experiments. All experimental procedures were in accord with the latest NIH guidelines and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Animal treatment

Mice weighing 16.0 ± 0.2 g (means ± SE) were randomly divided into two groups (n = 8 per group) and fed either a low-fat diet (LFD; D12450J, Research Diets, Inc., New Brunswick, NJ) or a high-fat diet (HFD; D12492, Research Diets) for a period of 6 weeks. The LFD diet provided 3.85 kcal/g of energy (70% carbohydrate, 20% protein, 10% fat, of which 22.7% saturated fatty acids [SAT], 29.9% monounsaturated fatty acids [MUFA], 47.4% polyunsaturated fatty acids [PUFA]). The HFD diet supplied 5.24 kcal/g of energy (20% carbohydrate, 20% protein, 60% fat, of which 32.0% SAT, 35.9% MUFA, 32% PUFA). The LFD diet was preferred over normal regular chow as the control diet because the former is appropriately matched in composition (micronutrients and carbohydrate [sucrose]) with our purified HFD diet (Warden and Fisler, 2008, Sasidharan et al., 2013). Moreover, in contrast to regular chow, the purified LFD or HFD diets does not contain phytoestrogen, which may affect the animal's food or water intakes, motor and non-motor behaviors, as well as the progression of metabolic disease states (Warden and Fisler, 2008, Sasidharan et al., 2013). Consequently, the purified LFD diets are being preferred as a control diet for majority of the high-fat dietary studies in rats and mice (El-Haschimi et al., 2000, Masuzaki et al., 2001, Posey et al., 2009, Hong et al., 2015). Body weight (BW) was recorded weekly. At the end of the study (6 weeks), mice were sacrificed and organs (brain, liver, spleen, kidneys and thymus) were harvested, weighed, and quickly frozen at -80°C for further analyses. In addition, visceral (mesenteric and retroperitoneal) adipose tissue and soleus muscle were collected and stored at -80°C until tissue-specific analyses.

Glucose tolerance test (GTT) and insulin sensitivity test (IST)

After 5 weeks on respective diets, GTT and IST were performed in succession (3 days apart) on the same animals over a 2-h window. The tests were performed after a 3 h fast (Cano et

al., 2013) and were completed 3 days prior to sacrifice. Glucose (2 g/kg BW; oral) and insulin (0.5 IU/kg; i.p.), both from Sigma (Sigma-Aldrich, St. Louis, MO) were administered and blood glucose levels were determined with a glucometer (TRUEresult®, Nipro Diagnostics, Fort Lauderdale, FL) by serial tail bleeds at various time points (GTT: 0, 15, 30, 60, 90, and 120 min; IST: 0, 15, 30, 60, 75, 90, 105, and 120 min). During IST, those animals with their blood levels lower than 20 mg/dl for two consecutive time points were given a single bolus of glucose to prevent them from developing severe signs of hypoglycemia; a blood glucose level of 20 mg/dl was assigned to these animals.

Intestinal permeability

Testing of intestinal wall integrity was based on (de La Serre et al., 2010a), with some modifications. Briefly, after 6 weeks on their respective diets, mice were fasted for 3 h and then gavaged with FITC-labeled dextran (4,000 kDa; Sigma) diluted in sterile saline (1000 mg/kg, 200 mg/ml). After 1 h, blood was collected by cardiac puncture, centrifuged (10,000 rpm for 3 min at 4°C) and a plasma aliquot was diluted 1:2 in PBS. Absorbance (485/535 nm, excitation/emission wavelengths) was measured with a spectrophotometer (SpectraMax M3; Molecular Devices, Sunnyvale, CA) and FITC-dextran plasma concentration was determined using a standard curve (8.0-0.125 ug/ml).

Western blot

Effect of HFD on pSer307 IRS1 (pIRS1), IRS1, IL-6, LBP and TLR4/MD2 expression was determined by western blot analysis according to a previously described procedure (de La Serre et al., 2010a). Briefly, tissues (liver, retroperitoneal adipose tissue, soleus muscle) were homogenized in lysis buffer (Life Technologies, #FNN0071, Carlsbad, CA) containing protease and phosphatase inhibitors (Roche, #1836170, #04906845001, Basel, Switzerland). 10 μg of

protein per sample was loaded onto a precast 10% Bis-Tris Plus gel. The PVDF (polyvinylidene difluoride) membranes (EMD Millipore) on to which protein was transferred (semi-dry transfer) were blocked with 10 % skim milk in TBST, washed (3 times), and incubated overnight at 4 °C with primary antibodies (β-actin #4970, GAPDH #2118, p-IRS1 #2381, IRS1#2382, Cell Signaling, Beverly, MA; IL-6 # ab6672, AbCAM, Cambridge, MA; LBP #14666, Santa Cruz, Dallas, TX; TLR4/MD2 #14-9924-81, eBioscience, Inc., San Diego, CA). Following incubation with appropriate secondary antibodies (HRP-linked anti-rabbit/mouse IgG, Cell Signaling) for 1 h at room temperature, the membranes were exposed to film (1- 30 min) and developed manually (GBX developer and fixer # 1901859, Kodak, Rochester, NY). The films were analyzed with Image J software (NIH) and bands of interest were normalized to GAPDH or β-actin (as appropriate) for statistical analysis.

Real-time quantitative PCR (qPCR)

Isolation and quantification of total RNA from liver (20 mg) and mesenteric/retroperitoneal fat (50 mg each) samples and cDNA synthesis were done as previously described (Krishna et al., 2014). Using 3 ng of cDNA per sample, expression of genes involved in inflammation, glucose homeostasis, and lipid metabolism was determined by qPCR using mouse-specific, certified primers from Life Technologies (Carlsbad, CA) and Real Time Primers (Elkins park, PA) (Table B1, Appendix B) and SYBR Green dye (Qiagen, Valencia, CA). Amplifications were performed on a Mx3005P qPCR machine (Stratagene) as described previously (Lin et al., 2013), with each sample run in duplicate. Treatment differences were calculated as a fold change using the $^{\Delta\Delta}$Ct method with 18S used as a house-keeping gene (HKG).

Hepatic triglyceride accumulation

Total lipid fraction was isolated using the Folch method (Folch et al., 1957). Briefly, liver samples were homogenized in a 2:1 chloroform:methanol solution and incubated overnight at 4°C. Samples were then centrifuged at 2,000 rpm for 15 min; the supernatant was collected and washed with 1% NaCl before being centrifuged at 2,000 rpm for 10 min. The subsequent top layer was discarded and the bottom layer was dehydrated and resupsended in 1% Triton X-100. Isolated triglycerides were quantified using the colorimetric Triglyceride (TAG) Reagent (Pointe Scientific, Canton, MI). Absorbance was read at a wavelength of 500 nm with a spectrophotometer (MRX Revelation, DYNEX Technologies, Inc., VA).

Histology

Sections (4 μm) from liver tissue samples were stained either using Oil Red O with hematoxylin as a counter nuclear stain or using hematoxylin and eosin (H & E) staining. Staining was performed at the University of Georgia (UGA) College of Veterinary Medicine's Pathology Laboratory. Sections were viewed under a light microscope (Nikon Eclipse E400, Tokyo, Japan) at 200X magnification.

Statistical analysis

Data were analyzed using Student's T-test. Two-way analysis of variance (ANOVA) was conducted to analyze the GTT/ITT (treatment x time) data. If ANOVA's overall main effect was found significant, treatment means were separated by Student-Newman-Keuls *post hoc* test. Prior to statistical analysis, the integrated areas under the curve (AUC) of GTT and ITT responses were calculated with the trapezoidal method (Rodriguez-Rivera et al., 2011). All results are presented as mean ± SEM and are considered significant at $P < 0.05$.

Results

Effect of HFD on organ weights

HFD feeding for 6 weeks resulted in a significant BW increase (LFD vs. HFD: 20.1 ± 0.4 vs. 25.0 ± 0.9 g, $P < 0.001$), but the absolute weights (g) of brain, liver, kidneys, spleen or thymus were not affected ($P > 0.05$; Fig. 5.1), suggesting visceral fat based weight gain. In light of this interpretation, the relative organ weights (g/kg BW; data not shown) of brain ($P < 0.001$), liver ($P = 0.01$), kidneys ($P < 0.01$) and spleen ($P < 0.05$) were decreased, but not of thymus ($P = 0.17$).

Effect of HFD on glucose tolerance

Five weeks of HFD consumption resulted in significant increase in fasting (0 min) baseline blood glucose level (LFD vs. HFD: 72.2 ± 6.2 vs. 106.2 ± 14.3 mg/dl, $P < 0.05$; Fig. 5.2A). Circulating glucose levels rose rapidly in both LFD and HFD groups after the oral glucose challenge and peaked at 15 min post challenge. Blood glucose levels in the LFD group started to decline 30 min after glucose administration; in the HFD group, circulating glucose remained elevated ($P < 0.05$; Fig. 5.2A). In HFD-fed mice, glycemia started to decline 60 min after glucose administration ($P < 0.001$; Fig. 5.2A), but it remained significantly higher than the LFD group at the remaining time points (90 and 120 min; Fig. 5.2A). The respective area under the GTT curve (AUC), an integrative measure of the animal's response to the glucose challenge, was significantly higher (79%) in the HFD group ($P < 0.001$; Fig. 5.2B).

Effect of HFD on insulin sensitivity

Because of the significant difference in the fasting glucose levels between LFD and HFD groups (LFD vs. HFD: 57.6 ± 10.5 vs. 111.5 ± 11.6 mg/dl, $P < 0.05$; Fig. 5.2C), we have presented the results from IST as a time-course of glucose levels, and not as the percentage of

basal glucose levels (Ayala et al., 2010). During the IST, both LFD and HFD-fed mice showed a similar early response to insulin (Fig. 5.2C). Specifically, both groups exhibited marked hypoglycemia at both 15 and 30 min post insulin injection (Fig. 5.2C). However, compared to the LFD group, which remained greatly hypoglycemic throughout the 2 h sampling period, the HFD-fed mice showed an early rebound in the blood glucose levels starting at 60 min post insulin challenge ($P < 0.05$; Fig. 5.2C); their blood glucose returned to almost normal level by 2 h (Fig. 5.2C). As a consequence, the AUC for IST in the HFD-fed mice was significantly greater (101%; $P < 0.01$; Fig. 5.2D).

Effect of HFD on IRS1 Ser307 phosphorylation

HFD-fed mice showed a significant increase in liver pSer307 IRS1/IRS1 ratio (LFD vs. HFD: 0.7 ± 0.1 vs. 1 ± 0.1, $P < 0.05$; Fig.5.3, A and B). HFD-fed animals exhibited non-significant trends towards an increase in the soleus muscle pSer307 IRS1/IRS1 ratio ($P = 0.21$; Fig. 5.3, C and D) and towards a decrease in the retroperitoneal adipose tissue pSer307 IRS1/IRS1 ratio ($P = 0.12$; Fig. 5.3, E and F). When analyzing the adipose tissue blot, it seemed to show a global decrease in IRS1 protein level in HFD-fed mice (Fig. 5.3F). However, when controlled for the total amount of protein present using GADPH, there was no difference in IRS1 protein levels in HFD-fed mice compared to LFD group ($P = 0.99$; data not shown).

Effect of HFD on adipose tissue gene expression

Notably, the *adipose tissue gene expression* response of mice within the treatment groups was variable. Mesenteric fat expression of IL-6, CD36, PPARα, PPARγ and IRS1 was not affected significantly by HFD ($P > 0.35$; Fig. 5.4A). Similar to mesenteric fat mRNA data, six weeks of HFD consumption did not significantly change the mRNA levels of IL-6, PPARα, and IRS1 in the retroperitoneal fat ($P > 0.15$; Fig. 5.4B). Compared to the LFD group, 6 weeks of

HFD feeding resulted in non-significant trends towards increases in the retroperitoneal fat mRNA levels of CD36 (~2.0-fold; $P = 0.15$) and PPARγ (>3.0-fold; $P = 0.13$); however these numerical increases did not reach statistical significance, likely due to the greater HFD group variability (Fig. 5.4B).

Effect of HFD on liver histology, total triglycerides, genes and protein expression

Compared to LFD-fed mice, six weeks of HFD feeding did not cause any apparent liver damage or increase in fat droplets (Fig. 5.5A); total liver triglyceride content was also unaffected by HFD ($P = 0.25$; Fig. 5.5B). With respect to gene expression, 6 weeks of HFD consumption significantly upregulated hepatic expression of the pro-inflammatory cytokine, IL-6 (2.1-fold, $P < 0.01$; Fig. 5.5C) and of the acute-phase protein haptoglobin (Hp, 1.9-fold, $P < 0.05$; Fig. 5.5C); TNFα levels were unaffected by HFD ($P = 0.51$; Fig. 5.5C). Additionally, compared to the LFD group, expression of CD36, a marker of activated macrophages and inflammation, was significantly upregulated in the livers of 6-week HFD-fed mice (1.7-fold, $P < 0.05$; Fig. 5.5C). Six-weeks HFD intake also significantly increased hepatic expression of PPARα (1.8-fold, $P < 0.05$; Fig. 5C) and PPARγ (2.8-fold, $P < 0.01$; Fig. 5.5C). Hepatic mRNA expression of IRS1 was not altered by HFD ($P = 0.45$; Fig. 5.5C). Elevated liver IL-6 was confirmed at the protein level (n-fold β-actin) by western blot (LFD vs. HFD: 0.1 ± 0.02 vs. 0.3 ± 0.05, $P < 0.05$; Fig.5.5, D and E).

Effect of HFD on gastrointestinal permeability and hepatic levels of LBP and TLR4

Compared to LFD-fed mice, 6 weeks of HFD feeding resulted in numerical increase (2.1-fold) in plasma FITC-dextran concentration ($P = 0.10$; Fig. 5.6A). However, this numerical increase did not reach statistical significance, likely due to the greater variability in the HFD group (Fig. 5.6A). HFD-fed mice also exhibited a non-significant trend for an increase in liver

LPS-binding protein (LBP) protein levels (P = 0.07; Fig. 5.6B). Quantification of the TLR4/MD2 complex was used to characterize TLR4 activation. HFD caused a significant increase in hepatic TLR4 (n-fold GAPDH) activation (LFD vs. HFD: 1.4 ± 0.07 vs. 2.1 ± 0.2, P < 0.01; Fig. 5.6, C and D).

Discussion

In the current study, we sought to investigate the glucose/insulin dyshomeostasis in key peripheral tissues (liver, muscle and adipose tissue) of female mice caused by HFD consumption for 5-6 weeks. We also determined HFD's effect on liver and white adipose tissue inflammatory profile. Female mice on HFD exhibited impaired glucose tolerance and insulin sensitivity. pSer307 IRS1, an inflammation-responsive marker of impaired insulin signaling (Rui et al., 2001), was only elevated in the liver with no significant differences seen in adipose or muscle tissues. Inflammation was also only evident in the liver with increased expression of IL-6, Hp and CD36; there were no significant inflammatory changes in adipose tissues. Taken together, these data suggest that in female mice, HFD feeding for shorter duration alters insulin sensitivity primarily via development of liver inflammation and liver-specific elevation of Ser307 IRS1 phosphorylation.

We found that high-fat feeding for 6 weeks was sufficient to induce an increase in body weight and consequently a decrease in the relative organ weights. There was no difference in liver absolute weight, which is concordant with the absence of hepatic lipid accumulation. The impaired glucose tolerance and insulin resistance, as shown by GTT and IST, in this study are in line with multiple studies with male (McDonald et al., 2011, Cano et al., 2013, Gao et al., 2014) and female rodents (Chalkiadaki and Guarente, 2012) fed HFD for durations ranging from 3 to 12 weeks. HFD consumption has been shown to induce inflammation in insulin-sensitive tissues,

such as hepatic, adipose and muscle (Williams et al., 2014), and it is believed to affect Ser307 IRS1 phosphorylation (Hotamisligil et al., 1996, D'Alessandris et al., 2007). We characterized Ser307 IRS1 phosphorylation in liver, muscle and retroperitoneal fat and found liver-specific increased phosphorylation caused by HFD feeding. Similarly, in these animals, there was evidence for hepatic-restricted inflammation. Expression of IL-6 and Hp, both involved in the acute phase response as well as the macrophage activation marker, CD36 were upregulated; however, TNFα expression was unchanged. Increased hepatic IL-6 levels could be viewed as an increased or decreased inflammatory response, owing its pro- or anti-inflammatory activities, respectively (Scheller et al., 2011). However, considering the simultaneous significant elevation in the hepatic expression of the other inflammatory markers, such as Hp and CD36, it could be inferred that, it is more likely the pro-inflammatory role of IL-6 that is dominating at this stage of HFD intake. Interestingly, although white adipose tissue has been thought to be the main source of inflammation in obesity (Xu et al., 2003, Bastard et al., 2006), we did not find any significant changes in pro-inflammatory gene expression in the retroperitoneal or mesenteric fat depots. We did not characterize the muscle inflammatory profile as muscle inflammation in mice is believed to occur after prolonged exposure to high-fat feeding (Kleemann et al., 2010). It should be noted that although IRS1 expression may be upregulated by inflammation, we did not observe that in this study. Increased IRS1 expression has been linked to TNFα upregulation (Jager et al., 2007, Lumeng et al., 2007a) and TNFα expression was unchanged in the livers of HFD-fed females in our study. This suggest that in female mice, after 6 weeks of HFD feeding, insulin homeostasis is dysregulated primarily in the liver and by an inflammation (non-TNFα) dependent increased phosphorylation of IRS1 Ser307.

A tissue/time response to HFD feeding has been described in mice before, although the timeline of events seem to differ between males and females. While, in vitro, Ser307 IRS1 phosphorylation takes place in different tissues, there is evidence for tissue-specific regulation of this pathway (Hotamisligil et al., 1996). For example, PI3K activity, which can promote Ser307 IRS1 phosphorylation, is upregulated in the liver of diabetic rats while being downregulated in muscle and adipose tissues (Anai et al., 1999). In male mice, a biphasic pattern of insulin resistance in response to high-fat feeding was recently reported (Williams et al., 2014). In (Williams et al., 2014) study, an acute transient response accompanied with liver inflammation developed after 3 days on HFD, this was followed by a second phase that developed after 12-16 weeks of HFD intake and it was associated with widespread (liver and adipose tissue) inflammation (Williams et al., 2014). During the acute phase, circulating IL-6, but not TNFα, was elevated (Williams et al., 2014). Liver inflammation seems required for this early onset insulin resistance as in male mice, Kupffer cells are activated within 3 days of HFD treatment and their deletion prevents the development of hepatic insulin resistance (Lanthier et al., 2010). In this study, we did not measure circulating IL-6, but found elevated expression of liver IL-6 and the acute-phase protein, Hp. In humans, C-reactive protein and serum amyloid A (acute phase proteins linked to systemic insulin resistance) showed an initial rise and rapid decline within 24-72 h following an inflammatory stimulus, whereas Hp remained elevated 10 days after the onset of inflammation (Gabay and Kushner, 1999). Increased plasma Hp levels that persisted several weeks after the onset of inflammation has also been demonstrated in mice (Ngure et al., 2008). Of note, liver Hp levels that increased after 1 week of HFD feeding remained elevated after 16 weeks of HFD intake in male mice (Williams et al., 2014). Our female mice data showing increased hepatic Hp levels after 6 weeks of HFD feeding are in line with this study. In

male mice, the hepatic acute phase response was observed within 3 days and did not resolve, but the early (3 days) hepatic inflammatory response was resolved by 1 week (Williams et al., 2014). We found signs of liver inflammation after 6 weeks of HFD, indicating that, in female mice, liver inflammation-driven insulin resistance in females may be delayed and/or may last for a longer period.

While liver damage and fat infiltration in male livers has been reported as early as after 4 days to one week of HFD feeding (Senthil Kumar et al., 2014, Williams et al., 2014), female livers maintained tissue integrity and showed no signs of inflammation after HFD intake (Senthil Kumar et al., 2014). There is also a report for increased liver infiltration of fat in the absence of liver damage or cholesterol accumulation in female mice after 5 weeks on a HFD (Fan et al., 2012). In the current study, we did not observe major fat infiltration or liver damage in HFD-fed mice; H and E and Oil Red O staining were identical, and so were the triglyceride contents. Use of more sophisticated measurements such as automated image analysis (Nativ et al., 2014) or other sensitive imaging modalities (computed tomography and magnetic resonance imaging) (Schwenzer et al., 2009) might have uncovered some low-grade liver damage (if at all present) that was not detectable with the methods we employed here.

Liver inflammation may be related to Kupffer cell activation, which, in part, is dependent on TLR4 activation (Rivera et al., 2007). TLR4 appears to play a critical role in the development of insulin resistance as its expression is dramatically increased in diabetic mice and it coincides with an increase in IL-6 expression (Ladefoged et al., 2013). In this study, liver TLR4 signaling, characterized by increased levels of the TLR4/MD2 complex, was upregulated. LPS is a ligand for TLR4 with its co-receptor CD14. Along with the accessory protein MD2, the TLR4/CD14/MD2 complex drives TLR4 signaling (Backhed and Hornef, 2003). We observed a

trend towards increased gastrointestinal permeability in the HFD-fed mice, potentially allowing LPS translocation from the gut to the circulation. In this regard, we also noted a trend towards increased liver LBP (LPS-binding protein) levels (LPS is transported with LBP to the liver for disposal). Although statistical significance was not reached for the liver LPB protein levels, we did observe a significant increase in hepatic TLR4 activation in HFD-fed females. Alternatively, TLR4 could also be activated by saturated fatty acids in addition to LPS (Lee et al., 2001, Shi et al., 2006). We did not characterize lipid plasma profile in this study, but HFD consumption has been shown to rapidly alter plasma lipids (Senthil Kumar et al., 2014).

Activation of CD36, a class B scavenger receptor expressed in macrophages, including Kupffer cells, also promotes inflammation (Kim et al., 2008a). CD36 activation has been shown to activate the NFκB pathway and pro-inflammatory cytokine secretions through its interaction with TLRs, including TLR4 (Cai et al., 2012b). We found a significant increase in liver CD36 expression caused by HFD. CD36 is also believed to be involved in hepatic triglycerides storage (Koonen et al., 2007), however, we did not find any evidence of triglycerides accumulation in the liver of the HFD-fed female mice, suggesting that its pro-inflammatory role dominates at this stage of HFD feeding duration.

CD36 upregulation can be induced by PPARγ (Inoue et al., 2005b, Moran-Salvador et al., 2011). We found significant increase in PPARγ expression in the livers of the HFD-fed female mice. Increased PPARγ expression has been associated with liver steatosis (Moran-Salvador et al., 2011). Of note, exogenous administration of a PPARγ agonist improves insulin sensitivity and reduces inflammation in adipocytes (Soares et al., 2013) via downregulation of TNFα and adipokines, such as resistin (Magliano et al., 2013). In our study, while the PPARγ/CD36 increases appear to promote inflammation, PPARγ may also curb TNFα as we did not observe

any changes in TNFα expression. Besides PPARγ, liver PPARα expression was also significantly upregulated by HFD feeding. Insulin treatment markedly reduces PPARα expression, thus PPARα upregulation in liver may be due to reduced insulin signaling (Patsouris et al., 2006). PPARα is believed to have an anti-inflammatory role in the liver. Indeed, in PPARα null mice there is a marked increase in apoptosis and hepatic TNFα in response to HFD (Abdelmegeed et al., 2011). This increase in PPARα may be compensatory and its potential associated beneficial effect may be sex-specific, i.e., in male mice, HFD consumption decreases PPARα expression (Schultz et al., 2013, Tapia et al., 2014), while estrogen treatment in rats increases hepatic liver PPARα expression (Abeles et al., 2012).

In conclusion, female mice develop glucose intolerance and insulin resistance after 5-6 weeks of HFD feeding; this seems to be driven by liver inflammation and dysregulated hepatic insulin homeostasis. These data are consistent with the two phase-tissue specific hypothesis: liver insulin resistance is believed to develop first, while adipose tissue and muscle insulin resistance might appear later in conjunction with adipose tissue inflammation (Kleemann et al., 2010, Williams et al., 2014). Studies in male rodents have reported an early onset of fat infiltration or inflammation in the liver, the latter being resolved within few days of HFD intake (Senthil Kumar et al., 2014, Williams et al., 2014). In our study with female mice, we found evidence of hepatic inflammation, but no signs of fat infiltration, even after 6 weeks of HFD feeding. Taken altogether, we conclude that, in female mice, after short-term HFD feeding, the hepatic phase might be either prolonged or delayed due to a potential protective role of PPARα.

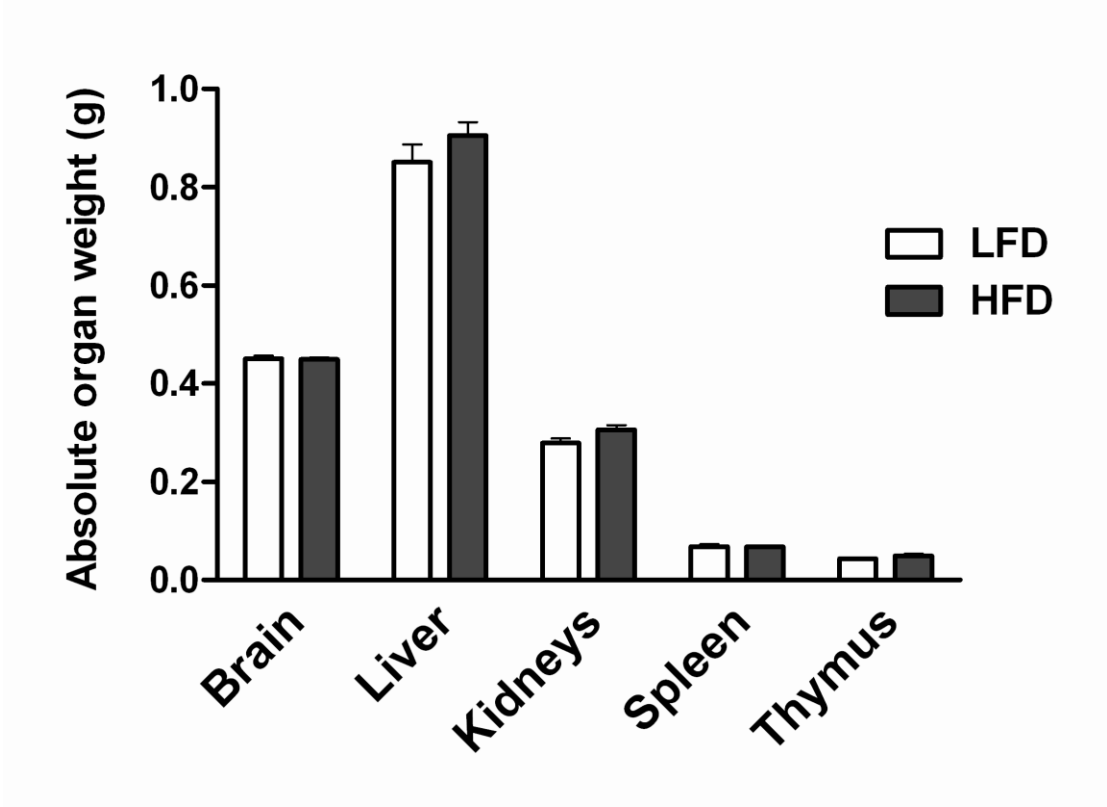

Figure 5.1. Absolute organ weights of LFD and HFD females after 6 weeks on their respective diets. Six weeks on HFD did not affect the absolute weights (g) of brain, liver, kidneys, spleen and thymus. Graphical representations are means ± SEM (*n* = 8 per group).

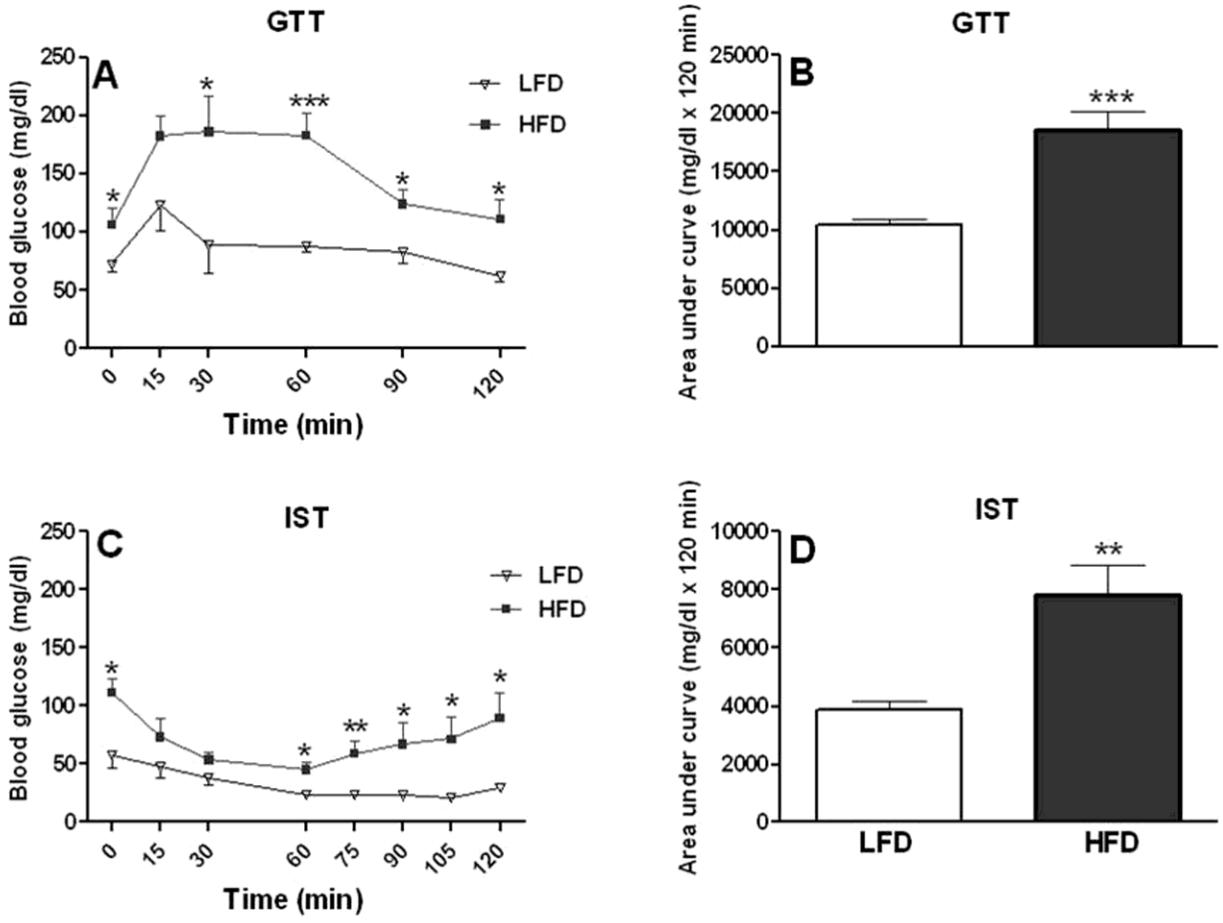

Figure 5.2. Glucose tolerance and insulin sensitivity in LFD and HFD-fed females after 5 weeks on their respective diets. A and B. Glycemia and area under the curve (AUC) following oral glucose gavage. Glycemia in both LFD and HFD groups peaked 15 min after glucose administration ($P = 0.06$). In HFD-fed mice, glycemia started to decline 60 min after glucose gavage and remained significantly higher than the LFD group (A). The respective AUC for GTT was significantly increased (79%) for the HFD group ($P < 0.001$; B). C and D. Glycemia and AUC following i.p. insulin injection. During IST, both LFD and HFD-fed mice responded similarly to insulin with reduction in blood glucose at 15 and 30 min post insulin injection (C). HFD-fed mice showed an early rebound in blood glucose level starting at 60 min post insulin

challenge ($P < 0.05$) with their glycemia almost returning to normal level by 2 h ($P < 0.05$; C). The respective AUC for IST was significantly increased (101%) for the HFD group ($P < 0.01$; D). Graphical representations are means ± SEM ($n = 8$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

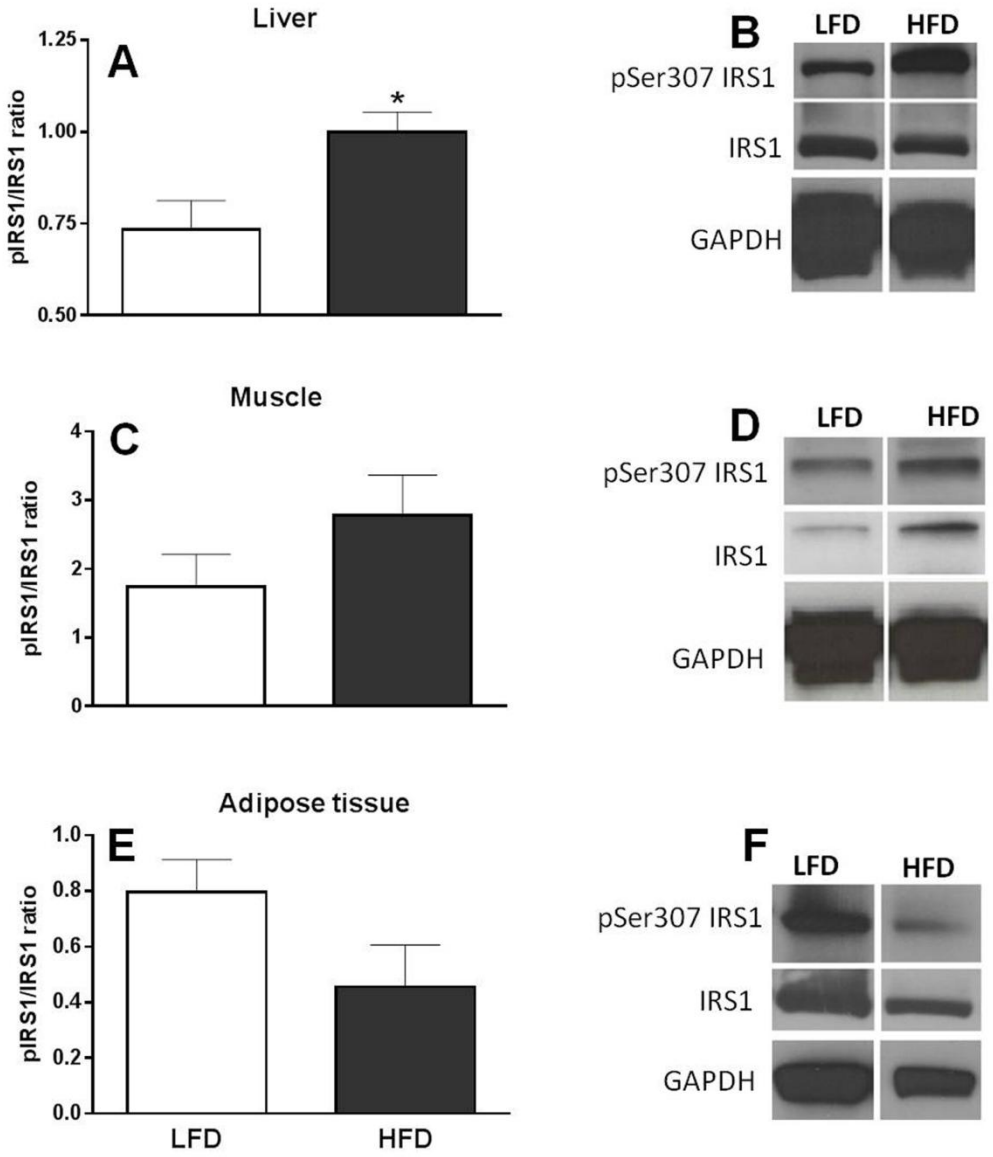

Figure 5.3. pSer307 IRS1/IRS1 ratio in liver (A and B), soleus muscle (C, D) and retroperitoneal adipose tissue (E, F) of LFD/HFD female mice after 6 weeks on respective diets. A. pIRS1/IRS1 ratio and B. Representative blot in liver. pSer307 IRS1/IRS1 ratio was significantly increased in the liver of the HFD-fed mice ($P < 0.05$). C. pIRS1/IRS1 ratio and D. Representative blot in muscle. There was a non-significant trend towards an increase in pIRS1/IRS1 ratio ($P = 0.21$) in HFD group. E. pIRS1/IRS1 ratio and F. Representative blot in adipose tissue. There was a non-significant trend towards a decrease in pIRS1/IRS1 ratio ($P = 0.12$) in HFD-fed mice. The blot

seemed to show a global decrease in IRS1 protein level in HFD group, however when controlled for the total amount of protein present using GADPH as a control, there was no difference in IRS1 protein levels between LFD and HFD groups ($P = 0.99$). Graphical representations are means ± SEM ($n = 8$ per group). *indicates significant effect of HFD ($P < 0.05$).

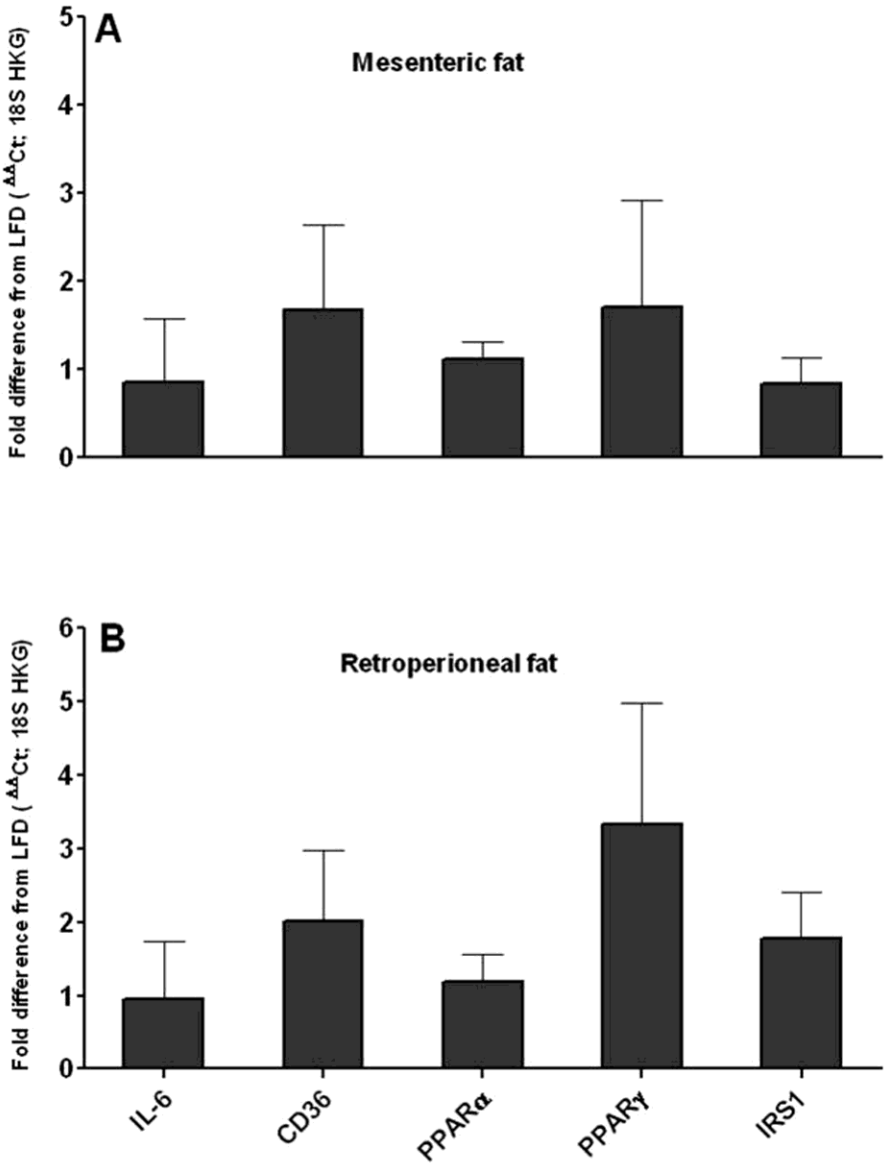

Figure 5.4. Adipose tissue gene expression in the LFD and HFD-fed female mice after 6 weeks on their respective diets. Mesenteric fat expression of IL-6, CD 36, PPARα, PPARγ and IRS-1 was unaffected by HFD ($P > 0.35$; A). HFD did not change the mRNA levels of IL-6, PPARα, and IRS-1 in the retroperitoneal fat ($P > 0.15$; B). HFD feeding resulted in a non-significant trend towards an increase in the mRNA levels of CD36 (~2.0-fold; $P = 0.15$) and PPARγ (>3.0-fold; $P = 0.13$) in the retroperitoneal fat; however this numerical increase did not reach statistical

significance, likely due to the greater variability in HFD group (B). The house keeping gene (HKG), 18 S was used to normalize the mRNA data and are presented as fold change relative to control. Graphical representations are means ± SEM (n = 8 per group).

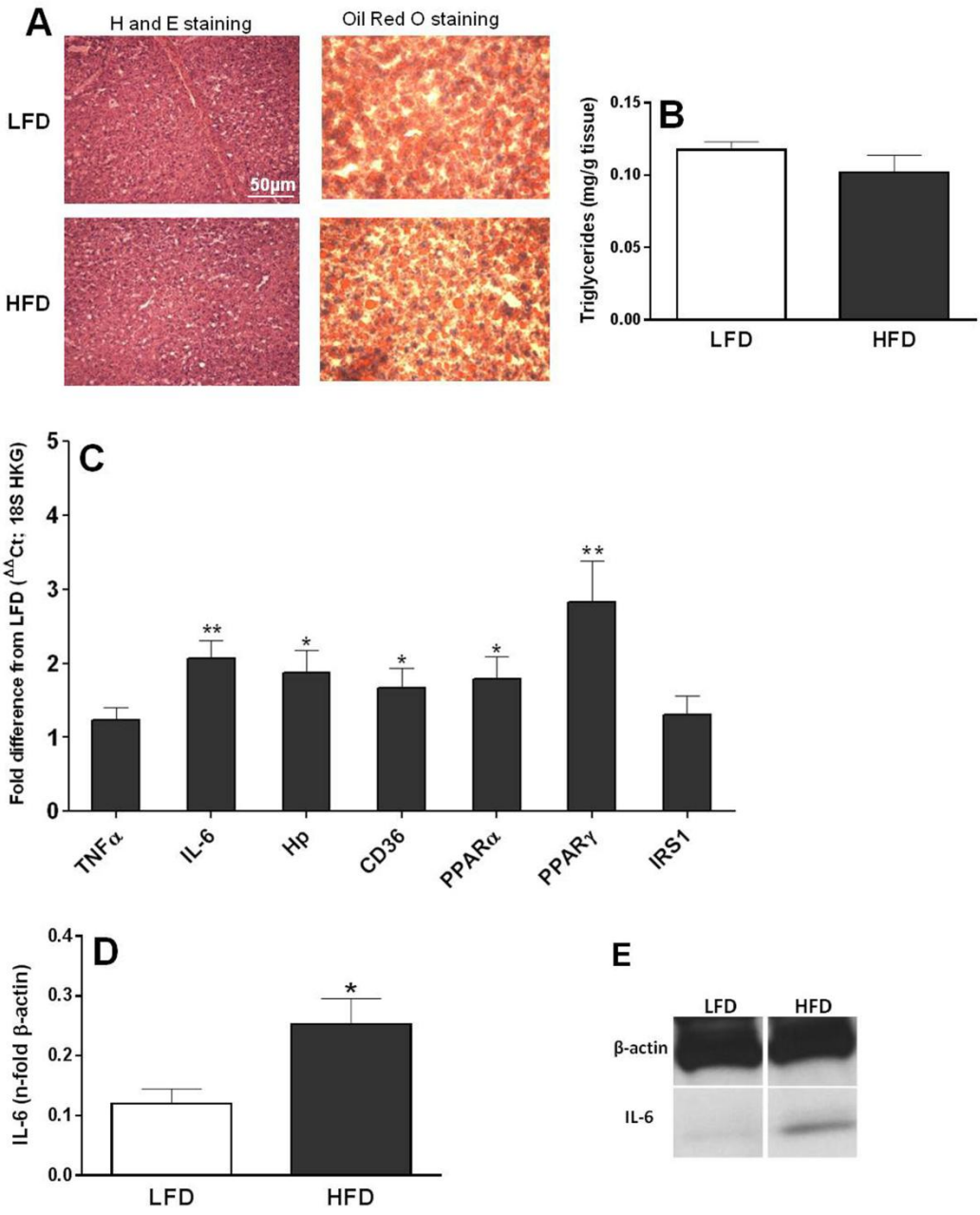

Figure 5.5. Liver histology (A), total triglycerides (B), genes (C) and protein (D and E) expression in LFD and HFD-fed female mice after 6 weeks on their respective diets. HFD did not cause any apparent liver damage; no significant difference was observed with respect to fat droplets (A) or total triglycerides (B) in the liver of the HFD-fed mice. C. Gene expression. HFD consumption significantly upregulated hepatic IL-6 (2.1-fold, $P < 0.01$) and haptoglobin (Hp,

1.9-fold, $P < 0.05$) expression; TNFα was not affected by HFD ($P = 0.51$). HFD intake significantly increased the hepatic expression levels of CD 36 (1.7-fold, $P < 0.05$), PPARα (1.8-fold, $P < 0.05$) and PPARγ (2.8-fold, $P < 0.01$). IRS1 mRNA level was unaffected by HFD ($P = 0.45$). The house keeping gene (HKG), 18S was used to normalize the mRNA data and are presented as fold change relative to control. D. Quantification of IL-6 protein level and E. Representative blot. Elevation in IL-6 was confirmed at the protein level by western blot ($P < 0.05$). Graphical representations are means ± SEM ($n = 8$ per group). * $P < 0.05$, ** $P < 0.01$.

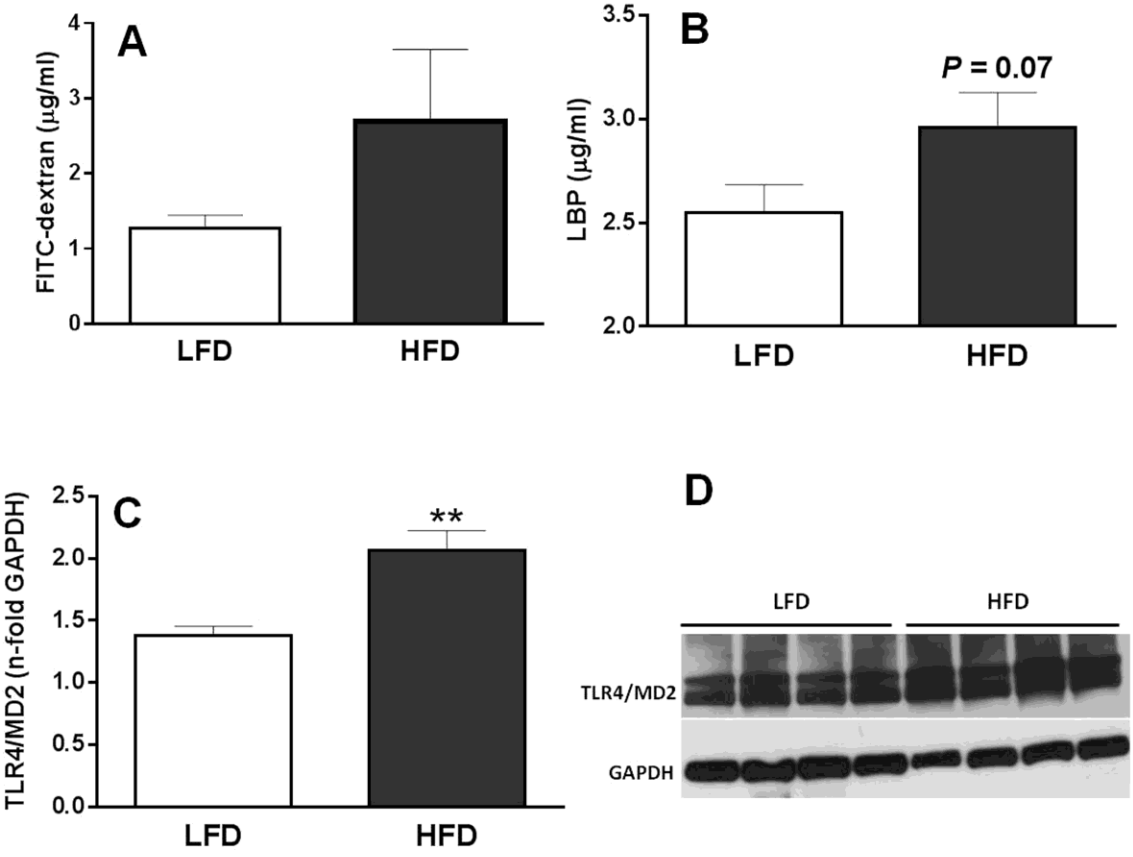

Figure 5.6. Gastrointestinal permeability (A), liver LPS-binding protein (LBP) (B) and liver TLR4 activation (C and D) in female mice fed LFD or HFD after 6 weeks on their respective diets. There was a non-significant trend ($P = 0.10$) towards an increase in the circulating fluorescent dextran following oral gavage (used as a marker of gastrointestinal permeability) in HFD-fed mice compared to LFD- fed animals (A). Six weeks of HFD feeding produced a non-significant ($P = 0.07$) increase in liver LPS-binding protein (LPB) protein levels (B). However HFD feeding triggered a significant increase in TLR4 activation, as shown via western blotting against the TLR4/MD2 complex (C and D). Graphical representations are means ± SEM ($n = 8$ per group). ** $P < 0.01$.

CHAPTER 6

NEUROBEHAVIORAL AND METABOLIC/INFLAMMATORY RESPONSES IN FEMALE MICE FED A HIGH-FAT DIET FOR UPTO 9 MONTHS [1]

[1]Krishna, S., Lin, Z., Weber, M., Pepples, L., Lee, S.H., Wagner, J.J., de La Serre, C.B., Harn, D.A., Filipov, N.M. To be submitted to *Physiology & Behavior*.

Abstract

Obesity, caused by excessive caloric intake due to consumption of high-fat diets (HFD) is associated with metabolic dysfunctions, low-grade chronic inflammation and behavioral deficits. In light of the limited information on the temporal changes in the neurobehavioral, inflammatory and metabolic dysregulation caused by chronic HFD feeding, we determined the time-dependent (22 and 36 weeks) effects of prolonged HFD intake on selected behavioral, metabolic and inflammatory markers and did so in female (C57BL/6) mice, which have been understudied. Chronic HFD feeding resulted in an obese phenotype associated with visceral (22 and 36 weeks) and intra-organ (liver and kidneys; 36 weeks) fat deposition. Peripherally, HFD intake impaired glucose tolerance, with the effect being greater at the later stage (33 weeks). HFD also impaired the sensitivity to insulin (20 weeks on HFD), but this response was not different between low-fat diet (LFD) and HFD at the later time point (33 week), likely due to age-related decreased sensitivity to insulin in the LFD mice. HFD intake for 36 weeks induced hepatic inflammation; hepatic mRNA expression of CD36, a key regulator of lipid metabolism was increased after 22 and 36 weeks of HFD consumption, with the effect being greater at 36 weeks. Behaviorally, 21-week HFD-fed mice did not exhibit locomotor deficits. However, after 32 weeks on HFD, mice were hypoactive which could be due to the excessive weight gain and it may be associated with the peripheral (hepatic) inflammation and dysregulated glucose metabolism we observed after chronic HFD consumption. Emotional (anxiety)/cognitive (short-term recognition memory) functions of mice were unaffected by chronic HFD feeding. Considering our prior findings of short-term (5-6 weeks) HFD-induced peripheral (glucose intolerance/insulin resistance/hepatic inflammation) and central (hyperactivity/anxiety) effects and our current results, it seems that in female mice, some metabolic/inflammatory dysregulations caused by HFD appear early and

persist, whereas others are exaggerated with continuous HFD feeding. In some cases, i.e., insulin sensitivity, HFD's effects are not evident at the later stages due to age-related alterations. Behaviorally, locomotor activity is affected the most with continued HFD feeding. Overall, it appears that in female mice, some of the HFD-induced behavioral and metabolic effects change over time, i.e., locomotor activity, whereas others, i.e., glucose tolerance, fat accumulation appear early and persist and/or are exacerbated with continuous HFD intake.
Key words: High-fat diet, Glucose intolerance, Hepatic inflammation, Hypoactivity.

Introduction

Obesity has reached epidemic proportions globally, affecting people, regardless of sex ethnicity, age, and socioeconomic groups (James et al., 2001), and both genetic and environmental factors contribute to its development (Hebebrand and Hinney, 2009). Among the environmental factors, consumption of energy-dense, high-fat diets (HFD), frequently paired with increasingly sedentary lifestyles, plays a key role in obesity pathogenesis (Swinburn et al., 2004, Jacobs, 2006). Obesity is a chronic and complex problem that has been associated with numerous secondary conditions, such as coronary heart disease, non-alcoholic fatty liver disease, cancer, etc. (Katzmarzyk et al., 2005, Fabbrini et al., 2010, Lumeng and Saltiel, 2011). Importantly, metabolic dysregulation, characterized by impaired glucose tolerance, insulin sensitivity and lipid metabolism is a hallmark feature of obesity that can lead to deleterious conditions, such as metabolic syndrome and diabetes (Lumeng and Saltiel, 2011). While the obesity epidemic is gender-independent and high-fat intake is of a long-term, chronic nature, most experimental studies with HFD have used male rodents and a single dietary exposure duration.

Low-grade chronic inflammation is a key obesity characteristic and it has been shown to contribute to, among others, glucose intolerance and insulin resistance (Komori et al., 2014). Impairments in glucose homeostasis and insulin sensitivity following short- (3-6 weeks) (Cano et al., 2013, Gao et al., 2014) and long-term (3 months) (Hedeskov et al., 1992, Chalkiadaki and Guarente, 2012) HFD consumption have been reported. Studies in male rodents have demonstrated time-dependent effects of continued HFD consumption on systemic glucose and insulin homeostasis (Raddatz et al., 2011, Dou et al., 2014); however, female data are lacking. Besides inducing systemic metabolic dyshomeostasis, HFD feeding leads to inflammatory and

metabolic dysregulations in key peripheral tissues, such as liver and adipose tissues (Kang et al., 2010, Komori et al., 2014). For example, 4 weeks of HFD feeding impaired insulin signaling, evidenced by decreased levels of insulin signaling pathway regulating genes, such as insulin receptor substrate 2 (IRS2), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt2) in the livers of male C57BL/6 mice (Gao et al., 2014). Similarly, hepatic inflammation, evidenced by increased levels of the pro-inflammatory cytokine interleukin-6 (IL-6) with an associated reduction in the levels of peroxisome proliferator-activated receptor alpha (PPARα; a key regulator of lipid metabolism) has been reported in male mice fed a HFD for 20 weeks (Kang et al., 2010). Besides upregulating proliferator-activated receptor gamma (PPARγ; another major lipid metabolic regulator), HFD feeding for 2-4 weeks increases the hepatic mRNA expression of one of its target genes, CD36, with an associated increase in the liver lipid accumulation (Inoue et al., 2005a, Yang et al., 2012). CD36 not only facilitates long-chain fatty acid uptake, but it also promotes macrophage lipid accumulation and inflammatory processes in peripheral tissues and subsequent development of peripheral insulin resistance (Koonen et al., 2007, Cai et al., 2012b). In addition to inflammatory changes in key peripheral tissues (liver, adipose tissue), HFD intake causes gastrointestinal inflammation and compromises the gut epithelial barrier function resulting in increased leakage of inflammatory mediators from the gut into the circulation, culminating in systemic inflammation (de La Serre et al., 2010b, Pendyala et al., 2012). Of note, experimental data regarding the temporal changes in the expression of key regulatory genes involved in glucose homeostasis, lipid metabolism and inflammation, in the face of continued HFD feeding are limited in males and absent in female rodents.

In addition to peripheral (metabolic and inflammatory) effects, HFD-induced neurological deficits have been previously reported. For example, several epidemiological

studies report increased mood disorders, such as anxiety and/or depression in obese individuals (Jorm et al., 2003, Simon et al., 2006). Emotional disturbances have also been demonstrated following short-term (1-5 weeks) (Chudasama and Bhatt, 2009, Kaczmarczyk et al., 2013) and long-term (23 weeks) (Heyward et al., 2012) HFD consumption in rodents. Short-term (3 weeks) HFD intake also leads to motor impairments, characterized by increased locomotor activity in male rodents (Sharma et al., 2012). Of note, there are no experimental data regarding the time-dependent progression in the behavioral (locomotor or emotional) changes in the event of continued HFD consumption.

Earlier we demonstrated that HFD feeding for a relatively short duration (5-6 weeks) leads to increased locomotor activity and anxiety (refer to chapter 4). Peripherally, mice on HFD for 5-6 weeks showed impairments in glucose tolerance and insulin sensitivity and a selective impairment of insulin signaling in the liver with associated increases in mRNA expression of genes related to inflammation and lipid metabolism. In light of the adverse central and peripheral effects observed after short-term HFD intake and considering the lack of a time-course data on the temporal progression of these effects, in this study, we aimed to evaluate the time-dependent changes in the central (behavioral) and peripheral (metabolic/inflammatory) effects in the female C57BL/6 mice at two different time points, an intermediate (20-22 weeks) and a late stage (32-36 weeks) of HFD consumption. To assess the potential neurobehavioral consequences of HFD consumption, we conducted several behavioral tests that could effectively assess the locomotor (open field, grip strength and pole tests), emotional (forced swim and marble burying tests) and cognitive alterations (novel object recognition test). To investigate the HFD-induced metabolic and inflammatory alterations in the periphery, metabolic tests such as glucose tolerance and insulin sensitivity tests (GTT and IST, respectively) were performed. Additionally, qPCR was

conducted to analyze the HFD-induced alterations in the expression of multiple metabolic and inflammatory markers in the liver.

Materials and methods

Animals

Female C57BL/6 mice (6-7 weeks old, Harlan, Indianapolis, IN) were housed (4-5 per cage) in an environmentally controlled room (22–24°C) with food and water available *ad libitum* on a 12h light/dark cycle in an AAALAC accredited facility throughout the study. All procedures were in accord with the latest National Institutes of Health (NIH) guidelines and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Animal treatment

Mice weighing 16.0 ± 0.20 g (mean ± SEM) were randomly divided into two groups (n = 6-8/group/time point) and placed on either a low-fat diet (LFD; 10% kcal from fat, D12450J, Research Diets, Inc., New Brunswick, NJ) or a high-fat diet (HFD; 60% kcal from fat, D12492, Research Diets) for a period of 22 or 36 weeks. Both diets were micronutrients and simple sugar (sucrose) balanced. Glucose tolerance (GTT) and insulin sensitivity (IST) tests were performed after 20 and 33 weeks of HFD treatment. Behavioral tests were carried out after 21 and 32 weeks of HFD consumption. GTT, IST and behavioral tests procedures are described in detail below. Mice were sacrificed at two time points (22 and 36 weeks), body weights were recorded and organs (brain, liver, spleen, kidneys and thymus) were harvested, weighed, and quickly frozen at -80°C. Liver samples were used for qPCR analysis. The experimental design is presented in Fig. 6.1.

Glucose tolerance test (GTT) and insulin sensitivity test (IST)

After 20 (n = 8/group) and 33 (n = 8/group) weeks on respective diets, GTT and IST were performed in succession (3 days apart) on the same animals over a 2-h time window and after a 3 h fast. Glucose (2 g/kg BW; oral) and insulin (0.5 IU/kg; i.p.) both from Sigma (Sigma-Aldrich, St. Louis, MO) were administered and blood glucose levels were determined with a glucometer (TRUEresult®, Nipro Diagnostics, Fort Lauderdale, FL) by serial tail bleeds at various time points (GTT: 0, 15, 30, 60, 90, and 120 min; IST: 0, 15, 30, 60, 75, 90, 105, and 120 min).

Intestinal permeability

Procedure was adopted for mice from (de La Serre et al., 2010b). Following 22 or 36 weeks on their respective diets, mice were fasted for 3 h and administered FITC-labeled dextran (4,000 kDa; Sigma) diluted in sterile saline (1000 mg/kg, 200 mg/ml) via oral gavage. After 1 h, mice were deeply anesthetized with CO_2, blood was collected by cardiac puncture, centrifuged (10,000 rpm for 3 min at 4°C) and plasma FITC-dextran concentration was determined with a spectrophotometer (SpectraMax M3; Molecular Devices, Sunnyvale, CA) at 485/535 nm excitation/emission wavelengths and a standard curve (8.0-0.125 μg/ml).

Behavioral tests

Behavioral tests were performed in succession over 3 days after 21 (n = 8/group) and 32 (n = 8/group) weeks on respective diets. Animals were naïve to the testing ambience at each time point of behavioral testing and all tests were performed by a treatment-blinded experimenter in a specially equipped behavioral testing room separate from the one where the mice were housed.

Open field. Mouse activity was monitored for a period of 30 min in an open field arena (25 cm x 25 cm x 40 cm; Coulbourn Instruments, Whitehall, PA) as described previously

(Krishna et al., 2014). Parameters evaluated using the Limelight software (Actimetrics, Wilmette, IL) included: (1) distance traveled (cm/5 min intervals); (2) time spent in defined regions, namely the center versus periphery of the square arena, analyzed per 5 min intervals; and (3) number of rearings during the first 5 min of open field testing.

Pole test. After 5 min resting period (following the open field test), mice were placed upright on a gauze-wrapped pole (1 × 55 cm; d × h) as detailed in (Lin et al., 2013). A total of four trials were completed with a 3-5 min inter-trial interval. The average times to turn, to descend, and the total time spent on the pole from the four trials were used for statistical analysis. The maximum turning time allowed was 60 s and the total time allowed per trial was 120 s (Krishna et al., 2014).

Grip strength. A strength gauge (Bioseb, France) with attached mouse-specific square wire grid (6 × 6 cm) was used to measure forelimb grip strength (10 min rest after the pole test) as previously described (Lin et al., 2013, Krishna et al., 2014). The average grip force (recorded in newtons [N]) of the four trials was used for statistical analysis.

Novel object recognition (NOR). The NOR was conducted at the beginning of day 2 of behavioral testing with the previous day open field test used as a habituation phase, as described in (Lin et al., 2013). Briefly, during the training phase, mice were placed in the open field arenas in the presence of two identical objects and were allowed to explore for 5 min. After 1 h rest in their home cages, mice were placed back into the arenas with one familiar and one novel object and allowed to explore for 5 min. The number of approaches towards the familiar or the novel object, as well as the time spent exploring the familiar or the novel object, were extracted using the Limelight software and used to determine novelty preference as in (Lin et al., 2013).

Forced swim test (FST). Following the NOR test and a 1.5-h home cage rest period, FST was carried out as detailed in (Lin et al., 2013). Mice were placed in a large beaker filled with 3L of water (29±1 °C) for 15 min. The total times spent swimming, climbing, or immobile was scored using the Limelight video tracking software (Actimetrics).

Marble burying test (MBT). This test was performed on day 3 as described previously, but with some modifications (Gaikwad et al., 2010, Lin et al., 2014). Mice were individually placed in cages containing 4-5 cm-thick pine bedding (American Wood Fibers, Columbia, MD) for 10 min (habituation phase). After a 40-min home cage resting period, mice were reintroduced into the cages which now contained twenty glass marbles (diameter ~10 mm, Panacea Products Corp., Columbus, OH), evenly placed on the bedding arranged in a 4 x 5 matrix for a 10 min testing phase. The number of marbles buried (≥ 70%) was counted based on images collected at times 0 and 10 min of the testing phase (Gaikwad et al., 2010, Lin et al., 2014) .

Real-time quantitative PCR (qPCR)

Total RNA from liver samples (20 mg) was isolated using a GeneJET ™ RNA Purification Kit (Thermo Fisher Scientific, Pittsburgh, PA) and quantified using a Take 3 plate and Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT). RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD) and a Peltier thermal cycler (Bio-Rad, Hercules, CA). Using 3 ng of cDNA per sample (with each sample run in duplicate), expression of genes involved in glucose homeostasis, lipid metabolism, and inflammation were determined by qPCR using mouse-specific primers (Real Time Primers, Elkins park, PA) and SYBR Green-based master mix (Qiagen, Valencia, CA). Amplifications were performed on a Mx3005P qPCR machine (Stratagene) and treatment differences were

calculated as a fold change by the $^{\Delta\Delta}$ Ct method with 18S used as a house-keeping gene (HKG) as described previously (Lin et al., 2013).

Statistical analysis

Two-way ANOVA (duration x treatment) was used initially to analyze the morphometric data, gut permeability and behavioral endpoints (except as specified below). Student's T-test was used to analyze the effect of HFD on these endpoints within a time point. Two-way analysis of variance (ANOVA) was conducted to analyze the open field (treatment x time [interval]) and GTT/IST (treatment x time) data within a time point. If ANOVA's overall main effect was found significant, treatment means were separated by Student-Newman-Keuls *post hoc* test. Prior to statistical analysis, the integrated areas under the curve (AUC) of GTT and IST responses were calculated using the trapezoidal method (Rodriguez-Rivera et al., 2011). These data were subjected to 2 way ANOVA (duration x treatment) analysis prior to post hoc. All results are presented as mean ± SEM and are considered significant at $P \leq 0.05$.

Results

Body weight and organ weights

LFD and HFD groups did not differ with respect to the initial BW (LFD vs. HFD: 16.0 ± 0.13 vs. 16.2 ± 0.11, $P > 0.30$; data not shown). After 22 weeks on respective diets, HFD-fed mice were significantly heavier ($P \leq 0.001$; Fig 6.2). Specifically, HFD feeding for 22 weeks resulted in a 29% greater increase in BW compared to mice fed LFD for 22 weeks. HFD consumption for another 14 weeks, i.e., a total of 36 weeks, resulted in an even greater difference (109%) between the LFD and HFD-fed mice ($P \leq 0.001$; Fig 6.2). Also, the BW of 36-week HFD mice was significantly higher than 22-week HFD mice ($P \leq 0.001$; Fig 6.2). Compared to a

69% weight increase in the LFD-fed mice over the 36 week period, HFD-fed mice's BW increased by 242% at the 36 week time point (Fig. 6.2).

HFD consumption for 22 or 36 weeks did not affect the absolute weights (g) of brain, spleen and thymus ($P > 0.10$; Fig. 6.3A); HFD intake for 36 weeks, but not 22 weeks, significantly increased the absolute weights of liver and kidneys ($P \leq 0.01$; Fig. 6.3A). Moreover, 36-week HFD mice had significantly greater absolute liver and kidney weights than 22-week HFD mice ($P \leq 0.001$; Fig. 6.3A). HFD feeding for 22 or 36 weeks significantly decreased the relative weights (g/kg BW) of brain, liver, kidneys and spleen ($P \leq 0.05$; Fig. 6.3B); relative weight of thymus was only significantly decreased after 36 weeks of HFD feeding ($P \leq 0.05$; Fig. 6.3B).

Glucose tolerance test (GTT)

The fasting (0 min) baseline blood glucose level of HFD mice was significantly ($P \leq 0.001$) greater than LFD after 20 weeks, but not after 33 weeks (Fig. 6.4A and B). Following oral glucose gavage, the blood glucose level of HFD mice after 20 or 33 weeks was significantly higher than LFD mice at the remaining time points (15, 30, 60, 90 and 120 min) recorded ($P \leq 0.01$; Fig. 6.4A and B); HFD-induced hyperglycemia was more prominent after 33 weeks as compared to 20 weeks (Fig. 6.4A and B). Compared to LFD mice at the respective time point, the integrated area under the curve (AUC) for GTT was 43% and 90% greater in mice fed HFD for 20 and 33 weeks, respectively ($P \leq 0.001$; Fig. 6.4C). Furthermore, the GTT AUC of 33-week fed HFD mice was significantly greater than their GTT AUC after 20 weeks ($P \leq 0.001$; Fig. 6.4C).

Insulin sensitivity test (IST)

Mice fed HFD for 20 weeks, but not 33 weeks, showed a significant increase in the baseline blood glucose level ($P \leq 0.001$; Fig. 6.4D and E). Although, both LFD and HFD-fed mice after 20 or 33 weeks showed a similar response to insulin characterized by the decreased blood glucose level at 15 and 30 min post insulin challenge (Fig. 6.4D and E), the blood glucose level of HFD mice at these time points remained significantly ($P \leq 0.01$) greater than LFD mice after 20 weeks, and not after 33 weeks (Fig. 6.4D and E). Starting at 60 min post insulin challenge, although both LFD and HFD fed mice after 20 weeks showed a rebound in the blood glucose level, which was almost normalized by 2 h, the blood glucose level of HFD mice remained consistently higher than the LFD group ($P \leq 0.05$; Fig. 6.4D). Similar to 20 weeks, we observed a rebound in the blood glucose level in both LFD and HFD groups beginning 60 min post insulin injection. However, unlike 20 weeks, the blood glucose level of LFD and HFD fed mice did not significantly differ at any of the time points recorded from 60 min to 120 min post insulin challenge ($P > 0.10$; Fig. 6.4E). Compared to LFD mice at the respective time point, the integrated area under the curve (AUC) for IST was significantly higher in the HFD group after 20 weeks (26%, $P \leq 0.001$; Fig. 6.4F), but not after 33 weeks ($P > 0.05$; Fig. 6.4F).

Intestinal permeability

Compared to LFD-fed mice, the plasma concentration of FITC-dextran was significantly increased after 22 (2.1-fold) and 36 (2.3-fold) weeks of HFD consumption ($P \leq 0.05$; Fig. 6.5), suggesting HFD-induced increased gastrointestinal permeability.

qPCR

Compared to the LFD-fed mice at each time point, CD36 mRNA expression was significantly upregulated in the liver after 22 (2.7-fold) and 36 (4.7-fold) weeks of HFD

consumption ($P \leq 0.01$; Fig. 6.6). Hepatic mRNA levels of the pro-inflammatory cytokines IL-6 and tumor-necrosis factor (TNFα), as well as the acute-phase protein haptoglobin (Hp) were unaffected by 22 week HFD ($P \geq 0.20$; Fig. 6.6). However, 36 weeks of HFD feeding resulted in non-significant trend towards decreased IL-6 mRNA levels ($P > 0.15$; Fig. 6.6) and significant upregulation of Hp levels ($P \leq 0.01$; Fig. 6.6); TNFα expression remained unaffected. HFD intake for 36 weeks, but not 22 weeks, resulted in significantly increased hepatic expression of PPARα (1.4-fold, $P \leq 0.05$; Fig. 6.6) and PPARγ (3.4-fold, $P \leq 0.01$; Fig. 6.6). Hepatic mRNA expression of IRS1 and GLUT4 was unaffected by HFD at both 22 and 36 week time points ($P > 0.35$; Fig. 6.6).

Behavior

Open field. The locomotor activity of mice fed HFD for 21 weeks did not differ from that of the LFD-fed mice ($P > 0.50$; Fig. 6.7A). As expected, both LFD and HFD-fed mice habituated to the arenas over time and their overall activity decreased (distance traveled: 4322.77 ± 212.76 vs. 4761.72 ± 375.96 cm in first 5 min and 3455.85 ± 173.62 vs. 3604.58 ± 362.26 cm in last 5 min of LFD and HFD groups, respectively).Vertical activity, measured by the number of rearings during the first 5 min of open field exploration was also unaffected after 21 weeks of HFD feeding ($P > 0.35$; data not shown). Similarly, the mean time spent per 5 min interval in the center and periphery was unchanged after 21 weeks of HD treatment ($P > 0.40$; Fig. 6.7B).

After 32 weeks of HFD intake, two-way ANOVA demonstrated overall significant main effects of diet ($P \leq 0.001$) and interval (5 min time period; $P \leq 0.05$), but without any significant interaction between the two ($P = 0.42$) with respect to the distance traveled in the open field arena; the locomotor activity of HFD-fed mice was significantly lower as illustrated by the decreased distance traveled per 5 min interval ($P \leq 0.001$; Fig. 6.7A). As expected, both LFD

and HFD-fed mice habituated to the arenas over time, but HFD-fed mice were hypoactive throughout the 30-min testing. For example, their locomotor activity during the 6th (LFD vs. HFD: 3882.11 ± 254.68 vs. 2919.32 ± 397.74 cm) 5-min interval compared to their activity during the 1st interval (first 5 min) of open field testing (LFD vs. HFD: 5237.61 ± 289.47 vs. 3584.66 ± 233.79 cm) was significantly lower. HFD-fed mice also showed a significant decrease in rearing (LFD vs. HFD: 21.6 ± 2.76 vs. 13.14 ± 1.35; $P < 0.05$). Additionally, the mean time spent in the center (LFD vs. HFD: 76.14 ± 5.01 vs. 38.28 ± 4.84 s per 5 min; Fig. 6.7B) and periphery (LFD vs. HFD: 223.76 ± 5.01 vs. 261.62 ± 4.84 s per 5 min; data not shown) was significantly ($P \leq 0.001$) decreased and increased, respectively, by HFD.

Pole test. HFD intake for 21 or 32 weeks produced robust, significant (3.1and 2.1-folds, respectively) increase in the mean turn time during the pole test ($P \leq 0.01$; Fig. 6.7C). The other two pole test parameters, time to descend and total time were not affected by HFD at either 21 or 32 weeks ($P > 0.15$; data not shown).

Grip strength. HFD intake for 21 weeks significantly increased the average forelimb grip strength ($P \leq 0.01$; Fig. 6.7D). However, after 32 weeks of HFD consumption, the mean grip strength was not different from that of mice fed LFD or HFD for 32 weeks ($P > 0.90$; Fig. 6.7D).

NOR. Mice exhibited normal NOR performance after 21 or 32 weeks of HFD consumption evidenced by the significantly increased preference of both LFD and HFD-fed mice towards the novel object in terms of time ($P \leq 0.05$; Fig. 6.8A) or approaches ($P \leq 0.05$; data not shown).

FST. Mice fed HFD for 21 or 32 weeks did not show any significant difference with respect to the swimming time compared to mice fed LFD ($P > 0.35$; Fig. 6.8B); the total time

spent climbing and immobile was also unaffected after either 21 or 32 weeks on HFD ($P > 0.10$; data not shown).

MBT. Compared to LFD-fed mice, 21 weeks on HFD did not result in a significant difference ($P > 0.30$) with respect to the number of buried marbles (Fig. 6.8C). By 32 weeks, mice from both LFD and HFD groups buried less marbles ($P \leq 0.01$; Fig. 6.8C). However, the HFD-fed mice performed much worse than the LFD-fed controls and they buried significantly fewer marbles ($P \leq 0.01$; Fig. 6.8C).

Discussion

Mounting epidemiological evidence has associated increased high-fat consumption and obesity with the development of metabolic syndrome and with behavioral abnormalities (Zivkovic et al., 2007, Petry et al., 2008, Gadalla, 2009). Despite the progressive and chronic nature of high dietary fat intake among humans, most of the experimental studies have evaluated the adverse effects of HFD consumption after a single time point and have done so in females. In this study, which is a continuum of our previous work which investigated the central (behavioral; refer chapter 4) and peripheral (metabolic and inflammatory; refer chapter 5) effects of relatively short-term (5-6 weeks) HFD consumption in female mice, we sought to determine the progression of behavioral, metabolic and inflammatory effects induced by chronic HFD consumption. The main findings from this work include: (i) long-term HFD consumption resulted in an obese phenotype associated with visceral (22 and 36 weeks) and intra-organ (liver and kidneys; 36 weeks) fat deposition; (iii) HFD consumption for 20 weeks impaired glucose tolerance and was greatest after 33 weeks; (iv) HFD consumption for 20 weeks induced insulin resistance, an effect not apparent after 33 weeks, likely due to the age-related alterations in insulin sensitivity in the LFD-fed counterparts; (v) HFD feeding for 36 weeks induced hepatic

inflammation; hepatic mRNA expression of CD36, a key regulator of lipid metabolism was upregulated after 22 and 36 weeks of HFD consumption, with the effect being greater at 36 weeks; (vi) while the locomotor activity was unaffected by HFD intake for 21 weeks, mice were hypoactive after 32 weeks of HFD intake.

HFD-induced accumulation of visceral fat and subsequent increase in body weight has been demonstrated by multiple animal studies (Murase et al., 2001, Chung et al., 2012). Although, in the female, HFD's effect on body weight was apparent as early as 6 weeks in our previous work, it became more pronounced with continued HFD consumption in this study. Chronic HFD consumption for 22 weeks increased intra-abdominal fat accumulation, as evidenced by the decrease in the relative organ weights. Prolonged HFD consumption for another 12 weeks resulted in an advanced obese phenotype characterized by exaggerated weight gain compared to the LFD-fed counterparts. Given the increased absolute liver and kidney weights along with an overall decrease in the relative organ weights at 36 weeks, it could be inferred that the exaggerated body weight gain is mainly via intra-abdominal and intra-organ fat deposition. Increased visceral fat accumulation was observed in female, but not male mice fed HFD for a chronic period, suggestive of the sex-specific differences in the visceral adiposity upon exposure to HFD (Yasmeen et al., 2013). Interestingly, similar sex-specific pattern of fat distribution has been observed in humans with obese females showing excess visceral deposition as compared to obese males (Yasmeen et al., 2013).

A close association between obesity and inflammation and inflammation in insulin-sensitive tissues, such as liver and adipose tissue is implicated in the development of obesity-induced insulin resistance (Zeyda and Stulnig, 2009). Obesity can also alter microflora composition and can compromise the integrity of the intestinal epithelial barrier, leading to an

increased passage of the bacterial components, such as lipopolysaccharide (LPS) from the gastrointestinal tract into systemic circulation (Cani et al., 2007, de La Serre et al., 2010b). Increased circulating LPS, through series of signaling events, can lead to insulin resistance (Jager et al., 2007, Lumeng et al., 2007b). In this study, prolonged HFD consumption compromised the gut permeability as evidenced by the increased plasma concentration of FITC-dextran after 22 and 36 weeks of HFD intake. Hence it is conceivable that the increased gut permeability might have facilitated the passage of LPS from the gut in to circulation, which can further trigger inflammation-related changes in the peripheral tissues, resulting in diminished glucose uptake and insulin resistance.

Similarly, the impaired glucose tolerance observed after 6 weeks of HFD feeding in our prior study persisted with continued HFD consumption and was greater after 33 weeks of HFD feeding in this study. Hence it is apparent that the impaired glucose homeostasis which appears even within few weeks of HFD feeding, persists, and it is exacerbated with continued HFD consumption. This is despite an apparent lack of difference between HFD and LFD's basal glucose levels (33 weeks), which is likely due to age related alterations (Escriva et al., 2007). Similarly, the insulin resistance induced by 6-week HFD feeding was evident even after 20 weeks of HFD consumption. However, surprisingly, by 33 weeks, HFD-fed mice did not show a significant difference in insulin sensitivity compared to the age-matched LFD-fed counterparts. Although not significant, the downregulation of IRS1 and GLUT4 observed after chronic HFD intake in the present study is suggestive of altered insulin signaling and decreased glucose uptake. Hence, the noticeable lack of insulin resistance after 33 weeks of HFD feeding could be due to the age-related, diet-independent loss of insulin sensitivity (Escriva et al., 2007) and may not be the accurate reflection of the effect of prolonged HFD consumption per se. It is

noteworthy that in our previous study, 6-week LFD-fed mice showed normal response to the glucose challenge (GTT) and were highly insulin sensitive, evidenced by their consistently lower blood glucose levels after the initial insulin challenge during IST. However, by 33 weeks, we found that the LFD-fed mice were relatively insulin insensitive, but still exhibited normal response to the glucose challenge, which is indicative of a non-insulin mediated uptake of glucose from the circulation (Baron et al., 1988). Administration of hormones, such as leptin or fibroblast growth factor 19 (FGF-19) into the brain ventricles restored glucose tolerance in insulin or leptin deficient diabetic rodent models; the ant-diabetic effect of these hormones were attributed to an increased glucose uptake in tissues such as skeletal muscle, heart and brown adipose tissue (Schwartz et al., 2013). In this regard, the normal glucose tolerance (in the absence of insulin sensitivity) exhibited by LFD mice at the later stage (33 weeks) could be likely due to a compensatory increase in hormones that are implicated in glucose disposal, such as leptin. Overall, our data is suggestive of an age-dependent differential mechanism in the regulation of peripheral glucose uptake between the early and later time points of the experimental period.

In order to assess whether the observed HFD-induced alterations in glucose homeostasis were associated with peripheral tissue inflammation, we evaluated the chronic HFD-induced inflammatory responses in the liver. Our previous study demonstrated increased inflammation and impaired insulin signaling in the hepatic tissue after 6 weeks of HFD intake. In this study, 22 weeks of HFD feeding accelerated macrophage infiltration in the liver as evidenced by the significantly increased CD36 expression. However, compared to 6 week, liver was relatively less vulnerable to the inflammatory/lipid profile alterations (evidenced by unaltered mRNA levels of pro-inflammatory cytokines, TNFα, IL-6 and the acute phase protein, Hp) after the intermediate

stage (22 weeks) of HFD feeding, suggesting the development of transient hepatic tolerance during different stages of HFD intake. Increased CD36 expression has been attributed to the development of insulin resistance in humans with type 2 diabetes as well as in animals fed HFD (Luiken et al., 2001, Koonen et al., 2007, Sheedfar et al., 2014). Interestingly, CD36 knockout mice displayed greater insulin tolerance and were protected from diet-induced obesity and adipose tissue inflammation (Cai et al., 2012b). Hence the significantly impaired glucose tolerance and insulin resistance observed after 22 weeks in this study could be attributed to the HFD-induced increase in hepatic CD36 mRNA expression.

Interestingly, continued HFD consumption for another 14 weeks (i.e. a total of 36 weeks) exacerbated alteration in the hepatic inflammatory profile evidenced by the increased CD36 and Hp mRNA levels and a non-significant trend towards a decrease in the hepatic IL-6 mRNA expression. Hepatic lipid metabolism was also altered, evidenced by the significantly increased PPARα and PPARγ mRNA levels. PPARs have been demonstrated to act on LPS/TLR4 signaling pathway and exert anti-inflammatory effects by preventing the nuclear translocation and activation of NFκB and subsequent production of pro-inflammatory cytokines (Wahli and Michalik, 2012). Given the increased gut permeability after 36 weeks of HFD intake, it could be presumed that the increased circulating LPS might have triggered the expression of PPARs to curb the overactivation of TLR4 pathway and subsequent production of pro-inflammatory cytokines. Hence the absence of altered TNFα levels and decreased hepatic IL-6 mRNA expression observed after 36 weeks of HFD feeding could be an end result of the HFD-induced upregulation of PPARα and PPARγ levels. CD36 plays an important role in the uptake of long-chain fatty acids (Wang et al., 2014); markedly enhanced hepatic CD36 expression accompanied with a significant increase in the absolute liver weight observed after 36 weeks of chronic HFD

consumption is indicative of delayed intra-hepatic fat accumulation. Besides regulating lipid metabolism, CD36 acts as an inducer of inflammation by interacting with TLR4 and subsequently activating the downstream inflammatory signaling pathways (Cai et al., 2012b). Hence, the increased expression of the acute phase protein, Hp, even in the absence of an increase in TNFα and IL-6 levels after the late stage of HFD intake is indicative of an ongoing chronic low-grade inflammation, which may have been mediated by the exaggerated hepatic CD36 mRNA expression.

Besides peripheral effects, previous studies have investigated the adverse neurobehavioral effects of HFD feeding. In our earlier study with female mice, we demonstrated that 6 weeks of HFD intake causes increased locomotor activity. However, here, we found that the HFD's effect on locomotor activity was no longer evident by 21 weeks which is in line with a previous report showing absence of locomotor deficits in mice fed HFD for similar duration (Heyward et al., 2012). Interestingly, mice with advanced obese phenotype caused by continued HFD consumption for another 11 weeks (total 32 weeks) displayed locomotor deficits, characterized by decreased locomotor activity in the open field test. Taken together, our previous and current findings demonstrate a biphasic effect of HFD consumption on locomotor activity in the female with the initial increased locomotor activity dissipating over time and further following an opposite trend (decreased locomotion) with continued HFD consumption, likely due to an advanced obese phenotype.

Previously we also found that 5 weeks of HFD feeding to female mice resulted in a trend towards increased grip strength. The significant increase in forelimb grip strength after the intermediate stage (21 weeks) of HFD feeding observed here is indicative of an increased skeletal muscle mass and muscle strength in addition to the increase in HFD-induced body fat

mass. This phenomenon has been observed in humans wherein overweight individuals exert greater absolute muscle strength and power compared to the lean counterparts (Hulens et al., 2001, Hulens et al., 2002). However, given the unaltered grip strength after the late stage (32 weeks) of HFD intake, it seems that, after a certain time point, the increase in body fat content does not translate into further increase of muscle mass and strength, which has also been observed in humans (Jackson et al., 2010, Stenholm et al., 2014).

Several studies with male rodents have demonstrated emotional disturbances, characterized by increased anxiety and/or depression following HFD consumption (Heyward et al., 2012, Sharma et al., 2012). Our prior work showed elevated anxiety in female mice fed a HFD for 5-6 weeks (chapter 4). In this study, we found that prolonged HFD consumption for 21 weeks did not induce anxiety-like behavior in the open field, FST or MBT tests. By 32 weeks, HFD-fed mice spent decreased time in the center and increased time in the corner of the open field arena, which is typically indicative of an increased anxiety level. However, given the decreased locomotor activity in the open field test and the lack of behavioral abnormalities in the FST, it could be inferred that the increased corner time could be merely a result of the overall decreased locomotion and is not necessarily a consequence of the altered anxiety level (Hiroi and Neumaier, 2009). Compared to 21 weeks, 32-week LFD or HFD-fed mice performed poorly in the MBT, which is suggestive of an age-related decline in mice's marble burying behavior (Deacon, 2006). Meanwhile, the drastic reduction in the number of marbles buried by 32-week HFD-fed mice as compared to the age-matched LFD controls could be attributed to their overall hypoactivity at that time point. Collectively, our previous 6-week data combined with the present data suggest that the increased anxiety phenotype may be associated with relatively early stages

in the development of diet-induced obesity, whereas in advanced obese female phenotypes, hypoactivity dominates.

Previously, we also found that 5 weeks of HFD feeding did not affect the short-term non-spatial object recognition memory (chapter 4). Here, we found that mice displayed intact short-term NOR memory even after 21 and 32 weeks of HFD feeding, which suggests that HFD consumption by female mice, regardless of the duration does not affect their short-term non-spatial memory. These findings are consistent with earlier studies in male rodents that demonstrated intact recognition memory after short- or long-term HFD feeding (Jurdak and Kanarek, 2009, Heyward et al., 2012, Tucker et al., 2012). However the decreased NOR performance observed in middle aged (9-month) male mice fed HFD for a chronic period (4 months) (Carey et al., 2014a) indicates that the HFD's effect on nonspatial recognition memory might have a different outcome in older animals.

Chronic low-grade central/peripheral inflammation is widely implicated in the development of neurodegenerative disorders/metabolic dysfunctions (Hotamisligil, 2006, Amor et al., 2010, Ferrari and Tarelli, 2011). In this study, prolonged (36 weeks) HFD consumption resulted in an advanced obese phenotype in female mice with associated increase in hepatic inflammation (increased CD36 and Hp levels) and subsequent development of metabolic dysfunction, such as glucose intolerance. Interestingly, metabolic dysregulation including impaired glucose metabolism and abnormal appetite regulation have been demonstrated to be comorbid with neurodegenerative disorders (Cai et al., 2012a, Dunn et al., 2014). Restoration of metabolic homeostasis has been demonstrated to improve motor deficits in neurodegenerative diseases (Cai et al., 2012a). In this regard, the neurological deficits characterized by decreased locomotor activity observed in 32-week HFD fed obese female mice in this study could be

attributed to the chronic HFD-induced peripheral (hepatic) inflammation and dysregulated glucose metabolism.

In summary, it appears that in female mice, some of the HFD-induced behavioral and metabolic effects change over time, i.e., locomotor activity, whereas others, i.e., glucose tolerance, fat accumulation appear early and persist and/or are exaggerated with continuous HFD feeding. Collectively, our findings imply that in females, constant intake of high-fat products over a chronic period leads to an exaggerated obese state, which is strongly associated with an increased peripheral inflammatory tone and dysregulation in the metabolic functions, and less robustly related to mood disorders/cognitive changes.

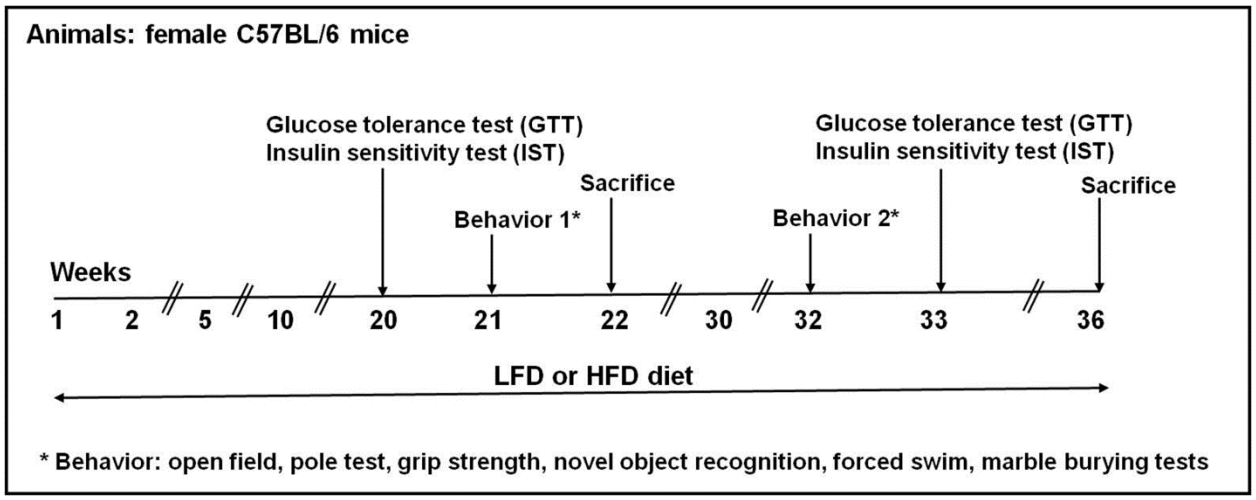

Figure 6.1. Detailed outline of the study's experimental design.

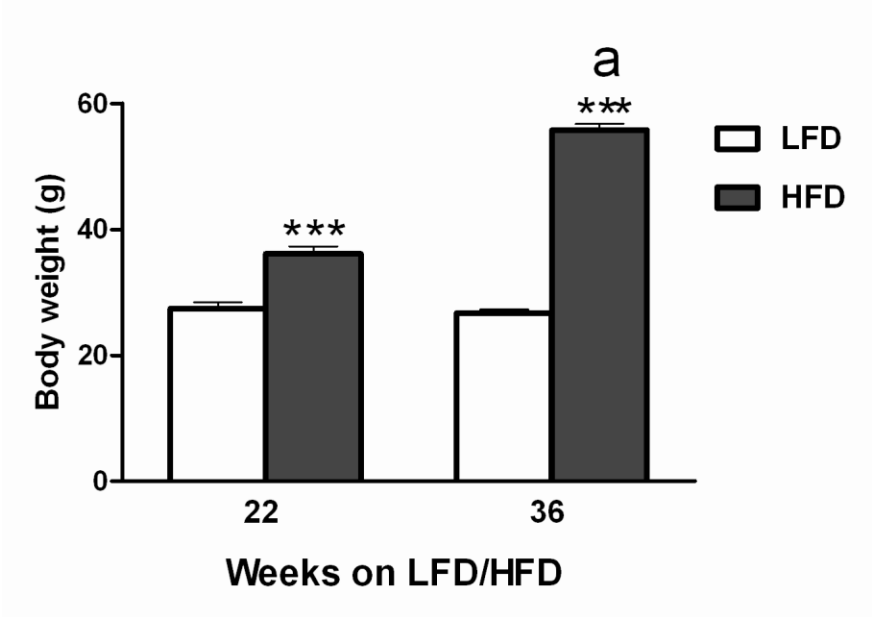

Figure 6.2. Effect of LFD or HFD consumption on mean body weights of female C57BL/6 after 22 (n = 8 per group) or 36 (n = 8 per group) weeks on respective diets. Body weights are expressed as mean ± SEM. *** $P \leq 0.001$ indicates significant difference from LFD within a time point. “a” indicates significant difference between time points within treatment ($P \leq 0.001$).

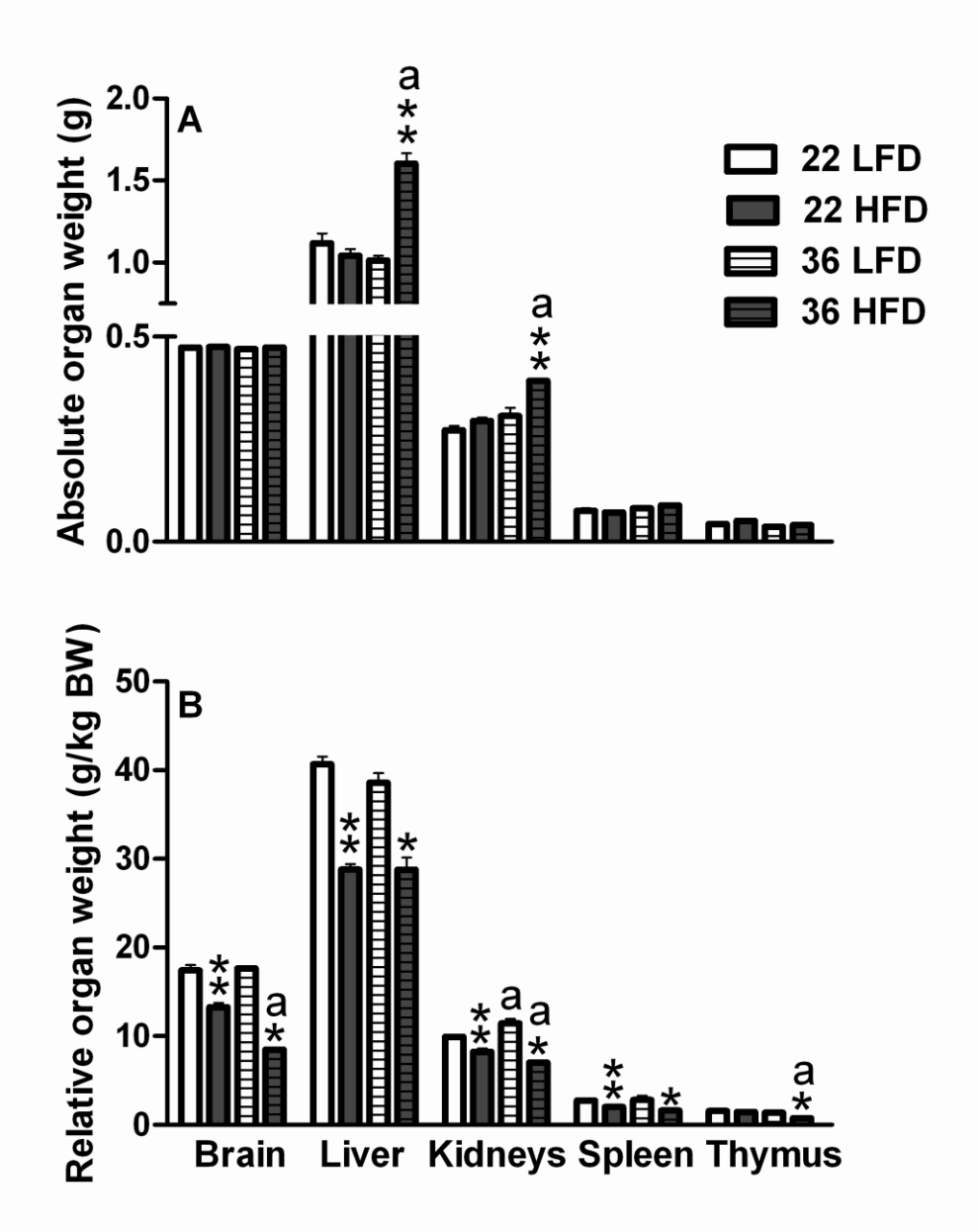

Figure 6.3. Absolute (g; A) and relative (g/kg BW; B) organ weights of female C57BL/6 mice fed either a LFD or HFD for 22 or 36 weeks. Graphical representations are mean ± SEM (n = 8 per group). * $P \leq 0.05$, ** $P \leq 0.01$ indicates significant difference from LFD within a time point. “a” indicates significant difference between time points within treatment ($P \leq 0.05$).

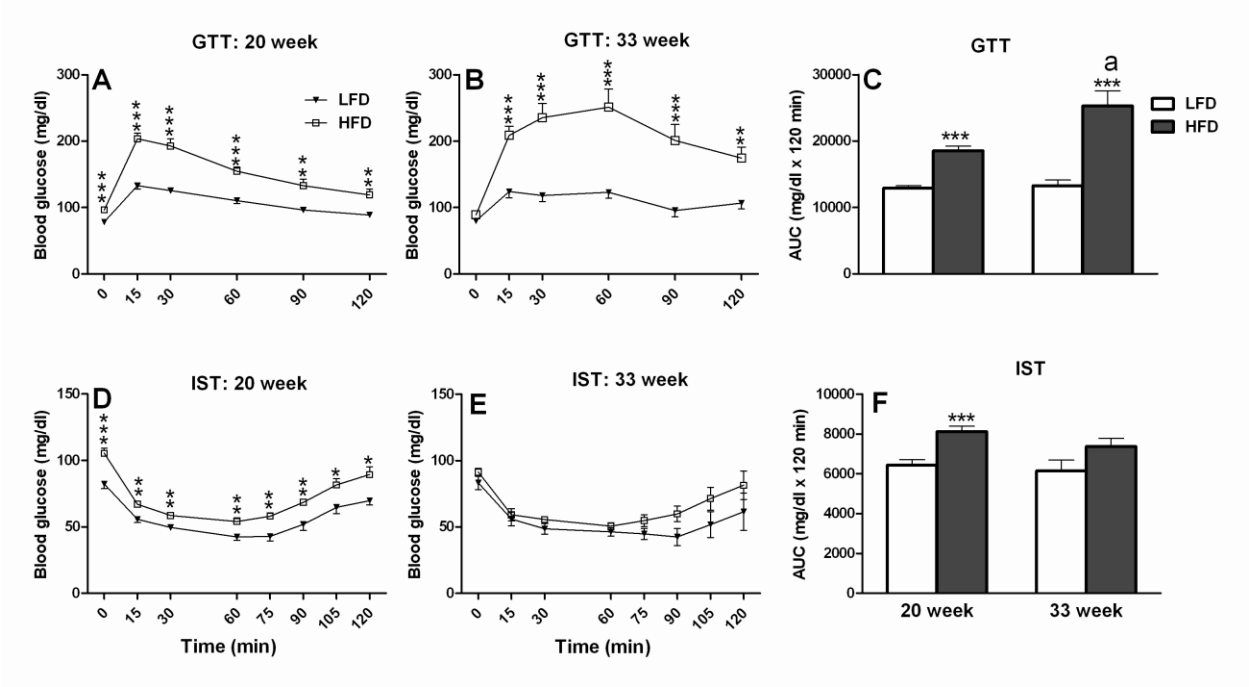

Figure 6.4. Glucose and insulin sensitivity tests (GTT; 2 g/kg BW, oral; A and B) and (IST; 0.5 IU, i.p.: D and E) of female C57BL/6 mice fed either a LFD or HFD diet for 22 (A, D), or 36 (B, E) weeks. Integrated area under the curve (AUC) of GTT and IST responses were also calculated and are presented in Fig. 3C and Fig. 3F, respectively. Graphical representations are mean ± SEM (n = 8 per group). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ represent effects within a time point (min, A, B, D and E; weeks C, F). Note: for simplicity, only the differences between LFD and HFD groups within a time point are represented in A, B, D and E panels. “a” indicates significant difference between time points within treatment ($P \leq 0.001$).

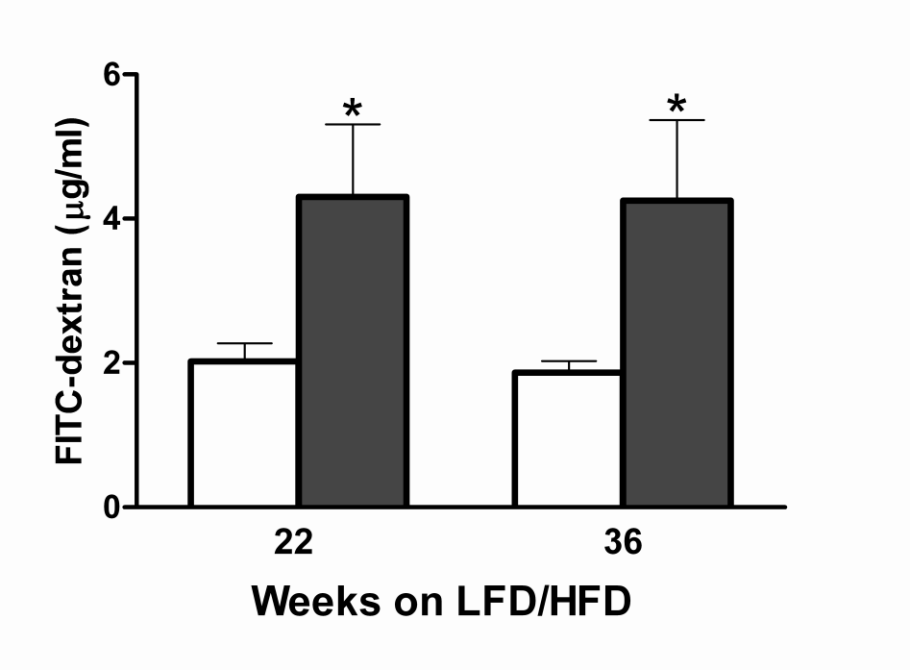

Figure 6.5. Effect of LFD or HFD consumption on gastrointestinal permeability (plasma FITC-dextran levels 1 h post oral gavage with FITC-labeled dextran) of female C57BL/6 mice after 22 (n = 8 per group) or 36 (n = 8 per group) weeks on respective diets. Graphical representations are mean ± SEM (n = 8 per group). * $P \leq 0.05$ represents treatment effect within feeding duration.

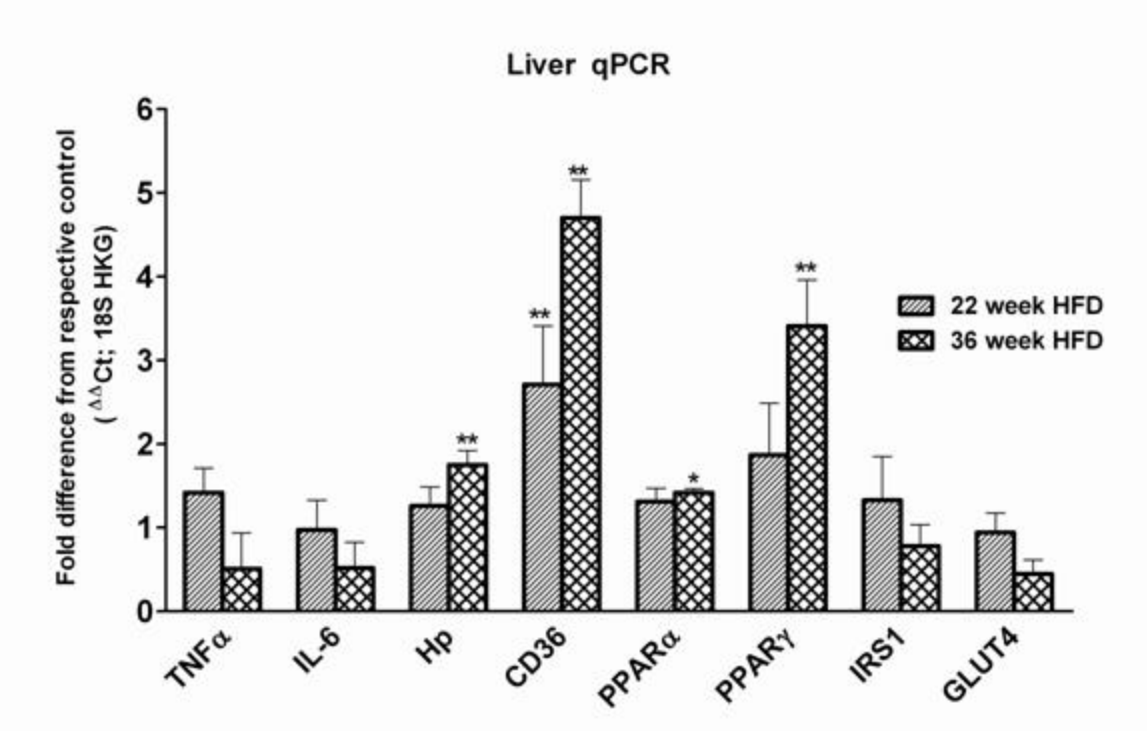

Figure 6.6. Effect of 22 or 36 weeks of HFD consumption on mRNA levels of tumor-necrosis factor alpha (TNFα), interleukin 6 (IL-6), haptoglobin (Hp), CD36, peroxisome proliferator-activated receptors-alpha and gamma (PPARα and PPARγ, respectively), insulin receptor substrate 1 (IRS1) and glucose transporter type 4 (GLUT4) in the liver of female mice. The house keeping gene (HKG), 18S was used to normalize the mRNA data and are presented as fold change relative to respective LFD group at each time point. Graphical representations are means ± SEM (n = 6-8 per group). * $P \leq 0.05$, ** $P \leq 0.01$ indicates treatment effect within feeding duration.

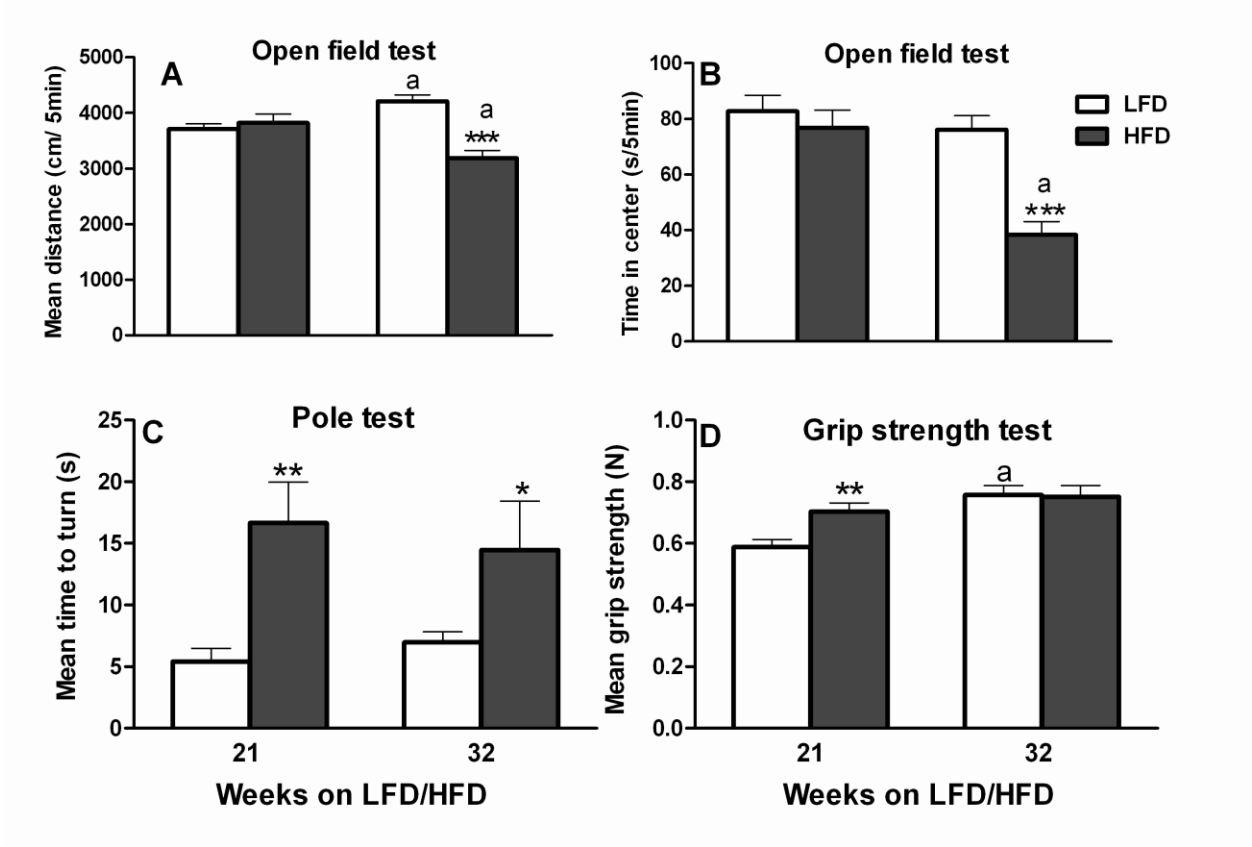

Figure 6.7. Effect of 21 or 32 weeks of HFD consumption on: (A) distance traveled (per 5 min interval) during open field testing; (B) time spent per 5 min interval in the center of the open field arena; and (C) average time to turn during the pole test; (D) mean forelimb grip strength. Graphical representations are mean ± SEM (n = 8 per group). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ indicates significant difference from LFD within a time point. “a” indicates significant difference between time points within treatment ($P \leq 0.05$).

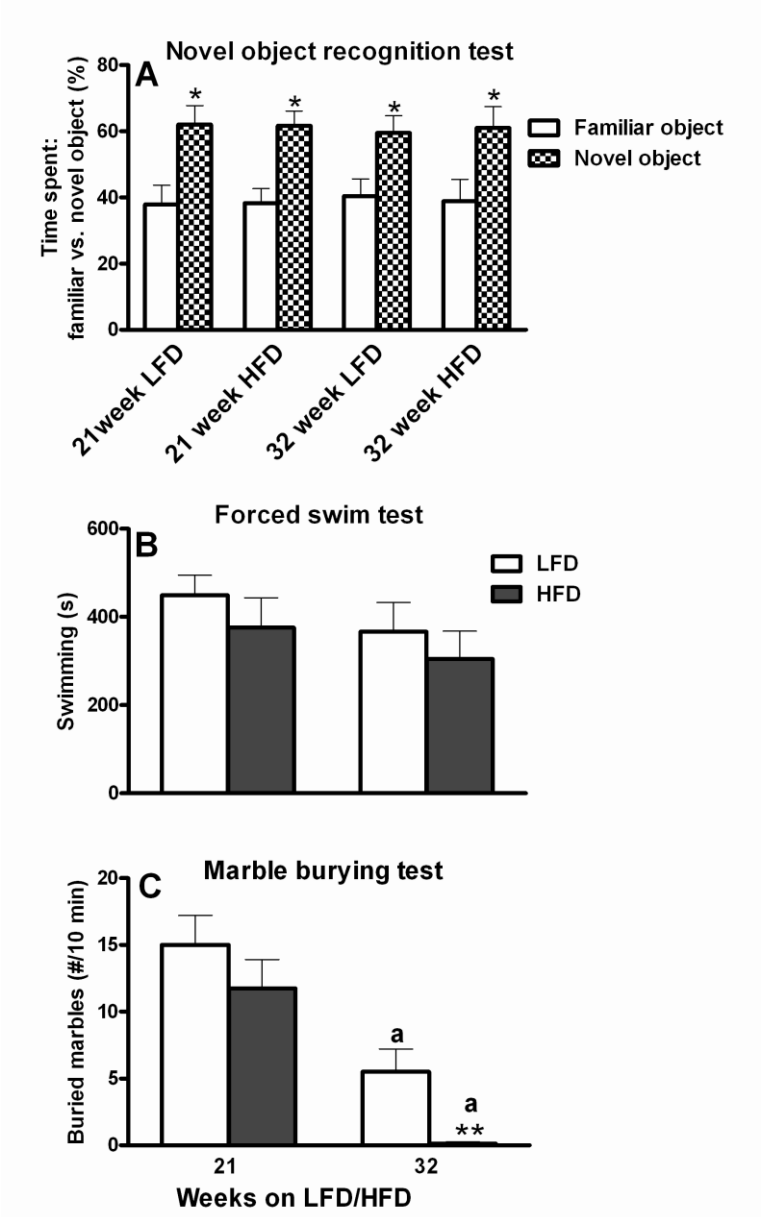

Figure 6.8. Effect of 21 or 32 weeks of HFD consumption on: (A) time spent with a familiar vs. a novel object (%) in a novel object recognition test; (B) total time spent swimming in a forced swim test; and (C) number of marbles buried (≥ 70%) during a marble burying test. Graphical representations are mean ± SEM (n = 8 per group). * $P \leq 0.05$ and ** $P \leq 0.01$ indicates significant difference from LFD within a time point. "a" indicates significant difference between time points within treatment ($P \leq 0.001$).

CHAPTER 7

NEUROLOGICAL DEFICITS IN ADOLESCENT OFFSPRING OF MOUSE DAMS FED A HIGH-FAT DIET: INTERVENTION BY MATERNAL ADMINISTRATION OF A NOVEL ANTI-INFLAMMATORY GLYCAN[1]

[1]Krishna, S., Lin, Z., Harn, D.A., Wagner, J.J., de La Serre, C.B., Filipov, N.M. To be submitted to *European Journal of Neuroscience.*

Abstract

Obesity is driven in large part by chronic ingestion of energy dense high-fat diets (HFD). Excessive weight gain before and/or during pregnancy leads to inflammatory changes that can harm the fetus. Maternal inflammation induced behavioral alterations in the offspring reminiscent of autism spectrum disorder (ASD) have been reported in rodents and children born to obese mothers have an increased ASD risk. Considering the paucity of experimental data on the role of diet-induced maternal obesity and ensuing maternal inflammation in the development of ASD or other behavioral abnormalities in the offspring as well as the lack of successful anti-inflammatory therapeutic interventions targeting the maternal environment, we conducted this study. Our objective was to determine whether maternal HFD consumption will produce ASD-like and/or other aberrant behaviors in adolescent offspring (postnatal day [PND] 35) and whether maternal administration of a potent anti-inflammatory glycan conjugate will ameliorate the adverse effects of the maternal HFD intake on the offspring. Female mice were fed either a low-fat diet (LFD) or HFD for 6 weeks before mating and throughout pregnancy and lactation. Beginning prior to mating and until weaning, subgroup of HFD-fed females were also given glycan-dextran conjugate. After weaning (PND21), offspring were gender separated and fed standard chow. Beginning on PND35, mice underwent multiple behavioral tests; one day after the last test, mice were sacrificed and brain regional neurochemical (monoamine) analysis was performed. Behaviorally, female offspring of glycan treated HFD-fed dams were more active in the open field test. Maternal HFD consumption increased anxiety (increased corner time in open field arena), and increased swimming time in a forced swim test; the former but not the latter being reversed by maternal glycan administration in female offspring. Maternal glycan treatment also produced a gender-independent enhancement of the short-term object recognition memory

in a novel object recognition test. Neurochemically, female PND35 offspring of HFD-fed dams showed a numerical increase in homovanillic acid (HVA; dopamine metabolite), indicative of increased DA utilization, in the prefrontal cortex (PFC) and ventral hippocampus (vHIP). Maternal glycan treatment counterbalanced this increase in DA utilization by increasing and decreasing the DA and HVA levels, respectively. In the vHIP, PND35 offspring of HFD-fed dams had increased serotonin and its major metabolite, which were further increased by maternal glycan administration. Overall, it appears that increased HFD consumption before/during pregnancy can induce sex-specific behavioral abnormalities, some of ASD-like nature and that intervention by maternal administration of a novel anti-inflammatory glycan can effectively reverse some of these behavioral abnormalities in part by regulating PFC and vHIP monoamine neurochemistry.

Key words: Maternal high-fat diet, Maternal inflammation, Glycan, ASD, anxiety

Introduction

The worldwide prevalence of overweight/obesity has risen dramatically over the past few decades and is a serious global public health issue at present (Leddy et al., 2008). Increased consumption of high-fat diet (HFD) and increasingly widespread sedentary life styles are considered major contributors to the obesity epidemic (Swinburn et al., 2004). Besides rising obesity rates in the general population, the prevalence of maternal obesity is on the rise. In the US, about 34% of women of child bearing age (20-44 years) is classified as obese (Krakowiak et al., 2012). In the UK, 33% of women are categorized as overweight and 23% are obese, accounting for a total of 56% over the recommended body mass index (BMI) (Bhattacharya et al., 2007).

Obesity during pregnancy is associated with long-term adverse health outcomes to children. For example, several epidemiological studies have reported increased risks for childhood and adulthood obesity among offspring of obese mothers (Oken et al., 2007, Ludwig and Currie, 2010, Janjua et al., 2012). Also, higher weight gain during pregnancy has been linked to an increased childhood and adulthood adiposity among offspring (Rooney and Ozanne, 2011). Moreover, obesity during pregnancy is associated with increased birth weight (Jones et al., 2009), which has been linked to an increased offspring risk of high BMI later in life (Boney et al., 2005a, Catalano and Ehrenberg, 2006).

Several lines of evidence suggest that the influence of maternal obesity on the offspring most likely starts in utero and can have long-term impact, increasing the risk of obesity and associated metabolic and/or behavioral abnormalities later in their life (Cottrell and Ozanne, 2008, O'Reilly and Reynolds, 2013). Maternal inflammation is among the several potential factors that can underlie the programming effects of maternal obesity (O'Reilly and Reynolds,

2013). For example, immune system activation and subsequent inflammation has been linked to an increased incidence of complex neurodevelopmental disorders, such as autism spectrum disorder (ASD), in the offspring of mothers who suffer infections during pregnancy (Patterson, 2002, Parker-Athill and Tan, 2010). Maternal inflammation and associated manifestation of ASD-like behavioral phenotype in the offspring has also been demonstrated in several rodent studies (Smith et al., 2007, Wolff and Bilkey, 2008). Besides ASD-like neuropathology, maternal inflammation may also lead to other neurological disorders, such as ADHD and schizophrenia (Mann and McDermott, 2011, Canetta et al., 2014).

HFD intake induces systemic inflammation in rodents (de La Serre et al., 2010b, Kim et al., 2012b, Pendyala et al., 2012) and obesity in pregnant women leads to inflammatory changes and immune alterations that can adversely affect the fetus (Challier et al., 2008). Interestingly, recent epidemiological studies demonstrated that pre-pregnancy obesity and excessive weight gain during pregnancy can increase the risk of ASD incidence in the offspring (Stein et al., 2006, Dodds et al., 2011, Krakowiak et al., 2012). Despite this epidemiological evidence, limited experimental studies have determined the role of maternal obesity in ASD or other neurodevelopmental disorders. More importantly, considering the significant contribution of HFD consumption in promoting obesity, it is noteworthy that animal studies on the specific role of diet-induced maternal obesity and ensuing maternal inflammation as a causal factor for ASD are non-existent.

Several treatment strategies, mostly symptomatic, have been adopted for neurodevelopmental disorders in general. For example, in the case of ASD, although the current medications used (two antipsychotics, namely, aripiprazole and risperidone) can alleviate the hyperactivity and irritability symptoms in autistic patients (McPheeters et al., 2011), they are not

effective in treating the core symptoms of ASD, including deficits in social interaction and verbal skills (Silverman et al., 2012). To date, medical interventions or therapeutic regimens in autism have not targeted the immune system, in spite of the proposed significant contribution of immune dysfunction in the pathogenesis of ASD (Vargas et al., 2005). Intriguingly, anti-inflammatory interventions, using the immunomodulatory glycan, lacto-N-fucopentaose III (LNFPIII; found in human milk and on schistosome parasites) have been effectively used to treat multiple sclerosis, which is a chronic neurological disorder (Zhu et al., 2012). Of note, administration of LNFPIII reduced white adipose tissue inflammation and improved metabolic functions in adult male obese mice fed a high-fat, high-carbohydrate diet for 6 weeks (Bhargava et al., 2012) .

Given the significant influence of the intrauterine environment on the normal fetal brain development, it is surprising that no studies, so far, have assessed the efficacy of maternal anti-inflammatory interventions in mitigating the development/progression of neurological deficits, such as ASD in the offspring. More specifically, no such approach has been tested in the context of ASD and HFD-induced maternal obesity.

Hence, in the current study, the main objectives were to investigate whether HFD consumption before and during pregnancy, as well as throughout lactation, by C57BL/6 female mice will produce inflammation-dependent ASD-relevant behaviors in adolescent offspring (postnatal day [PND] 35) and to determine whether maternal-only administration of a potent anti-inflammatory glycan can ameliorate offspring's neurochemical and behavioral abnormalities caused by the maternal HFD consumption.

Symptoms of neurodevelopmental disorders, including ASD are initially manifested during infancy and then progress during the pre-pubertal period (Ozonoff et al., 2010), which is

why we focused our studies on PND35 offspring. To assess the potential neurobehavioral consequences of maternal HFD consumption, we conducted a series of behavioral tests (open field, pole and grip strength, marble burying and forced swim and novel object recognition) that could effectively evaluate motor function, emotional disturbances, such as anxiety or depression, and cognitive changes; perturbations in the performance in some of these tests, i.e., open field, forced swim test, marble burying, novel object recognition tests has been observed in animal models of ASD (Brielmaier et al., 2012, Sampino et al., 2014). Perinatal exposure to a HFD has been reported to affect the development of neural pathways important in behavioral regulation, such as dopaminergic and serotonergic systems; dysregulation in these systems can lead to anxiety, depression and ASD-like behaviors in the offspring (Sullivan et al., 2014). Hence, in this study, the maternal HFD-induced changes in the levels of dopamine (DA), serotonin (5-HT), norepinephrine (NE) and their metabolites were determined in a brain region-specific manner (prefrontal cortex [PFC], nucleus accumbens [NAc], striatum, dorsal hippocampus [dHIP], ventral hippocampus [vHIP] and cerebellum [CB]).

Materials and methods

Animals and treatment

Female mice were fed either a control low-fat (LFD; 10% kcal from fat, D12450J, Research Diets, Inc., New Brunswick, NJ) or a high-fat diet (HFD; 60% kcal from fat, D12492, Research Diets, Inc.) for 6 weeks prior to mating and were maintained on respective diets throughout gestation and lactation. Both diets are micronutrients and simple sugar (sucrose) balanced. Prior to mating and until weaning, HFD-fed females were divided into two groups: one was treated with dextran (40 kDa; Sigma-Aldrich, St. Louis, MO) vehicle, the other was given an anti-inflammatory glycan (LNFPIII conjugated to dextran) (Bhargava et al., 2012). The LFD-fed

females also received the dextran vehicle. Both dextran and glycan (G) were administered subcutaneously at a dose of 35 μg/mouse dissolved in 100 μl sterile saline twice weekly. The dose of glycan conjugate was selected based on a previously published study wherein *in vivo* activation of murine peritoneal macrophages by systemic glycan conjugate administration successfully produced an anti-inflammatory effect via increased production of anti-inflammatory cytokines (Atochina et al., 2008) . Offspring (LFD/HFD/HFD-G) were sexed and all litters with more than n = 8 were reduced to eight pups (4 male, 4 female, whenever possible) on PND5. Female and male pups were weaned on PND21, gender-separated and placed on a standard chow (Purina, St. Louis, MO) until PND35. Female and male offspring (each pair from separate litters; n= 8-12/group/sex) underwent behavioral tests (described in detail below) beginning on PND35 and were sacrificed (24 h after the last behavioral test); body weight (BW) was recorded, organs (brain, liver, spleen, kidneys and thymus) were harvested, weighed, and quickly frozen at -80 °C for potential further analyses.

Behavioral tests

Behavioral tests were performed on 3 consecutive days as in (Lin et al., 2014). Animals were naïve to the testing environment prior to testing initiation and all tests were performed in a specially furnished behavioral testing room adjacent to the one where animals were housed. In all behavioral tests, the experimenter was blinded to the treatment groups.

Open field. Mouse locomotor activity was monitored for 30 min in an open field arena (25 cm x 25 cm x 40 cm, divided into 16 square grids; Coulbourn Instruments, Whitehall, PA) using Limelight software (Actimetrics, Wilmette, IL) as detailed in (Krishna et al., 2014). The distance traveled (cm) and number of crossings were analyzed per 5 min intervals. Additionally,

the time spent in the periphery, center and corner was recorded and analyzed per 5 min interval to determine the anxiety level of mice (Dulawa et al., 2004, Bailey and Crawley, 2009).

Pole test. Motor coordination of mice was assessed using pole test as previously described (Lin et al., 2013). Briefly, mice were placed vertically facing upward on the top of a vertical gauze-wrapped pole (d × h; 1 × 55 cm). Each mouse underwent four trials and the average times to turn, to descend, and total time spent on the pole from the four trials was used for statistical analysis. The maximum turning time allowed was 60 s and the maximum total time per trial was 120 s (Krishna et al., 2014).

Grip strength. A strength gauge (Bioseb, France) with attached mouse-specific square wire grid (6 × 6 cm) was used to measure forelimb grip strength as previously described (Lin et al., 2013, Krishna et al., 2014). The maximum grip force per trial was recorded in newtons (N) for a series of four trials (1 min apart) to obtain an average value that was used for statistical analysis.

Novel object recognition (NOR). The NOR was conducted on day 2 of behavioral testing with the previous day 30-min open field testing used as a habituation phase, as detailed in (Lin et al., 2013). Briefly, mice were placed in the arena and allowed to explore two identical objects for 5 min. After 1 h rest period (in home cages), mice were placed back into the arenas for 5 min with one familiar and one novel object. The number of approaches towards the familiar (N_f) versus the novel object (N_n) was extracted using the Limelight software. To determine novelty preference, the percent of approaches towards the familiar vs. the novel object and the percentage of time spent with the familiar vs. the novel object were calculated as we have described it previously (Lin et al., 2013, Lin et al., 2014).

Forced swim test (FST). Following the NOR test and a 1.5-h home cage rest period, FST was completed as in (Krishna et al., 2014). Mice were gently placed in a 3L cylindrical container (18 × 25 cm; d × h) filled with tap water that was maintained at 29±1 °C. Mice were allowed to swim for 15 min and the total times spent swimming, climbing, or immobile were scored using the Limelight video tracking software and analyzed per 5 min intervals.

Marble burying test (MBT). This test was performed on day 3 as described previously (Gaikwad et al., 2010), but with some modifications. Mice were individually placed in cages containing 4-5 cm-thick pine bedding (American Wood Fibers, Columbia, MD) for 10 min (habituation phase). After the habituation phase, twenty glass marbles (diameter ~10 mm, Panacea Products Corp., Columbus, OH) were laid gently on the top of the bedding, arranged in a 4 x 5 matrix. After a 40-min home-cage resting period, animals were reintroduced into the testing cages and the number of marbles at least two-thirds covered by bedding material in 10 min was counted (Gaikwad et al., 2010).

Neurochemistry

Brain regional punching and determination of monoamines and their metabolites was performed as in (Coban and Filipov, 2007). Briefly, micropunches (1.5-mm diameter) from prefrontal cortex (PFC), nucleus accumbens (NAc), striatum (STR), dorsal hippocampus (dHIP), ventral hippocampus (vHIP) and cerebellum (CB) were collected from 500-μm thick brain sections and sonicated in 100 μl of 0.2 N perchloric acid. Prior to analysis, the samples were centrifuged and an aliquot (20 μl) of the supernatant was injected into HPLC with an electrochemical detector (Waters Alliance, Waters Co., Milford, MA) for determination of: (i) dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT); (ii) norepinephrine (NE) and its metabolite 3-methoxy-

4-hydroxyphenylglycol (MHPG; only in PFC); (iii) serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). Prior to statistical analysis, all neurochemistry data were normalized on per mg of protein basis as previously described (Coban and Filipov, 2007, Krishna et al., 2014).

Statistical analysis

Two-way analysis of variance (ANOVA) was used for body and organ weights, behavioral and neurochemistry data analysis (treatment [maternal diet] x sex [offspring]). In addition, two-way ANOVA was conducted to analyze the effect of treatment and time (interval) within a sex for the open field data. If ANOVA's overall main effect was found significant, treatment means were separated by Student-Newman-Keuls *post hoc* test. All results are presented as mean ± SEM and are considered significant at $P \leq 0.05$.

Results

Body weight and organ weights

There was a significant main effect of sex ($P \leq 0.001$), but not treatment ($P = 0.58$) with respect to offspring BW; PND35 males were heavier than females ($P \leq 0.001$; Fig. 7.1A). Similarly, there were overall significant main effects of sex ($P \leq 0.05$) but not of treatment ($P > 0.25$) with respect to the brain, liver, kidney and spleen weights; the mean weights (g) of these organs were slightly, but significantly, greater in the PND35 males compared to the females ($P \leq 0.05$, Fig. 7.1B, C, D and E). No significant main effects of sex ($P = 0.06$) or treatment ($P > 0.15$) was observed with respect to the offspring's thymus weight, but within female offspring, the HFD and HFD-G females had smaller thymuses ($P \leq 0.05$; Fig. 7.1F).

Behavior

Open field. There were overall significant main effects of treatment ($P \leq 0.01$) and interval (5 min time period; $P \leq 0.001$), but without any significant interaction between the two ($P = 0.96$) with respect to the distance traveled in the open field arena. HFD-G PND35 offspring traveled significantly more (per 5 min interval) than the LFD or HFD groups ($P \leq 0.05$). The overall treatment effect was driven exclusively by the HFD-G females which exhibited greater locomotor activity ($P \leq 0.05$; Fig. 7.2A). There was also significant effect of sex ($P \leq 0.001$) with respect to the distance traveled in the open field arena; PND35 females traveled longer than the males ($P \leq 0.001$; Fig. 7.2A). With respect to the mean time spent in the arena center, compared to LFD females, HFD and HFD-G females showed only numerical trends towards a decrease ($P = 0.11$) and increase ($P = 0.13$), respectively (Fig. 7.2B). The center time of the open field arena was unaffected by the maternal treatment in the male PND35 offspring (Fig. 7.2B). Female offspring showed significant difference regarding the time spent in the corner; compared to LFD females, HFD and HFD-G females showed a significant increase and decrease, respectively, in the corner time ($P \leq 0.05$; Fig. 7.2C). No significant difference between the treatment groups with respect to the corner time was observed in male PND35 offspring ($P \geq 0.45$; Fig. 3C).

Pole test. Pole test performance was not affected by sex or treatment, i.e., mean turn time, time to descend and total time ($P \geq 0.60$; data not shown).

Grip strength test. Similar to the pole test data, there were no significant effects of sex ($P > 0.60$) or treatment ($P > 0.10$) with respect to the mean forelimb grip strength (data not shown)

NOR. PND35 female offspring in the LFD, HFD and HFD-G groups exhibited normal NOR performance evidenced by the increased number of approaches during the novel object

testing phase ($P \leq 0.01$; Fig. 7.3A); in the males, the PND35 offspring from LFD ($P = 0.14$) and HFD ($P = 0.21$) dams only trended towards greater novel object preference. However, HFD-G males's preference for the novel object was highly significant ($P < 0.01$; Fig. 7.3A). Compared to the LFD and HFD groups, female and male offspring in the HFD-G groups showed an increased tendency in exploring the novel object ($P \leq 0.05$; Fig. 7.3A).

FST. There was no significant main effect of sex ($P \geq 0.06$) or treatment ($P \geq 0.06$) with respect to the total times spent swimming, climbing or immobile in the FST during the overall 15 min trial, but there was a trend for increased swimming in females and males from HFD and HFD-G dams. During the first 5 min (interval 1), HFD and HFD-G females swam significantly more than the LFD female offspring ($P \leq 0.05$; Fig. 7.3B); there was a concomitant decrease in the immobility time ($P \leq 0.05$; data not shown). Contrary to the effects in the females, male offspring performance in the FST was unaffected even during the first interval ($P > 0.45$; Fig. 7.3B).

MBT. The overall number of buried marbles was quite low in all groups and it was unaffected by treatment ($P > 0.50$) or sex ($P > 0.40$; Fig. 7.3C).

Neurochemistry

In the PFC, DA and DOPAC (DA metabolite) levels were affected by treatment ($P \leq 0.05$) but not by sex ($P > 0.80$). Specifically, PFC DA levels were increased in the HFD-G PND35 offspring compared to the rest ($P \leq 0.05$; Fig. 7.4A). DOPAC levels on the other hand were decreased in both the HFD and HFD-G groups ($P \leq 0.05$; Fig. 7.4B). As a result, the DA turnover expressed as DOPAC/DA ratio was significantly decreased in the female HFD and HFD-G groups ($P \leq 0.05$; data not shown). Although not significant, compared to LFD, HFD and HFD-G female offspring showed numerical increase (1.4-fold) and decrease (1.3-fold),

respectively, in the levels of the other DA metabolite, HVA ($P > 0.35$; data not shown). Consequently, the DA turnover expressed as HVA/DA ratio was numerically increased and decreased in the female HFD and HFD-G groups, respectively ($P > 0.10$; data not shown).

There was a significant ($P \leq 0.05$) interaction between sex and treatment regarding NE and MHPG (NE metabolite) levels in the PFC; NE was significantly decreased in HFD and HFD-G female offspring ($P \leq 0.05$; Fig. 7.4C) whereas no significant difference was observed between the treatment groups in the males. Compared to males, MHPG level was significantly decreased in the female offspring ($P \leq 0.05$; data not shown); within PND35 males, MHPG was significantly decreased in both HFD and HFD-G groups ($P \leq 0.05$; data not shown). There were no significant effects of sex ($P > 0.08$) or treatment ($P > 0.10$) with respect to 5-HT or 5-HIAA (data not shown).

In the NAc, DA, DOPAC, HVA and 5-HT levels were unaffected by sex ($P > 0.09$) or treatment ($P > 0.35$; Table 7.1). 5-HIAA levels were affected by sex (higher in females; $P \leq 0.01$), but not by treatment ($P > 0.60$; Table 7.1). No effects of sex ($P > 0.06$) or treatment ($P > 0.06$) was observed with respect to DA, 5-HT or their metabolites in the striatum (Table 7.1).

DA level in dHIP were affected both by sex ($P \leq 0.01$) and treatment ($P \leq 0.05$). Female offspring had higher dHIP DA than males ($P \leq 0.01$; Table 7.2). Within females, the HFD-G group showed an apparent trend towards DA decrease that was almost significant ($P = 0.07$; Table 7.2); in the males, DA levels were significantly reduced in the HFD-G group ($P \leq 0.05$; Table 7.2). dHIP levels of DOPAC or HVA were not affected by sex or treatment ($P > 0.30$; Table 7.2). 5-HT was affected by sex (higher in female offspring; $P \leq 0.001$, Table 7.2), but not by treatment ($P > 0.90$; Table 7.2). Levels of 5-HT metabolite, 5-HIAA, were not affected by sex

or treatment ($P > 0.07$; Table 7.2). NE level was affected by sex (decreased in female offspring; $P \leq 0.01$), but not by treatment ($P > 0.35$; Table 7.2).

In the vHIP, DA was altered by treatment ($P \leq 0.05$), but not by sex ($P > 0.45$). Within sex, HFD-G females had significantly higher DA ($P \leq 0.05$; Table 7.2); whereas in the males vHIP DA levels were not affected by the maternal diet ($P > 0.15$; Table 7.2). DOPAC or HVA levels in the vHIP were unaltered by sex or treatment ($P > 0.35$; Table 7.2). 5-HT levels were increased significantly in both male and female offspring from the HFD and HFD-G groups with the increase in the HFD-G groups being greater than the increase in the HFD groups ($P \leq 0.001$; Table 7.2); there was no effect of sex on vHIP 5-HT ($P > 0.80$; Table 7.2). Regarding 5-HIAA, two-way ANOVA showed an overall main effect of sex (greater 5-HIAA in females; $P < 0.05$) and treatment ($P \leq 0.001$), but without any significant interaction between two ($P > 0.45$). Female HFD and HFD-G groups had higher 5-HIAA levels ($P \leq 0.01$; Table 7.2; whereas only the HFD-G male offspring had significantly higher 5-HIAA ($P \leq 0.01$; Table 7.2). There were no significant effects of sex ($P > 0.25$) or treatment ($P > 0.75$) with respect to vHIP NE (Table 7.2).

No significant effects of sex ($P > 0.50$) or treatment ($P > 0.08$) were observed on any of the monoamines and their metabolites in the CB (Table 7.2).

Discussion

Apart from the increasing obesity rates in the general population, the prevalence of maternal obesity has risen significantly in the last two decades (Maric-Bilkan et al., 2011). Importantly, mounting evidence from epidemiological studies demonstrates an association between maternal obesity and adverse effects on the long-term health of the offspring (Maric-Bilkan et al., 2011, O'Reilly and Reynolds, 2013). For example, pre-pregnancy obesity, as well as excessive weight gain during pregnancy has been linked to an increased offspring risk of high

BMI and of an increased likelihood of developing neurodevelopmental disorders, such as ASD (Dodds et al., 2011, Krakowiak et al., 2012, Tanne, 2012, Sridhar et al., 2014). Besides maternal obesity, maternal infection and associated inflammatory changes have been shown to increase the ASD risk (Patterson, 2002, Parker-Athill and Tan, 2010). Interestingly, increased systemic inflammation in pregnant obese women (Challier et al., 2008) and heightened neuroinflammation in rodents perinatally exposed to HFD have been demonstrated (Bilbo and Tsang, 2010, Sasaki et al., 2013), but role of diet-induced maternal obesity (DIMO) and resulting maternal inflammation as a causal factor for ASD has not been studied experimentally. Importantly, interventions targeting the maternal immune system/maternal inflammation in the context of maternal obesity and ASD are non-existent.

In this study, we evaluated the effect of maternal HFD consumption on the development of ASD-like behavioral endophenotype, other behavioral aberrations and the associated neurochemical alterations in adolescent offspring; we also assessed the efficacy of maternal glycan conjugate administration in mitigating/preventing the HFD's adverse effect on the offspring. Our main findings include: (i) a sex-specific effect on locomotor activity with female PND35 offspring showing overall increased locomotor activity than males; (ii) female offspring-specific glycan effect, increased activity in the open field test; (iii) increased anxiety (corner time) in female offspring by maternal HFD which was reversed by maternal glycan administration; (iv) sex-independent enhancement of short-term object recognition memory by maternal glycan treatment; (v) selective alterations of monoamine homeostasis by maternal HFD consumption; (vi) counterbalancing or enhancing effects on some of the HFD-induced monoamine alterations by maternal glycan treatment.

Maternal HFD/glycan treatment did not produce any significant difference in the body weight of adolescent offspring. Overall, male offspring were heavier than females; similar finding (maternal HFD) has been reported recently (Sasaki et al., 2014). Interestingly, while offspring perinatally exposed to HFD were heavier than chow-exposed offspring throughout the pre-weaning period; they were not during the post-weaning stage, i.e., adolescence and adulthood (Sasaki et al., 2013, Sasaki et al., 2014). Given the absence of notable BW differences between adolescent offspring that were maternally exposed to LFD or HFD in this study, it could be inferred that the behavioral alterations we observed are not BW-dependent.

Perinatal overnutrition has been shown to increase the offspring risk of developing behavioral disorders, such as anxiety, depression and impaired social interaction, with some of the effects being sex-specific (Sasaki et al., 2013, Sullivan et al., 2014). However, it is noteworthy that majority of the reports on the adverse neurological outcomes are in sexually mature adult offspring that were perinatally exposed to HFD (Bilbo and Tsang, 2010, Peleg-Raibstein et al., 2012); limited information exist regarding the influence of maternal HFD intake on adolescent offspring (Sasaki et al., 2014). Given the notable differences in the brain structure and function, as well as the distinct manifestation of anxiety between adolescents and adults (Pine et al., 1998, Spear, 2000, Stein et al., 2001, Casey et al., 2008), it is imperative to generate more data on the impact of maternal HFD consumption on adolescent neurological functions.

Excessive in utero exposure to polyunsaturated fatty acid resulted in increased locomotor activity of adolescent CD-1 female and male offspring in an open field test (Raygada et al., 1998). Conversely, reports of decreased locomotor activity or absence of locomotor deficits have been observed in adult C57BL/6 female and male offspring exposed to maternal HFD (Samuelsson et al., 2008, Peleg-Raibstein et al., 2012). In the current study, maternal HFD did

not affect juvenile offspring's locomotor activity, which is in line with the studies from the adult offspring from the same strain (Peleg-Raibstein et al., 2012). Interestingly, female offspring of glycan treated HFD-fed dams showed an increase in exploratory behavior in the open field test in our study; this may also be suggestive of a reduced anxiety level (Gao et al., 2011).

An association between maternal obesity and neurodevelopmental disorders, such as ASD in the offspring characterized by altered emotional and psychiatric outcomes, has been suggested (Mehta et al., 2014). Emotional disorders, including increased anxiety and/or depression have also been demonstrated in rodent and non-human primate models developmentally exposed to HFD (Bilbo and Tsang, 2010, Peleg-Raibstein et al., 2012, Sullivan et al., 2014). Elevated anxiety and decreased social interaction are considered two core ASD endophenotypes (Laushey and Heflin, 2000, Smith et al., 2007, Hallett et al., 2013). In this study, female offspring of HFD-fed dams exhibited elevated anxiety as evidenced by the increased time spent in the corner of open field test and increased swimming time (first 5 min) during the FST. However, the increased anxiety level observed in the open field and FST tests was not reflected in the MBT as female offspring of dams exposed to LFD or HFD showed similar marble burying behavior. This may be due to their young age and/or the short time (10 min) allotted to bury the marbles. For example, MBT has been used successfully in PND21 and adolescent mice previously, but the normal for this strain testing session of 10 min was extended to 30 min (Westmark et al., 2011, Barnum et al., 2012). Interestingly, maternal HFD consumption did not affect the anxiety response in the male offspring. Similar finding has been observed in rodent and non-human primates wherein HFD intake during gestation and lactation produced a female-restricted anxiety response in adolescent offspring (Sullivan et al., 2010, Kang et al., 2014). Interestingly, sex-specific effect of maternal HFD on anxiety level has been demonstrated in

adult offspring; however, during adulthood, males from HFD-fed dams have been reported to be more anxious (Bilbo and Tsang, 2010, Sasaki et al., 2013, Bellisario et al., 2014). In total, these data are indicative of a distinct anxiety profile during adolescence versus adulthood in the offspring perinatally exposed to HFD.

Intriguingly, maternal glycan administration reversed the anxiety-like response in the female offspring of HFD-fed dams as evidenced by the significantly decreased corner time in the open field arena. However, this maternal glycan-induced reversal of open field anxiety response was not evident in the FST as female offspring of both HFD-fed dams and glycan-treated HFD dams showed similar response (increased swimming) in the FST. It has to be noted that female offspring of glycan treated HFD dams were hyperactive in the open field test and hence the increased swimming time could be a reflection of their increased locomotor activity, rather than their anxiety level (Perona et al., 2008). Besides maternal overnutrition, maternal infection and associated immune system activation have been demonstrated to cause ASD-like behavioral phenotype characterized by increased anxiety and deficits in social interaction (Smith et al., 2007, Parker-Athill and Tan, 2010). Interestingly, genetic (deletion) or pharmacologic (neutralizing antibody) inhibition of the pro-inflammatory cytokine interleukin (IL)-6 in pregnant mice prevented the maternal immune activation-induced social and emotional deficits in the adult offspring (Smith et al., 2007); this finding indicates that maternal inflammation plays a crucial role in causing ASD-like behavioral deficits in the offspring.

The anti-inflammatory effects of the glycan conjugate we used in our study have been demonstrated previously, i.e. in its ability to mitigate the severity of experimental autoimmune encephalomyelitis and associated neuroinflammation in adult female C57BL/6 mice (Zhu et al., 2012). The immunomodulatory role of the glycan conjugate in (Zhu et al., 2012) study was

attributed to the decreased infiltration of macrophage and T cell surface activation markers, such as F4/80 and CD4, respectively, in the brain, and to an increased production of the anti-inflammatory T-helper type (Th)-2 cells in the periphery (Zhu et al., 2012). Reduced central nervous system (CNS) inflammation with an associated increase and decrease in the anti-inflammatory (IL-4) and pro-inflammatory (interferon gamma) cytokines, respectively, following systemic administration of *Schistosoma mansoni* and *S. japonicum* has been reported (Sewell et al., 2003, Zheng et al., 2008).

At a molecular level, glycan regulates anti-inflammatory signaling in macrophages through Toll-like receptor-4 (TLR-4) (Zhu et al., 2012). Specifically, upon TLR4 activation, glycan conjugate causes transient activation of the nuclear factor (NF)-κB pathway, resulting in reduced signaling of downstream targets, such as c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases and increased expression of extracellular signal-regulated kinase (ERK) (Thomas et al., 2003, Thomas et al., 2005). Importantly, this unique signaling pattern induced by glycan conjugate was found to be necessary for the maturation of myeloid dendritic cells (DC) in the CNS to a DC2 phenotype that can drive anti-inflammatory Th2 response (Thomas et al., 2005). That being said, it is possible that the above molecular mechanisms may be involved in the glycan's anti-inflammatory effect observed in our study; however the exact mechanism is unclear at this stage. Regardless, given the observed decrease in anxiety-like response (decreased time in the corner of the open field arena) in the female offspring of HFD-G dams, it could be inferred that the glycan conjugate prevented ASD-like behavior in the offspring, perhaps by mitigating the effects of the HFD-induced maternal inflammation. However, maternal inflammatory status in response to HFD and its modulation by the glycan conjugate administration needs to be ascertained in detail in the future.

Besides altering emotional functions, maternal HFD consumption is linked to cognitive deficits in the offspring. For example, several studies have demonstrated spatial memory impairment in adult offspring following maternal exposure to HFD (Bilbo and Tsang, 2010, Page et al., 2014). Of note, limited information exists on the impact of maternal HFD exposure on offspring's non-spatial memory. Interestingly, recent study demonstrated sex-specific effect on the short-term non-spatial object recognition memory in adult offspring following maternal exposure to a hypercaloric cafeteria diet only during lactation (Wright et al., 2014). However, in our study, maternal HFD exposure did not affect the short-term object recognition memory of adolescent offspring regardless of sex. The varied behavioral outcome could be due to the differences in the diet composition (HFD vs. cafeteria diet) and the timing of dietary fat introduction (entire perinatal period vs. lactation-only). In contrast to the lack of HFD effects on offspring's NOR memory, we found that regardless of sex, offspring of glycan treated HFD-fed dams gravitated more towards the novel object as opposed to the familiar one. Moreover, within the novel object exploration, offspring of HFD-G dams were significantly different (greater) than that of the LFD/HFD groups. Overall, it appears that maternal glycan administration may have a potential beneficial role in enhancing offspring's recognition memory. Additionally, we found that, compared to the significant increase in the novel object preference (as opposed to familiar ones) by the female offspring in LFD or HFD groups, the male counterparts showed only a trend towards greater exploration of the novel object, which is suggestive of a delay in the recognition memory development in males compared to females. Interestingly, similar results, wherein females outperformed males, in recognition memory tasks, have been reported in rodents (Sutcliffe et al., 2007, Bettis and Jacobs, 2009) as well as in humans (Heisz et al., 2013). Although sex hormones have been implicated in the different cognitive profile between males

and females (Rena, 2014), the gender disparity in the cognitive memory is more likely to start during early developmental stage and is mainly attributed to the differences in the neural projections and global brain connectivity, with female brains showing more interconnections between cerebral hemispheres than males (Schulte and Muller-Oehring, 2010, Zaidi, 2010).

In addition to the neurobehavioral abnormalities, the adverse consequences of maternal HFD on dopaminergic and serotonergic brain circuitries that regulate key offspring behaviors, have been reported. Maternal HFD consumption decreased behavioral sensitization to amphetamine in adult male offspring by altering DA transmission in the nucleus accumbens (Naef et al., 2008). Chronic HFD consumption during pregnancy also decreased central serotonergic signaling in the fetal offspring of non-human primates (Sullivan et al., 2010). Of note, the impact of maternal HFD exposure on underlying brain region-specific neurochemical (monoamine) changes associated with the behavioral alterations in the offspring remains poorly understood. In this study, maternal HFD decreased PFC NE (tissue content) levels only in the female offspring and this effect was not modified by the glycan administration; there was only sex difference with females showing decreased PFC NE levels than males. NE release from the PFC is involved in locomotion; increased synaptic NE is associated with increased locomotor activity (Snoddy and Tessel, 1985, Blanc et al., 1994). In this regard, the overall increased locomotor activity of female offspring observed in the open field test in our study could be attributed to an increased noradrenergic transmission, characterized by increased synaptic NE release and decreased cortical levels of tissue NE.

Maternal HFD intake also altered DA homeostasis in a brain region- and sex-specific manner. Specifically, female offspring of HFD-fed dams showed a numerical increase in the HVA (DA metabolite) level and DA turnover (HVA/DA ratio) in the PFC, which is indicative of

increased DA utilization. Interestingly, maternal HFD decreased the DOPAC and DA turnover (DOPAC/DA) in PFC that was not modified by the glycan treatment. Decreased tissue DOPAC level in the presence of a numerical increase in HVA indicates decreased intracellular DA and is in turn suggestive of an increased synaptic DA release or DA utilization. Maternal HFD produced a similar increase in DA utilization in the vHIP of female offspring as evidenced by the numerical increase in HVA level. The maternal HFD induced augmentation in DA utilization might be due to an increase in synaptic DA release and it is likely that the increased synaptic DA level may have contributed to the heightened anxiety-like behavior in the female offspring. Given the synchronized activity between PFC and vHIP in regulating anxiety (Adhikari et al., 2010), it could be inferred that the increased anxiety observed in female offspring of HFD-fed dams could be due to the impaired DA homeostasis in both brain regions. Interestingly, we found that maternal glycan treatment produced a compensatory effect on the increased DA utilization induced by maternal HFD. Specifically, maternal glycan treatment induced an increase and decrease (numerical) in the DA and HVA, respectively, in the PFC and vHIP of female offspring, which is indicative of decreased synaptic DA release or reduced DA utilization. In this regard, the reversal of the increased anxiety in the female offspring by the maternal glycan treatment could be attributed to glycan-driven maintenance of DA homeostasis in the face of the increased demands imposed by maternal HFD; however synaptic DA needs to be measured using sensitive measures such as microdialysis or voltammetry (Yang and Michael, 2007, Chefer et al., 2009) to confirm this assumption, plus the exact mechanism (s) the glycan uses to have this effect are unknown at this time.

Maternal HFD exposure significantly increased the serotonergic activity in the vHIP of the PND35 offspring (with effects being more prominent in females) as evidenced by the

significantly increased 5-HT and 5-HIAA levels. Consequently, the 5-HT turnover, expressed as 5-HIAA/5-HT ratio was significantly decreased in the PND35 offspring. The maternal HFD-induced increase in serotonergic activity was further enhanced by the glycan treatment. Altered serotonergic neurotransmission characterized by increased serotonin turnover and decreased serotonin in brain regions, including PFC and hippocampus is increasingly associated with depression (Barton et al., 2008, Mosienko et al., 2012, Mahar et al., 2014). FST has been widely used to assess the efficacy of antidepressant drugs in animals and those antidepressants that augment serotonergic activity have been found to significantly increase the swimming behavior in the FST (Cryan et al., 2005). Of note, the increased swimming time exhibited by female offspring of HFD or HFD-G dams during (first 5 min) FST indicates that they were less depressed. Given the inverse association of brain serotonin level and depression, the less depressive (increased swimming in FST) behavior displayed by female HFD and HFD-G offspring observed in this study could be attributed to their increased brain (vHIP) serotonin and decreased serotonin turnover.

In summary, we found that maternal HFD consumption can induce sex-selective ASD-like behavioral aberrations, with effects being more noticeable in females as opposed to male adolescent offspring. It is interesting to note, however, that in humans, ASD is more prevalent in males than females, which averages at approximately 4:1 (male-female) ratio (Kothari et al., 2013). Intriguingly, recent reports indicate that the male preponderance of ASD could be due to a bias in the diagnostic criteria currently used, wherein females with ASD may go under-diagnosed unless presented with additional intellectual deficits or behavioral problems, such as hyperactivity (Dworzynski et al., 2012). Moreover, sex-specific differences in the manifestation of ASD symptoms (ASD girls being more anxious and passive than boys) (Hartley and Sikora,

2009) as well as the differences in the developmental trajectories (social difficulties in ASD boys manifesting earlier in life, whereas girls exhibiting greater impairments during early adolescence) may also account for the gender bias in ASD diagnosis.

Importantly, our finding of emotional deficit in female adolescent offspring even after being placed on regular chow at weaning provides indirect support for the developmental programming hypothesis wherein environmental influences during critical windows of development can induce long-term impact on the health of the offspring. Importantly, the fact that anti-inflammatory treatment with a glycan-conjugate of HFD dams during pregnancy and lactation reversed the maternal diet's negative effects on the female offspring suggests that maternal inflammation mediates altered developmental programming and that intervention targeting the maternal immune system can be beneficial in rescuing some of the adverse health events on the offspring.

Table 7.1. Concentrations of monoamines or their metabolites in different brain regions of PND 35 offspring (n = 5-6/group/sex) from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate.

Table 7.1. Brain neurotransmitter and neurotransmitter metabolites

		NAc			
Treatment/Sex	DA	DOPAC	HVA	5-HT	5-HIAA
LFD/F	34.69 ± 5.98	17.63 ± 1.83	7.43 ± 1.05	1.32 ± 0.12	**3.00 ± 0.23***
LFD/M	40.67 ± 8.63	19.66 ± 1.38	8.41 ± 0.86	1.14 ± 0.13	2.38 ± 0.32
HFD/F	27.04 ± 3.83	14.17 ± 0.94	6.84 ± 0.35	1.45 ± 0.12	3.18 ± 0.25
HFD/M	49.24 ± 4.02	17.70 ± 1.44	7.86 ± 0.77	1.22 ± 0.10	1.79 ± 0.17
HFD-G/F	44.81 ± 6.23	17.96 ± 3.66	8.32 ± 1.13	1.29 ± 0.11	2.91 ± 0.43
HFD-G/M	41.52 ± 4.13	14.96 ± 1.44	7.41 ± 0.28	1.49 ± 0.15	2.54 ± 0.12
		STR			
LFD/F	65.87 ± 3.24	23.34 ± 2.33	10.94 ± 0.95	1.41 ± 0.14	2.87 ± 0.28
LFD/M	60.30 ± 8.20	18.77 ± 3.95	10.94 ± 1.56	1.26 ± 0.12	2.98 ± 0.46
HFD/F	53.36 ± 6.26	17.29 ± 1.35	9.12 ± 1.10	1.39 ± 0.20	2.48 ± 0.36
HFD/M	57.98 ± 3.03	21.63 ± 1.29	9.11 ± 0.93	1.09 ± 0.07	1.90 ± 0.19
HFD-G/F	75.52 ± 5.34	22.95 ± 2.72	12.28 ± 1.20	1.66 ± 0.18	2.40 ± 0.24
HFD-G/M	59.47 ± 9.04	17.61 ± 3.20	9.90 ± 1.77	1.41 ± 0.11	2.17 ± 0.26

NAc: nucleus accumbens; STR: striatum; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid. Data are presented as mean ± SEM; unit: ng/mg protein. * indicates significant overall effect of sex ($P \leq 0.05$). Note: for the sake of simplicity, the * is designated on the LFD-female group.

Table 7.2. Concentrations of monoamines or their metabolites in different brain regions of PND 35 offspring (n = 5-6/group/sex) from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate.

Table 7.2. Brain neurotransmitter and neurotransmitter metabolites

			dHIP			
Treatment/Sex	DA	DOPAC	HVA	NE	5-HT	5-HIAA
LFD/F	**1.43 ± 0.11***	2.73 ± 0.65	0.31 ± 0.10	**2.83 ± 0.25***	**2.05 ± 0.34***	3.18 ± 0.43
LFD/M	1.03 ± 0.23	2.39 ± 0.56	0.34 ± 0.06	3.11 ± 0.15	1.08 ± 0.10	2.80 ± 0.25
HFD/F	1.33 ± 0.20	3.11 ± 0.55	0.28 ± 0.07	2.39 ± 0.09	1.77 ± 0.19	3.11 ± 0.38
HFD/M	1.13 ± 0.21	2.29 ± 0.52	0.39 ± 0.14	3.00 ± 0.27	1.18 ± 0.16	2.29 ± 0.23
HFD-G/F	1.09 ± 0.13	2.56 ± 0.48	0.23 ± 0.04	2.43 ± 0.16	1.64 ± 0.21	3.24 ± 0.26
HFD-G/M	**0.49 ± 0.04[a]**	2.91 ± 0.39	0.21 ± 0.08	3.07 ± 0.23	1.39 ± 0.16	2.99 ± 0.29
			vHIP			
LFD/F	1.83 ± 0.11	3.60 ± 0.14	0.66 ± 0.13	6.00 ± 0.21	0.85 ± 0.04	**2.96 ± 0.23***
LFD/M	1.89 ± 0.27	3.11 ± 0.37	0.82 ± 0.08	5.50 ± 0.17	0.82 ± 0.06	2.32 ± 0.15
HFD/F	2.56 ± 0.38	3.38 ± 0.29	0.87 ± 0.13	6.04 ± 0.27	**2.76 ± 0.64[a]**	**6.87 ± 1.77[a]**
HFD/M	2.03 ± 0.16	3.32 ± 0.32	0.62 ± 0.12	5.66 ± 0.14	**2.73 ± 0.75[a]**	4.08 ± 1.09
HFD-G/F	**2.60 ± 0.29[a]**	3.59 ± 0.39	0.58 ± 0.13	5.57 ± 0.39	**4.45 ± 0.59[b]**	**9.21 ± 0.66[a]**
HFD-G/M	2.60 ± 0.35	3.44 ± 0.35	0.85 ± 0.22	5.79 ± 0.20	**4.78 ± 0.29[b]**	**6.85 ± 0.61[a]**
			CB			
LFD/F	0.87 ± 0.11	1.55 ± 0.09	NA	4.62 ± 0.31	0.66 ± 0.21	1.37 ± 0.27
LFD/M	0.83 ± 0.07	1.55 ± 0.11	NA	5.35 ± 0.32	0.62 ± 0.23	1.71 ± 0.54
HFD/F	0.89 ± 0.08	1.32 ± 0.13	NA	5.20 ± 0.55	0.74 ± 0.18	2.01 ± 0.47
HFD/M	0.84 ± 0.05	1.34 ± 0.07	NA	4.42 ± 0.19	0.54 ± 0.25	1.17 ± 0.19
HFD-G/F	0.80 ± 0.09	1.41 ± 0.12	NA	4.15 ± 0.23	0.27 ± 0.05	1.18 ± 0.30
HFD-G/M	0.87 ± 0.09	1.40 ± 0.14	NA	4.71 ± 0.21	0.29 ± 0.02	1.32 ± 0.35

dHIP: dorsal hippocampus; vHIP: ventral hippocampus; CB: cerebellum; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; NA: not analyzed. Data are presented as mean ± SEM; unit: ng/mg protein. * indicates significant overall effect of sex ($P < 0.05$). Note: for the sake of simplicity, the * is designated on the LFD-female group. Different superscript letters indicate significant difference between treatments within a sex ($P \leq 0.05$).

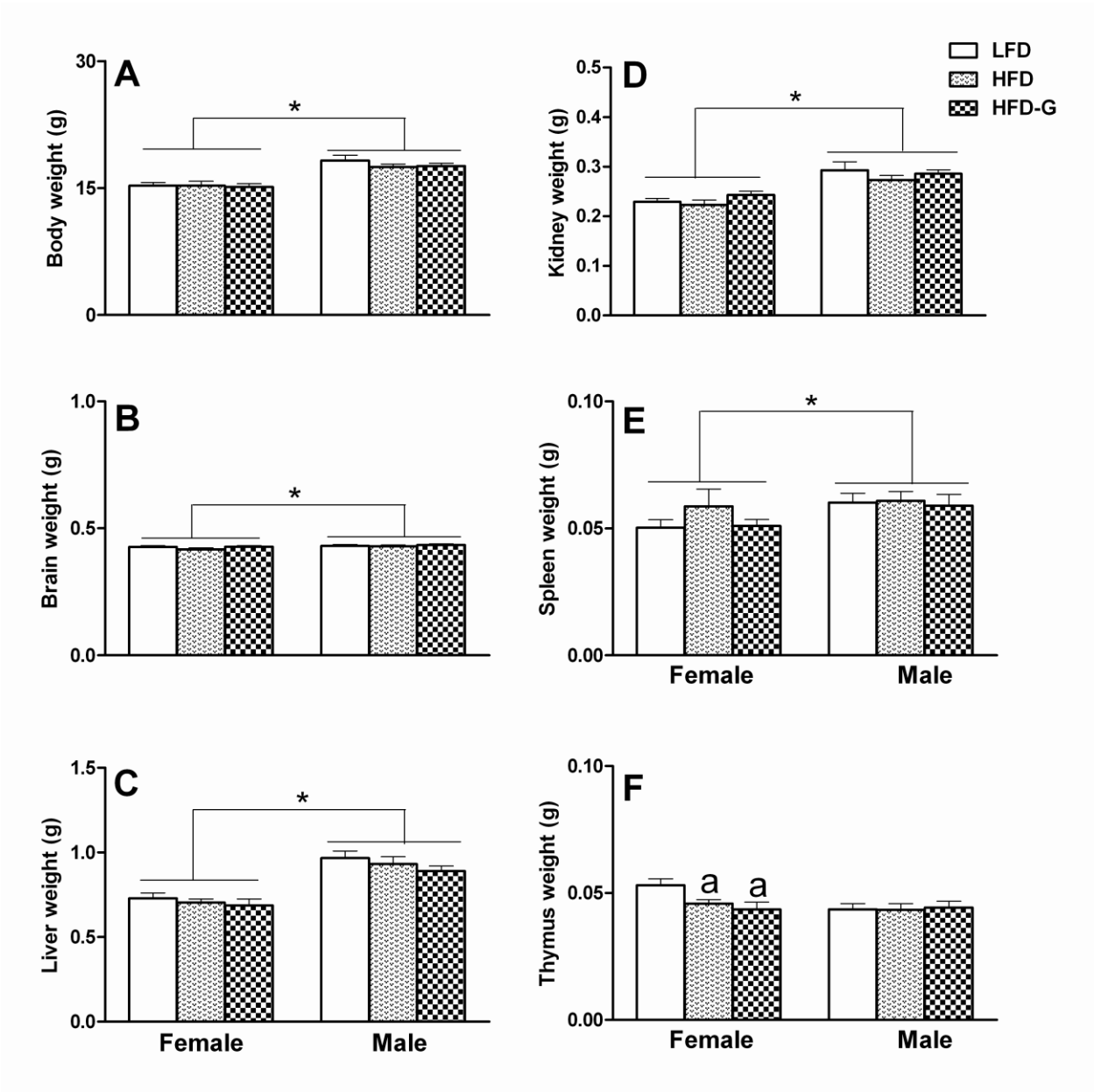

Figure 7.1. Mean body weight (g; A) and absolute brain (B), liver (C), kidney (D), spleen (E) and thymus (F) weights (g) of PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate. * indicates significant sex difference ($P \leq 0.001$). "a" indicates significant difference from LFD within a sex ($P \leq 0.001$). Data are expressed as means ± SEM (n = 8-12/group/sex).

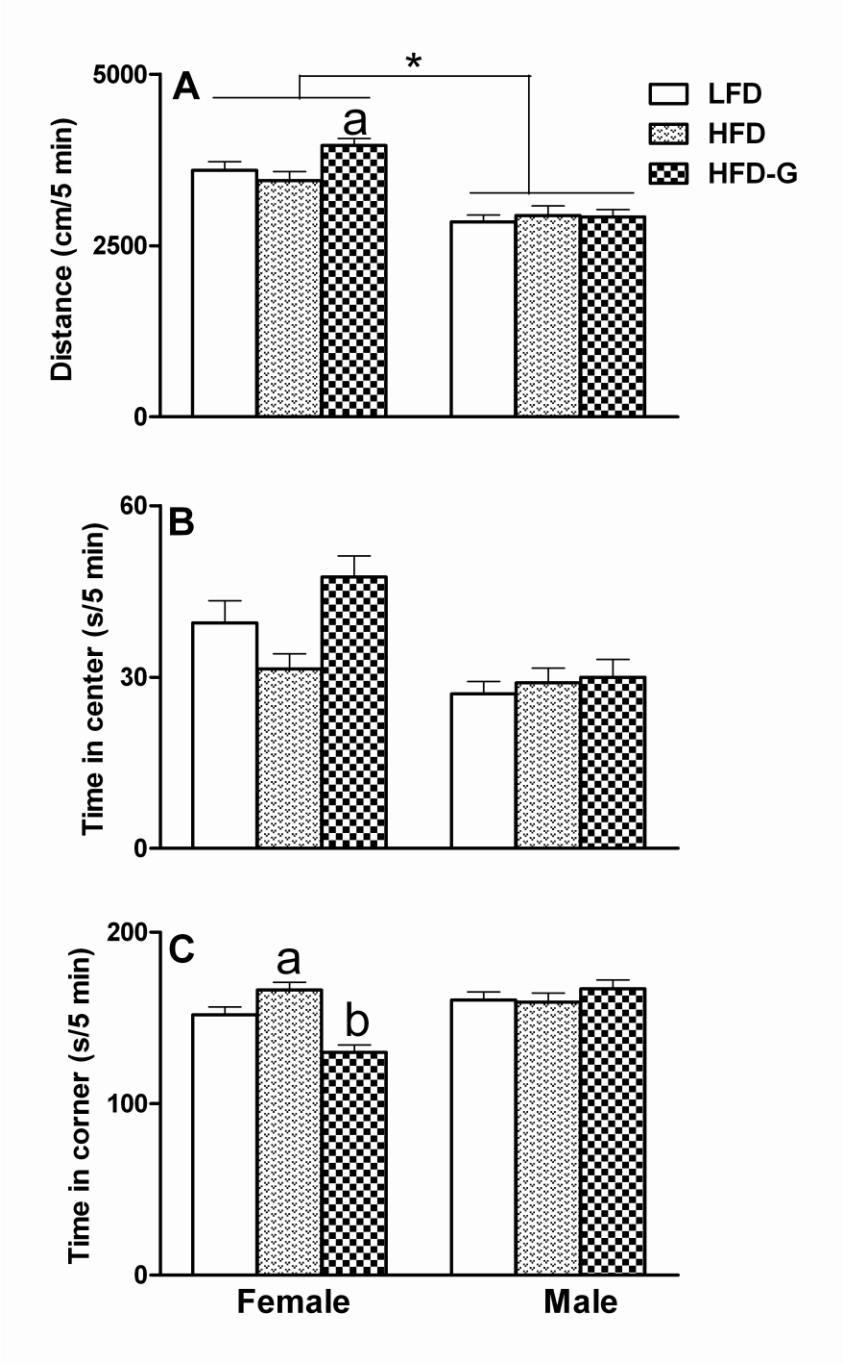

Figure 7.2. Distance traveled (per 5 min interval) during open field testing (A), time spent per 5 min interval in the center (B) and corner of the open field arena (C) by PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate.

* indicates significant sex difference ($P \leq 0.001$). Different letters indicate significant difference between treatments within a sex ($P \leq 0.001$). Data are expressed as means ± SEM (n = 8–12/group/sex).

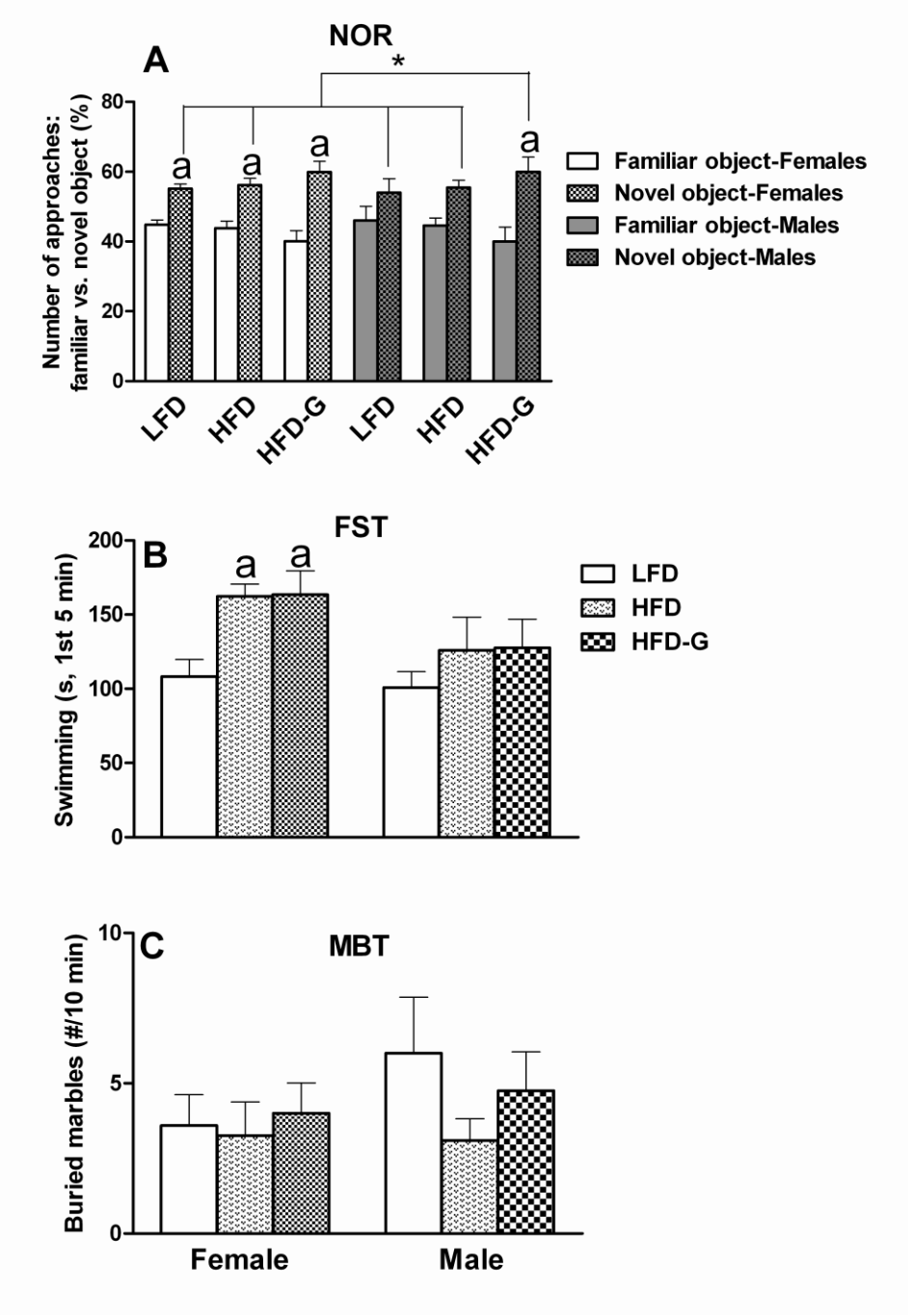

Figure 7.3. Number of approaches towards a familiar vs. a novel object (%) in a novel object recognition test (NOR; A), time spent swimming during the first 5 min (interval 1) in a forced swim test (FST; B) and number of marbles buried (≥ 70%) during a marble burying test (MBT; C) by PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6

weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate. * indicates significant effect of glycan within the novel object ($P \leq 0.05$). “a” indicates significant difference from the control (LFD) group within a sex ($P \leq 0.001$). Data are expressed as means ± SEM (n = 8–12/group/sex).

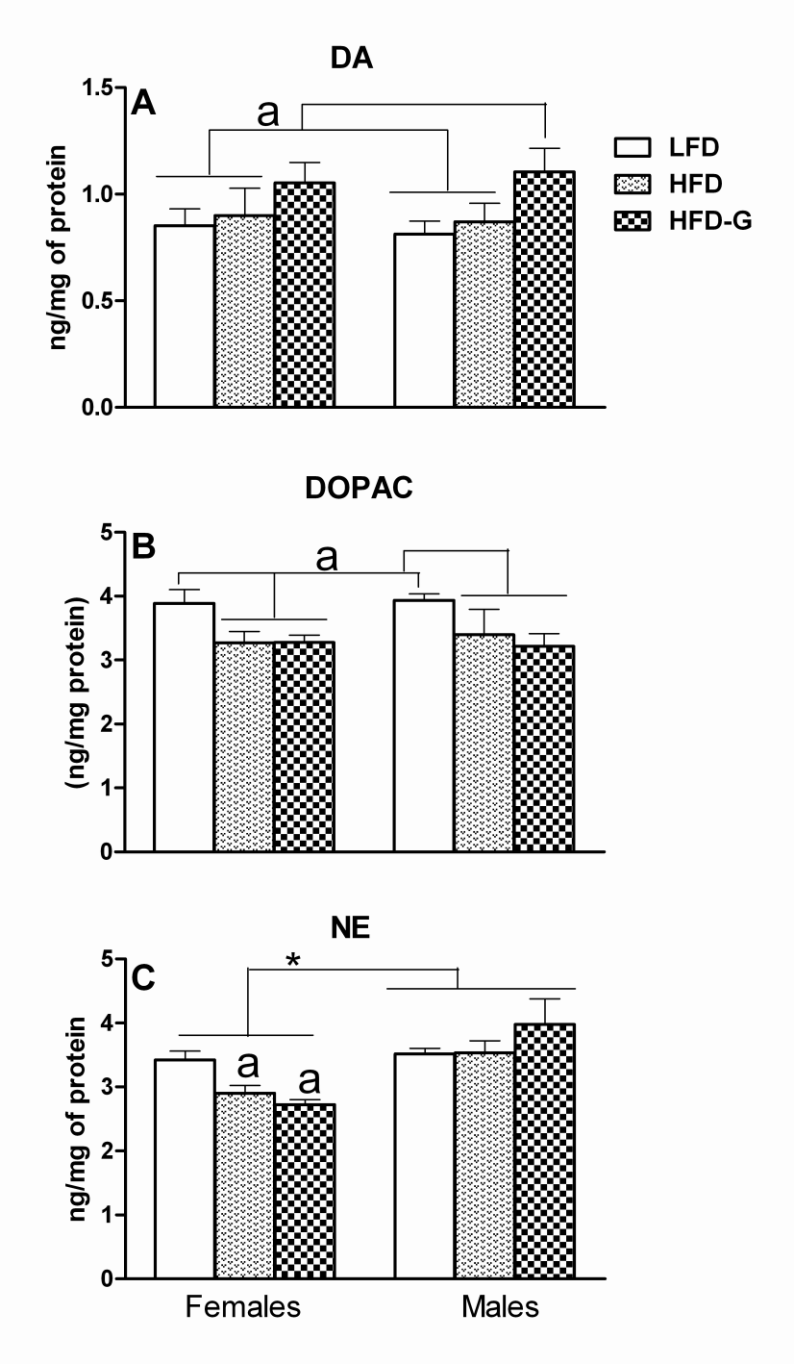

Figure 7.4. Dopamine (DA; A), dihydroxyphenylacetic acid (DOPAC; B) and norepinephrine (NE; C) in the prefrontal cortex (PFC) of PND35 offspring from dams fed a high-fat diet (HFD) or control (low-fat diet; LFD) 6 weeks prior to pregnancy and throughout gestation and lactation. A subset of HFD-fed dams was given a glycan (G) conjugate. * indicates significant sex

difference ($P < 0.05$). a" indicates significant main effect of treatment ($P \leq 0.001$). Data are expressed as means ± SEM (n = 5-6/group/sex).

CHAPTER 8

OVERALL SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Overall summary

It is estimated that 1 billion people worldwide are affected by neurological diseases, which is an enormous disease burden (Gourie-Devi, 2008). Several environmental factors can contribute significantly to the risk of developing neurological disorders of neurodegenerative or neurodevelopmental nature (Brown et al., 2005, van Loo and Martens, 2007). One common mechanism they use is activation of resident microglial populations in the CNS and subsequent release of neuroinflammatory mediators leading to chronic neuroinflammation (Frank-Cannon et al., 2009, El-Ansary and Al-Ayadhi, 2012). Besides neuroinflammation, immune dysfunction outside the CNS, i.e., peripheral inflammation and associated metabolic derangements is also increasingly recognized to modulate the disease progression and neuropathology of neurological disorders. The goals of this dissertation project were to determine the neurological/metabolic consequences induced by environmental factors, namely manganese (Mn), lipopolysaccharide (LPS) and high-fat diet (HFD) as well as to evaluate the role played by inflammation (central or peripheral) in inducing the neuronal and/or metabolic dysregulation.

To accomplish these goals, six Specific Aims were conducted. Aim 1 determined the molecular, neurochemical and behavioral effects of subchronic drinking water Mn exposure in adult male mice. Aim 2 evaluated specific neurochemical responses, molecular changes and behavioral alterations, including their persistency, in adult male mice with low-grade chronic inflammation caused by repeated peripheral LPS administration. Aim 3 investigated the

relatively early central (neurochemical, behavioral and electrophysiological) effects of HFD consumption in adult female mice. Aim 4 evaluated the metabolic/inflammatory effects induced by relatively short period of HFD consumption in adult female mice. Aim 5 determined the time-dependent central (behavioral) and peripheral (metabolic and inflammatory) effects of chronic HFD consumption in adult female mice. Aim 6 investigated the neurochemical and autism spectrum disorder (ASD)-like behavioral deficits of the offspring that are caused by maternal HFD consumption and also assessed the efficacy of maternal-only administration of a potent anti-inflammatory glycan in ameliorating offspring's neuropathology caused by maternal HFD consumption. Major findings from the completion of these Specific Aims are illustrated in Fig. 8.1 and can be summarized as follows.

One of the major findings from Aim 1 is that low-level Mn exposure via the drinking water causes increased brain Mn deposition and produces motor and emotional deficits associated with altered monoamine homeostasis and region-specific glial activation in the brain. Previous studies in rodents have demonstrated increased brain Mn deposition following Mn overexposure via different (non-drinking water) routes (Dorman et al., 2006b, Finkelstein et al., 2008); our findings have shown that lower levels of Mn in the drinking water can result in a region and time-independent increase in brain Mn deposition. Importantly, our findings suggest that MRI can be used successfully in mice to determine longitudinal brain Mn deposition following DW exposure and that MRI can be employed as a useful tool in future analyses of brain Mn deposition dynamics. Consistent with previous findings, Mn-induced locomotor impairments observed in our study occurred in the absence of alterations in striatal DA levels or TH/D2DR mRNA (nigra) or protein levels (striatum) (Witholt et al., 2000, Guilarte et al., 2006a). Thus, in spite of the differences in exposure routes and duration, available studies,

including ours, consistently support the hypothesis that low-level Mn exposure does not impact dopaminergic terminals structurally, but may produce its neurotoxic effects by causing functional deficits of the nigrostriatal dopaminergic system (Guilarte et al., 2006a, Guilarte et al., 2008a). In this study, Mn altered striatal serotonergic activity, suggesting that at lower levels of Mn exposure, 5-HT signaling in the striatum is either more sensitive or is affected earlier than DA signaling. Finally, Mn-induced increase in astrocytic activation (substantia nigra pars reticulata) and subsequent neurological deficits, including locomotor and emotional alterations demonstrated in our study supports the mounting evidence that Mn can also indirectly mediate its neurotoxic effect by producing activation of glial cells and associated release of neuroinflammatory mediators in the basal ganglia.

In Aim 2, we evaluated the time-dependent changes in motor and non-motor (emotional) behavioral domains in male mice following chronic LPS-induced peripheral administration. We found that chronic LPS administration produced early hypoactivity, which was later followed by hyperactivity that persisted even long after the termination of the LPS treatment. These results suggest that chronic LPS administration causes locomotor deficits, with effects being biphasic and long-lasting. Of note, the initial hypoactivity demonstrated by LPS-treated mice occurred in the absence of DA imbalance in prefrontal cortex, striatum and hippocampus. Similar to the effects on locomotor activity, chronic LPS treatment produced a biphasic effect on emotional functions, with mice exhibiting initial increased anxiety that was later followed by decreased anxiety after the discontinuation of chronic low-dose LPS treatment. Another important finding from this study is that prolonged low-dose LPS administration produced chronic depressive-like behavior that was apparent early on and it persisted even after the termination of the LPS treatment, indicating the ability of chronic peripheral inflammation to induce a long-lasting

depressogenic effect. This finding is consistent with earlier studies in humans that found worsening of depressive symptoms even after the termination of chronic inflammation induced by IFN-α therapy via enhanced production of pro-inflammatory cytokines and subsequent downregulation of serotonin synthesis (Janssen et al., 1994, Hoyo-Becerra et al., 2014). The locomotor and emotional deficits observed during the LPS treatment were associated with an increase in microglial activation in the substantia nigra; however, LPS-induced nigral glia activation was not accompanied with an increased pro-inflammatory cytokine (IL-6 and IL-1β) output. As we only assessed IL-6 and IL-1β, and only in the substantia nigra, other microglial derived cytokines, such as IL-2 and IFN-α (Meyers, 1999, Moreau, 2002) or inflammation in other brain regions, such as the hippocampus or PFC (Drevets, 2000, Koenigs and Grafman, 2009) might have been involved in the depressive-like behavior induced by chronic LPS.

The neurological deficits and peripheral (metabolic/inflammatory) effects induced by relatively short-term HFD consumption, was respectively assessed in Aims 3 and 4. In Aim 5, we evaluated the time-dependent progression in the central (behavioral) and peripheral (metabolic/inflammatory) effects induced by chronic HFD consumption. Although, 5-6 weeks, 20-22 weeks and 32-36 weeks have been indicated as initial, intermediate and late stages of LFD or HFD feeding in the respective chapters, for the sake of simplicity, here, we are referring the initial stage as 6 weeks, intermediate stage as 22 weeks and late stage as 36 weeks. We found that the duration of HFD exposure had a profound influence on the body weight gain in female mice. Although the body weight of mice was significantly increased as early as 6 weeks (Aim 4) after HFD feeding, they exhibited an obese phenotype after prolonged HFD consumption for 36 weeks (Aim 6), characterized by both visceral and intra-organ fat accumulation. HFD-induced impairments in glucose homeostasis have been demonstrated by several animal studies

(Chalkiadaki and Guarente, 2012, Cano et al., 2013, Gao et al., 2014); however data regarding the time-dependent changes in the glycemic profile during HFD consumption are limited. HFD feeding even for a relatively short period impaired glucose tolerance in male mice that persisted throughout the experimental period (Raddatz et al., 2011, Dou et al., 2014, Williams et al., 2014). Consistent with these findings, we found that the female mice were glucose intolerant within relatively short period (6 weeks; Aim 5) of HFD intake and remained hyperglycemic throughout the experimental period. The impairment in glucose tolerance was greatest in obese mice that were fed HFD for 36 weeks (Aim 6). Taken together, our results are in agreement with previous studies which reported that obesity induced by a HFD can promote glucose intolerance in a short period of time and that these effects are prolonged. Besides being glucose intolerant, mice were insensitive to insulin after short-term (6 weeks; Aim 5) HFD feeding as demonstrated elsewhere (Chalkiadaki and Guarente, 2012, Cano et al., 2013). Interestingly, we found that mice remained insulin resistant after prolonged HFD consumption for 22 (Aim 6), but not after 36 weeks (Aim 6). The lack of HFD effect after 36 weeks (Aim 6) may be due to an age-dependent, diet-independent loss in insulin sensitivity, which masks the effects of HFD consumption in older animals.

Besides glucose and insulin dyshomeostasis, we found that 6 weeks (Aim 5) of HFD intake increased inflammation and altered lipid metabolism in the liver of female mice. However, the inflammatory/metabolic markers were unchanged in the adipose tissue at this time point. By 22 weeks (Aim 6) of continued HFD consumption, hepatic mRNA expression of CD36 (involved in inflammation and fatty acid transport) was significantly elevated, while others involved in inflammation and lipid metabolism (PPARs, TNFα, IL-6 and the acute phase protein, Hp) was relatively unaffected. Increased CD36 expression and associated increase in

inflammatory signaling has been also attributed to the development of insulin resistance in humans with type 2 diabetes as well as in animals fed HFD (Luiken et al., 2001, Koonen et al., 2007, Sheedfar et al., 2014). Hence the significantly impaired glucose tolerance and insulin resistance observed after 22 weeks (Aim 6) in this study could be a an end result of the altered inflammatory response associated with the HFD-induced hepatic CD36 mRNA upregulation. Interestingly, with continued HFD feeding for additional 14 weeks (a total of 36 weeks; Aim 6), the impaired glucose homeostasis was associated with pronounced alterations in hepatic inflammatory/lipid profile. Overall, our data indicates a biphasic pattern of the HFD-induced peripheral inflammatory response, with the initial inflammatory/lipid metabolic changes becoming relatively less noticeable during the intermediate stage of HFD feeding, but later on, reappearing with continued chronic HFD feeding.

HFD-induced neurobehavioral (motor and emotional) alterations have been demonstrated by multiple animal studies (Chudasama and Bhatt, 2009, Sharma et al., 2012). However majority of these studies have assessed the neurological deficits after a single exposure to HFD; experimental data regarding the time-dependent progression of HFD-induced neurobehavioral deficits are extremely limited. We found that HFD fed mice were hyperactive after 6 weeks (Aim 4) of HFD feeding. However with prolonged HFD consumption (22 weeks; Aim 6), the initially observed locomotor deficit was no longer evident. Intriguingly, HFD feeding for several more weeks (total 36 weeks; Aim 6) resulted in hypoactivity which coincided with marked adiposity. Taken together, our results suggest that the duration of HFD exposure has a significant influence on the locomotor activity, with mice showing a biphasic pattern characterized by initial increased locomotor activity that disappears over time and further pursuing an opposite trend (decreased locomotion) with continued HFD consumption. Besides having locomotor deficits,

the HFD-fed mice were anxious after 6 weeks (Aim 4) of HFD feeding. However, this initial increased anxiety level did not persist with continued HFD exposure, suggesting a transient response. In this regard, studies in male rodents have reported that HFD can act as an anxiogenic or as an anxiolytic agent depending on the duration of HFD exposure (Del Rosario et al., 2012). Specifically, in male rodents, short-term HFD feeding has been found to decrease anxiety (Prasad and Prasad, 1996, Alsio et al., 2009), whereas, long-term HFD consumption increases it (Buchenauer et al., 2009, Del Rosario et al., 2012). In comparison, our data suggest that the female response to HFD in terms of anxiety may differ from the one in males.

In specific aim 6, we determined the effect of maternal HFD consumption on the neurobehavioral/neurochemical deficits in PND 35 offspring and also evaluated the ability of the maternal-only administration of an anti-inflammatory glycan to reduce the maternal HFD-induced neurological deficits in the offspring. Within females, PND35 offspring of HFD-fed dams treated with anti-inflammatory glycan were more active in the open field test. Similarly, PND35 offspring also exhibited a sex-specific effect with respect to the time spent in the center and corner of the open field arena. Maternal HFD consumption resulted in increased anxiety in females, but not male offspring, an effect that was reversed by maternal glycan administration. Taken together, our data indicate that HFD consumption before and during pregnancy negatively impacts the emotional behavior of PND35 offspring in a sex-specific manner and the maternal glycan treatment can normalize the altered emotional response. Unlike the sex-specific effect of maternal HFD on emotional function, the cognitive behavior of the PND 35 offspring was not affected by maternal HFD consumption. However, intriguingly, both female and male offspring of HFD dams treated with the glycan conjugate showed a greater tendency exploring the novel

object in the NOR test, which is suggestive of a potential role of maternal glycan treatment in enhancing the offspring's recognition memory.

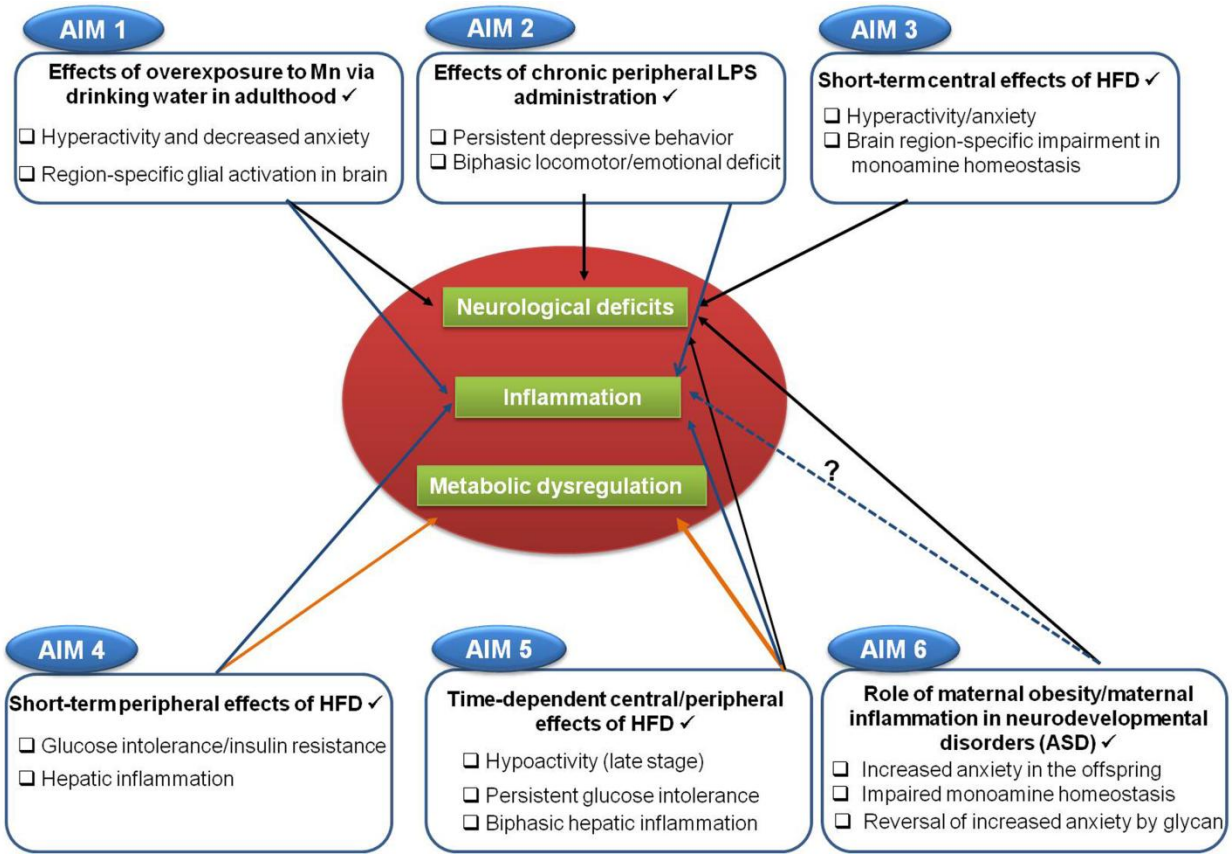

Figure 8.1. Overall summary. Black, orange and blue arrows indicate neurological deficits, metabolic derangements, and inflammation, respectively. Solid arrows represent confirmed major findings from each aim with the differences in the thickness of arrows representing the variations in the robustness of the effects observed; thicker arrows indicate persistent robust effect, while thinner arrows indicate relatively transient effects. Question mark on dashed black arrow indicates potential influences for which specific experimental data are currently lacking.

Conclusions

In this dissertation research, we studied the role of central/peripheral inflammation in the development of neurological deficits/metabolic dysregulation caused by three disparate environmental triggers: including manganese (Mn; neurotoxic metal), lipopolysaccharide (LPS; bacterial infection mimic) or high-fat diet (HFD) in C57BL/6 mice. Regardless of the exposure routes or durations, we observed significant locomotor abnormalities in mice that were accompanied with the monoamine homeostasis perturbations following exposure to Mn, LPS, or HFD. However, it is noteworthy that the manifestation of observed locomotor alterations varied with each of the environmental triggers, with mice showing increased locomotor activity following short-term Mn or HFD treatment and decreased locomotor activity after short-term LPS administration. Intriguingly, chronic LPS or HFD treatment produced a biphasic locomotor activity pattern, indicative of environmental trigger-independent time-dependent shift of the locomotor deficits. Besides locomotor deficits, we found emotional abnormalities in adult mice exposed to Mn, HFD, or LPS, as well as in adolescent offspring developmentally exposed to HFD. Overall, our data showed that in mice, short- or long-term exposure to disparate environmental triggers can induce overlapping neurological dysfunctions characterized by locomotor or emotional abnormalities.

Metabolic dysfunction associated with inflammation has been demonstrated to be comorbid with neurological disorders. Hence, we investigated whether the neurological deficits induced by environmental triggers are also accompanied with peripheral metabolic abnormalities. We found that besides inducing neurological deficits, short-term dietary fat intake also induces peripheral inflammation and metabolic dysfunctions, with some of the abnormalities

persisting and being augmented with continued dietary fat consumption, while others becoming unnoticeable due to the age-related changes.

Most intriguingly, we found that the neurological deficits and/or metabolic dysregulation observed in mice following exposure to different environmental triggers were accompanied by altered inflammatory responses either within in the brain or in the periphery; short-term Mn drinking water exposure produced brain region-specific astrocytic activation, while chronic LPS exposure resulted in time-dependent microglia and astrocytic activation. Interestingly, the observed metabolic dysregulation following short- or long-term exposure to HFD consumption was accompanied by peripheral (hepatic) inflammation. In light of the overlap in the central/peripheral inflammation we observed and the noticeable commonalities among the neurological/metabolic outcomes caused by the three different environmental agents, it is conceivable that the inflammation that occurs either within in the brain or periphery act as a convergence point for disparate environmental triggers to induce adverse health outcomes.

Taken together, this dissertation research identifies inflammation (central or peripheral) as the common denominator for the development of the neurological or metabolic abnormalities in exposed animals to disparate environmental triggers and hence our findings emphasize the need to devise novel inflammation-targeted therapeutic strategies, including during neurodevelopment that can help in the treatment/prevention of inflammation-associated CNS and metabolic disorders.

Unanswered questions and future directions

The sub-chronic low-dose Mn drinking water study demonstrated neurological deficits and brain region specific glial activation in exposed adult animals. In order to make these

findings more pertinent to the general population, future studies could use a long-term low-dose drinking water exposure paradigm.

We observed long-lasting emotional alteration (depression), region-specific neurochemical changes and altered splenic monoamine homeostasis following chronic LPS-induced peripheral inflammation; however, these changes did not parallel well with the inflammatory status in the brain region we assessed (substantia nigra). Hence, whether the observed chronic depressive behavior and altered central/peripheral monoamine homeostasis is a consequence of an increased neuroinflammatory response in other brain regions that are prominently implicated in depression, such as prefrontal cortex or hippocampus needs to be determined in future. Additionally, the involvement of microglial-derived cytokines in meditating the chronic LPS-induced neuroinflammation (other than what we assessed: IL-1β and IL-6), such as IL-2 or IFN-α needs to be ascertained in future.

The present dietary study demonstrated increased anxiety in the initial stages of HFD feeding in female mice, which did not persist towards the later stages of HFD consumption. These findings are not in line with earlier studies demonstrating greater anxiety level in experimental animals with continued HFD consumption (Heyward et al., 2012, Sharma and Fulton, 2013). Therefore future studies using more sensitive behavioral tests for anxiety, such as elevated plus maze test are necessary to confirm our findings of short- or long-term effect of HFD on anxiety response in females. In contrast to the hyperactivity observed during the initial stage, mice were hypoactive during the late stage of HFD feeding. Although, the latter could be explained by the observed excessive adiposity and associated peripheral (hepatic) inflammation and dysregulated glucose metabolism, the involvement of other key insulin-sensitive peripheral tissues, such as muscle or adipose tissue in mediating the neurobehavioral changes needs to be

determined. In addition, the influence of peripheral (hepatic) inflammation in altering the brain monoamines, involved in locomotion such as dopamine, needs to be ascertained. Finally, the involvement of central inflammation, i.e., whether the chronic HFD-induced behavioral or neurochemical (if any) effects is also associated with an increased neuroinflammatory response in brain regions, implicated in locomotion, such as striatum or substantia nigra, needs to evaluated.

The present maternal HFD study revealed sex-specific ASD-like behavioral (increased anxiety) alterations in the adolescent offspring which were reversed by the maternal administration of an anti-inflammatory glycan. Whether maternal HFD-induced neurological deficits observed in PND 35 offspring will persist to the adulthood (PND 70) and whether maternal glycan administration will continue to exert an ameliorating effect on altered adulthood behavior needs to be analyzed further. By evaluating maternal HFD's neurological effects on offspring at multiple time points, we can identify the most sensitive postnatal developmental endpoint of maternal HFD exposure and thereby initiate effective therapies targeting the critical period of neurological alterations. Also, further studies are required to understand the detailed mechanism by which diet-induced maternal obesity and maternal inflammation leads to ASD-like behavioral phenotype in the offspring. Importantly, investigation of the exact mechanistic role of glycan in diminishing the ASD-relevant behavioral phenotype in offspring, i.e., whether the protective effects of glycan are due to alteration of the maternal inflammatory environment or due to a direct influence in the developing offspring needs to be analyzed in future.

In summary, the present dissertation research provides comprehensive information of the neurological impairments and/or metabolic derangements caused by different environmental

factors that are inflammatory in nature. However there are still some important data gaps that need to be addressed in future studies.

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APPENDIX A

SUPPLEMENTAL FIGURE FOR CHAPTER 2

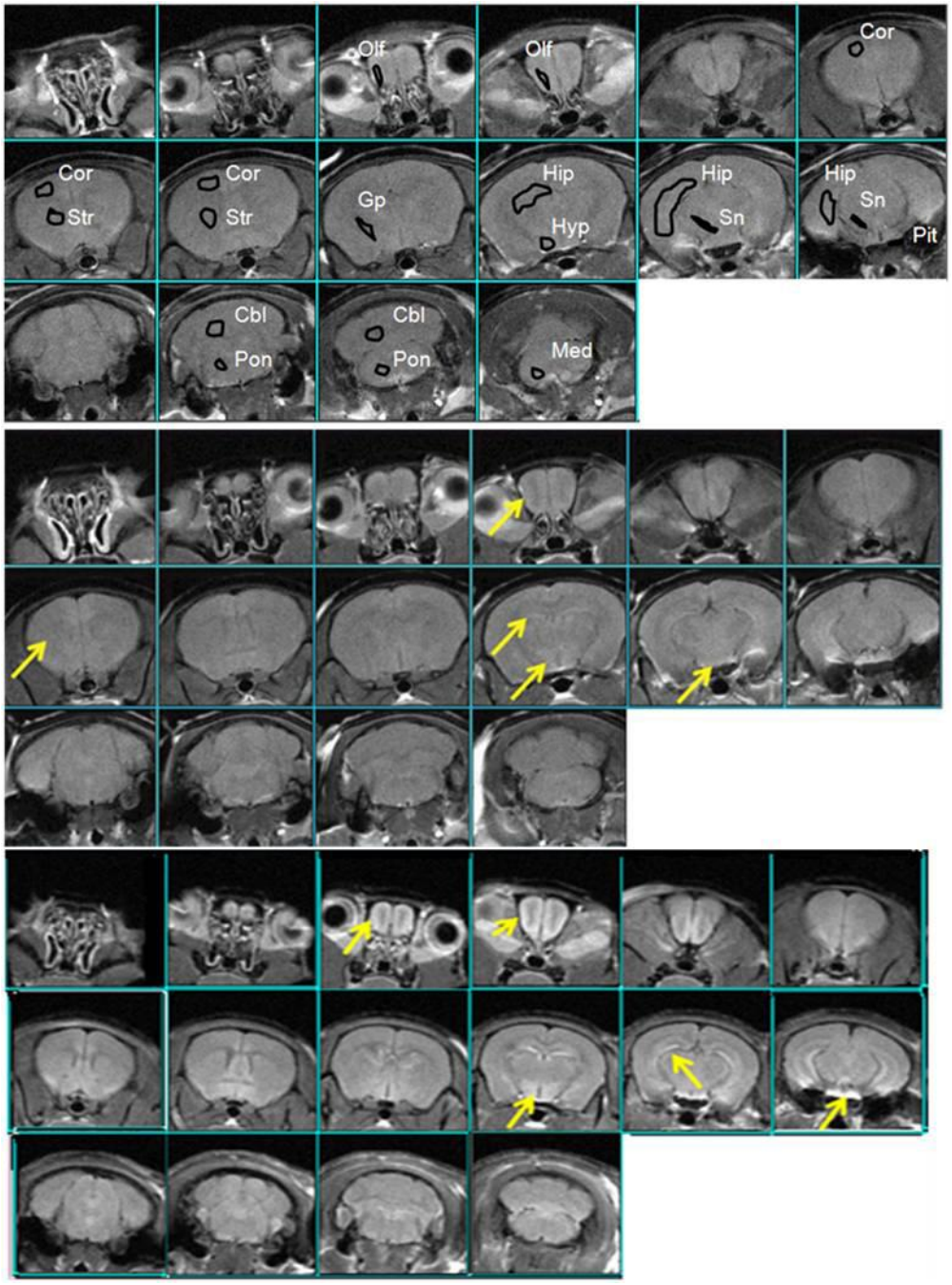

Figure A1. Coronal T1-weighted 2D MRI images. Regions of Interest (ROIs)- olfactory bulb (Olf), cortex (Cor), striatum (Str), globus pallidus (Gp), hippocampus (Hip), hypothalamus (Hyp), substantia nigra (Sn), pituitary gland (Pit), cerebellum (Cbl), pons (Pon) and medulla (Med) on each slice of control mouse brain (top) acquired after 5 week exposure to a vehicle (0.4 g Na/l in deionized water) are labeled. Representative ROIs of a Mn-exposed mouse brain (middle) acquired after 5 week exposure to 0.4 g Mn/l in deionized water and a positive control mouse brain (bottom) acquired 24 h after Mn SC injection (50 mg/kg) indicative of Mn accumulation are marked with yellow arrows.

APPENDIX B

SUPPLEMENTAL TABLE FOR CHAPTER 5

Table B1. List of primers used for qPCR.

Genes	Taqman reference number	
TNFα*	Mm00443258_m1	
IL-6 *	Mm00446190_m1	
Hp*	Mm00516884_m1	
PPARα*	Mm00440939_m1	
PPARγ*	Mm01184322_m1	
	Forward primer	Reverse primer
IL-6$	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
CD36$	TCCTCTGACATTTGCAGGTCTATC	AAAGGCATTGGCTGGAAGAA
PPARα$	AACATCGAGTGTCGAATATGTGG	CCGAATAGTTCGCCGAAAGAA
PPARγ$	CCACCAACTTCGGAATCAGCT	TTTGTGGATCCGGCAGTTAAGA
IRS1$	GGTGCTGCAGCTGATGAATA	CGAGATCTCCGAGTCAGTCC
18S$	GGGAGCCTGAGAAACGGC	GGGTCGGGAGTGGGTAATTT

* indicates primers from Life Technologies (Carlsbad, CA)
$ indicates primers from Real Time Primers (Elkins park, PA)