THE INFLUENCE OF DIET AND EXERCISE ON EARLY LIFE STRESS-INDUCED CO-MORBID DISORDERS IN MICE

By

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Abstract

Individuals with functional pain disorders such as irritable bowel syndrome, interstitial cystitis/painful bladder syndrome, migraine, and fibromyalgia often present with symptoms of or are diagnosed with more than one disorder. Furthermore, these patients with co-morbid pain disorders are also more likely to present with mood disorders, including depression and anxiety, as well as with obesity-related metabolic syndrome. Exposure to stress or adversity early in life increases the risk of developing co-morbid chronic pain, mood, and obesity-related metabolic syndrome in adulthood. This may be due to altered functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response, alters our perception of pain, and has downstream metabolic effects. The overall goals of this dissertation are to expand our studies on the many consequences of early life stress and investigate potential non-pharmacological treatment options for patients with these disorders including exercise and an anti-inflammatory diet (AID). To do this, I use a mouse model of early life stress, neonatal maternal separation (NMS), which exhibits increased urogenital sensitivity as well as altered limbic regulation of the HPA axis. This dissertation investigates the susceptibility of female NMS and non-stressed (naïve) sedentary (Sed) and exercised (Ex) mice to evoked migraine like-behaviors and the susceptibility of male NMS- and naïve-Sed and –Ex mice to the development of high-fat/high-sucrose diet-induced obesity-related metabolic syndrome. I focus on migraine and obesity-related metabolic syndrome due to the high clinical incidence of these disorders in chronic urogenital pain patients. Finally, I evaluate the effect of AID on putative early life stress0induced co-morbid disorders in female and male mice.

This work provides insight into the significant influence that environmental factors such as early life stress, diet, and exercise have on the development of chronic disorders as well as the complex interactions of these factors. Additionally, this research reveals sex differences in stress, pain, and metabolic pathways. The incidence of most chronic disorders differs in females and males, which makes sex another important factor to consider when studying these health conditions and potential therapeutic treatments. These studies are novel because each combines multiple environmental factors in an attempt to mimic human lifestyle choices to study chronic pain disorders, obesity-related metabolic syndrome, and co-morbidity.

Dedication

This dissertation is dedicated to my Grandma Cookie, whose strength, love, and constant smile still inspire me every day.

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Table of Contents

Chapter I: Introduction to dissertation

Parts of this chapter were previously published as an open access article (CC-BY) and reprinted here with additional, never-before published text. Eller-Smith, OC, Nicol, AL, Christianson, JA. Potential Mechanisms Underlying Centralized Pain and Emerging Therapeutics Interventions. *Frontiers in Cellular Neuroscience.* eCollection 2018 (1).

1.1 Early life stress

Stress is defined as an alteration in homeostasis that can be caused by a psychological, environmental, or physiological threat (2). It has long been known to affect the perception of pain, in both acute and chronic settings. Acute stress is crucial for the survival of an organism: individuals are alerted to dangerous and life-threatening situations and can subsequently respond to the perceived or anticipated stress. However, stress can become detrimental when experienced in the long-term, especially early in life such as when children experience maltreatment, neglect, physical or emotional abuse, and witnessing parental turmoil. Early life stress is associated with the development of co-morbid chronic pain (e.g. migraine, fibromyalgia, and chronic pelvic pain), mood, and metabolic disorders in adulthood (3-8). This has been attributed to improper functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response and plays a role in pain perception (9, 10). Unfortunately, the prevalence rate of childhood maltreatment is shockingly high and is increasing (11). In the U.S Department of Health and Human services annual Child Maltreatment report from 2016, 676,000 children were victims of maltreatment with 74.8% of these children suffering from neglect, 18.2% being physically abused, and 8.5% sexually abused. The age group that showed the greatest maltreatment rate per 1000 children were those under 1-year old (12). This finding is particularly distressing because the HPA axis is not fully developed when children are born and therefore stressful events at this stage of development can permanently affect both the regulation and output of the stress response system, including its downstream effects on nociceptive processing and metabolic regulation in the periphery (13, 14).

1.2 The Hypothalamic Pituitary Adrenal (HPA) Axis

Under normal conditions (schematized in Figure 1.1), an acute stressor will signal the paraventricular nucleus (PVN) of the hypothalamus to release corticotropin-releasing factor (CRF) and arginine vasopressin into the hypophyseal portal veins, which causes the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). Circulating ACTH signals the adrenal cortex to release glucocorticoids (GCs; cortisol in humans and corticosterone in rodents) that have downstream metabolic effects including glucose and fat metabolism, control of the cardiovascular system, and regulation of the immune response (15-17). A negative feedback loop is established to turn off activation of the HPA axis by suppressing the production of CRF and ACTH upon cessation of the initial stressor (18, 19).

Regulation of the HPA axis is driven in part by receptors that bind CRF family members or GCs and are located at every level of the HPA axis as well as in regulatory limbic regions. Corticotropin-releasing factor receptor 1 (CRF_1) and 2 (CRF_2) work in opposition to one another to enhance and reduce HPA output, respectively (20). This has been demonstrated by studies that have deleted CRF_1 or CRF_2 and measured anxiety- like behaviors in rodents. Deletion of CRF_1 results in a significant decrease in anxiety-like behaviors (21) while deleting CRF_2 results in a significant increase in these behaviors (22). GC-mediated regulation occurs via two receptors, mineralocorticoid receptor (MR) and GC receptor (GR), which are slow acting and function both as transcriptional regulators (23) and through GC-mediated retrograde endocannabinoid release from parvocellular neurons, which suppresses the release of excitatory glutamatergic molecules from pre-synaptic terminals and subsequently inhibits the

Figure 1.1

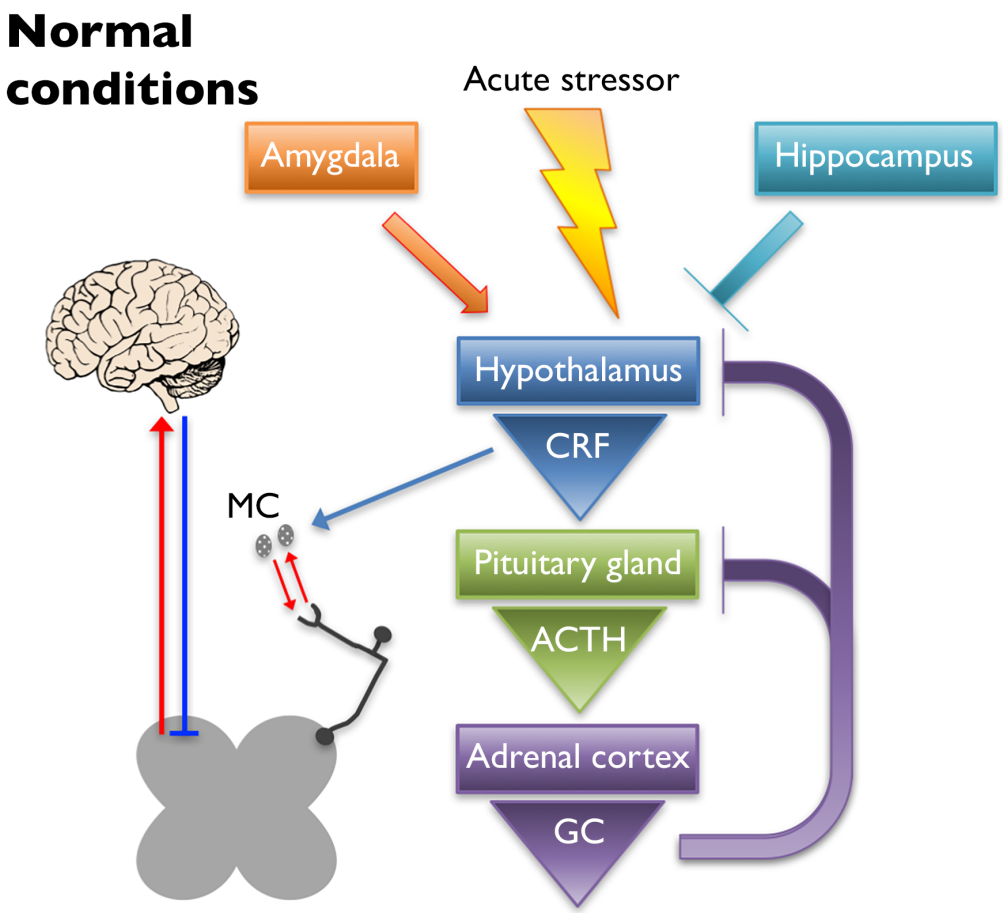
Normal conditions
Acute stressor
Amygdala
Hippocampus
Hypothalamus
CRF
MC
Pituitary gland
ACTH
Adrenal cortex
GC

Figure 1.1 Under normal conditions, an acute stressor will signal the paraventricular nucleus of the hypothalamus to release corticotropin-releasing factor (CRF) into the hypophysial portal veins, which causes the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). Circulating ACTH signals the adrenal cortex to release GCs (GC) that have downstream metabolic effects. A negative feedback loop is established to turn off activation of the HPA axis by suppressing the production of CRF and ACTH upon cessation of the initial stressor. The hippocampus and the amygdala play inhibitory and excitatory roles in regulation of the HPA axis, respectively. CRF released upon HPA axis activation also has peripheral effects. Mast cells (MC) can become activated by CRF, causing the release of cytokines and growth factors that have reciprocal interactions with peripheral nociceptors. Nociceptor activation signals through the dorsal horn of the spinal cord, leading to activation of supraspinal somatosensory brain regions. The descending pain pathway also plays a role in the regulation of painful experiences.

hypothalamic release of CRF (24).

Limbic structures including the hippocampus, amygdala, and prefrontal cortex, assist in resetting the HPA axis following a stressful event, as well as regulate its tone. Neural projections from the hippocampus and prefrontal cortex are mostly glutamatergic and synapse on GABAergic interneurons within the PVN, thereby dampening HPA axis activation (16, 25). Lesioning the hippocampus leads to increased stress-induced HPA axis activation as evidenced by increased CRF immunoreactivity in the PVN, GC hypersecretion, and behavioral evidence of heightened anxiety in rats (26). Disruption of GR expression in the forebrain of mice resulted in heightened stress-induced locomotor activity and acute stress exposure increased ACTH secretion, plasma corticosterone levels, and CRF expression in the PVN (27). The amygdala works to activate the HPA axis through disinhibition, sending GABAergic projections to the GABAergic neurons of the PVN (16, 25). Administration of corticosterone to the amygdala in rats resulted in an increase in anxiety-like behaviors as well as somatic and visceral hypersensitivity (28). These observations were likely caused by GR and/or MR signaling as it was shown that repeated exposure to water avoidance stress (WAS) induced an increase in plasma corticosterone and visceral hypersensitivity that was inhibited in rats that received a GR (mifepristone) or MR (spironolactone) antagonist applied to the amygdala (29). Further evidence that the amygdala plays a role in HPA axis regulation comes from a study where either direct application of corticosterone onto the amygdala or exposure to WAS increased CRF expression in the amygdala, which coincided with visceral and somatic hypersensitivity (30). These effects were attenuated after engineered reduction of CRF gene expression in the central amygdala. Taken together, these studies highlight the

importance of inhibition/activation coming from the limbic structures, which plays a significant role in regulating normal stress responses from the HPA axis and ultimately affects the perception of pain.

1.3 Downstream effects of the HPA axis

1.3.1 Pain signaling

While activation of the HPA axis does not directly initiate pain signaling, downstream mediators can influence the neuroimmune status of peripheral tissues and increase nociceptive tone. In human tissue, CRF_1 has been observed in adrenal tissue, adipose, gonads, endometrium, myometrium, placenta, skin, spleen, and various immune cells; whereas CRF_2 has been found in skin and all three types of muscle tissue. Immunoreactivity for both CRF receptors has been observed in rat colon, primarily in the mucosal layer, inflammatory cells, and enteric innervation for CRF_1 and on goblet cells and in submucosal blood vessels for CRF_2 (31). CRF signaling influences both contractility (32) and transepithelial resistance (33) of the gastrointestinal tract. Feline urothelial cells express functionally-active CRF_1 and CRF_2, as well as their intrinsic ligands CRF and Ucn1 (34). The naturally-occurring feline interstitial cystitis model shows altered CRF signaling in the urothelium, indicating a potential role for CRF in the etiology of IC.

Exposure to chronic stress causes a decrease in GR expression in the hippocampus, which reduces the normal descending inhibition onto the PVN and therefore increases CRF release and propagates GC production (15, 16). In the amygdala, the GCs released upon chronic stress exposure activate GR receptors, which increase CRF release in the PVN and also GC production (35).

Mast cells (MCs) are a critical part of the innate immune system and are highly responsive to activation of the HPA axis, as they express five isoforms of the CRF_1 receptor, a single isoform of the CRF_2 receptor, and contain one of the largest peripheral stores of CRF (36). They are found in highly vascularized tissues, most predominantly in areas with direct contact to the environment: skin, airway, gastrointestinal and urinary tracts. Mast cells are increased in a number of chronic pain disorders including irritable bowel syndrome (37), interstitial cystitis (38), migraine (39), and fibromyalgia (40). They are derived from hematopoietic stem cells, circulate in the blood stream as immature cells, and mature upon entry into peripheral tissues (41, 42). Their differentiation depends on the presence of cytokines and growth factors. The c-kit tyrosine kinase receptor and its ligand stem cell factor (SCF) are important in the migration and distribution of MC precursors (43). MC are filled with granules that contain histamine, heparin, tryptase, as well as other proteases and cytokines (44). They are activated by immune and non-immune signals, including endogenous neuropeptides such as CRF and substance P (SP), and cause hypersensitivity reactions (45, 46). Although the hallmark form of MC activation is evidenced by the partial or complete release of granular stores, stress-activated release of cytokines and growth factors from MC can occur in the absence of degranulation (36, 46).

MCs are observed adjacent to unmyelinated nerves throughout the body, including the skin (47), trachea (48), and intestine (49) as well as in direct contact with nerve fibers in the dura mater (50). These afferents express receptors involved in nociception, including transient receptor potential vanilloid 1 (TRPV1), transient receptor potential ankyrin 1 (TRPA1), and protease-activated receptor 2 (PAR2) (51-53). PAR2

is a G protein-coupled receptor activated by MC tryptase, trypsin, and coagulation protease FVIIa, and FXa (54). Activation of PAR2 initiates downstream sensitization of TRPV1 and TRPA1 through several mechanisms including phosphorylation by protein kinase C (PKC) (55) and protein kinase A (PKA) (56), and TRPV1 channel release from phosphatidylinositol 4,5-bisphosphate (PIP2)- dependent inhibition through phospholipase C (PLC) activation (57). All three receptors are expressed on neurons with cell bodies in the dorsal root ganglia (DRG), trigeminal ganglia (TG), and nodose ganglia (58-60) and on peripheral projections to the skin and deeper tissues, such as muscle and viscera (61, 62). Both TRPV1 and TRPA1 have been shown to be involved, if not required, for the generation of visceral hypersensitivity (63-66). Increased activation of these receptors enhances pain-related afferent input to the central nervous system; however, they also generate and maintain peripheral neurogenic inflammation by releasing neuropeptides, including SP and CGRP, which perpetuates inflammatory mediator release in the proximate milieu. Therefore, increased peripheral CRF release due to dysregulated HPA axis activity (schematized in Figure 1.2) could result in MC activation and, in turn, sensitization of nearby sensory nerve endings and lowered pain thresholds.

1.3.2 Glucocorticoid influence on metabolism

Excessive GC secretion affects almost every tissue in the body due to the ubiquitous expression of GC cytosolic receptors (67). After binding to their receptor, GCs dimerize and translocate to the nucleus and subsequently influence gene transcription (68). Therefore, increased GCs are associated with deleterious changes in many different systems including increased visceral adiposity, myopathy, insulin

Figure 1.2

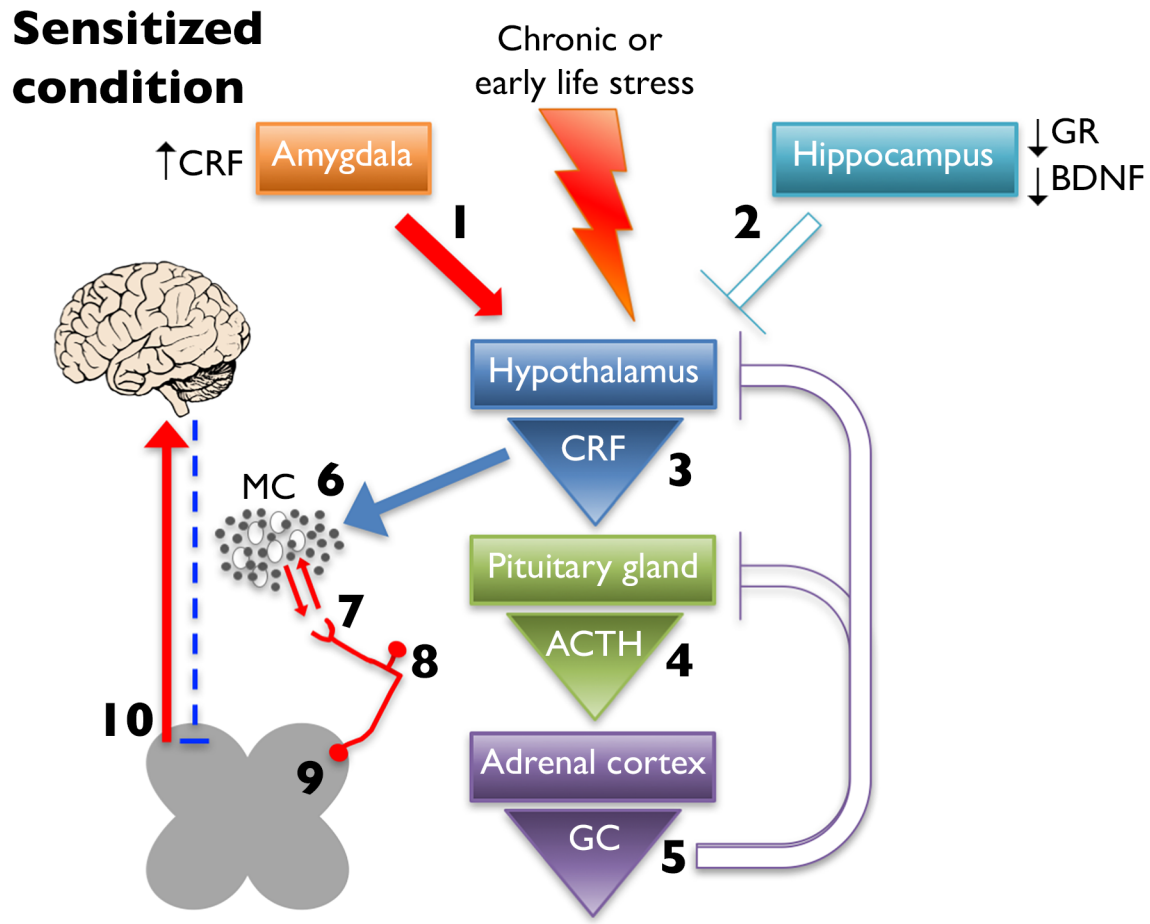
Sensitized condition
Chronic or early life stress
↑CRF
Amygdala
Hippocampus
↓GR
↓BDNF
1
2
Hypothalamus
CRF
3
MC
6
Pituitary gland
ACTH
4
7
8
10
9
Adrenal cortex
GC
5

Figure 1.2 Chronic early life or adult stress leads to alteration in limbic regulation of the HPA axis. This is due to increased corticotropin-releasing factor (CRF) expression and drive from the amygdala (1) and decreased GC receptor (GR) and brain-derived neurotrophic factor (BDNF) expression in hippocampus, which dampens inhibition (2). These changes ultimately lead to increased CRF release from the hypothalamus (3), increased and prolonged release of adrenocorticotrophic hormone (ACTH) after cessation of the stressor (4), and increased GC (GC) production (5) with decreased negative feedback at higher structures. Increased CRF release leads to greater mast cell (MC) activation and infiltration (6) leading to enhanced peripheral nociceptor interaction (7). Increased peripheral drive can lead to hyperalgesic priming (8) and/or wind-up (9), eventually increasing ascending pain signaling, while simultaneously decreasing descending inhibition (10).

resistance, glucose intolerance, dyslipidemia, hypertension, and immunosuppression. These symptoms are seen in humans with endogenous Cushing's disease, which is associated with increased cortisol levels (69), and in individuals receiving treatment with exogenous steroids (70). Many of these symptoms are also associated with metabolic syndrome, discussed in more detail below.

One tissue that plays a significant role in metabolism is adipose tissue. When there is excess energy, either due to abundant calorie intake, low physical activity, or a combination of the two, this energy is stored as triglycerides in adipose tissue. Alternatively, when there is an energy deficit such as during fasting or exercise, triglycerides are broken down and fatty acids and glycerol are released into circulation. If dysregulation of this usually balanced system occurs there can be either an excess or deficit in the storage of triglycerides. Stress hormones, including both GCs and catecholamines, are key regulators of this energy balance (71).

Glucocorticoids have been shown to be both lipolytic and adipogenic by causing the mobilization of lipids or differentiation of pre-adipocytes to adipocytes in body fat, respectively. However, they appear to favor adipogenesis (72). GCs' adipogenic effects work at the tissue level as well as at the whole body level. At the tissue level, adipose tissue can grow in size through two different mechanisms: hyperplasia, or an increase in the number of adipocytes, and hypertrophy, or an increase in the size of existing adipocytes. For example, cortisol and dexamethasone have been shown to cause the differentiation of pre-adipocytes into mature adipocytes *in vitro*, demonstrating hyperplasia (73), while human Cushing's patients (74) and rodents given exogenous steroids (75) have hypertrophic adipocytes. Another way that GCs work at the tissue

levels is by increasing the activity of lipoprotein lipase, which is an enzyme that promotes the uptake and storage of fatty acids (76). At the whole-body level, GCs increase adipose tissue by increasing food intake, specifically of palatable higher calorie 'comfort foods'. This has been demonstrated in rats (77, 78) as well as in humans (79, 80). In one study evaluating this phenomenon, women with higher stress-induced cortisol levels displayed a greater caloric intake, particularly from high-fat/high-sugar foods, after novel stress exposure compared to those with lower cortisol levels. This effect of greater food consumption was not observed on the control day when participants were not stressed (79). It is hypothesized that this occurs due to GCs influence on other molecules such as neuropeptide Y (NPY) and leptin. Cortisol increases NPY Y2 receptor in abdominal fat (81) and its activation by NPY increases food intake as well as fat storage. GCs also cause the release of leptin from adipocytes, a molecule that usually serves as a satiety signal to the hypothalamus (82). However, when there is a long-term increase in GC signaling, leptin resistance can occur whereby the efficacy of leptin is reduced (83). Therefore, HPA axis hyperactivity could be involved in the leptin resistance that is often seen in human obesity.

There are more GC receptors found in visceral compared to subcutaneous adipose in both humans (84) and rodents. This could explain why individuals with increased GC levels show significant increases in visceral adiposity in relation to other fat depots. Furthermore, inactive GCs, cortisone in humans and 11-dehydrocorticosterone in rodents, are converted to their active forms, cortisol and corticosterone, by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is upregulated in the adipose tissue in obese individuals (85, 86). This increase in

11β-HSD1 can lead to a subsequent increase in active GCs and therefore increase GCs' actions in adipose tissue. This is supported by pre-clinical research carried out by Masuzaki et al. where they overexpressed 11β-HSD1 in the adipose tissue of a transgenic mouse model and found that these mice developed visceral obesity as well as elevated corticosterone levels in their adipose tissue (87).

Taken together, these data demonstrate that the output of the HPA axis, CRF, ACTH, and GCs, can have many downstream pain-related and metabolic effects. Therefore, when regulation of the HPA axis is disturbed, such as when individuals experience early life stress, there are many negative consequences. We are interested in studying these consequences by utilizing a mouse model, neonatal maternal separation (NMS), which is described in the following section.

1.4 Neonatal maternal separation

Dysregulation of the HPA axis is an established outcome of neonatal maternal separation (NMS), a model of early life stress that has predominantly been performed in rats. This is evidenced by higher basal plasma corticosterone concentrations and increased CRF_1 expression in the PVN of NMS rodents (88, 89), decreased action of the negative feedback loop leading to prolonged release of ACTH and GCs after a stressful event (90, 91), changes in anxiety-like behaviors (92), and increased colorectal sensitivity (93, 94). In line with these observations, Aisa et. al. 2008 found that NMS female rats are more susceptible to chronic stress in adulthood compared to normally reared female rats. Additionally, augmented early life experiences (maternal licking and grooming) were found to reduce excitatory input onto CRF expressing neurons in rats. This leads to reduced CRF mRNA and protein levels in the hypothalamus and an

attenuated behavioral response to stress (95).

Our lab has adapted a NMS paradigm in mice to investigate the impact of early life stress on the molecular regulation of the HPA axis and downstream histological and behavioral outcomes. We found that hypothalamic mRNA levels of CRF, Unc2, and CRF_1 trend toward an increase in NMS female mice while CRF_2 levels are significantly decreased (96). In the hippocampus, female NMS mice have significantly decreased GR and MR mRNA levels and display a trend toward decreased CRF_1 and CRF_2 mRNA levels. This suggests NMS alters the development and function of important regulators of the stress response. To investigate the consequences of HPA axis disfunction, we have measured mechanical hypersensitivity and MC activation in urogenital organs of NMS and naïve mice. We found that NMS significantly increases urinary bladder (97) and vaginal sensitivity (96) in females and urogenital sensitivity in males(98, 99). We also saw an increase in the percent of degranulated MCs in the female (97) and male (100) urinary bladder, the vagina (unpublished observation), and the prostate (98). These results are similar to symptoms seen in patients with chronic urogenital pain disorders.

Due to the fact that individuals with urogenital pain disorders often suffer from other disorders co-morbidly (3-8), we are now interested in investigating if NMS mice display evidence of evoked migraine (Chapter 2) or obesity-related metabolic syndrome (Chapter 3). And if these consequences of early life stress can be attenuated by non-pharmacological interventions such as exercise (Chapters 2 & 3) or an anti-inflammatory diet (Chapter 4). The following sections give more detail about the effect that dysfunction of the HPA axis has on the development of centralized pain disorders

as well as obesity-related metabolic syndrome. Therapeutic interventions commonly used to treat these disorders are also discussed.

1.5 Centralized Pain Disorders

Altered functioning of the HPA axis has been observed in patients suffering from a number of centralized pain disorders (101). Approximately 20%–25% of patients with stress-related disorders have hypocortisolism, which has been postulated to come about as a compensatory response to a preceding period of hypercortisolism and excessive GC release (102). GC resistance, either through reduced availability of GCs or impaired function of GR, has also been proposed to contribute toward co-morbidity of inflammatory disorders, including centralized pain syndromes (103). A history of abuse or early life stress is linked to both HPA abnormalities and chronic pain syndromes; however, a clear and convincing connection between all three has yet to be fully established in a clinical setting. Conflicting studies have shown both hypercortisolism (104, 105) and hypocortisolism (9, 106) in adults that report a history of childhood abuse or stress. It is likely that the form of abuse and sex of the patient may influence the eventual effect on GC production and more work is needed to determine these genetic and environmental interactions. The potential role of the HPA axis in three major centralized pain disorders, chronic pelvic pain, fibromyalgia, and migraine, is discussed in more detail below.

1.5.1 Chronic Pelvic Pain

Alterations in HPA axis output have been reported for all chronic pelvic pain syndromes, although the impact, in terms of hyper- or hypocortisolism, largely depends on the type of syndrome. Patients with irritable bowel syndrome (IBS) have increased basal and

evoked cortisol release that, in some cases, is linked to early life adverse events (107, 108). Treatment with CRF_1 antagonist has shown mixed clinical results with no effect on stooling symptoms in diarrhea-predominant IBS patients (109), but a positive impact on significantly reducing the blood oxygen level-dependent signal in the hypothalamus in IBS patients (with average or high levels of anxiety) during the expectation of abdominal pain (110). Men with CP/CPPS have greater waking cortisol levels (111) and delayed ACTH release in response to an acute stressor, which correlates with significant psychological disturbances (112). Finally, women with vulvodynia have blunted serum cortisol cycles and reported higher symptoms of stress compared to healthy controls (113). Despite showing disparate cortisol levels, chronic pelvic pain syndromes all have evidence of increased activation downstream of the HPA axis in affected peripheral tissues. Biopsies from patients with IBS (114), CP/CPPS (44, 115), IC/PBS (116, 117), and vulvodynia (118) all revealed increased MC infiltration and altered granular structure including a reduced proportion of intact MC. Serum (119) and urine (120) samples from IC/PBS patients also had elevated MC granule components, including nerve growth factor (NGF), histamine, and pro-inflammatory cytokines, indicating an increase in MC activation. Tissue biopsies from IC/PBS patients revealed increased MC infiltration in close proximity to densely-populated SP-immunopositive nerve fibers (121). Expressed prostatic secretions from CP/CPPS patients had elevated MC tryptase and NGF levels (122) and urine samples also had increased tryptase, as well as carboxypeptidase A3, a marker of MC activation (123). In line with these observations in humans, MC activation, histamine release, NGF expression, and associated pelvic organ hypersensitivity have all been shown to be increased by stress exposure in adult

male rats (124). We demonstrated that NMS increased vaginal (96), bladder (97), and referred prostatic (100, 125) sensitivity in mice, which corresponded to an increased percentage of degranulated MC in urogenital tissues, compared to naïve mice. Exposure to WAS further increased MC degranulation in the bladder of NMS and naïve female mice (97) and the prostate of NMS and naïve male mice (125). The limited nesting material method was also employed for assessing visceral sensitivity. Adult male rats that were exposed to limited nesting material as neonates exhibited increased colonic sensitivity and anxiety behaviors, which were not present in female littermates (126). The finding is particularly intriguing considering the larger number of female chronic pelvic pain patients, as well as the preponderance of preclinical research that has been done in male rodents. Both restraint stress and central administration of CRF caused MC degranulation in the colon in rats (127). The role of MC activation in stress induced pain is further illustrated by studies using MC stabilizers. When injected 30-minutes before restraint stress, the MC stabilizer doxantrazole attenuated the stress-induced increase in abdominal contractions during CRD (128). Pre-treatment with a non-specific CRF antagonist, a-helical-CRF, also prevented stress-induced visceral hypersensitivity in NMS rats following WAS, in a MC-dependent manner (129). Treatment with a MC stabilizer, but not a-helical-CRF, was also capable of reversing WAS-induced visceral hypersensitivity, suggesting that non-CRF dependent factors are involved in the maintenance of post-stress hypersensitivity. In a study of a transgenic autoimmune cystitis mouse model of IC/PBS that displays bladder inflammation, increased number of MC, and urinary tract dysfunction, treatment with the MC stabilizer cromolyn reversed these symptoms (130). Furthermore, crossing this transgenic model

with MC-deficient mice produced mice with reduced bladder inflammation and no urinary tract dysfunction. However, both of these symptoms were re-established upon MC reconstitution.

1.5.2 Fibromyalgia

Hypocortisolism has overwhelmingly been reported in fibromyalgia patients. A meta-analysis and meta-regression of 85 case-control comparisons reported on HPA axis involvement in functional somatic disorders, including CFS, fibromyalgia, and IBS, and showed a significant reduction in basal cortisol in all CFS and female fibromyalgia patients compared to healthy controls (131). When compared to patients with shoulder and neck pain or healthy controls, fibromyalgia patients had significantly lower waking cortisol levels (132). Strikingly, the patients with shoulder and neck pain had waking cortisol levels higher than either control or fibromyalgia patients and the authors suggest that this group may represent an intermediate step in the progression from regional to widespread musculoskeletal pain similar to the proposed mechanism of hypercortisolism leading to hypocortisolism described by Fries et al. (2005) (102). A recent study compared basal and stress-evoked salivary cortisol levels between fibromyalgia and control patients with and without a history of early childhood abuse (133). They reported a decrease in stress-evoked cortisol release in fibromyalgia patients that was largely driven by increased cortisol release in control patients with a history of early childhood abuse. This observation again underscores the disparate effects of childhood experiences on later output of the HPA axis and its involvement in pain processing. Rodent models of adult stress are commonly used to induce behaviors and molecular changes similar to what is observed in fibromyalgia patients. These

models include intermittent cold stress (134), unpredictable sound stress (135), WAS (136), and restraint stress (137) and induce long-lasting widespread mechanical allodynia and hyperalgesia. An early life stress model of fibromyalgia that incorporates limited nesting material during the pre-weaning period produces adult rats that display mild muscle hyperalgesia that worsens following sound stress (138). We have reported hind paw mechanical hypersensitivity in both male (139) and female (96) mice that also demonstrate urogenital hypersensitivity (discussed above) following neonatal maternal separation (NMS). Interestingly, we observed evidence of decreased HPA axis output in male (125) and increased HPA axis output in female (97) NMS mice, suggesting that regardless the effect of early life stress on GC production, widespread allodynia is a common final pathway of HPA axis dysregulation in this model. The most commonly employed rodent models of fibromyalgia involve direct activation of the peripheral nervous system via intramuscular injection of carrageenan (140) or acidic saline (141). Carrageenan is a chemical nociceptive stimulus that evokes inflammation and excites muscle nociceptors (142). When injected intramuscularly, grip strength is reduced in the ipsilateral limb (140). Repeated intramuscular injection of low pH saline causes ipsilateral and contralateral mechanical hyperalgesia lasting 4-weeks after the second injection (141), likely via activation of acid-sensing ion channels (ASIC) present on primary afferent fibers (143). The use of these stress- or nociceptor-induced models can be used to tease out the various ‘‘bottom-up’’ and ‘‘top-down’’ mechanisms that likely contribute to the disparate etiologies underlying fibromyalgia (144).

1.5.3 Migraine

Stress is a commonly-reported trigger for migraine attack (145, 146) and while evidence of altered cortisol release, compared to other centralized pain syndromes, is less prevalent among migraine patients, many migraineurs have shown abnormal HPA axis activity (147-149). A recent literature review of seven cross-sectional studies largely showed no baseline differences in cortisol level between migraineurs and healthy controls (150). A potential for increased HPA axis responsiveness was noted, as nitroglycerin- and CRF-evoked cortisol levels were reportedly higher in migraineurs, compared to healthy controls, across four observational studies. Another study looking at blood cortisol levels over a 12-hour period revealed a greater total cortisol release and peak in migraineurs compared to control patients (147). One prospective study of 17 migraine patients reported on stress related parameters prior to and during a migraine attack (151). While a subgroup of patients did report an increase in perceived stress, which also reportedly triggered their migraine attacks, no objective measures of increased cortisol or other measures of a biological stress response were present either prior to or during a migraine attack. The mouse model of Familial Hemiplegic Migraine type 1 (FHM1), which was generated by knocking-in an R192Q missense Cav2.1 Ca2C channel mutation, displayed an increase in cortical spreading depression following treatment with corticosterone, which was blocked by pretreatment with a GR antagonist (152). The same FHM1 mice did not show increased cortical spreading depression following an acute stress. The authors explained this discrepancy as a direct effect of corticosterone on glutamatergic neurotransmission, via a GR-mediated mechanism, that is otherwise counteracted during bouts of acute stress. This observation suggests that

future studies on the effect of stress and the HPA axis on migraine should take into account the type of stressor and etiology of the patient, as migraineurs represent a heterogeneous population of patients. It has been known for several decades that patients with migraine have increased plasma histamine levels, an indicator of MC activation, both during migraine attack and at rest, when compared to healthy controls (153). MC reside in the dura and have been hypothesized to release their pro-inflammatory contents following activation by neuropeptides released from nearby sensory nerve endings, thereby generating neurogenic inflammation (154). A recent study revealed that cultured human MCs do not express receptors for either calcitonin gene-related peptide (CGRP) or pituitary adenylate cyclase-activating polypeptide (PACAP), the two neuropeptides most commonly associated with migraine, but rather express and release PACAP upon activation (155). Animal models of migraine have investigated the role of MCs more thoroughly and shown that application of MC mediators can sensitize dural afferents and evoke migraine-like behaviors (156). Restraint stress has also been shown to induce dural MC degranulation and increase protease I levels in the cerebrospinal fluid (157). These outcomes could be attenuated by pretreatment with antisera to CRF or neonatal treatment with capsaicin to deplete peptidergic innervation, further supporting the role of neuropeptide release in MC activation and the generation of migraine.

1.6 Obesity related metabolic syndrome

The development of obesity involves a complex interaction of both genetic and environmental factors (158). One environmental factor found to be significantly associated with the development of obesity is stress (6) and dysregulation of the HPA

axis is often seen in obese individuals (159). Childhood neglect and maltreatment have both been shown to be associated with the development of obesity in adulthood (160-162). Furthermore, research utilizing early life stress paradigms in non-human primates (163) and rodents (164) also show this same effect. Obesity is generally considered a pre-requisite in the development of metabolic syndrome (165). However, there is a subset of individuals that are metabolically obese but normal weight (166). Metabolic syndrome is diagnosed when an individual suffers from 3 of the following 5 criteria: central obesity, insulin resistance, hypertension, high triglycerides, and low HDL-cholesterol (167).

Adipose tissue distribution and composition is important in predicting the development of obesity-related diseases because it plays a key role in regulating systemic metabolic function and inflammation (168). Visceral fat accumulation is associated with a greater risk of metabolic dysfunction (169) and leads to chronic low-grade inflammation (170). This low-grade inflammation is associated with increased macrophage infiltration (171) and has been demonstrated in both obese mice and humans (172). MCs were also increased in visceral adipose tissue of obese humans compared to non-obese (173, 174). Furthermore, Liu et. al (2009) investigated the role of MCs in diet-induced obesity. They found that when fed a Western diet for 12-weeks, MC-deficient mice gained significantly less body weight and had less body fat than wild-type controls. Additionally, wild type mice treated with a MC stabilizer, also gained less weight and body fat compared to controls. Interestingly, when control mice originally fed a western diet were switched to a control diet and treated with a MC stabilizer, they had improved glucose tolerance and body weight compared to groups only switched to

control diet (175). These data suggest that MC have a role in the development of obesity.

1.7 Therapeutic interventions

Non-pharmacological therapeutic intervention is a growing area of research for treating chronic pain and metabolic disorders. These therapies include exercise, cognitive behavioral therapy (CBT), trigger point injection, physical therapy, neuromodulation (176, 177), and diet alterations (178-181). The advantage of these therapeutic interventions is that they generally result in symptom improvement without the harmful side effects commonly associated with pharmacological treatments. Another benefit is that these forms of intervention can be used singly or in combination with each other. This allows treatment plans to be personalized on a case-by-case basis depending on what results in the greatest improvement for a particular patient. Evidence of these interventions for the treatment of centralized pain syndromes and obesity related-metabolic syndrome are listed in Table 1.1.

1.7.1 Cognitive Behavioral therapy (CBT)

One form of non-pharmacological therapy is CBT, which consists of educating patients about their pain, techniques on how to cope with their pain, such as relaxation training, and how to implement these cognitive coping techniques in real-life situations (182). Although this technique cannot be used in rodents, it is particularly useful in stress-induced pain syndromes in humans as the premise behind CBT is to reduce stress and emotional responses and thereby subsequently reduce symptom severity or frequency. The use of CBT for improving symptoms of depression and anxiety has been

Table 1.1 Evidence of non-pharmacological therapies for the treatment of centralized pain symptoms and associated co-morbidites

Therapeutic treatment	Disorder	Outcomes measured	References
Exercise (walking, aerobic strength training, yoga, pilates, or swimming)	Psychological disorders	Depression scores; anxiety scores; mood	(183)
	Irritable bowel syndrome	Irritable bowel specific quality of life, GI symptoms (constipation, diarrhea, pain)	(184, 185)
	Chronic prostatitis and/or chronic pelvic pain	Pain scores; quality of life	(186-188)
	Migraine	Headache frequency, headache intensity, number of headache days, disability, quality of life, depression, anxiety	(189-192)
	Fibromyalgia	Fibromyalgia Impact Questionnaire score, 6-minute walk test, self-efficacy, grip strength, pain severity, social functioning, quality of life, psychological distress, brain response and pain rating to heat stimuli	(193-198)
	Metabolic syndrome	Percent body fat, abdominal visceral fat, fasting insulin, insulin sensitivity, metabolic risk factors, quality of life	(199-201)
Cognitive behavioral therapy	Psychological disorders	Depression scores, self-esteem scores, anxiety scores	(202-204)
	Chronic pelvic pain	Pelvic pain, widespread pain, dyschezia, dyspareunia, quality of life, disability, depression, anxiety	(205, 206)
	Migraine	Headache frequency, headache duration, headache intensity, anxiety, depression, self-efficacy	(207)
	Fibromyalgia	Fibromyalgia Impact Questionnaire score, 6-minute walk test, self-efficacy, quality of life, social functioning, psychological distress, McGill ratings of pain, physical functioning	(195, 197, 208)
	Metabolic syndrome	Adherence to a Mediterranean diet, waist circumference, blood pressure, lipid and glycemic profiles	(209)
Anti-inflammatory diet	Metabolic syndrome	Weight, lipid and glucose parameters, systemic inflammation makers, endothelial function	(210, 211)
	Chronic headache	Frequency of headache, quality of life	(179)
	Inflammatory joint pain	Patient and physician assessed pain, number of painful joints, duration of morning stiffness, NSAID drug consumption	(180)
	Knee osteoarthritis	Osteoarthritis index, pain, stiffness, and function scores	(181)

well-established (202-204). In a study evaluating CBT as a form of fibromyalgia treatment, a greater percentage of patients displayed improvement in physical functioning after CBT compared to patients that received standard care in the form of pharmacological management of symptoms (208). Although CBT is most commonly used in treating symptoms in fibromyalgia patients, it has also been used effectively to treat chronic pelvic pain symptoms. In a study of women with endometriosis-associated pelvic pain, CBT was implemented in addition to somatosensory stimulation in the form of acupuncture and this combination of treatments resulted in a decrease in pelvic pain and an increase in quality of life (206). Additionally, a recent review categorized studies that used Internet-delivered CBT into headache and non-headache chronic pain conditions. They found that pain, disability, depression, and anxiety were reduced in non-headache chronic pain conditions and there was insufficient evidence to make conclusions in headache conditions due to having only two studies to analyze (205). However, another review focused on CBT in headache disorders found that CBT reduced headache activity 30%–60% on average across studies but notes that there are a fair number of patients who were non-responders (40%–70%; (207)). It is hypothesized that the positive effects of CBT seen in the treatment of chronic pain are due to structural changes in the gray matter in regions of the brain associated with pain management and/or in the functional connectivity of these regions *(*Figure 1.3*)*. CBT induced changes in gray matter volume have been observed when implemented to treat patients with chronic pain disorders (212), CFS (213), and posttraumatic stress disorder (214). One study found that 11-weeks of CBT in a mixed group of chronic pain patients resulted in significant gray matter differences in sensory, motor, and affective brain

Figure 1.3

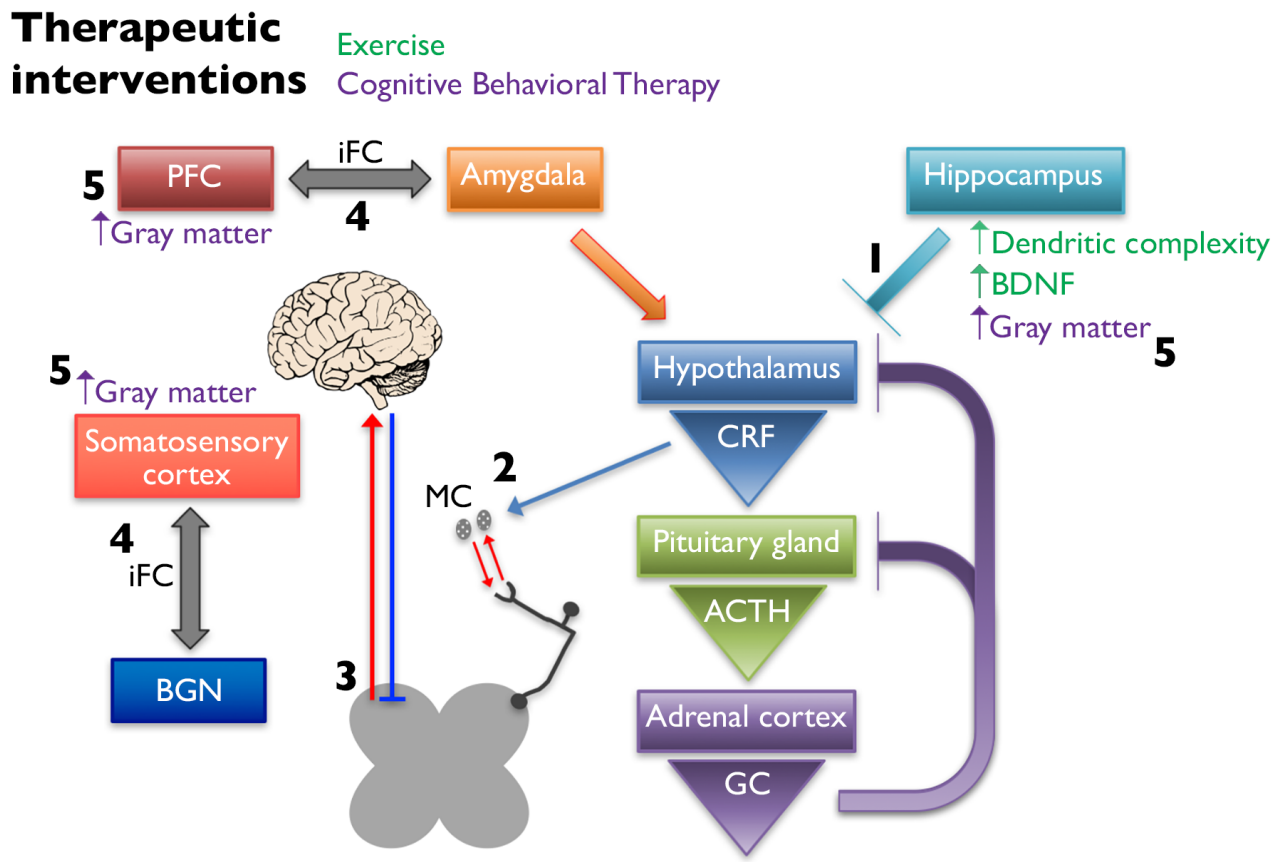
Therapeutic interventions
Exercise
Cognitive Behavioral Therapy
5
PFC
Gray matter
iFC
4
Amygdala
Hippocampus
1
Dendritic complexity
BDNF
Gray matter
5
5
Gray matter
Somatosensory cortex
4
iFC
BGN
MC
2
3
Hypothalamus
CRF
Pituitary gland
ACTH
Adrenal cortex
GC

Figure 1.3 Non-pharmaceutical interventions restore proper signaling within the hypothalamic-pituitary-adrenal (HPA) axis and between higher structures. Exercise increases dendritic complexity and brain-derived neurotrophic factor (BDNF) expression in the hippocampus, which restores negative input onto the hypothalamus to restore proper HPA axis output (1). Decreased CRF release stabilizes mast cell (MC) activation and infiltration associated with chronic pain disorders (2), thereby reducing peripheral nociceptive input. Exercise influences the descending pain pathway, likely through release of endogenous opioids, increasing neuronal activity, and balancing excitatory and inhibitory transmission (3). Cognitive behavioral therapy (CBT) alters the intrinsic functional connectivity (iFC) between brain regions associated with pain management, including connections between the prefrontal cortex (PFC) and amygdala and somatosensory cortex and the basal ganglia network (BGN) (4). Cortical and hippocampal gray matter densities are also increased in patients following CBT (5).

areas as measured using voxel-based morphometry (212). The majority of the CBT induced changes were increases in gray matter, however one region, the supplementary motor area (SMA), showed a significant gray matter decrease after CBT. The patients in this study showed significant improvements in pain measurements as well as region specific positive and negative correlations in gray matter changes and pain catastrophizing. Changes in intrinsic functional connectivity (iFC) are also seen in areas of the brains of chronic pain patients. Baliki et al. found that functional connectivity patterns between the medial prefrontal cortex and nucleus accumbens could predict whether a patient with sub-acute back pain would recover or develop persistent pain (215). They also describe iFC differences in other brain regions including the insula and basal ganglia in these patients (215). In a study investigating the effect of CBT on iFC, 11-weeks of CBT in patients with various types of chronic musculoskeletal pain led to significant pre-post changes in iFC. Specifically, they found a decrease in iFC between the medial prefrontal cortex and amygdala and PAG and an increase in iFC between the basal ganglia network (BGN) and the right secondary somatosensory cortex. These changes were correlated with significant improvements in clinical measures of pain. CBT patients that showed the greatest improvement in self-efficacy and pain symptoms exhibited the greatest pre-post change in iFC. These results were not seen in an active control group who received educational materials that did not include CBT (216).

Cognitive behavioral therapy is used most commonly in obesity caused by binge-eating-disorder (BED) (217). CBT has been shown to reduce BED, however it does not usually produce actual weight loss (218). Behavioral weight loss strategies, such as

changes in physical activity and diet, are typically necessary to produce physiological changes (219). One study measured the efficacy of using CBT to assist with adherence to a Mediterranean Diet in patients with metabolic syndrome. They found significant improvements in waist circumference and triglyceride levels compared to the control group (209). Therefore, combining CBT with behavioral changes could prove to be the most effective in treating obesity related-disorders and maintaining weight loss.

1.7.2 Exercise

Another form of non-pharmacological intervention is exercise. Exercise is defined as physical activities that are planned, structured, repetitive, and centered on an improvement in physical health (220). In humans, exercise therapy can take the form of a wide range of activities such as walking, aerobic strength training, yoga, Pilates, or swimming. This variety is important so that a patient can find an activity that they enjoy and therefore are more likely to view exercise as a long-term lifestyle change rather than a quick-fix treatment. Exercise has been shown to relieve stress and reduce depression and anxiety (183) and has successfully been implemented in the treatment of chronic pain disorders including chronic pelvic pain (184), migraine (189), and fibromyalgia (198) as well as in the treatment of symptoms associated with metabolic syndrome (221). In a 12-week exercise intervention in patients with IBS, exercise led to improvements in the IBS Severity Scoring System score, which includes measurements in pain severity and pain frequency, and IBS-quality of life score, which measures qualities related to emotional functioning compared to patients that received usual care for IBS (185). Similarly, in a cohort of women with chronic pelvic pain, an 8-week yoga intervention resulted in improvement in pain and quality of life scores compared to the

control group that was treated with non-steroidal anti-inflammatory drugs (NSAID) (187). The frequency, intensity, and duration of migraine pain was significantly reduced when 12-weeks of outdoor walking was added to the treatment regime of migraine patients taking amitriptyline (192) and Nordic walking (walking with poles) for 15-weeks resulted in improvements in the Fibromyalgia Impact Questionnaire score compared to control fibromyalgia patients (196). A study evaluating the effect of exercise on body composition in adult females with metabolic syndrome found that high-intensity exercise training reduced abdominal and subcutaneous fat compared to sedentary or low-intensity exercise groups (199). Another study found that moderate-intensity exercise was able to improve insulin resistance and hyperinsulinemia in obese individuals (200).

Not only has exercise been used as a therapeutic intervention for chronic pain and obesity-related metabolic disorders, being more physically active has also been shown to reduce the chance of developing these disorders while a sedentary lifestyle is associated with a greater risk of developing these disorders. Specifically, higher levels of both moderate- and high-intensity physical activity were associated with a lower risk of developing CP/CPPS in older men (188) and endometriosis in young adult women (186). Additionally, a prospective cross-sectional study found an association between low levels of physical activity and an increase in prevalence of migraine and non-migraine headache (222). Furthermore, a study investigating glucose tolerance and insulin sensitivity in young and old lifelong athletes and untrained men demonstrated that the older lifelong athletes were protected from worsening glucose tolerance and insulin insensitivity that is often associated with ageing (223). Finally, it has been shown

that physically active individuals are 30-50% less likely to develop non-insulin dependent diabetes mellitus compared to sedentary individuals (224).

In rodents, exercise has been shown to have positive effects on neurodevelopment (225), increase neuronal survival and resistance to brain insult (226), and stimulate brain vascularization (227). In a rodent model of high-fat diet induced metabolic syndrome, exercise improved visceral obesity, hyperglycemia, dyslipidemia, and hypertension (228). Furthermore, exercise in rodents has been shown to be beneficial in the prevention of development of symptoms similar to those seen in chronic pain disorders. Previous research from our lab has demonstrated this for chronic pelvic pain symptoms (100, 229). Exercise has also been shown to prevent the development of autonomic dysfunction and paw and muscle allodynia in a mouse model of fibromyalgia (230). Exercise protocols in rodents can take the form of resistance training or aerobic exercise such as wheel running, treadmill running, and swimming. Forced treadmill running in rodents could be considered a stressor due to the fact that it generally involves the use of an aversive stimulus, such as probing or foot shock, to provoke the rodent to continue running. In contrast, voluntary wheel running is considered rewarding, as most rodents will choose to run when provided a running wheel (231) and therefore this form of exercise is generally not viewed as a stressor. In support of this, corticosterone levels and anxiety behaviors have been shown to be elevated after forced treadmill running compared to sedentary controls, but these effects were not seen in rodents following voluntary wheel running (232-234). Furthermore, voluntary exercise induced higher hippocampal brain-derived neurotrophic factor

(BDNF) concentration compared to rats subjected to forced exercise or sedentary controls (233).

A potential explanation for the beneficial effects of exercise is that it influences gene expression and structural complexity in the limbic structures that regulate the HPA axis (Figure 1.3). Specifically, running wheel access has been shown to normalize hippocampal GR and BDNF mRNA levels in NMS rats (235), and increase neurogenesis and dendritic spine density in the hippocampus of adult rats (236). Other groups have also evaluated the effects of exercise on stress-induced changes in rodents. It was shown that chronic unpredictable stress induced a depressive phenotype in rats, decreased hippocampal BDNF and GR mRNA levels, and increased circulating corticosterone, while 4-weeks of voluntary wheel running attenuated these effects (237). Similarly, 3-weeks of voluntary wheel running before exposure to immobilization stress was able to prevent the decrease in BDNF protein levels caused by the stressor (238). These findings are relevant to the study of treatment of chronic pain as well as obesity-related metabolic syndrome because of the association of stress causing or exacerbating symptoms of these disorders as described above.

Another hypothesis for the mechanism underlying the positive influence of exercise on pain specifically is through improved conditioned pain modulation (CPM), formerly referred to as diffuse noxious inhibitory control (DNIC). CPM is a ‘‘pain inhibits pain’’ mechanism and represents a measure of the function of descending analgesic pathways. This system has been shown to be defective in chronic pain patients (239) and these deficits in pain inhibitory pathways are involved in the development of central sensitization (240). The relationship between exercise and improved CPM was

demonstrated in a study evaluating the ability of self-reported physical activity to predict thermal sensitivity as well as pain facilitatory and inhibitory function, tested by temporal summation (TS; perceived increase in pain intensity to repeated stimulation at a constant stimulus intensity, reflecting central sensitization) and CPM respectively. Results indicated that individuals reporting greater total physical activity showed reduced TS of pain and greater CPM (241).

There are different mechanisms that could explain the beneficial influence of exercise on CPM including causing an increase in endogenous opioids, stimulating brain structures involved in descending analgesic pathways, and/or maintaining the balance between excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the CNS (241). Exercise increases endogenous opioids in the CNS (242) and the effects of this increase are often compared to effects following administration of the opioid receptor antagonist naloxone. One study assessing changes in pain threshold after swimming found that there was a significant increase in hind limb hot plate withdrawal threshold in exercised mice that received saline injection before exercise but no increase in pain threshold in exercised mice that received naloxone (243). Similar results were seen in humans when saline or naloxone was administered to long-distance runners after a run. Long-distance running produced thermal and ischemic hypoalgesia and mood elevation, but most of these effects were attenuated after administration of naloxone (244). Further evidence of the influence of exercise on CPM comes from studies evaluating brain activity in areas involved in descending pain modulation pathways. Ellingson et al. (194) examined brain activity during administration of painful heat stimuli following moderate intensity cycling exercise or

quiet rest in fibromyalgia patients. They found that exercise, but not quiet rest, prior to heat stimulation, elicited increased activity in brain regions involved in the anterior insula and dorsolateral prefrontal cortex, as well as lower pain ratings. A final potential mechanism regarding the beneficial role of exercise on CPM is that exercise can influence the balance of excitatory and inhibitory transmission in pain pathways. Four weeks of voluntary wheel running in rats resulted in significant changes in the forebrain GABAergic system compared to sedentary control rats (245). All of the previously described results suggest that exercise significantly alters structures and signals involved in CPM and these alterations could begin to explain why exercise has been shown to reduce pain symptoms in many chronic pain disorders.

Regarding the positive influence of exercise on obesity-related metabolic syndrome, there are many mechanisms involved and considerable research is still necessary to understand them all. However, it is known that during exercise, the body works through many systems to maintain energy homeostasis. For example, the liver increases glucose output through gluconeogenesis and adipose tissue increases lipolysis and free fatty acid (FFA) mobilization, while skeletal muscle increases glucose and FFA uptake (246). Whether carbohydrates or lipids are preferentially metabolized depends on many different factors including the intensity of the exercise, diet, sex, and prior training conditions (247). Exercise also increases resting metabolic rate (RMR), which is the metabolic cost of maintaining all body systems at rest. RMR can stay elevated for several hours after the end of high-intensity exercise (248). Furthermore, exercise, unlike dietary interventions alone, preserves lean body mass (249), which is important because the largest component of overall energy expenditure is fat free mass

(FFM) (250). Utilization of energy stores by skeletal muscle and increasing overall energy expenditure is important in creating an energy deficit that can eventually lead to weight loss, especially when paired with a healthy diet.

Because obesity is generally considered the first step in developing obesity-related metabolic syndrome, loss of visceral adipose tissue can lead to improvement in the other symptoms of metabolic syndrome including increased insulin sensitivity, improved glucose tolerance (251), and reduced inflammation. Exercise reduces inflammation by reducing the accumulation of visceral fat and its associated pro-inflammatory macrophage population (252), as well as through the release of anti-inflammatory cytokines and/or “myokines”, which are cytokines and peptides produced by muscle tissue (253). These myokines can directly influence adipose tissue metabolism as well as exert their own anti-inflammatory effects. The first myokine released during exercise is IL-6 followed by the anti-inflammatory myokines IL-1ra and IL-10. Another way exercise creates an anti-inflammatory environment is by inhibiting the production of the pro-inflammatory cytokine TNFα (254). Creating an anti-inflammatory environment is important because many metabolic and chronic pain disorders are associated with a chronic state of low-grade inflammation (255, 256).

It is important to note that the length of exercise protocol, as well as intensity of exercise, have been shown to have differential outcomes. In regards to duration of exercise intervention in rodents, both short- and long-term periods of voluntary wheel running increased cell proliferation in the rat hippocampus, but a longer-term exercise protocol was required to increase neurogenesis (14- days) and LTP (56- days; (257)). In a study comparing the effects of low-intensity running, high-intensity running, or

sedentary conditions in rats, it was found that low-intensity running increased BDNF levels and dendritic complexity and branching in the hippocampus while not significantly affecting corticosterone levels. In contrast, high-intensity exercise did not increase hippocampal BDNF levels or induce structural hippocampal changes but did cause a significant increase in corticosterone. This suggests that the high-intensity exercise elicited a stressful response and therefore did not have the beneficial effects that the low-intensity exercise did (258). These preclinical results are similar to what is seen in humans, where there appears to be a narrow ‘‘therapeutic window’’ for the use of exercise in chronic pain treatment. For example, the majority of studies evaluating the efficacy of exercise for fibromyalgia treatment have found that low or moderate levels of aerobic training reduces tender point pain compared to before training or a control group. However, more strenuous exercise regimes often result in an increase in fibromyalgia pain (101). Additionally, it has been shown that the greatest improvement in fibromyalgia patients is seen after long-term (12-weeks) moderate intensity aerobic training (193). There is evidence that exercise can be a trigger or increase pain during migraine attacks (191). However, it is suggested that exercise treatment for migraine be implemented with a slow increase in intensity and duration to allow the patient to habituate to exercise and eventually see benefits (190). Therefore, in the instances where exercise has been found to have detrimental effects on chronic pain symptoms, it is likely due to improper application of exercise treatment. This highlights the importance of tailoring exercise regimes specifically to individual patients to achieve the most benefit and avoid exacerbation of pain symptoms due to overly intense exercise prescription.

The benefits of exercise on metabolic syndrome, (increased RMR, improvements in insulin sensitivity and glucose tolerance) will return to baseline after a few days of sedentary behavior (259, 260). This finding highlights the importance of making exercise a lifestyle change rather than an occasional activity. The American College of Sports Medicine (ACSM), recommends that most adults “engage in moderate-intensity cardiorespiratory exercise training 30min/day for 5- days a week, vigorous-intensity cardiorespiratory exercise training for 20min/day for 3-days a week, or a combination of moderate and vigorous-intensity exercise to achieve a total energy expenditure of 500-1000 MET min/week.” MET is an index of energy expenditure and is a ratio of the rate of energy expended during activity to the rate of energy expended at rest. They also recommend incorporating resistance training and flexibility exercises into an exercise routine. The interaction between frequency, intensity, and duration of exercise is important in reaching the appropriate ‘overload stimulus’ that will allow an individual to see an effect. Although a lower stimulus is correlated with a lower training effect, health benefits from exercise can be achieved even at this lower stimulus (261). This is important because many individuals with metabolic syndrome are not able to complete the recommendations for activity described above. However, if an exercise plan is appropriately developed to match an individual’s abilities, they will still see results and eventually be able to increase their activity over time.

Regarding which type of exercise therapy is the most efficient for the treatment of metabolic syndrome specifically, multiple studies have been published as part of the Studies Targeting Risk Reduction Interventions Through Defined Exercise-Aerobic Training and/or Resistance Training (STRRIDE/AT/RT). These studies compare various

metabolic syndrome diagnostic criteria between 3 treatment groups: aerobic training, resistance training, or a combination of aerobic and resistance training (an exact combination of both). These studies have found that aerobic training and aerobic training + resistance training showed greater improvement of metabolic syndrome score than resistance training alone (262). Improvements were seen in reduced visceral, subcutaneous, and liver fat, as well as in fasting insulin levels (263). These data suggest that consistent aerobic training might be the most efficient way to treat metabolic syndrome. However, as recommended by the ACSM, a variety of types of exercise is important for overall health (261).

The previously described benefits of exercise should not be too surprising. Humans evolved during a time when energy dense foods were not readily abundant and required greater physical activity to attain. This led to the evolution of the most efficient way to store energy, or the ”thrifty genes” (264). This lifestyle was much more active than most human lifestyles today, where energy dense foods are actually the more convenient option. Likewise, rodent activity in research settings is greatly restricted compared to what is experienced in the wild. Therefore, although exercise in both human and rodent research is viewed as a ‘treatment’, if society could get back to seeing exercise as the normal human condition, the development of many of the debilitating diseases caused by sedentary lifestyle could be avoided. Considerable research is still needed to understand all the pathways that are influenced by exercise. Hopefully the trend of discovering the plethora of benefits associated with exercise will encourage this research.

1.7.3 Anti-inflammatory Diet

One problem commonly seen in prescribing exercise for the treatment of chronic pain, depression, or metabolic syndrome is lack of adherence. If progress is not seen instantly or if exercise is perceived as too difficult or time consuming, patients often stop exercising. Another lifestyle change that could be paired with exercise or stand alone is improving one's diet. Studies often find that exercise + diet interventions show the greatest benefit for individuals (265). For example, increasing physical activity and consuming a healthier diet has been shown to be effective in the prevention of type 2 diabetes in over-weight individuals with impaired glucose tolerance (266). However, it has also been found that diets with strict rules or complete elimination of certain food groups can lead to weight loss followed by weight regain after cessation of the diet. Therefore, adapting lifestyle changes that can be maintained long-term is important (267).

Many chronic pain, mood, and metabolic disorders are associated with a chronic state of low-grade inflammation (255, 256, 268). Therefore, a diet high in anti-inflammatory components could be a good diet treatment for these patients (269). An anti-inflammatory diet, which is very similar to the Mediterranean diet, is high in fruits, vegetables, lean protein or plant based protein, whole grains, high fiber, and healthy fats such as omega-3 fatty acids (FAs) (270). Diets with a high content of fruits and vegetables have been shown to lower inflammatory markers in the blood (271). For an animal protein source to be considered anti-inflammatory the source of fat within the protein needs to be from omega-3 FAs, such as in certain fish, as opposed to omega-6 FAs (272). Plant-based proteins (e.g. soy legumes, tempeh, and tofu) should be a

significant source of protein and have been shown to have anti-inflammatory properties (273). Whole grains (e.g. buckwheat, barley, and rye) should predominate over refined carbohydrates because they are high in fiber, an important property to be considered in regards to inflammation (274). Other dietary components found to have anti-inflammatory properties include tea, specifically green or white, red wine, because it contains resveratrol, and spices such as ginger and turmeric (275). Long-term consumption of a Mediterranean diet was associated with a significant reduction in mortality in a Greek population (276-278).

Anti-inflammatory diets have been shown to improve many of the symptoms of metabolic syndrome in humans. For example, patients with metabolic syndrome on a Mediterranean diet for 2- years showed decreased weight, cardiovascular benefits, and increased insulin sensitivity compared to the non-intervention group (279). An anti-inflammatory diet also elicits a widespread reduction in inflammation (280). This form of diet has also been shown to reduce the development of diabetes by around 20% (210). In the treatment of pain disorders, most studies focus on increasing a particular anti-inflammatory ingredient rather than a full dietary intervention. For example, an increase in omega-3 FA consumption paired with a reduction in omega-6 FA reduced headache pain and improved quality of life in a study of chronic headache patients (179). Goldberg and Katz (2007) found that adding omega-3 FAs to the diet of patients with rheumatoid arthritis, as well as joint pain associated with inflammatory bowel diseases and dysmenorrhea, reduced joint pain intensity, number of painful joints, and NSAID consumption for the treatment of joint pain (180). A study comparing treatment of knee osteoarthritis patients with curcumin, the main active ingredient in turmeric, versus

ibuprofen found that both treatments were effective but curcumin had less reported gastrointestinal problems (181). There appears to be a lack of studies investigating an anti-inflammatory diet as a therapeutic intervention for the treatment of depression and anxiety in humans. However multiple studies have found an association of increased inflammatory diet pattern and the development of mood disorders (281).

Limited studies investigating the benefits of an entire anti-inflammatory diet in rodents exist, as most studies focus on the addition of one anti-inflammatory ingredient. An exception to this is one study in mice that demonstrated that an anti-inflammatory diet shortened the recovery time after an injection with the inflammatory solution complete Freund's adjuvant (282). A different study looked at the effect of various tocochromanol phytochemicals, which are the different groups of Vitamin E compounds and are thought to have anti-inflammatory and other health promoting properties, in reducing symptoms associated with diet-induced metabolic syndrome in rats. They found that delta-tocotrienol in particular was beneficial in reducing diet-induced inflammation, normalizing glucose tolerance, reducing total fat mass, and improving heart and liver function in high-fat high-sucrose fed rats (283). A study investigating the effect of omega-3 FA supplementation in high-fat diet induced obese mice showed that including omega-3 FAs in the diet prevented the increased macrophage infiltration in adipose tissue normally seen in a high-fat diet not supplemented with omega-3 FAs. Interestingly, body weight was higher in the mice whose diet was supplemented by omega-3 FAs but their plasma triglyceride concentrations were significantly lower (284).

1.8 Western Diet

A western or high-fat/high-sucrose (HFS) diet is high in saturated fat, such as omega-6 FAs, and refined carbohydrates both of which are considered pro-inflammatory (269, 285). This type of diet is also low in fiber, fruits, and vegetables (286, 287). HFS diet consumption has grown due to the convenience and lower cost of fats and sugars compared to healthier foods, such as fruits and vegetables (288). Long-term consumption of HFS diet has many health consequences in humans including the development of obesity (178, 289), type 2 diabetes (290), cardiovascular disease (291), and adverse impacts on brain function and behavior (292). The brain functions particularly impacted are those involved in memory (293) and reward processing (294).

Rodent studies find similar results, such that a HFS diet leads to a significant increase in visceral body fat and the subsequent development of other symptoms associated with metabolic syndrome (164, 252, 282). It also appears that early life stress paired with HFS diet consumption has an even more detrimental effect on weight gain. For example, in a mouse model of early life stress using limited nesting material, after 8-weeks on a HFS diet, the stressed mice had a 1.9 fold increase in body fat percentage compared to non-stressed mice that had a 1.6 fold increase. Plasma leptin levels were also influenced by early life stress. Therefore, the authors suggest that leptin could be one mediator of early life stress and HFS diet induced obesity and subsequent development of metabolic syndrome (164). Memory impairment (295) and reduced inhibitory control on a long-term HFS diet is also demonstrated in rodents. It has been suggested that these behaviors are due to a reduction in BDNF in the

hippocampus and prefrontal cortex, which has been shown to be caused by a long-term HFS diet (296).

1.9 Sex differences

Sex differences have been found in animal responses to stress (297). Females display higher levels of basal circulating corticosterone compared to males, which could be due to estrogen interacting with the HPA axis (298). Furthermore, MC activation has been shown to be influenced by estradiol and progesterone (299) and dural MCs have stained weakly for the estrogen receptor (300). Therefore, differences in estrogen levels could be causing the sex differences seen in stress responses. In humans, women are more susceptible to stress-related illnesses, such as migraine, than men (301). Additionally, metabolic syndrome is more prevalent in women than men (302). However, female mice are resistant to diet-induced weight gain compared to male mice (303). Therefore, the evoked migraine study in this dissertation uses female mice (Chapter 2) and the Western diet study uses male mice (Chapter 3). Both sexes were used in the anti-inflammatory diet study to investigate if there are differences in this particular diet intervention (Chapter 4).

1.10 Central Hypothesis and Specific Aims

Early life stress has many long-term consequences including increased susceptibility to developing co-morbid pain and obesity-related metabolic syndrome in adulthood. Other risk factors for developing these conditions include a sedentary lifestyle and poor diet. Research is needed to investigate the interaction between stress-

induced co-morbid disorders and lifestyle factors to find appropriate therapeutic interventions.

The studies in the following chapters were focused on investigating if our mouse model of early life stress, NMS, that shows behavioral and molecular evidence similar to symptoms experienced by chronic urogenital pain patients also shows evidence of susceptibility to evoked migraine and HFS diet-induced obesity-related metabolic syndrome. Additionally, exercise or an anti-inflammatory diet is used to evaluate the use of these therapeutic interventions in the treatment of these putative stress-induced disorders. The central hypothesis of this dissertation is that early life stress in mice elicits symptomologies indicative of co-morbid widespread chronic pain and obesity-related metabolic syndrome, which are exacerbated by long-term HFS diet consumption, and can be prevented by non-pharmacological interventions such as exercise or an anti-inflammatory diet. This hypothesis was investigated with the following three aims:

Aim 1: Investigating the long-term consequences of early life stress and exercise on evoked migraine in mice.

Aim 2: Investigating the influence of early life stress, exercise, and a long-term high-fat/high-sucrose diet on the development of obesity-related metabolic syndrome in mice.

Aim 3: Investigating the effect of an anti-inflammatory diet intervention on early life stress-induced co-morbid disorders in mice.

Chapter II: The effect of early life stress and exercise on evoked migraine-like behaviors in mice

2.1 Abstract

Migraine is a complex chronic pain disorder that is highly prevalent in today's society. This neurological disorder affects three times more women than men and can be triggered by many endogenous and exogenous factors. Migraineurs are often diagnosed with other chronic pain disorders as well as mood disorders. Although the exact cause of this co-morbidity is not fully understood, a commonality in many of these patients is that they have a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis, which helps regulate the stress response and influences the perception of pain. The HPA axis is programmed during the formative years. Therefore, adverse childhood experiences, such as stress, can have adverse long-term consequences on the appropriate development of the HPA axis. In this chapter, I use a mouse model of early life stress, neonatal maternal separation (NMS), which exhibits urogenital hypersensitivity and altered limbic regulation of the HPA axis, to determine if female NMS mice are more susceptible to evoked migraine-like behaviors compared to female non-stressed (naïve) mice. Additionally, I investigate the influence of exercise in the form of voluntary wheel running as a therapeutic intervention for evoked migraine-like behaviors. This non-pharmacological intervention was used because exercise successfully reduces frequency, duration, and intensity of migraines in humans and voluntary wheel running attenuates urogenital hypersensitivity in NMS mice. In this study, I first assessed mast cell (MC) characteristics in the dura mater, a tissue important in migraine pathophysiology. When MCs are activated, they release pro-inflammatory cytokines and proteases that can lead to migraine-pain. It was discovered

that female NMS-Sedentary mice had increased MC activation and male NMS-Sedentary mice had an increase in the number of MCs compared to naïve-sedentary or exercised mice. I then used two methods to evoke migraine in female mice: a novel non-surgical method to apply an inflammatory soup (IS) directly onto the dura mater and intraperitoneal nitroglycerin (NTG) injection. Following administration of the noxious solutions, mouse grimace, paw mechanical withdrawal threshold, and photophobia, or sensitivity to light, were measured. I found that dural application of IS resulted in a decrease in forepaw withdrawal threshold in all groups of mice and that NTG increased mouse grimace in all groups of mice. NMS-Sedentary mice appeared more susceptible to NTG induced hind paw sensitivity and photophobia-like behavior compared to Naïve-Sedentary or NMS-Exercised mice. Interestingly, in both naïve and NMS mice, exercise increased mouse grimace following dural application of IS and saline as well as in mice with no dural stimulation. Taken together, these findings demonstrate that selecting the appropriate method to evoke migraine as well as the assessment of proper migraine-like behaviors are important when designing a migraine study in rodents. Additionally, these results suggest that female NMS-Sedentary mice are more susceptible to NTG induced hind paw hypersensitivity and photophobia-like behavior, potentially due to their increased dural MC activation. Future studies in our laboratory will further investigate the mechanism behind this association.

2.2 Introduction

Migraine is the 3rd most prevalent and 6th most debilitating illness in the world (304). It often starts around puberty and affects three times more women than men (305, 306). Migraine is a neurological disorder that presents as throbbing cranial pain, sensitivity to light (photophobia) and sound (phonophobia), nausea, fatigue, irritability, muscle tenderness, and cutaneous allodynia (307). It can be triggered and exacerbated by both endogenous and exogenous factors including hormonal changes, stress, sleep deprivation, certain foods and smells, alcohol, and noise (308). Migraine pain is thought to originate from chemical activation of sensory nerves that interact with intracranial blood vessels and the meninges, primarily the dura mater, which is the outermost covering of the brain (309). The trigeminovascular pathway facilitates nociceptive communication from the meninges to the brain. Sensory neurons from the trigeminal ganglia (TG) have peripheral axons that innervate the dura and cerebral arteries and their central projections terminate at the spinal trigeminal nucleus (SpV) (310, 311) as well as the upper cervical spinal cord (C1-C2) (312), which together are referred to as the trigeminocervical complex (TCC). The innervation associated with migraine pathophysiology is predominately through one branch of the trigeminal nerve, the ophthalmic (V1) division. The other two divisions of the trigeminal nerve, maxillary (V2) and mandibular (V3), play a lesser role in migraine (313). The TCC also receives signals from periorbital skin and pericranial muscles (314). Second order TCC neurons then project to higher pain processing centers including the thalamus, as well as to brainstem nuclei, hypothalamic nuclei, and basal ganglia nuclei (315).

The origination of migraine pain has not been established. One proposed mechanism is that stimulation of the trigeminal nerve causes the release of peptides, including calcitonin gene-related peptide (CGRP) and substance P (SP) (316), from trigeminal nerve endings. These peptides then cause meningeal vasodilation and activation of dural mast cells (MC). Alternatively, meningeal MCs could become activated first and lead to subsequent sensitization of meningeal nociceptors (317, 318). When MCs become activated, they release vasoactive, nociceptive, and inflammatory molecules (e.g. histamine, bradykinin, tryptase, serotonin, CGRP, SP) (44). Despite the uncertainty regarding the origination of migraine, it is generally accepted that MCs play a role in the propagation of migraine pain. MCs are increased in migraineurs (39) and they can expand their population during immune and inflammatory responses by increasing recruitment, survival, and maturation of MC precursors in the area (319). They reside close to sensory nerve endings (47-49) and dural MCs have even been demonstrated to be in direct contact with nerve fibers (50). Additional evidence of MC involvement in migraine is that histamine levels, a major component of MC granules released upon activation, are higher in the serum of migraineurs during migraine attacks (153).

One-third of migraineurs experience migraine with aura, which is described as transient neurological symptoms including visual and speech deficits usually experienced before other migraine symptoms and is hypothesized to be caused by cortical spreading depression (CSD) (320). This can be modeled in rodents by electrical, mechanical, or chemical stimulation of the cortex (321). While CSD is an important factor in migraine pathophysiology, it is beyond the scope of this study.

Stress and migraine

Migraine is often triggered or exacerbated by stress (145, 146) and experiencing multiple stressful life events has been linked to the transformation of episodic migraine to chronic migraine (322). Furthermore, stress experienced early in life is associated with increased susceptibility to developing migraine in adulthood (323-325). One of the first studies to document this relationship was published using data from the Adverse Childhood Experiences (ACE) study. The ACE study used a sample of 9,500 adults and investigated the prevalence rate of early life stress. It included questions about childhood maltreatment including neglect, physical, sexual, and emotional abuse, witnessing parental substance abuse, and parental divorce. They found that half of the individuals surveyed reported at least one ACE and one-fourth reported two or more ACEs (323). From this original cohort of individuals, over 50 studies have been published investigating the long-term consequences of early life stress. Anda et al., (324) used childhood maltreatment data from the ACE study and looked into the prevalence of headaches in these individuals as adults. They found that as the ACE score increased, so did the prevalence of frequent headaches in both males and females The American Migraine Prevalence and Prevention study also found an association between early life stress and headaches in adults. This association was particularly strong for childhood emotional abuse and migraine compared to tension type headache (326). Finally, Brennenstuhl and Fuller-Thomson (325) used data from the 23,000 individuals that participated in the Canadian Community Health Survey-Mental Health, which measured three types of self-reported childhood stress: sexual abuse, physical abuse, and witnessing parental violence. In this cohort of individuals, they found that all three types of early life stress were associated with the development

of migraine in adulthood in both men and women. They also found that as the number of childhood adversities increased, so did the odds of developing migraine in adulthood.

The exact mechanism underlying the relationship between early life stress and migraine has not been fully established. However, many individuals that suffer from migraine show signs of a dysfunctional hypothalamic-pituitary-adrenal axis (HPA) (147-149), which is described in detail in the following section.

Hypothalamic pituitary adrenal (HPA) axis and migraine

Early life stressors, such as childhood neglect and abuse or witnessing parental discord in the home, can negatively impact neurodevelopment resulting in lifelong physical and physiological changes. Specifically, early life stress causes a disruption in the functional output of the HPA axis (105, 106), which regulates the stress response and influences the perception of pain (9, 10). Under normal conditions, an acute stressor will signal the paraventricular nucleus (PVN) of the hypothalamus to release corticotropin-releasing factor (CRF) and arginine vasopressin, which causes the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH signals the adrenal cortex to release glucocorticoids (cortisol in humans and corticosterone in rodents) that have downstream metabolic effects (15, 16). CRF also works in the periphery by stimulating MC degranulation, causing neurogenic inflammation (45, 46). A negative feedback loop is established to turn off activation of the HPA axis by suppressing the production of CRF and ACTH upon cessation of the initial stressor (18, 19). Limbic structures, including the hippocampus, amygdala, and prefrontal cortex, regulate the HPA axis as well (Figure 1.1*)*. The hippocampus and prefrontal cortex usually dampen the HPA axis activation, while the amygdala usually activates it (25).

Chronic migraine sufferers have shown abnormal HPA axis activity (147-149). A study evaluating HPA axis response in patients with chronic migraine and medication-overuse headache found that after administration of CRF, both ACTH and cortisol were significantly elevated in migraineurs compared to control patients (147). A different study administered nitroglycerin (NTG), a known migraine trigger, to migraineurs and controls and measured plasma cortisol levels. 22 out of 30 migraineurs developed a migraine and their plasma cortisol levels significantly increased compared to the controls, none of which developed a migraine (149). These studies suggest that migraineurs might have a hyperactive HPA axis and that stress hormones could play an important role in migraine pathogenesis. These findings could also explain the association between individuals that suffer from early life stress being more susceptible to the development of migraine in adulthood.

Migraineurs often suffer co-morbid mood disorders, such as depression and anxiety, as well as other chronic pain disorders, including interstitial cystitis, irritable bowel syndrome, fibromyalgia, arthritis, and chronic fatigue syndrome (8). Similar to migraine, these disorders are also more prevalent in individuals that have experienced early life stress (3-7) and it appears that one common underlying mechanism in these disorders is a dysfunctional HPA axis (9, 105, 108). The association between early life stress and the development of migraine and other chronic disorders in adulthood is particularly worrying because the rate of childhood maltreatment in the United States is increasing (11). This implies that the incidence of early life stress induced disorders will also increase. Therefore, finding an appropriate way to study this association as well as finding treatment options is important.

Neonatal maternal separation (NMS)

NMS is a method used in rodents that mimics early life stress in humans. NMS protocols vary in length of separation, but the general concept is that pups are removed from the dam for a set amount of time each day over a pre-determined time period. For example, our lab's NMS paradigm is separation of the whole litter from the dam for 3-hours/day from post-natal day 1(P1)-P21 (98). Dysregulation of the HPA axis is an established outcome of NMS. This is evidenced by higher basal plasma corticosterone concentrations and increased CRF_1 expression in the PVN of NMS rodents (88, 89), decreased action of the negative feedback loop leading to prolonged release of ACTH and glucocorticoids after a stressful event (90, 91), and changes in anxiety-like behaviors (92). In line with these observations, Aisa et. al. found that NMS female rats are more susceptible to chronic stress in adulthood compared to normally reared female rats (88). Our lab has found that in NMS mice, hypothalamic and hippocampal mRNA levels of CRF receptors are dysregulated (96, 97). Additionally, we have measured mechanical hypersensitivity and MC activation in urogenital organs of NMS and naïve mice and found that NMS significantly increases urinary bladder (97) and vaginal (96) sensitivity in females and urogenital sensitivity (98) in males. We also saw an increase in the percentage of degranulated MCs in the female (97) and male (100) urinary bladder, the vagina (unpublished observation), and the prostate (98).

The goal of this study was to investigate susceptibility to evoked migraine in NMS mice. To my knowledge, this is the first study to combine early life stress with evoked migraine to evaluate the relationship between early life stress and the susceptibility to migraine in adulthood in rodents.

Models of migraine

Many methods to study migraine in rodents exist, including evoked and genetic models (327). One rodent model of evoked migraine involves administration of various noxious solutions to the dura mater. These noxious solutions include calcitonin-gene-related peptide (CGRP) (328), inflammatory soup (IS; bradkyinin, histamine, 5-HT, and prostaglandin E_2) (329, 330), mustard oil (MO), and the environmental irritant umbellulone (331). These substances are applied with the goal of stimulating the TG afferents that innervate the dura, thus activating the trigeminovascular system. These inflammatory agents are used because they have been shown to be algesic in humans (332). Following noxious dural stimulation, rodents display behavioral and electrophysiological evidence indicative of migraine-like symptoms including facial and extracephalic allodynia, or sensitivity to something that does not normally evoke a painful response, (329-331, 333, 334), increased motivated behavior to seek pain relief (335), the suppression of exploratory behavior (336), light and sound aversion, grimacing behaviors, and alterations in activation of meningeal and trigeminal pain-sensitive neurons that can last for many hours (337, 338). Until recently, rodent dural stimulation had only been accomplished with a surgically implanted cannula. However, a novel non-surgical method has been developed in mice that uses a modified injection cannula to penetrate the skull without puncturing the dura mater (339). This non-surgical method was adapted in this study to attempt to evoke migraine-like behaviors.

Another form of evoked migraine in rodents is intraperitoneal or intravenous administration of pharmacological solutions including nitroglycerin (NTG) (340) and CGRP (341), which trigger migraines in human migraineurs (342, 343). NTG is a nitric

oxide donor that evokes a delayed severe headache along with other migraine symptoms in migraineurs, and occasionally a milder headache without the associated migraine symptoms in healthy subjects (344). In rodents, NTG administration has been shown to induce photophobia-like behavior (345) and mechanical and thermal hind paw allodynia (340, 346). Similar to the delayed effect of NTG seen in humans, the effects of NTG on mechanical allodynia in rodents start between 30-60- minutes after injection and are still present 4- hours later (347). Reuter et al. studied the effects of intravenous NTG administration on the rat meninges (348). They found that NTG infusion caused a delayed increase in dural plasma protein extravasation as well as increased inflammatory markers IL-1β and IL-6. Furthermore, NTG caused significant dural MC degranulation. These findings suggest that NTG evokes migraine symptoms by increasing dural inflammation. IP NTG injection is another method used in this study to attempt to evoke migraine-like behaviors.

Transgenic mouse models of migraine have also been developed and are still being characterized. These include models of monogenic migraine mutations for familial hemiplegic migraine (FHM), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and familial advanced sleep-phase syndrome (FASPS) (349). All of these models show increased susceptibility to CSD (350-352). FHM mice show signs of grimace behavior (353), photophobia, increased head grooming, and increased blinking with one eye closed (354). The transgenic FASPS mouse model has been shown to be more susceptible to NTG induced mechanical and thermal allodynia compared to wildtype mice (352). In addition to these mouse models created from human mutations, a transgenic mouse model with

increased expression of a subunit of the CGRP receptor has also been created. This model consistently displays photophobia-like behaviors that are further exacerbated after CGRP administration and can be blocked by a CGRP receptor antagonist (355, 356).

Despite the association between stress and migraine in humans, a limited number of pre-clinical studies have investigated this. One study evaluated the effect of both acute and chronic restraint stress prior to IP NTG injection on latency of response in a tail-flick test. They found that in non-stressed mice, NTG injection caused hyperalgesia, or greater sensitivity to a painful stimulus, in the tail-flick test compared to non-injected mice. They then found that acute restraint stress caused an analgesic state in the tail-flick test both before and after NTG injection compared to non-stressed controls, while chronic immobilization stress over 7- days caused a hyperalgesic state in the tail-flick test that was exacerbated by NTG injection (357). Another study used both 14- day social defeat stress (SDS) and 40- day chronic variable stress (CVS) paradigms and evaluated hind paw mechanical allodynia before and after the stress as baseline measurements and then 75- and 120- minutes after NTG injection. They found a significant reduction in baseline mechanical withdrawal threshold in the CVS paradigm but not in the SDS paradigm or control mice. After NTG injection, control and both stressed groups showed a significant reduction in hind paw mechanical allodynia at both time points with no significant differences between the groups (346). Theoharides et al., (1995) demonstrated that dural MC degranulation is increased following restraint stress and pretreating rats with polyclonal antiserum to CRF significantly reduced the percent of MC degranulation during restraint stress (157). Finally, a transgenic mouse

model of FHM showed an increase in CSD following treatment with corticosterone, which was blocked by pretreatment with a glucocorticoid receptor antagonist. However, this same model did not show increased CSD following acute stress (152). Taken together, these data demonstrate that stress plays a role in the development of some migraine-like symptoms in rodents and that the type of stressor as well as the outcome measured are important variables to consider.

Migraine Treatment

There are two broad classifications of pharmacological treatments of migraine. These are drugs that are taken as acute treatment during a migraine attack or drugs that are taken as a daily preventative treatment to reduce frequency and severity of attacks. The first class of drugs specifically developed to be taken during migraine attacks are the triptans, which are 5-HT (serotonin) receptor agonists (358). However, they are only efficacious in some migraineurs (359). Other drugs used acutely for the treatment of migraine include ergot alkaloids and non-steroidal anti-inflammatory drugs (360). These drugs are also not universally successful. Until recently, the most well accepted preventative treatments have been β–adrenergic-receptor antagonists, however these are not specific to migraine and they have several unpleasant side effects (360). In 2018, a new class of preventative drugs specific to migraine was approved by the FDA: monoclonal antibodies targeting calcitonin gene-related peptide (CGRP) and its receptor (361). These antibodies are novel because they are too large to cross the blood-brain barrier and therefore work peripherally (313). So far, these drugs have been well tolerated with none of the substantial side effects associated with other preventative treatments (362). However, long-term data are still needed.

Anti-migraine drugs have shown some success in rodent migraine models. For example, sumatriptan, an acute migraine treatment, attenuated IP NTG induced mechanical and thermal hind paw allodynia (347). Additionally, Pradhan et al., (2014) studied sumatriptan as well as topiramate, a migraine preventative therapy. They found that sumatriptan was able to reduce acute NTG induced mechanical hyperalgesia, but was not successful in their chronic migraine model of multiple NTG injections. However, topiramate was able to attenuate both acute and chronic mechanical hyperalgesia (340). Finally, a CGRP anti-body was administered to a medication over-use headache rodent model. They found that this anti-body was able to inhibit NTG or bright light stress evoked cutaneous allodynia (363).

In addition to the pharmacological treatments described above, several non-pharmacological treatments for migraine exist. Most research in this area has focused on behavioral headache management including relaxation training, biofeedback, and cognitive behavioral therapy (177). Exercise has also been investigated as a treatment for migraine prevention. A prospective cross-sectional study found an association between low levels of physical activity and an increase in prevalence of migraine and non-migraine headache (222). Aerobic exercise improves migraine frequency and intensity as well as stress level in migraineurs (364, 365). Additionally, Varkey and colleagues (2011) found that there were no significant differences in headache frequency between individuals that participated in aerobic exercise and those that received either topiramate or relaxation treatment (366), suggesting that exercise could be as effective as pharmacological treatment of migraine in some individuals. There are some studies that have found exercise-induced migraine. However, the majority of

these studies use unusually strenuous exercise (367) coupled with other factors that are known to precipitate migraine such as drinking a large amount of caffeine while consuming little food (368). These are not the types of exercise that would be prescribed as therapy for migraineurs. It is usually suggested that migraineurs slowly habituate to exercise to avoid exercise becoming a migraine trigger (369).

Due to the multitude of effects that exercise has on the body, there are multiple mechanisms that could underlie exercise's effect on migraine symptoms. The influence of exercise on pain and stress are of particular relevance to our work. One hypothesis regarding the development of chronic migraine is that long-term stress can decrease the efficacy of the endogenous antinociceptive system, leading to chronicity of symptoms (322). Exercise can improve descending analgesic pathways by altering conditioned pain modulation (CPM) or the "pain-inhibits-pain" mechanism (241) by increasing endogenous opioids (242), stimulating brain structures involved in descending analgesic pathways (194), and/or maintaining the balance between excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the CNS (245). In support of this, aerobic exercise is associated with an analgesic response in both humans and animals (85). Another hypothesis as to how exercise benefits migraineurs is through the reduction of depression, anxiety, and stress (370), which as described previously is a common migraine trigger. Dientsbier (1989) suggests that long-term exercise can help an individual develop a greater stress tolerance and adapt quicker to stressful situations (371). Exercise induced endorphin production could also decrease stress and anxiety (244).

Exercise has been shown to influence the regulation of the HPA axis, specifically in the hippocampus, which is important in dampening HPA axis activation. If migraineurs have a hyperactive HPA axis, increasing inhibitory control could be important in treating this disorder. We have previously used exercise in the form of voluntary wheel running in our mice and have shown that it increases brain-derived-neurotrophic factor (BDNF) mRNA in the hippocampus (229). Other groups have also found that exercise normalizes NMS-induced changes in hippocampal mRNA including glucocorticoid receptor and BDNF (233, 235). Furthermore, exercise has been shown to increase neurogenesis and dendritic spine density in the hippocampus (236). In addition to changes in the hippocampus, we have demonstrated that exercise normalizes NMS induced urogenital hypersensitivity and increased MC degranulation (100, 229). Therefore, in this study voluntary wheel running is used to evaluate if exercise is also beneficial in preventing the development of migraine-like behaviors in evoked migraine models.

Due to the fact that patients with chronic urogenital pain disorders frequently suffer co-morbidly from migraine, the goal of this study is to evaluate evoked migraine-like symptoms in a mouse model of early life stress, NMS, which has previously displayed behavioral and molecular evidence similar to what is experienced by chronic urogenital pain patients. I hypothesized that NMS mice would display behaviors indicating that they are more susceptible to evoked migraine compared to non-stressed mice. Additionally, because we have seen that voluntary wheel running is beneficial in preventing the development of chronic urogenital pain symptoms in our NMS mice, I

also evaluated the influence of voluntary wheel running on evoked migraine-behaviors. I hypothesized that exercise would prevent the proposed sensitization caused by NMS.

2.3 Methods

Three different cohorts of mice (A, B, and C) were used in the following experiments. All groups were subject to NMS and late (A) or early (B & C) exercise paradigms. The evoked migraine experiments that were implemented are different between the groups. A timeline of events is displayed in Figure 2.1 for clarity.

NMS: Pregnant C57Bl/6 dams at 14-16- day gestation were ordered from Charles River and housed at the Department of Laboratory Animal Resources at the University of Kansas Medical Center. Litters were divided equally into NMS and naïve groups. NMS pups were removed as whole litters from their home cage for 180- minutes (11am-2pm) daily beginning at postnatal day 1 (P1) until P21. During separation, pups were placed in a clean glass beaker with bedding from their home cage. The beaker was placed in an incubator maintained at 33°C and 50% humidity. Naïve mice remained undisturbed in their home cage except for normal animal husbandry. All mice were weaned on P22 and housed 2-5/cage with same sex litter mates and *ad libitum* access to food and water.

Exercise: Group A: At 8-weeks of age, male and female NMS and naïve mice were equally divided into exercised (Ex) and sedentary (Sed) groups. Groups B & C: We have found that an earlier exercise intervention is associated with greater therapeutic

Figure 2.1

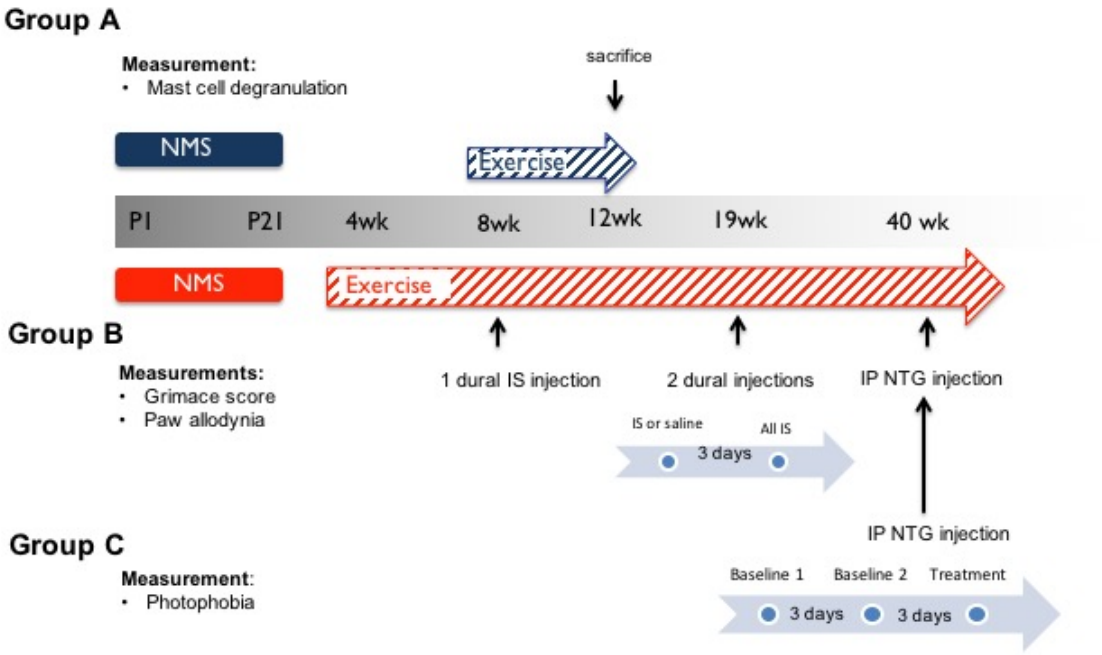
Group A
Measurement:
• Mast cell degranulation
sacrifice
NMS
Exercise
P1
P21
4wk
8wk
12wk
19wk
40 wk
NMS
Exercise
Group B
Measurements:
• Grimace score
• Paw allodynia
1 dural IS injection
2 dural injections
IP NTG injection
IS or saline
All IS
3 days
IP NTG injection
Group C
Measurement:
• Photophobia
Baseline 1
Baseline 2
Treatment
3 days
3 days

Figure 2.1: Group A: Female and male mice either underwent NMS from postnatal day 1 (P1) to P21 or remained in their home cage until weaning (naïve). At 8-weeks of age NMS and naïve mice were further divided into exercised (Ex) or sedentary (Sed) groups. At 12-weeks of age, all mice were sacrificed and dura mater was collected to evaluated mast cell characteristics. **Group B:** Female mice underwent NMS from P1 to P21 or were naïve. At 4-weeks of age NMS and naïve mice were divided into Ex or Sed groups. At 8-weeks of age, mice received either inflammatory soup (IS) or saline applied to the dura mater. At 19-weeks of age, a two application method to the dura was used: First application: either IS or saline and 3-days later the second application: all IS. At 40-weeks of age, mice either received an IP nitroglycerin (NTG) or saline injection. At each treatment time point mouse grimace score and paw mechanical withdrawal threshold were measured. **Group C:** Female mice underwent NMS from P1 to P21 or were naïve. At 4-weeks of age NMS and naïve mice were divided into Ex or Sed groups. At 40-weeks of age, all mice received an IP NTG injection and photophobia-like behavior was measured.

benefit. Therefore, in these groups at 4-weeks of age, female NMS and naïve mice were divided equally into Ex or Sed groups. To avoid the added stress of singly housing, all Ex mice were pair housed with a litter mate of the same sex in cages equipped with a stainless steel running wheel (STARR Life Sciences Corp, Oakmont, PA) and Sed mice remained in their home cage with no access to a running wheel. Distance ran was recorded by STARR Life Sciences VitalView Activity Software version 1.1.

Dural mast cells: Group A: Mice were sacrificed at 12-weeks of age and perfused with 4% cold paraformaldehyde (PFA). Dura was removed from the skull and post-fixed in 4% cold PFA for 1- hour then placed in 30% sucrose. Dura was whole mounted on a glass microscope slide and stained for 10- minutes with 1% toluidine blue (TB) solution acidified with 1M HCL. Slides were allowed to dry for 2- hours in a 37°C oven, washed in 95% then 100% EtOH, fixed in xylene, and cover slipped. Light microscopy (Nikon eclipse 90i, Nikon Instruments, Inc., Melville, NY) was used to take images of each dura sample (QIClick digital CCD Camera, QImaging, Surrey, BC, Canada). The total number and the number of degranulated MCs were counted in 10 non-adjacent fields (800 um^2 per field) per tissue. The percent of degranulated MCs was quantified using the following equation: (Degranulated MC/total MC) x 100.

Timelines on experiment days: **Group B experiment 1:** Naïve- and NMS-Sed and Ex mice received a dural application (described below) of inflammatory soup (IS) or saline. Mice were then allowed to recover from anesthesia for 1- hour before their behavior was recorded to evaluate facial behaviors for the mouse grimace scale (MGS) for 1- hour. Forepaw mechanical withdrawal threshold was then measured. **Group B experiment 2**:

Naïve- and NMS-Sed and Ex mice either received a dural application of IS or saline and were allowed to recover for 1- hour. Then, behavior was recorded to evaluate facial behaviors for 1- hour and forepaw mechanical withdrawal threshold was measured. 72-hours later, all groups received an IS application to the dura and the same behavior was recorded again. **Group B experiment 3:** Naïve- and NMS-Sed and Ex mice received an IP NTG or saline injection (described below). 30- minutes after injection behavior was recorded for 1- hour to evaluate facial behaviors and then hind paw mechanical withdrawal threshold was measured. **Group C:** Naïve- and NMS- Sed and Ex mice received an IP NTG injection and 30- minutes later photophobia-like behavior was measured.

Dural injection: Under inhaled isoflurane a modified cannula injector (Plastics One) was inserted at the lamboidal suture without penetrating the dura (339). 10 microliters IS (1mM histamine, 1mM 5-hydroxytryptamine, 1 mM bradykinin, and 0.1 mM prostaglandin E2 in PBS) or saline was slowly dispersed onto the dura and the injector was removed.

NTG injection: Mice received an IP NTG or saline injection at 10 mg/kg.

Mouse grimace scale (MGS): The MGS is a measure of facial expressions indicating 'pain' in mice. It is a set of five behaviors: orbital tightening, nose bulge, cheek bulge, ear position, and whisker change. Each facial expression is rated as "not present (scored 0), moderate (scored 1), or severe (scored 2)" and then an overall score is assigned (353). Mice were placed on top of a wire mesh screen elevated 55cm above a table and enclosed under an over-turned 500 mL glass beaker. Behavior was video

recorded for 1- hour. Facial screen shots were then taken from the videos every 5-minutes. Photographs of each mouse were randomized and grimace score was assigned to each picture according to a modified version of the MGS established by Langford et al 2010 (353). Only orbital tightening and ear position were scored due to the difficulty of scoring the other features (nose bulge, cheek bulge, and whisker change) on a C57Bl/6 mouse. An average grimace score of each mouse was quantified.

Paw sensitivity: Before testing for paw mechanical sensitivity, mice were acclimated to a sound proof room and the testing table for 2- days before the day of testing. This acclimation consisted of 30- minutes within the sound proof room followed by 30-minutes inside of a clear plastic chamber (11x5x3.5cm) on a wire mesh screen elevated 55cm above a table. Paw mechanical withdrawal threshold was measured using a standard set of graded von Frey monofilaments (1.65, 2.36, 3.22, 3.61, 4.08, 4.31, 4.74g; Stoelting, Wood Dale, IL) following the up-down method. The 3.22g monofilament was used to apply force to one paw. If there was no response, the next larger grade of monofilament was used on the next round of application. If there was a positive response (e.g. raising of the forepaw from the table, licking forepaw), the next smaller grade of monofilament was used on the next round of application. After the first positive response, the up-down method was continued on alternating paws for four more applications with a minimum of five or a maximum of nine applications. The withdrawal threshold of each mouse was then quantified as a 50% g threshold for each mouse (372).

Photophobia: Group C: A modified force plate actimeter was used to assess light aversion behavior. An opaque insert with an opening in the middle was placed in the

center of the box on the actimeter. This allowed the mice to move freely between both sides. The light side of the chamber was equipped with lights affixed to the ceiling and controlled by a single dimmer with low, medium, and high settings. The high setting was used and the intensity on the light side was 950 +/- 20 lux. The light level in the home cage was 170 +/- 20 lux. Two mice could be tested simultaneously in a 20- minute session with a maximum of 15 mice tested in one day between 0800 and 1200- hours, during the light cycle. Mice were tested 6- days and 3- days before the day of treatment to establish a baseline. Mice were always acclimated to the room for 1- hour before testing. During the testing period, mice were placed in the lit compartment facing away from the opening and allowed to freely move between the light and dark compartments for 20- minutes. Movement was recorded using FPARun software (Bioanalytical Systems Inc. West Layfette, IN).

Serum corticosterone ELISA: Group C: Trunk blood was collected at the time of sacrifice between 0900-1100- hours, allowed to clot for 1- hour on ice, and centrifuged at 10,000 rpm for 10- minutes. Serum was collected and stored at -20°C until analysis. Serum corticosterone was quantified using an ELISA kit according to the manufacturer's instructions (ALPCO, Salem, NH).

Statistical analysis: Calculations of the measurements described above were made in Excel (Microsoft, Redmond, WA) and statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA) or IBM SPSS Statistics (IBM Corporation, Armonk, NY). Differences between groups were determined by non-repeated or repeated-measure multi-variate ANOVA and Fisher's LSD or Bonferroni posttest. Statistical significance was set at $p<0.05$.

2.4 Results

NMS influences dural MC characteristics in female and male mice and exercise attenuates these effects.

Experiment A: The total number of MCs and the number of degranulated MCs in 10 non-adjacent fields (800 um^2 per field) per dura were quantified in female and male Naïve- and NMS-Sed and –Ex mice. In female mice, there were no significant differences between groups for total number of dural MC, but there was a trend for NMS-Sed to have a greater number than the other groups (Figure 2.2A). There was a significant overall NMS/exercise interaction effect on the percent degranulated dural MCs in female mice ($p<0.05$, Figure 2.2B). Female NMS-Sed mice had a significantly higher dural MC degranulation rate compared to female Naïve-Sed mice ($p<0.05$) and NMS-Ex mice ($p<0.05$). In male mice, there was an overall NMS/exercise interaction in the total number of dural MCs ($p<0.05$; Figure 2.2C). Male NMS-Sed mice had a significantly greater number of dural MCs compared to male Naïve-Sed ($p<0.01$), but this effect was not seen in male Naïve- and NMS-Ex mice (Figure 2.2C). There were no significant differences in the percent of degranulated dural MCs in the male mice (Figure 2.2D).

Exercise increases MGS in female NMS and naïve mice after dural application of IS or saline, while dural application of IS evokes a decrease in forepaw withdrawal threshold in female mice.

Experiment B1: Under inhaled isoflurane, IS or saline was applied to the dura in female

Figure 2.2

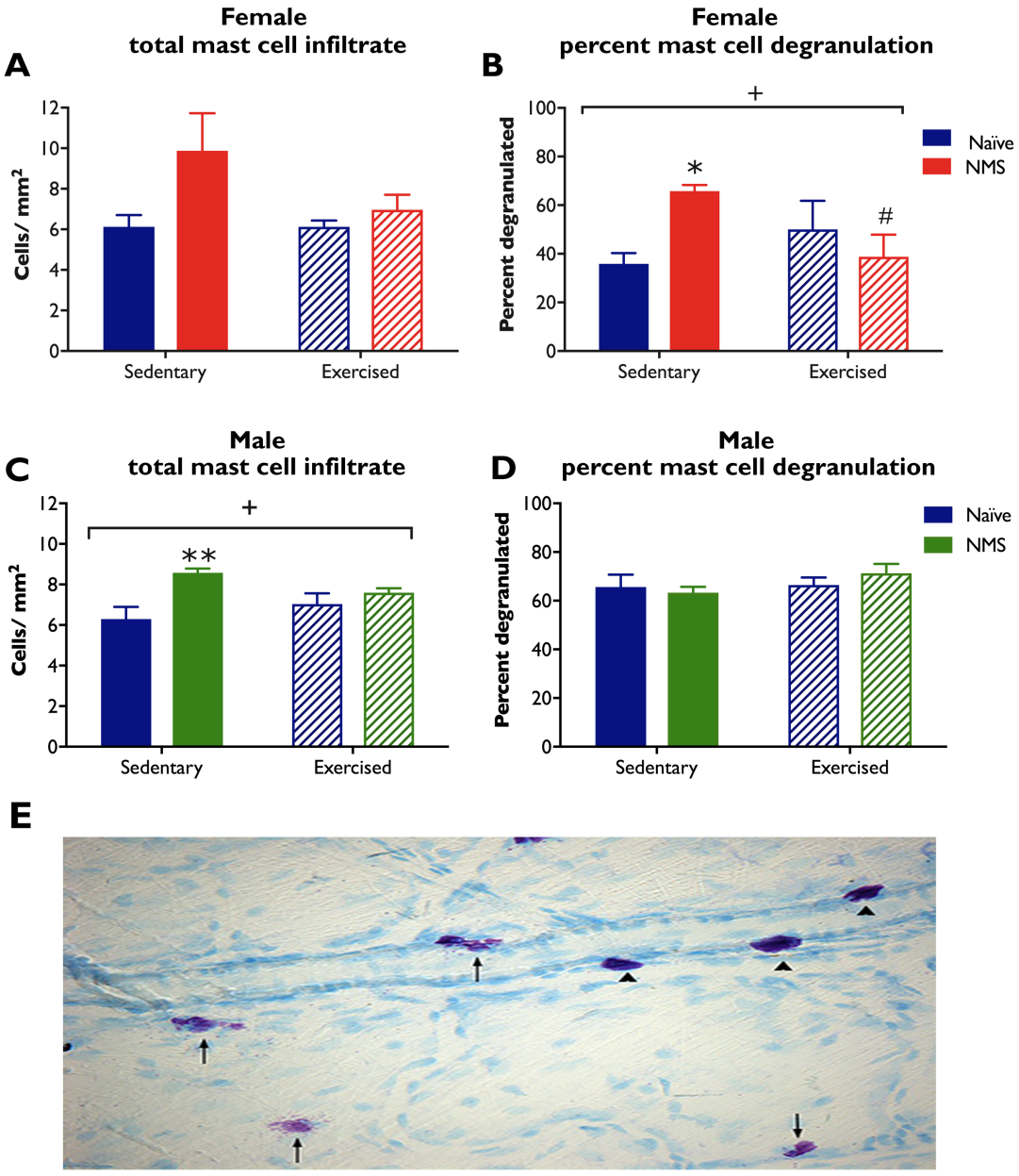
A
Female
total mast cell infiltrate
Cells/ mm²
12
10
8
6
4
2
0
Sedentary
Exercised
B
Female
percent mast cell degranulation
Percent degranulated
100
80
60
40
20
0
+
*
#
Naïve
NMS
Sedentary
Exercised
C
Male
total mast cell infiltrate
Cells/ mm²
+
**
Sedentary
Exercised
D
Male
percent mast cell degranulation
Percent degranulated
Naïve
NMS
Sedentary
Exercised
E

Figure 2.2. Dura mater was stained with toluidine blue to visualize mast cells (MCs) and the total number and percent of degranulated MCs were quantified in female **(A & B)** and male **(C & D)** mice. **A)** Female NMS-Sed mice trended toward a greater number of MC in the dura compared to female Naïve-Sed and NMS-Ex mice but this did not reach significance. **B)** There was a significant NMS/exercise interaction on the percent of degranulated dural MCs in female mice. Female NMS-Sed mice had a significantly greater percent of degranulated dural MC compared to female Naïve-Sed. This effect was attenuated by exercise, as NMS-Ex mice had a significantly lower percent of degranulated MC compared to Naïve-Sed mice. **C)** There was a significant NMS/exercise interaction on the total number of dural mast cells in male mice. Male NMS-Sed mice had a significantly greater number of dural MC compared to male Naïve-Sed mice and this effect was attenuated by exercise. **D)** There were no significant differences in percent of degranulated MC in male mice. **E)** Representative image of dura mater stained with toluidine blue. Arrow heads indicate intact MCs and arrows indicate degranulated or activated MCs. Bracket indicates a significant NMS/exercise interaction (+ $p<0.05$), 2-way ANOVA; *, ** $p<0.05$, 0.01 vs. naïve, # $p<0.05$ vs. sedentary, Bonferroni posttest.

naïve- and NMS-Sed and –Ex mice. 1- hour later, they were evaluated for grimace behavior and forepaw mechanical sensitivity. Following the application of either IS or saline to the dura of female naïve- and NMS-Sed and –Ex mice there was an overall effect of exercise (p<0.0001) on MGS score. Exercise significantly increased MGS score in naïve-Ex-Saline, NMS-Ex-Saline, and NMS-Ex-IS compared to their sedentary counterparts (p<0.05; Figure 2.3A). There was also an overall effect of IS on lowering forepaw withdrawal thresholds (p<0.05; Figure 2.3B). **Experiment B2:** In this experiment, IS or saline was applied to the dura and grimace and forepaw withdrawal threshold were measured. 72- hours later, IS was applied to the dura of all mice and grimace and forepaw withdrawal threshold were measured again. Following the first application of either IS or saline to the dura there were no significant differences in MGS (p>0.05; Figure 2.4A). The NMS-Sed-IS group had a significantly lower forepaw withdrawal threshold compared to the NMS-Ex-IS group (p<0.05; Figure 2.4B). After the second application, where all groups received IS, there was a significant overall effect of exercise on MGS (p<0.001; Figure 2.4C). All exercised groups had significantly greater MGS compared to their sedentary counterparts (p<0.05; Figure 2.4C). Additionally, there was an overall significant interaction effect of exercise and drug on forepaw mechanical withdrawal threshold (p<0.05). Naïve-Sed-IS-IS mice had a significantly higher withdrawal threshold compared to Naïve-Sed-Saline-IS mice (p<0.05) and Naïve-Ex-IS-IS mice (p<0.05; Figure 2.4D).

Exercise increases MGS in female NMS and naïve mice that have not received any form of dural stimulation

Due to the consistent observation that exercised mice, regardless of NMS status,

Figure 2.3

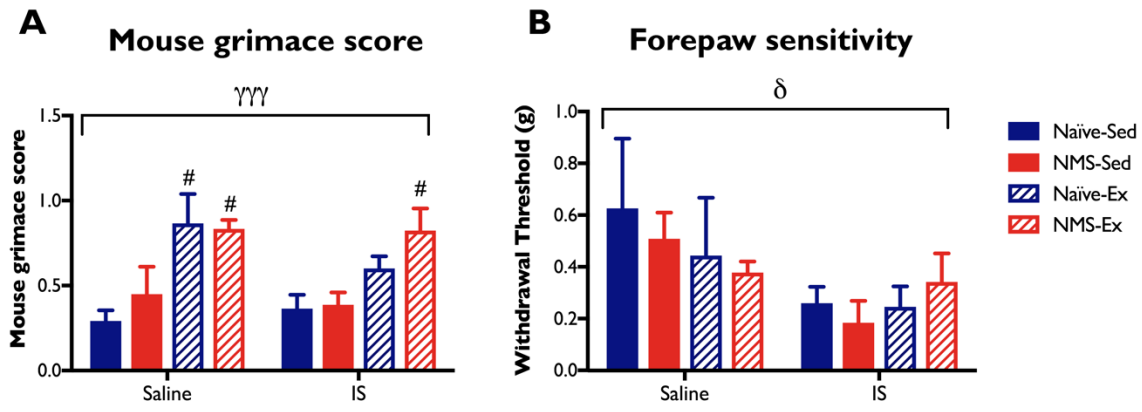
A
Mouse grimace score
γγγ
Mouse grimace score
1.5
1.0
0.5
0.0
#
#
#
Saline
IS
B
Forepaw sensitivity
δ
Withdrawal Threshold (g)
1.0
0.8
0.6
0.4
0.2
0.0
Saline
IS
Naïve-Sed
NMS-Sed
Naïve-Ex
NMS-Ex

Figure 2.3. Inflammatory soup (IS) or saline was applied to the dura mater of female mice through a modified injection cannula. 1- hour after injection, mouse grimace score (MGS) was evaluated by recording facial features for 1- hour. Forepaw mechanical withdrawal threshold was then measured. **A)** There was an overall effect of exercise on MGS. Naïve-Ex-Saline, NMS-Ex-Saline, and NMS-Ex-IS all had significantly higher MGS compared to their sedentary counter parts. **B)** There was an overall effect of IS on forepaw mechanical withdrawal threshold. IS groups generally had lower forepaw mechanical withdrawal thresholds compared to saline groups. Bracket indicates a significant effect of exercise ($\gamma\gamma\gamma$ $p<0.001$) or IS (δ $p<0.05$), 3 way ANOVA; # $p<0.05$ vs. sedentary, LSD posttest.

Figure 2.4

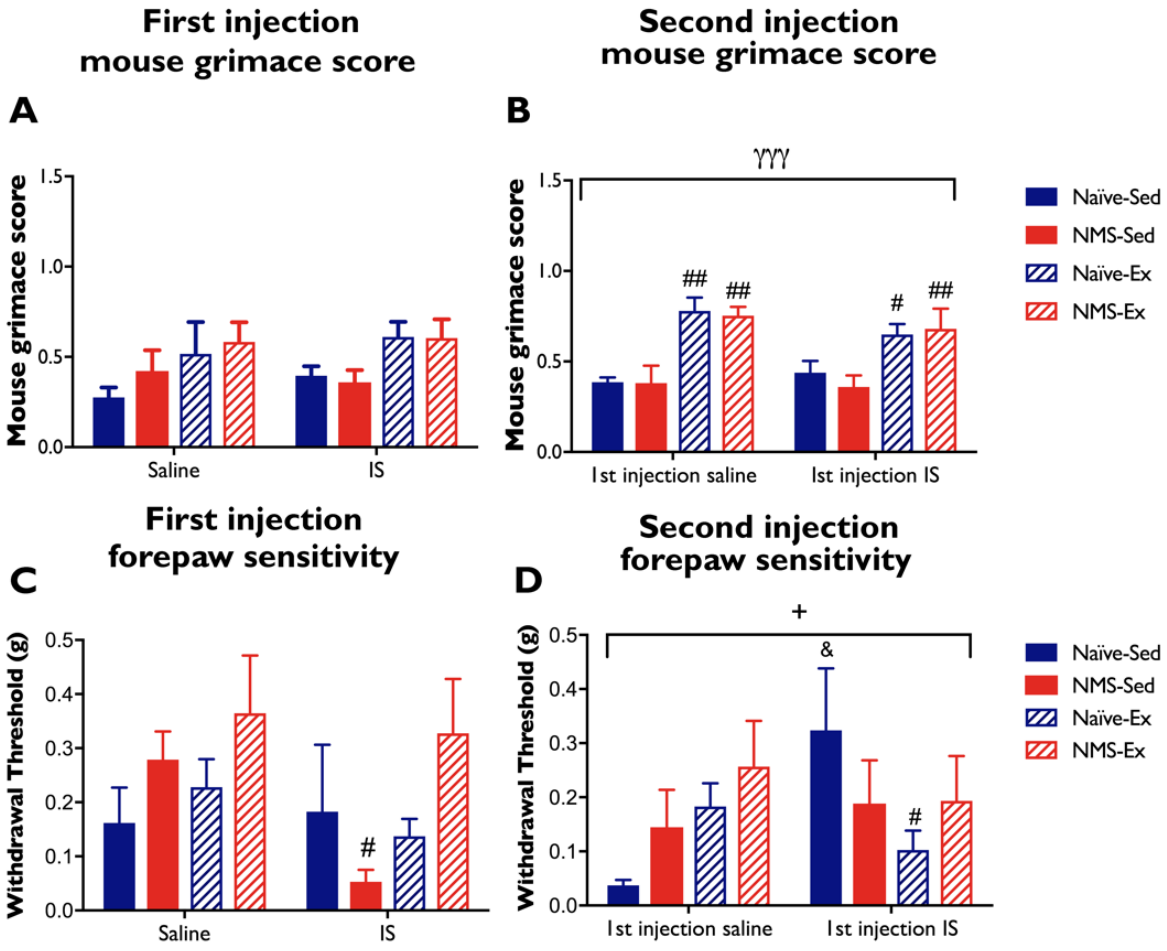
First injection
mouse grimace score
A
Mouse grimace score
1.5
1.0
0.5
0.0
Saline
IS
Second injection
mouse grimace score
B
ϒϒϒ
##
##
#
##
1st injection saline
1st injection IS
Naïve-Sed
NMS-Sed
Naïve-Ex
NMS-Ex
First injection
forepaw sensitivity
C
Withdrawal Threshold (g)
0.5
0.4
0.3
0.2
0.1
0.0
#
Saline
IS
Second injection
forepaw sensitivity
D
+
&
#
1st injection saline
1st injection IS
Naïve-Sed
NMS-Sed
Naïve-Ex
NMS-Ex

Figure 2.4: Inflammatory soup (IS) or saline was applied to the dura of female mice through a modified injection cannula. 1- hour after injection, mouse grimace score (MGS) was evaluated by recording behavior for 1- hour. Forepaw mechanical withdrawal threshold was then measured. 3- days later, all groups received a dural application of IS and the same behaviors were measured. **A)** There were no significant differences in MGS after the first application of IS or saline. **B)** There was a significant effect of exercise on MGS after the 2nd application, where all groups received IS. All exercised groups had significantly higher MGS compared to their sedentary counterparts. **C)** NMS-Sed-IS mice had a significantly lower forepaw withdrawal threshold compared to Naïve-Sed-IS after the first application. **D)** There was a significant overall exercise/drug interaction after the 2nd application, where all groups received IS. Naïve-Sed-IS-IS had a higher forepaw withdrawal threshold then Naïve-Sed-Saline-IS. Naïve-Ex-IS-IS had a significantly lower forepaw withdrawal threshold compared to Naïve-Sed-IS-IS. Bracket indicates a significant effect of exercise (γγγ $p<0.001$) or exercise/drug interaction (+, $p<0.05$), 3-way ANOVA; #, ## $p<0.05$, 0.01 vs. sedentary, & $p<0.05$ vs. saline, LSD posttest.

had increased MGS scores after dural application of either IS or saline, the effect that exercise has on MGS in mice in the absence of dural stimulation was also evaluated. Interestingly, even after no dural stimulation, there was an overall effect of exercise on MGS score (p<0.001; Figure 2.5). Naïve-Ex mice had significantly greater MGS score than Naïve-Sed (p<0.01) and NMS-Ex mice trended toward an increase in MGS score compared to NMS-Sed but it did not reach significance (p=0.0601 Figure 2.5).

Intraperitoneal NTG injection increases MGS and alters hind paw mechanical sensitivity

Experiment B3: Female Naïve- and NMS-Sed and -Ex mice received an IP injection of either NTG or saline and mouse grimace and hind paw mechanical withdrawal threshold were measured 30- minutes later. There was an overall effect of drug on MGS (p<0.0001; Figure 2.6A). Naïve-Ex-NTG had a significantly higher MGS score compared to Naïve-Sed-NTG (p<0.05), Naïve-Ex-Saline (p<0.0001), and NMS-Ex-NTG (p<0.05). NMS-Ex-NTG had a significantly higher MGS score compared to NMS-Ex-Saline (p<0.05). There was a significant overall effect of NMS (p<0.05) and a NMS/exercise interaction (p<0.05) on hind paw withdrawal threshold (Figure 2.6B). Naïve-Sed-Saline had a significantly higher withdrawal threshold than Naïve-Ex-Saline (p<0.05) and NMS-Sed-Saline (p<0.05). There were no significant differences between the NTG groups, but the NMS-Sed-NTG group had a lower withdrawal threshold than Naïve-Sed-NTG and NMS-Ex-NTG groups.

IP NTG injection evokes photophobia-like behavior in NMS mice

Experiment C: Female Naïve- and NMS-Sed and –Ex mice were exposed for 20-minutes to a light/dark box on a force place actimeter on days 3 and 6 prior to the

Figure 2.5

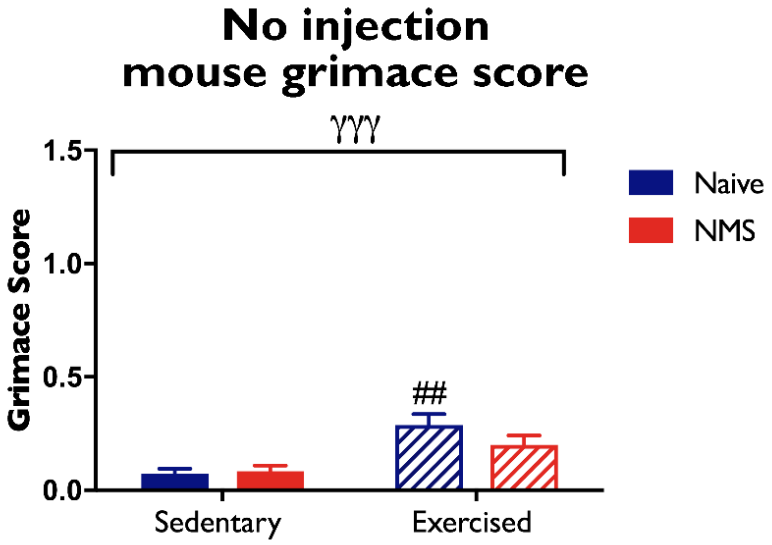
No injection
mouse grimace score
γγγ
1.5
1.0
0.5
0.0
Grimace Score
##
Sedentary
Exercised
Naive
NMS

Figure 2.5: Female mice were briefly anesthetized and 1- hour later behavior was recorded for 1- hour to evaluate mouse grimace score (MGS). There was a significant overall effect of exercise (γγγ $p<0.001$; 2-way ANOVA). Naïve-Ex had a significantly greater MGS compared to Naïve-Sed (## $p<0.01$; Bonferroni posttest).

Figure 2.6

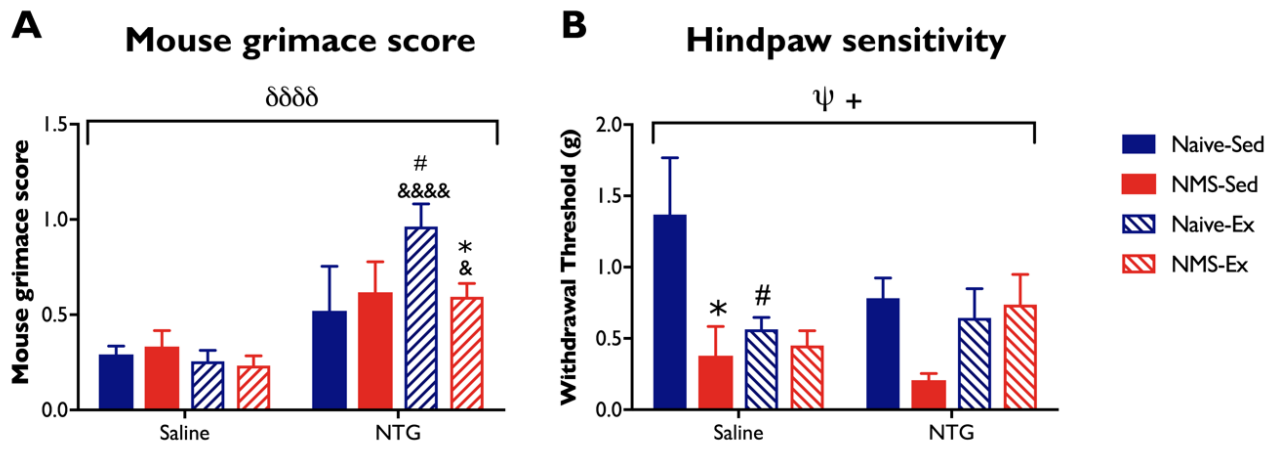
A
Mouse grimace score
δδδδ
Mouse grimace score
1.5
1.0
0.5
0.0
#
&&&&
*
&
Saline
NTG
B
Hindpaw sensitivity
ψ +
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
*
#
Saline
NTG
Naive-Sed
NMS-Sed
Naive-Ex
NMS-Ex

Figure 2.6: Nitroglycerin (NTG) or saline was injected intraperitoneally in female mice. 30- minutes after injection, mouse grimace score (MGS) was evaluated by recording facial features for 1- hour. Hind paw mechanical withdrawal threshold was then measured. **A)** There was a significant overall effect of NTG on MGS. Naïve-Ex-NTG had a significantly higher MGS then Naïve-Sed-NTG and Naïve-Sed-Saline. NMS-Ex-NTG had a significantly lower MGS compared to Naïve-Ex-NTG and a significantly higher MGS than NMS-Ex-Saline. **B)** There was a significant overall effect of NMS and a NMS/exercise interaction on hind paw mechanical withdrawal threshold. NMS-Sed-Saline and Naïve-Ex-Saline had significantly lower hind paw withdrawal thresholds compared to Naïve-Sed-Saline. Bracket indicates a significant effect of NTG (δδδδ $p<0.0001$), NMS (ψ $p<0.05$) or a NMS/exercise interaction (+ $p<0.05$), 3-way ANOVA; # $p<0.05$ vs. sedentary, &, &&&& $p< 0.05, 0.0001$ vs. saline, * $p<0.05$ vs naïve, LSD posttest.

experiment to obtain baseline photophobia data. On the day of the experiment, all groups received an IP injection of NTG and were placed in the light/dark box for 20-minutes to assess photophobia-like behavior. Time spent in the light and distance traveled were quantified at each time point. There was a significant overall effect of time on the percent of time spent in the light side of the box over the 3 experiment days ($p<0.0001$; Figure 2.7A). Naïve-Sed, NMS-Sed, and NMS-Ex all spent significantly less time in the light on the NTG treatment day compared to the first baseline day (6- days before the experiment; $p<0.05$; Figure 2.7A). NMS-Sed mice was the only group that had a significantly lower percent time in light on the NTG treatment day compared to the second baseline day (3- days before the experiment; $p<0.01$; Figure 2.7A). There was a significant overall effect of time on distance traveled during the 2- acclimation days and the treatment day ($p<0.001$; Figure 2.7B). From baseline day 1 to baseline day 2 all groups trended towards traveling more but the only group that reached significance was NMS-Ex ($p<0.05$). The only group that did not decrease the distance traveled between baseline 2 and treatment day was Naïve-Ex, however these decreases were not significant ($p>0.05$; Figure 2.7B). On the treatment day, there was a significant overall effect of NMS on percent time spent in the light ($p<0.05$; Figure 2.7C). Both NMS groups spent less time in the light compared to their naïve counterparts ($p<0.05$). The second baseline day was used to calculate a change from baseline for the percent time spent in light and there was an overall effect of NMS on decreasing the percent of time spent on the light side of the box ($p<0.05$; Figure 2.7D).

Figure 2.7

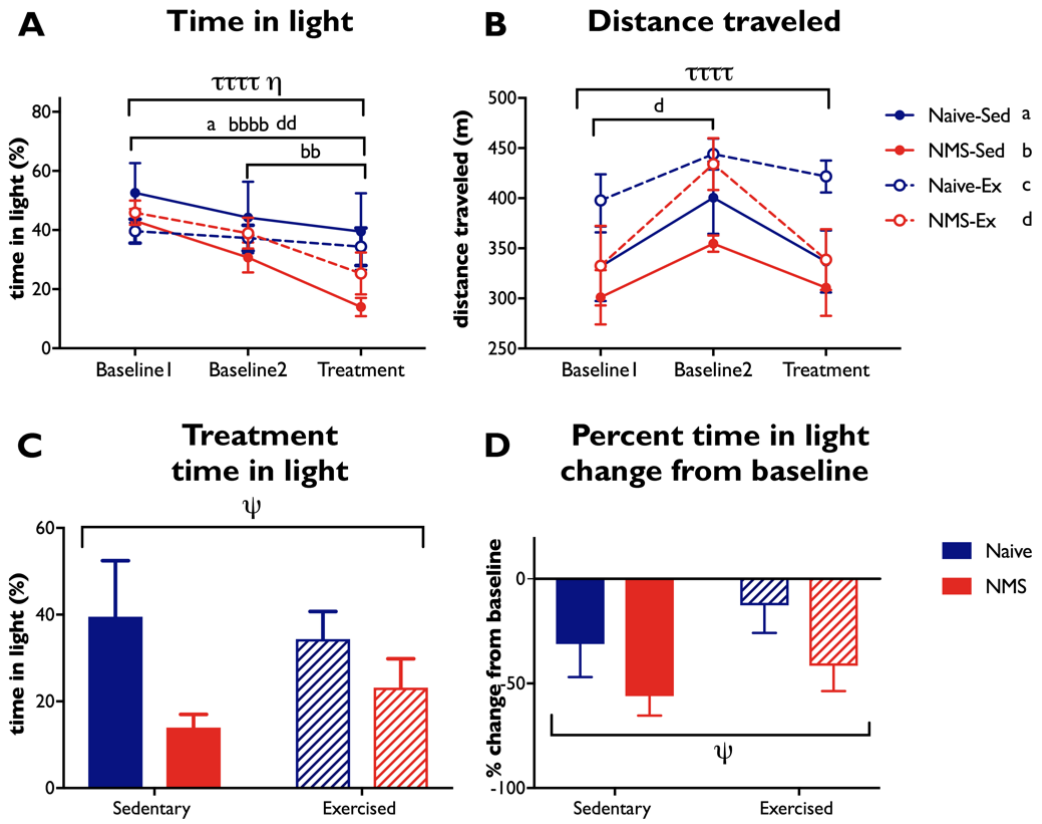
A
Time in light
τττη
a bbbb dd
bb
time in light (%)
80
60
40
20
0
Baseline1
Baseline2
Treatment
B
Distance traveled
τττ
d
distance traveled (m)
500
450
400
350
300
250
Baseline1
Baseline2
Treatment
Naive-Sed a
NMS-Sed b
Naive-Ex c
NMS-Ex d
C
Treatment time in light
ψ
time in light (%)
60
40
20
0
Sedentary
Exercised
D
Percent time in light change from baseline
% change from baseline
0
-50
-100
ψ
Sedentary
Exercised
Naive
NMS

Figure 2.7: Photophobia-like behavior was measured in female mice following IP NTG injection. 3- and 6- days before the NTG treatment day, mice were placed in a light/dark box placed on a force plate actimeter for 20- minutes to obtain baseline measurements of time spent in light and distance traveled. On the treatment day, NTG, was administered by IP injection to all mice. 30- minutes after NTG injection mice were placed in the light/dark box for 20- minutes. **A)** The percent of time spent on the light side of the box was quantified at each time point. There was a significant overall effect of time and a NMS/time interaction on the time spent in the light. Between the first baseline day and the treatment day Naïve-Sed, NMS-Sed, and NMS-Ex mice decreased their time spent in the light. Between the second baseline day and the treatment day, only the NMS-Sed group significantly decreased their time spent in the light. **B)** Total distance traveled was also quantified at each time point and there was a significant overall effect of time. From the first baseline day to the second baseline day, NMS-Ex significantly increased their distance traveled. From the second baseline day to the treatment day, the only group that did not decrease their distance traveled was Naïve-Ex group. **C)** On the NTG treatment day, there was a significant overall effect of NMS on the time spent on the light side of the box. NMS groups spent less time in the light on treatment day compared to naïve groups. **D)** There was a significant overall effect of NMS on the percent change from the second baseline day on the time spent in the light. Bracket indicates a significant effect of time ($\tau\tau\tau\tau$ $p<0.0001$), a NMS/time interaction (η $p<0.05$), or NMS (ψ $p<0.05$), 2-way RM ANOVA or 2-way ANOVA. a, $p<0.05$ Naïve-Sed between time points, bb, bbbb $p<0.01$, 0.0001 NMS-Sed between time points, d, dd $p<0.05$, 0.01 NMS-Ex between time points, Bonferroni posttest.

Serum corticosterone is not significantly influenced by NMS or exercise after IP NTG injection

Experiment C: Mice were sacrificed 2- hours after IP NTG injection. Trunk blood was collected and serum was frozen until analysis using a corticosterone ELISA kit. There were no significant differences in serum corticosterone levels overall or between groups (Figure 2.8).

2.5 Discussion

Migraine is a debilitating neurological disorder that affects 9.7% of males and 20.7% of females in the United States (373). It is a complicated condition with many symptoms that can be triggered by both endogenous and exogenous factors (45, 46), making it difficult to understand and treat. One trigger of migraine is stress and early life stress is associated with the development of migraines in adulthood (323-325). Different preclinical migraine models have been reported in the literature to study the pathophysiology of migraine. This research has led to discoveries of pharmacological migraine treatments that show some success, but there are often harmful off-target side effects associated with these drugs. Therefore, it is important to develop safer therapeutic interventions for migraine, such as exercise (364-366). This study used a mouse model of early life stress, NMS, to investigate the susceptibility of NMS mice to evoked migraine-like behaviors compared to non-stressed mice and if voluntary wheel running had any influence on these behaviors.

Figure 2.8

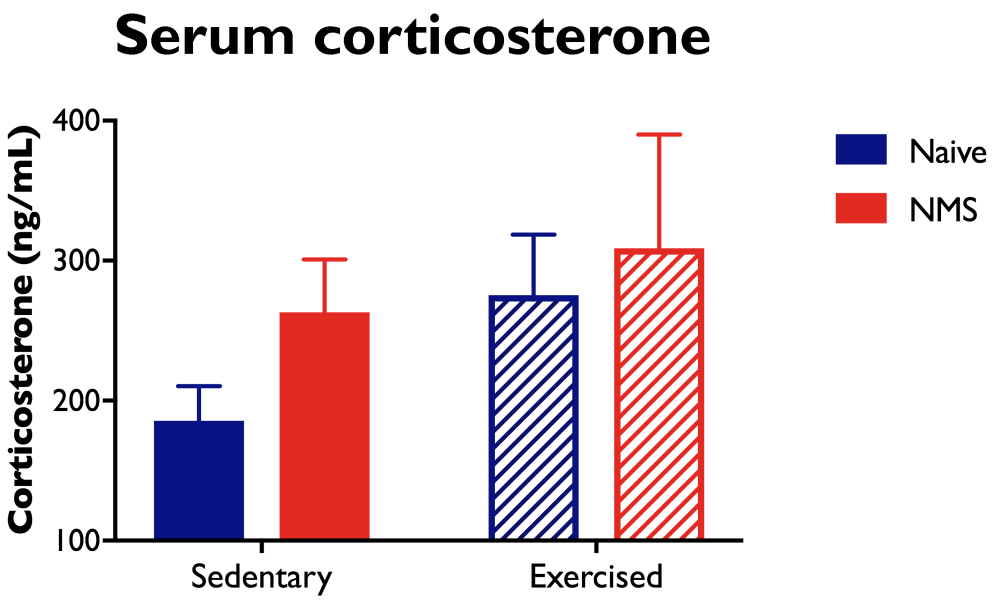
Serum corticosterone
Corticosterone (ng/mL)
400
300
200
100
Sedentary
Exercised
Naive
NMS

Figure 2.8 Mice were sacrificed 2- hours after NTG injection and after assessment of photophobia-like behavior. Trunk blood was collected and serum was frozen until analysis using a corticosterone ELISA. There were no significant overall differences (2-way ANOVA) or differences between groups (Bonferroni posttest).

One tissue that plays an important role in migraine pathophysiology is the dura mater. It contains MCs and is highly innervated by TG neurons (309-311). Dural MCs can become activated by several molecules including CGRP and substance P (45, 46). Furthermore, they are highly responsive to activation of the HPA axis, as they express five isoforms of the CRF_1 receptor, a single isoform of the CRF_2 receptor, and contain one of the largest peripheral stores of CRF (36). This could explain why stress is a common trigger of migraine; increased peripheral CRF release during a stressful event results in dural MC activation and the subsequent release of inflammatory cytokines causing a hypersensitivity reaction (45, 46) that leads to migraine pain. In support of this, I found that early life stress caused an increase in the percent of degranulated MCs in the dura mater of female sedentary mice and an increase in the total number of dural MCs in male sedentary mice. Another group has shown increased dural MC degranulation in rats subject to restraint stress, but treatment with polyclonal antiserum to CRF reduced this effect (157). An additional finding in this study was that exercise normalized the NMS-induced increases in MC degranulation and MC number in NMS sedentary female and male mice, respectively (Figure 2.2). This effect could be due to voluntary wheel running lowering rodent susceptibility to stress, which has been demonstrated in a rodent model of uncontrollable tail shock (374). In this study, compared to rats that had access to running wheels for 6-weeks, sedentary rats displayed more shock-elicited freezing behavior and escape deficits. This effect is similar to exercise reducing stress and frequency of migraine symptoms in migraineurs (364, 365). I did not evaluate MC degranulation in our studies of evoked migraine because the substances administered cause MC degranulation (348). Therefore, it

would not be possible to determine if the MCs were degranulated because of previous stress or because of the trigger used to evoked migraine.

Facial and extracephalic hypersensitivity are commonly seen during a migraine attack in migraineurs (329, 375-377) and after evoked migraine in rodents (329, 330, 334). Facial allodynia is likely caused by sensitization of second order trigeminovascular neurons found in the TCC that receive input from the dura and the periorbital skin (338), while extracephalic allodynia is likely caused by central sensitization (329, 375, 376). I hypothesized that NMS mice would display paw hypersensitivity after evoked migraine compared to naïve mice and that exercise would attenuate this effect. However, after one dural IS application, there was an overall effect of IS lowering forepaw withdrawal thresholds with no significant differences between groups (Figure 2.3). In the IP NTG injection-evoked migraine paradigm, I found that there was a significant overall effect of NMS on hind paw sensitivity, and NMS-Sed mice had a lower withdrawal threshold compared to naïve-Sed, however this did not reach significance (Figure 2.6B). Similar to these findings regarding a lack of significance between NMS and naïve groups, Kaufmann et al., (346) found that both stressed and non-stressed rats developed hind paw mechanical hypersensitivity after NTG injection. This suggests that stress may not be a factor in the development of IS or NTG evoked widespread hypersensitivity. However, I only measured forepaw or hind paw sensitivity at one time point. It would be interesting to see if withdrawal thresholds changed over time in NMS and naïve mice and if exercise had any influence on this. It is possible that NMS mice could develop IS- or NTG-evoked mechanical hypersensitivity quicker than naïve mice or that it takes longer for NMS mice to return to baseline measurements. By only measuring withdrawal

threshold at one time point, I could have missed significant differences throughout the time course of the IS and NTG effects.

I also employed an evoked migraine paradigm where we administered saline or IS then 3- days later administered IS to all groups. I chose to explore this method because it has been shown that one migraine-like event can lead to meningeal sensitization, or priming, to subsequent events (378). However, I did not see consistent patterns in mechanical withdrawal thresholds with this method (Figure 2.4). The method of multiple administrations of migraine-evoking solutions has also been used with IP NTG administration in mice and has demonstrated the progression of widespread hypersensitivity as evoked migraine becomes chronic (340). I only evaluated behavioral responses after one NTG injection. But, if multiple NTG injections had been administered to mimic the multiple migraines that migraineurs usually experience throughout their life, widespread allodynia might have developed, similar to what is seen in migraineurs. I hypothesize that NMS mice would be more susceptible to this chronic NTG administration compared to naïve mice, however it has yet to be tested. Finally, another factor that could be altered in these studies to see if there are differences in widespread sensitivity between stressed and non-stressed groups is to use a lower dose of NTG. For the previously described studies 10 mg/kg NTG was used, but 5 mg/kg and 10 mg/kg NTG are both commonly used in the literature for evoked migraine in rodents. If the lesser dose of 5 mg/kg was used, it is possible that NMS mice could develop widespread allodynia while the naïve mice do not.

Paw mechanical sensitivity is a form of evoked pain-like behavior. I also measured mouse grimace, which is an accepted measure of spontaneous pain-like

behavior (353), and hypothesized that NMS-sed mice would have a higher MGS score compared to naïve or exercised mice. Interestingly, there was consistently an increase in MGS score in both naïve- and NMS-Ex groups after dural IS and saline application (Figure 2.3A and 2.4B), as well as in non-injected mice (Figure 2.5). A higher MGS is associated with more spontaneous painful behaviors. However, in previous studies in our lab, exercise has mitigated chronic urogenital pain symptoms (100, 229). The differences could be due to the fact that past measurements were of evoked pain and this was our lab's first evaluation of spontaneous pain. One factor that could influence these MGS results is that only two measurements of the MGS were evaluated: eye squint and ear position. However, there are three other behaviors that were not evaluated: whisker position, cheek bulge, and nose bulge. Evaluating these three behaviors in black mice proved to be very challenging. Available software to automatically evaluate these measures has only been developed for white rodents (379). An automated 'eye squint' assay has also been developed that highly correlates with MGS (380), suggesting that eye squint alone is predictive of what all 5 MGS behaviors will reveal and that the limited analysis in this study is likely a valid measure.

The significant impact that exercise had on MGS, even in the absence of dural stimulation, was a novel finding. It is unknown what impact voluntary wheel running has on mouse facial features and how this may impact scoring, particularly in terms of eye squint and ear position. In some instances, exercise has been shown to elicit migraine in humans. However, this is usually after strenuous exercise (367) or if exercise is novel and therefore it is suggested that migraineurs be slowly habituated to exercise (369). Our exercised mice had access to running wheels for an extended amount of time

before evaluating grimace, and therefore likely did not elicit a migraine attack because of novelty. Further support of this is that exercise decreased dural MC degranulation in our NMS mice (Figure 2.2).

Mechanical hypersensitivity and MGS are not behaviors specific to migraine; therefore, to follow up these initial studies, photophobia, or sensitivity to light, was measured, which is one symptom that meets the diagnostic criteria of migraine according to the International Classification of Headache Disorders (381). Using a light/dark box placed on a force plate actimeter, similar to the method published by Rossi et al., (382), the location of the mouse was continuously measured during each 20- minute testing period in order to quantify distance traveled and the percent time spent in the light. All groups, with the exception of Naïve-Ex, significantly decreased their time spent on the light side of the box from the first baseline day to the treatment day. However, the only group that significantly decreased their time in the light from the second baseline day to the treatment day was the NMS-Sed group (Figure 2.7A). The percent change in time spent in the light on the treatment day from the second baseline day was then quantified and there was an overall effect of NMS reducing the time spent in light (Figure 2D). These data suggest that the NMS-Sed mice were more sensitive to the light after NTG injection compared to naïve or exercised mice. Total distance traveled during the two baseline measurements and on the treatment day were also evaluated. Interestingly, the only group that did not decrease their distance traveled from the second baseline day to the treatment day was the Naïve-Ex group (Figure 2.7B). Other groups have evaluated photophobia-like behavior in migraine models (341,

345, 356); however, to our knowledge this is the first study to combine a stress and exercise component.

Serum corticosterone after IP NTG injection and subsequent photophobia assessment was also measured. Human plasma cortisol levels are increased in migraineurs after NTG injection and this increase significantly correlated with the development of migraine (149). Due to the fact that NMS and exercise have been shown to influence HPA axis activity, I hypothesized that the combination of NMS and NTG would evoke an increase in circulating corticosterone compared to naïve mice, and exercise would prevent this effect. However, there was no significant effect of NMS or exercise on serum corticosterone level. NMS-Sed mice trended toward an increase in corticosterone, however it did not reach significance. Although this result implies that corticosterone may not have an effect on evoked migraine-like behaviors, corticosterone level was only measured at one time point. Circulating corticosterone is known to follow a circadian rhythm, which peaks in the early evening just before the dark cycle in laboratory rodents and is lowest at the beginning of the light cycle (383). This is opposite to the circadian rhythm of cortisol in humans, which peaks in the early hours of the morning and decreases throughout the day (384). Migraineurs have been shown to have a greater peak cortisol level compared to control patients (147). In this study, serum corticosterone was measured when it should have been in a trough. If corticosterone had been measured in our mice during peak hours, there might have been different results.

In conclusion, these results indicate that female and male NMS-Sed mice have altered dural MC degranulation characteristics compared to naïve mice and that

voluntary wheel running attenuates this. Furthermore, female NMS-Sed mice appear to be more susceptible to NTG induced photophobia-like behavior compared to naïve or exercised mice. Increased MC degranulation and photophobia are two characteristics are also seen in migraineurs. Therefore, these data suggest that female NMS-Sed mice might be more susceptible to the development of migraine-like symptoms compared to naïve or exercised mice. Although the mechanism behind this has yet to be investigated, future work in our laboratory will involve c-fos staining in the TCC as well as phosphorylated extracellular signal related kinase (pERK) staining in the TG to determine if NMS-Sed mice have greater activation in these tissues, which are both relevant to migraine pathophysiology (308). If NMS-Sed mice have increased activation in these tissues, this would provide molecular evidence coinciding with our behavioral photophobia data and make a stronger argument that NMS-Sed mice are in fact more susceptible to evoked migraine and that exercise is able to prevent this stress-induced effect.

Chapter III: The influence of early life stress, exercise, and a long-term high-fat/high-sucrose diet on the development of obesity-related metabolic syndrome in mice

3.1 Abstract

The development of obesity-related metabolic syndrome involves a complex interaction of genetic and environmental factors. An individual is diagnosed with this disorder when they meet at least 3 of the following 5 criteria: central obesity, insulin resistance, hypertension, high triglycerides, and low HDL-cholesterol. One environmental factor found to be significantly associated with the development of obesity-related metabolic syndrome is early life stress. Early life stress is known to cause a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which helps regulate the stress response, influences our perception of pain, and impacts downstream metabolic functions including glucose and fat metabolism, control of the cardio vascular system, and regulation of the immune response. In this chapter I use a mouse model of early life stress, neonatal maternal separation (NMS), which displays urogenital hypersensitivity and altered limbic regulation of the HPA axis, to expand our research on the many consequences of early life stress and investigate if NMS mice can also be used as a model of early life stress-induced obesity-related metabolic syndrome. Additionally, I use various lifestyle conditions, including a high-fat/high-sucrose (HFS) diet and exercise, to investigate if these environmental factors influence NMS and non-stressed (naïve) mice differently in the development of obesity-related metabolic syndrome. Results indicate that adult female and male NMS-Sedentary (Sed) mice have altered body composition compared to female and male Naïve-Sed or exercised (Ex) mice on a standard chow diet. Next, male naïve- and NMS- Sed and -Ex mice were given *ad libitum* access to a HFS or control diet starting at 16-weeks of age for 11-weeks. Results indicate that all of the HFS diet groups gained a significant

amount of weight and body fat compared to the control diet groups, with the exception of the Naïve-Ex-HFS group, which appeared to be protected from HFS diet-induced obesity compared to the other groups. Naïve-Ex-HFS mice were also more protected from a HFS diet-induced increase in pro-inflammatory macrophages in visceral adipose tissue compared to NMS-Ex-HFS mice. I hypothesize this is due to the fact that naïve mice run significantly more than NMS mice and therefore could attain more benefit from exercise. However, exercise was still partially protective in our NMS-HFS mice. For example, both Naïve- and NMS-Ex-HFS groups had significantly lower fasting insulin level and were more glucose tolerant compared to Naïve- and NMS-Sed-HFS groups. Interestingly, the NMS-Sed-HFS group displayed the highest fasting insulin level as well as the greatest increase in pro-inflammatory macrophage, pro-inflammatory cytokine TNFα, and mast cell tryptase mRNA in visceral adipose. These data suggest that this group of mice was particularly susceptible to diet-induced alterations in metabolic function. Finally, NMS-Sed-Control group had greater feed efficiency, which is the ability to turn food consumed into weight gain, was more glucose intolerant, had more pro-inflammatory macrophage mRNA in visceral adipose tissue, and was leptin resistant. These findings are particularly interesting because they demonstrate that before any form of diet or exercise intervention, mice subjected to early life stress have altered metabolic functioning compared to naïve mice. Taken together, this study demonstrates that NMS in mice models early life stress-induced changes in metabolic function and that exercise is an effective therapeutic intervention for the prevention of some of the symptoms associated with obesity-related metabolic syndrome.

3.2 Introduction

An estimated 39.6% of individuals aged 20 and over in the United States are obese and 7.7% are morbidly obese according to the 2015-2016 National Health and Nutrition Examination Survey (NHANES). These statistics have consistently been rising over the past decade and represent significant increases from the 2007-2008 NHANES, in which the prevalence of obesity was 33.7% and morbid obesity was 5.7% (385). Obesity is generally considered a pre-requisite to the development of metabolic syndrome (165), which is diagnosed when an individual suffers from 3 of the following 5 criteria: central obesity, insulin resistance, hypertension, high triglycerides, and low HDL-cholesterol (167). However, there is a subset of individuals that are metabolically obese but normal weight (166). According to NHANES from 2003-2012, overall prevalence of metabolic syndrome in the US was 33% (302). Additionally, individuals with metabolic syndrome are three times more likely to develop cardiovascular disease and five times more likely to develop type 2 diabetes. These data indicate that obesity is a serious public health problem that can lead to the development of other severe and life-threatening diseases.

Obesity is more prevalent among women (386), while type 2 diabetes mellitus is diagnosed at a lower age and body mass index in men (387). These differences have been partially linked to adipose tissue distribution. Central adiposity, particularly visceral adipose tissue, is considered more detrimental to one's health compared to lower body adipose accumulation in the form of subcutaneous adipose tissue (388). Women tend to have more subcutaneous adipose and less visceral adipose compared to males (389). It has been suggested that sex chromosomes are responsible for the differences in fat

distribution and the development of diet-induced obesity and/or diet-induced insulin resistance (390).

Metabolic complications associated with obesity can be attributed to systemic processes, such as widespread metabolic inflammation, and changes at the cellular level, such as mitochondrial dysfunction (391). An obesogenic environment is one in which the amount of energy available exceeds the lipid storage capacity of adipocytes. Therefore, free fatty acids and cytokines can be released into circulation (392) and cause inflammation that leads to the development of health complications including endothelial dysfunction, non-alcoholic fatty liver disease, and insulin resistance (393, 394). This chapter is focused on factors that influence the risk of developing widespread metabolic inflammation as well as other symptoms involved in obesity-related metabolic syndrome.

Stress and obesity-related metabolic syndrome

The development of obesity involves a complex interaction of both genetic and environmental factors (158). One environmental factor found to be significantly associated with the development of obesity is stress (6). Although acute stress is necessary for survival, long-term stress has detrimental consequences. The hypothalamic-pituitary-adrenal (HPA) axis is one neuroendocrine system that helps control the stress response (10). During a stressful event, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing factor (CRF), which signals the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH then causes the adrenal cortex to release glucocorticoids (GCs), which include cortisol in

humans and corticosterone in rodents (15). GCs have many downstream metabolic effects including glucose and fat metabolism as well as control of the cardiovascular system and the immune response (17). Upon cessation of the stressor, GCs work to create a negative feedback loop to turn off the HPA axis at both the pituitary and hypothalamic level (19). Limbic structures, including the hippocampus, amygdala, and prefrontal cortex also play a role in regulating the HPA axis. The hippocampus and prefrontal cortex reduce HPA axis activity while the amygdala increases it (25). CRF can also work in the periphery by activating mast cells (MC), which release cytokines and proteases that contribute to the formation of an inflammatory environment (45, 46).

The HPA axis is not fully developed when children are born and therefore stressful events at this stage of development can cause long-term permanent damage on the regulation and output of this system leading to health problems in adulthood, including obesity (6). Childhood neglect and maltreatment are two forms of early life stress that are associated with the development of obesity in adulthood in humans (160-162). Additionally, HPA axis hyperactivity is seen in patients with visceral obesity (395, 396). Research in non-human primates (163) and rodents (164) also show early life stress-induced alterations in metabolic functions.

According to the 2016 U.S Department of Health and Human services annual Child Maltreatment report, 676,000 children were victims of maltreatment, which shows a 3% increase from data reported 5- years previously. In 2016, 74.8% of these maltreated children were neglected, 18.2% were physically abused, and 8.5% were sexually abused. The age group that showed the greatest maltreatment rate per 1000 children were those under 1 year old (12). This finding is particularly distressing with the

knowledge that during this time, the stress system is still developing (13) and therefore is vulnerable to alterations that can have long-term consequences such as dysregulation of the HPA axis in adulthood. One such form of dysregulation is impaired inhibitory control of the HPA axis by the hippocampus. The hippocampus has been shown to be particularly sensitive to stress-induced atrophy (397). This brain region expresses both glucocorticoid and mineralocorticoid receptors (GR and MR, respectively), which bind the GCs that are released during stress. During a time of chronic stress, such as early life stress, these receptors are constantly occupied. This can lead to hippocampal dendritic atrophy (397), which causes altered function within the hippocampus (398). Without appropriate hippocampal inhibition of the HPA axis, hypersecretion of GC stress hormones can occur. Increased circulating GCs has been associated with the development of obesity, particularly in the visceral region, and other symptoms of metabolic syndrome including insulin resistance, dyslipidemia, and hypertension (399).

Neonatal maternal separation

Dysregulation of the stress system is not only associated with the development of obesity, but also with a plethora of other disorders, including chronic pain and mood disorders, which are often experienced co-morbidly with obesity (3-7). To investigate the complicated relationship between early life stress and the subsequent development of obesity-related metabolic syndrome in adulthood, I used a mouse model called neonatal maternal separation (NMS). In this model, pups are separated as a litter from their mom for 3-hours/day from postnatal day (P) 1 to P21. Our laboratory previously used NMS to study the association between early life stress and the development of symptoms

similar to those experienced by chronic urogenital pain patients (96, 98). The goal of this study is to see if mice subjected to NMS also develop characteristics of obesity-related metabolic syndrome. Furthermore, I evaluate whether or not lifestyle conditions such as exercise or diet have an influence on symptoms associated with this syndrome.

Lifestyle factors and obesity

A sedentary lifestyle (400-402) and a diet with a high proportion of saturated fat and refined sugars (HFS), referred to as a 'western diet' (286, 287) are significant environmental risk factors associated with obesity and related chronic diseases. Unfortunately, HFS diet consumption has grown due to its convenience and lower cost compared to healthier foods such as fruits and vegetables (288). Long-term consumption of HFS diet has many negative health consequences including the development of obesity (178, 289), type 2 diabetes (290), cardiovascular disease (291), and adverse impacts on brain function and behavior (292). Sedentary behavior is also highly prevalent in the United States, where most individuals do not meet the recommended requirements for daily physical activity (403). Sedentary behavior is associated with 35 chronic diseases, including metabolic syndrome, obesity, insulin resistance, hypertension, cognitive dysfunction, depression, and anxiety (402).

Due to the fact that lifestyle factors are significantly associated with the development of obesity-related metabolic syndrome and other chronic health conditions, making lifestyle changes such as a healthier diet and increased physical activity may be the best way to prevent or treat the symptoms associated with these disorders. To successfully treat obesity, it is essential to reverse the positive energy balance that

underlies obesity into an energy deficit by decreasing energy intake and/or increasing energy expenditure. Exercise improves symptoms of metabolic syndrome in humans (221, 287) and rodents (228, 404), while the combination of diet and exercise interventions has the greatest therapeutic effect (405). There are pharmacotherapies for the treatment of obesity-related metabolic syndrome that can be used in addition to lifestyle changes, but these have been shown to be only marginally efficacious (406, 407). 'Exercise mimetics' are also being studied, which attempt to mimic some of exercise's influence on the body including mitochondrial remodeling, increased mitochondrial oxidative phosphorylation, and fatty acid metabolism (408). However, due to the wide-range of effects that exercise has on the body (246), these are unlikely to be able to completely replace physical activity, suggesting exercise still remains the best option for the treatment of some of these chronic disorders. Additionally, heat therapy is being investigated as a treatment option for individuals with obesity-related metabolic syndrome or type 2 diabetes because of the significant role that heat shock proteins play in metabolic regulation (409). Gupte et al., (410) demonstrated that weekly heat treatments (41°C for 20 mins) in high-fat diet fed rats improved many measures related to metabolic syndrome. Specifically, heat-treated rats had reduced epididymal fat pad weight and fasting insulin level as well as improved glucose tolerance compared to non-heated treated high-fat diet fed rats. This group is now evaluating the influence of heat-treatments in clinical studies to determine if their pre-clinical findings can be translated to humans. This would provide a novel therapeutic intervention that would be easier for many individuals with obesity-related metabolic syndrome to partake in compared to some exercise regimens.

Models of obesity and exercise intervention in rodents

Both genetic and diet-induced rodent models of obesity are highly prevalent in the literature (411). Diet-induced obesity is a particularly relevant model due to the current lifestyle trends in today's society. These experimental designs usually allow rodents free access to calorie-dense foods that are high in fats, sugar, or both, to mimic the Western diet consumption of humans. The development of obesity and metabolic disorders is then monitored (411). Many different factors can influence the results of these experiments, including strain, sex, and age of the rodent. Certain strains of mice are more susceptible to diet-induced obesity and related disorders than others. For example, C57Bl/6J are highly susceptible to these disorders (412) while A/J mice are less susceptible (413). The sex of mice used is also an important factor to consider because male mice are more susceptible to diet-induced obesity-related metabolic syndrome compared to female mice (303, 414). Finally, age should be considered as well. In general, body weight in C57Bl/6J increases over time and older mice have increased fat mass compared to younger mice (415). Additionally, Nishikawa et al., (2007) demonstrated that starting a high-fat diet at an older age in C57Bl/6J produced more robust changes in adipose tissue and hepatic lipid accumulation compared to starting the high-fat diet in younger mice (416).

Genetic mouse models of obesity include knockout models of genes in the hypothalamic leptin-melanocortin feeding pathway such as *Lepr, Mc4r, Pomc,* and *Pcsk1*. These models have been shown to have altered energy homeostasis and body weight (417). Other genetic models of obesity include the *ob/ob* and *db/db* mice, which have deficiencies in leptin or leptin receptor production, respectively (418). *ob/ob* mice

develop severe obesity and subsequently develop mild hyperglycemia and insulin resistance. *db/db* mice develop severe hyperinsulinemia early on but eventually insulin levels drop significantly as pancreatic β– cells atrophy, leading to a hyperglycemic state (411).

Exercise interventions in rodents include both resistance training and aerobic exercise such as wheel running, treadmill running, and swimming. Forced treadmill running is considered a stressor, while voluntary wheel running is considered rewarding (232-234), as most rodents run when provided a running wheel (231). Our laboratory has incorporated voluntary wheel running into our studies and have previously shown that it can prevent or attenuate NMS-induced urogenital MC degranulation and pain-like behaviors (100, 229). Therefore, in this chapter voluntary wheel running is used to evaluate the influence of exercise on diet-induced obesity-related metabolic syndrome.

Significance

This study is novel because it is investigating the influence of three factors, early life stress, voluntary wheel running, and HFS diet, on the development of obesity-related metabolic syndrome. I adapted paradigms similar to the human experience in an attempt to make our studies as translatable as possible. Early life stress mimics childhood neglect, which is the most prevalent form of childhood maltreatment, voluntary wheel running mimics humans choosing to exercise and avoids the added stress of forced exercise paradigms, and a HFS diet mimics the Western diet that has increased in consumption in recent years. This work is important in advancing our knowledge regarding the prevention of potentially modifiable risk factors, such as early

life stress, poor diet, and sedentary behavior, in the development of obesity-related metabolic syndrome.

3.3 Methods

Neonatal maternal separation: Pregnant C57Bl/6 dams at 14-16- day gestation were ordered from Charles River and housed in the Department of Laboratory Animal Resources at the University of Kansas Medical Center. Litters were divided equally into NMS and naïve groups. NMS pups were removed as whole litters from their home cage for 180- minutes (1100-1400- hours) daily beginning at postnatal day 1 (P1) until P21. During separation, pups were placed in a clean glass beaker with bedding from their home cage. The beaker was placed in an incubator maintained at 33°C and 50% humidity. Naïve mice remained undisturbed in their home cage except for normal animal husbandry. All mice were weaned on P22 and pair-housed with same sex litter mates and *ad libitum* access to food and water.

Exercise: At 4-weeks of age, female and male NMS and naïve mice were equally divided into exercised (Ex) or sedentary (Sed) groups. Ex mice were pair housed with a same sex litter mate in cages equipped with a stainless steel running wheel (STARR Life Sciences Corp, Oakmont, PA) and Sed mice were pair housed with a same sex litter mate but no access to a running wheel. Distance ran/pair was recorded by STARR Life Sciences VitalView Activity Software version 1.1.

Experimental designs: **Female mice:** At 40-weeks of age, NMS- and Naïve- Sed and -Ex mice body composition was evaluated; they were then sacrificed and adipose tissue was removed. **Male mice:** At 14-weeks of age, NMS- and Naïve- Sed and -Ex mice

body composition was evaluated. At 16-weeks of age, mice were further divided into control diet or high-fat/high-sucrose (HFS) groups. Body composition was measured every 2-3-weeks while food consumption and running distance were measured weekly throughout the experiment. At 27-weeks of age, fasting insulin and glucose tolerance were measured. Mice were sacrificed the morning after these measurements and adipose tissue was removed.

Body composition analysis: Mice were weighed and then placed in an EchoMRI 2015 to measure lean mass and fat mass. Total weight, percent body fat, and free fat mass were quantified every 2-3-weeks.

Periovarian adipocyte size: Female mice at 40-weeks of age were over dosed with inhaled isoflurane. Periovarian adipose tissue was dissected out and a piece was post-fixed in 4% PFA for 48-hours then placed in 70% EtOH. It was then paraffinized, sectioned, and stained with hematoxylin and eosin to assess adipocyte size.

High-fat/high-sucrose diet (HFS): At 16-weeks of age, male naïve- and NMS-Sed and -Ex groups were divided equally into HFS and control diet groups. HFS diet was composed of 20% protein, 35% carbohydrates, and 45% fat (Research Diets Inc; New Brunswick, NJ; Table 3.1).

Table 3.1: Control and HFS diet components

	Control diet Research diets D12110704		HFS diet Research diets D12451	
	gm%	**kcal%**	**gm%**	**kcal%**
Protein	19.2	20	24	20
Carbohydrate	67.3	70 (3.5% sucrose)	41	35 (15% sucrose)
Fat	4.3	10	24	45
Total		100		100
Kcal/g	3.85		4.73	

Food consumption and feed efficiency: Food consumption per pair of mice was measured weekly. Feed efficiency was quantified every week using the following equation: ((weight change per pair)/(calories consumed per pair))*1000. It is presented as the average feed efficiency over the 11-weeks of the experiment after the start of the HFS diet.

Glucose Tolerance Test: At 27-weeks of age, following a 6-hour fast, mice were given an intraperitoneal injection of glucose at 1 g/kg body weight. Blood glucose levels were measured via tail clip immediately before glucose injection and 15, 30, 60, and 120-minutes thereafter (glucose diagnostic reagents, Sigma).

Fasting insulin level: At 27-weeks of age, following a 6- hour fast, blood was collected via tail-clip, placed on ice for 1-hour, and centrifuged at 10,000 rpm for 10-minutes. Serum was collected and frozen until analysis using an insulin *ELISA* kit.

mRNA extraction and RT-PCR: Mice were overdosed with inhaled isoflurane. Epididymal adipose tissue was dissected and immediately frozen in liquid nitrogen. Frozen tissue was then crushed (Cellcrusher, Portland, OR) and total RNA was isolated using QIAzol Lysis Reagent and the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA)**.** The concentration and purity were determined using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) and cDNA was synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) and a Bio-Rad iCycler IQ real time PCR system with indicated 20uM primers (Table 3.2; Integrated DNA Technologies, Coralville, IA). Samples were run in triplicate and negative control

Table 3.2: Forward and reverse primers used for RT-PCR

Gene	Forward (5'-3')	Reverse (3'-5')
PPiB	GGCTCCGTCGTCTTCCTTTT	CCTCTACTTAGACATCCTGCTCA
F4/80	TGACTCACCTTGTGGTCCTAA	CCTTTCTGACCTAAGACCCTTC
CD68	CCTCGCCTAGTCCAAGGTC	TCGGGTTTAAGTTTAGGCTTAGG
CD11b	GGGAGGACAAAAACTGCCTCA	TACGACGCTTCTAGGATCAACA
CD11c	CCAAGACATCGTGTTCCTGATT	ACGACCTGAAACAATTTCGACA
TNFα	CAGGCGGTGCCTATGTCTC	GATGACTTGAAGCCCCACTAGC
IL-6	TCTATACCACTTCACAAGTCGGA	TTTCTCAACACGTTACCGTTAAG
IL-1B	GCAACTGTTCCTGAACTCAACT	TCAACTGCCTGGGGTTTTCTA
INFγ	ACAGCAAGGCGAAAAAGGATG	AGTAGGCTCACCAGGTGGT
Tryptase	GCCAATGACACCTACTGGATG	GTTGTTCCAGTCTCATGTCGAG
Leptin	GTGGCTTTGGTCCTATCTGTC	CCTAGTTACTGTAAAGTGTGTGC
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
11βHSD1	GGAGCCCATGTGGTATTGACT	TACCTTCTGTACTGTAAACGCC

reactions were run with each amplification series. To reduce variability among efficiency due to fluctuations in baseline fluorescence, the raw PCR data was imported to the LinRegPCR software and PCR efficiency values were derived for each individual sample. Threshold cycle values were subtracted from that of the housekeeping gene PPiB and the percentage of fold change over Naïve-Sed-Control was calculated using the Pfaffl method (419).

Statistical analysis: Calculations of the above measurements were made in Excel (Microsoft, Redmond, WA) and statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA) or IBM SPSS Statistics (IBM Corporation, Armonk, NY). Differences between groups were determined by 2- or 3-way ANOVA or 4-way RM ANOVA. Fisher's LSD or Bonferroni posttest were used. Statistical significance was set at $p<0.05$.

3.4 Results

Adult NMS female Sed mice have altered body composition compared to naïve and Ex female mice

Female mice were divided into sedentary (Sed) or exercised (Ex) groups at 4-weeks of age and were allowed *ad libitum* access to a standard chow diet. At 40-weeks of age, EchoMRI was used to assess female NMS- and naïve- Sed and Ex body composition. There was a significant overall effect of NMS ($p<0.001$) and NMS/exercise interaction ($p<0.05$) on body weight (Table 3.3). NMS-Sed mice weighed significantly more than naïve-Sed mice ($p<0.01$) and this effect was prevented by exercise. There was a significant effect of NMS ($p<0.01$), exercise ($p<0.01$), and a NMS/exercise

Table 3.3 Female body composition at 40-weeks of age

Variable	Overall analyses	Naïve-Sed	NMS-Sed	Naïve-Ex	NMS-Ex
Body weight (g)	ψψψ +	24.483 ± 1.047	30.440 ± 1.192 *	25.088 ± 0.724	26.589 ± 0.871 #
Body fat percentage (%)	ψψ γγ +	15.292 ± 1.405	25.977 ± 3.240 *	14.037 ± 1.293	15.816 ± 1.930 ##
Fat free mass (g)	ψ	20.675 ± 0.606	22.442 ± 0.902	21.511 ± 0.397	22.278 ±0.422
Fat mass (g)	ψψ γγ +	3.808 ± 0.499	7.998 ± 1.221 **	3.576 ± 0.426	3.768 ± 0.345 **
Periovarian fat mass (g)		0.755 ±0.134	1.496 ± 0.273 *	0.743 ± 0.114	0.963 ± 0.187
Retroperitoneal fat mass (g)		0.235 ± 0.049	0.524 ± 0.111 *	0.229 ± 0.047	0.316 ± 0.066

Data are displayed as mean ± S.E.M. Overall analyses were performed using two-way ANOVA. Significant effect of NMS, ψψ, ψψψ $p< 0.01, 0.001$, exercise, γγ $p<0.01$, or a NMS/exercise interaction + $p<0.05$ are denoted.*,** $p<0.05, 0.01$ vs. naïve; #, ## $p<0.05, 0.01$ vs. sedentary; Bonferroni posttest.

interaction (p<0.05) on body fat percentage and total fat mass. NMS-Sed had a significantly higher body fat percentage (p<0.05) and greater total body fat (p<0.01) compared to naïve-Sed and NMS-Ex (p<0.01). There was a significant effect of NMS on fat free mass (p<0.5) but no significant differences between groups. After sacrifice, periovarian and retroperitoneal adipose tissue was removed and weighed. NMS-Sed mice had significantly more periovarian and retroperitoneal adipose tissue compared to naïve or exercised mice (p<0.05; Table 3.3). Additionally, NMS-Sed mice had larger periovarian adipocytes compared to naïve and exercised mice (Figure 3.1).

Adult male NMS mice have greater body fat percentage compared to adult male naïve mice

Male mice were divided into Sed or Ex groups at 4-weeks of age and were allowed *ad libitum* access to a standard chow diet. At 14-weeks of age, EchoMRI was used to assess male NMS- and naïve- Sed and -Ex body composition. There was a significant overall effect of exercise on body weight (p<0.05; Table 3.4). There was a significant overall effect of NMS (p<0.05) and exercise (p<0.001) on fat mass and body fat percentage. NMS-Ex had significantly less fat mass and a lower body fat percentage compared to NMS-Sed (p<0.01) and a significantly higher body fat percentage compared to Naïve-Ex (p<0.05). Naïve-Ex had significantly less fat mass and a lower body fat percentage compared to naïve-Sed (p<0.01). No significant differences in fat free mass were observed.

Figure 3.1

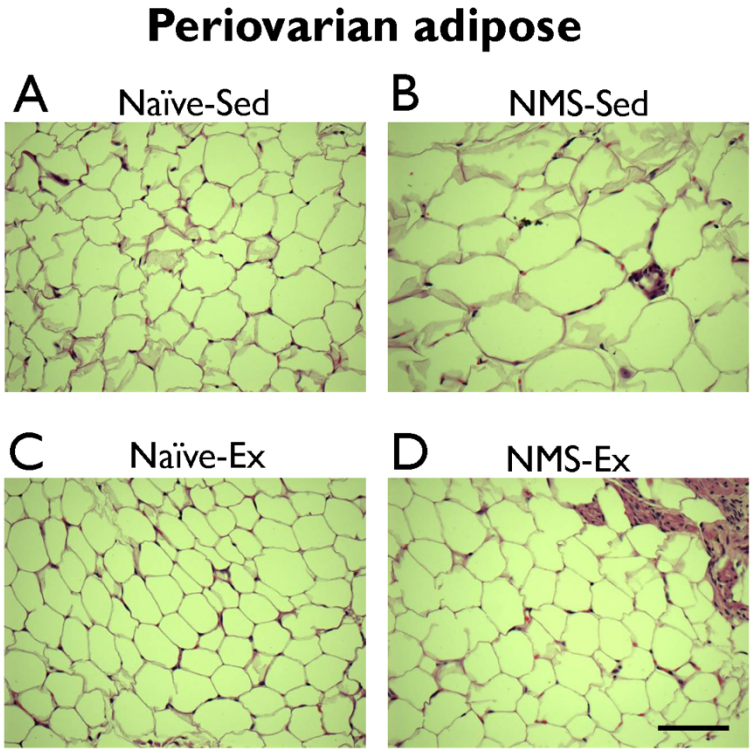

Figure 3.1 Periovarian adipose tissue from 40-week-old female mice on a standard chow diet was paraffinized, sectioned, and stained with hematoxylin and eosin to observe the size of the adipocytes. NMS-Sed (B) had larger adipocytes compared to naïve-Sed (A) and this effect was not seen in exercised mice (C-D). Scale bar equals 100μm.

Table 3.4 Male body composition at 14-weeks of age

Variable	Overall analyses	Naïve-Sed	NMS-Sed	Naïve-Ex	NMS-Ex
Body weight (g)	γ	27.650 ± 0.636	28.100 ± 1.454	25.470 ± 0.558	25.380 ± 1.007
Body fat percentage (%)	ψ γγγ	16.451 ± 0.578	19.745 ± 2.712	7.651 ± 0.747 ##	12.526 ± 1.409 *##
Fat free mass (g)		22.891 ± 0.421	22.279 ± 0.464	23.514 ± 0.513	22.113 ± 0.675
Fat mass (g)	ψ γγγ	4.473 ± 0.179	5.821 ± 1.004	1.956 ± 0.198 ##	3.267 ±0.468 ###

Data are displayed as mean ± S.E.M. Overall analyses were performed using two-way ANOVA. Significant effect of NMS ψ $p<0.05$, or exercise, γ, γγγ $p<0.05$, 0.001; #, ## $p<0.05$, 0.01 vs. sedentary; Bonferroni posttest.

Long-term HFS diet and exercise alter body composition in male mice

To determine if NMS increases obesogenic susceptibility to a HFS diet, male NMS- and naïve-Sed and -Ex groups were placed on high-fat/high-sucrose (HFS) diet or control diet at 16-weeks of age. All mice were sacrificed at 27-weeks of age, which corresponded with 11-weeks after the introduction of the HFS diet. There was a significant overall effect of diet ($p<0.0001$) on body weight, body fat percentage, and total fat mass as well as on fat free mas ($p<0.05$; Table 3.5). Exercise had a significant overall effect on body weight ($p<0.05$), body fat percentage ($p<0.0001$), and total fat mass ($p<0.0001$). Finally, there was a significant diet/exercise interaction on body fat percentage ($p<0.05$). HFS groups weighed significantly more ($p<0.01$), had significantly higher body fat percentage ($p<0.01$), and greater total fat mass ($p<0.0001$) than control diet groups. Exercise attenuated body fat gain in naïve- and NMS- control diet groups, however this effect was greater in naïve mice. Exercise also prevented naïve-HFS mice from gaining as much body fat as naïve-Sed-HFS mice ($p<0.05$), however this effect of exercise was not observed in NMS-HFS mice.

Body weight, body fat percentage, fat free mass, and fat mass were measured every 2-3-weeks using EchoMRI (Figure 3.2). A significant effect of time was observed for all measurements ($p<0.0001$). Diet also significantly impacted body weight, body fat percentage, and total fat mass over time ($p<0.0001$), such that the HFS diet groups gained significantly more body weight and body fat percentage over time compared to control diet groups. Exercise influenced body weight ($p<0.05$), body fat percentage ($p<0.0001$), and total fat mass ($p<0.0001$), such that exercised groups gained less weight and body fat percentage over time compared to sedentary mice. NMS influenced

Table 3.5 Male Body composition at 27-weeks of age

Variable	Overall analyses	Naïve-Sed-Control	NMS-Sed-Control	Naïve-Ex-Control	NMS-Ex-Control	Naïve-Sed-HFS	NMS-Sed-HFS	Naïve-Ex-HFS	NMS-Ex-HFS
Body weight (g)	γ δδδδ	32.55± 1.247	32.675 ± 3.066	38.5 ± 1.404	28.875 ± 2.256	45.1 ± 1.431 **&&&**	45.6 ± 1.44 **&&&&**	39.3 ± 2.317 **&&&**	42.983 ± 2.223 **&&&&**
Body fat percentage (%)	γγγγ δδδδ ϕ	27.402 ± 2.351	27.416 ± 6.316	7.129 ± 1.524 **###**	17.410 ± 4.997 **# ***	42.920 ± 0.585 **&&**	41.948 ± 0.657 **&&**	33.359 ± 3.387 **&&&& #**	41.054 ± 0.135 **&&&&**
Fat free mass (g)	δ	23.568 ± 0.607	23.148 ± 0.454	26.438 ± 1.042	23.615 ± 1.419	25.743 ± 0.852	26.483 ± 0.977	24.812 ± 0.633	25.195 ± 1.314
Fat mass (g)	γγγγ δδδδ	8.983 ± 1.013	9.528 ± 2.649	2.063 ± 0.488 **##**	5.260 ± 1.862	19.358 ± 0.678 **&&&&**	19.118 ± 0.572 **&&&**	13.4 ± 1.917 **&&&& ##**	17.788 ± 0.993 **&&&& ***

Data are displayed as mean ± S.E.M. Overall analyses were performed using multi-variate ANOVA. Significant effect of exercise, γ, γγγγ *p*<0.05, 0.0001; diet δ, δδδδ *p*<0.05, 0.0001; or exercise/diet interaction ϕ *p*<0.05;
&&, &&&, &&&& *p*<0.01, 0.001, 0.0001 vs. control diet; #, ##, ### *p*<0.05, 0.01, 0.0001 vs. sedentary; * *p*<0.05 vs. naïve; LSD posttest.

Figure 3.2

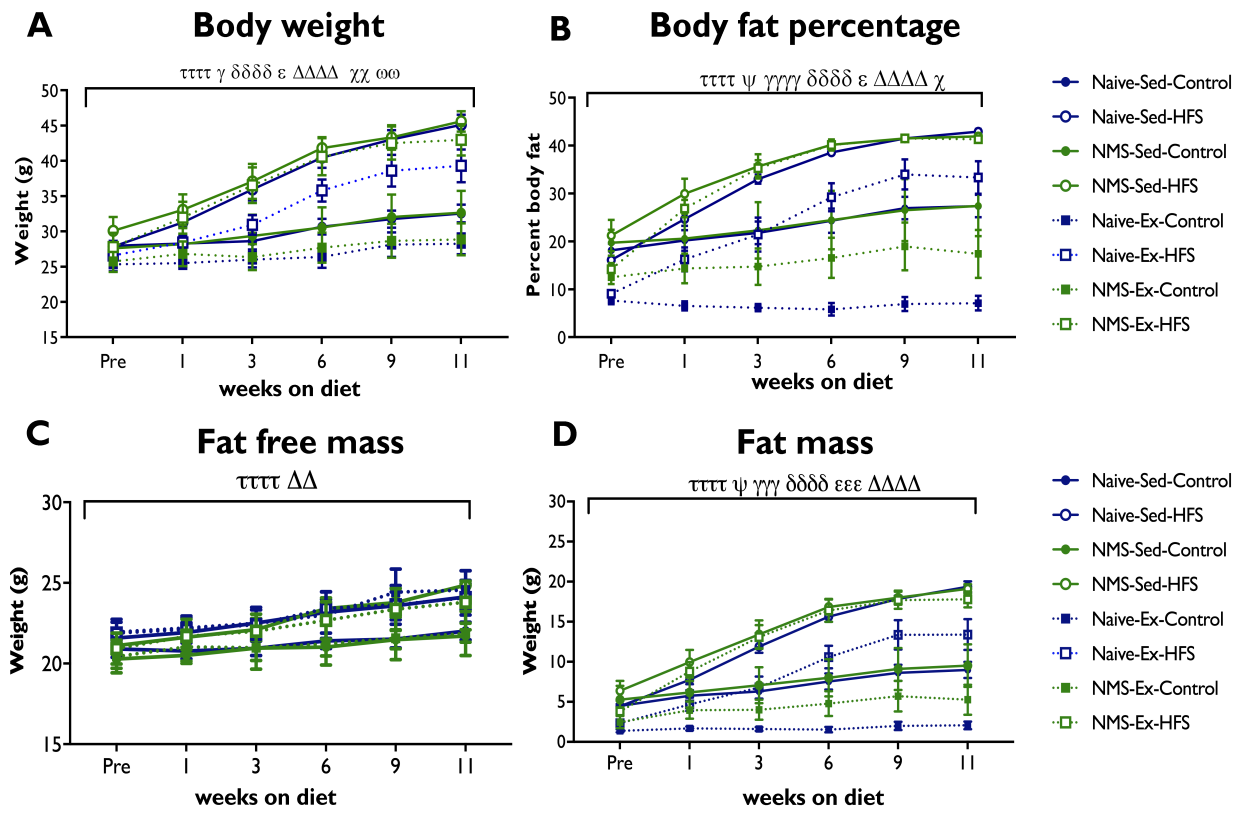
A
Body weight
Weight (g)
weeks on diet
B
Body fat percentage
Percent body fat
weeks on diet
C
Fat free mass
Weight (g)
weeks on diet
D
Fat mass
Weight (g)
weeks on diet
Pre
1
3
6
9
11
Naive-Sed-Control
Naive-Sed-HFS
NMS-Sed-Control
NMS-Sed-HFS
Naive-Ex-Control
Naive-Ex-HFS
NMS-Ex-Control
NMS-Ex-HFS

Figure 3.2 EchoMRI was used to assess body composition of NMS- and naïve- Sed and -Ex mice immediately prior to and following the initiation of HFS and control diet interventions. **A**) A significant overall effect of time, exercise, a time/exercise interaction, a time/diet interaction, a time/stress/exercise/diet interaction, and a time/exercise/diet interaction, was observed on body weight. **B**) A significant overall effect of time, NMS, exercise, diet, a time/exercise interaction, a time/diet interaction, and a time/stress/exercise/diet interaction was observed on body fat percentage. **C**) A significant overall effect of time and a time/diet interaction was observed on fat free mass. **D**) A significant effect of time, NMS, exercise, diet, a time/exercise interaction, and a time/diet interaction was observed on fat mass. Bracket indicates a significant effect of time (ττττ, $p<0.0001$), exercise (γ, γγγ, γγγγ $p<0.05$, 0.001, 0.0001), diet (δδδδ $p<0.0001$), NMS (ψ $p<0.05$), a time/exercise interaction (ε, εεε $p<0.05$, 0.001), a time/diet interaction (ΔΔ, ΔΔΔΔ $p<0.01$, 0.0001), a time/exercise/diet interaction (ωω $p<0.01$), or a time/NMS/exercise/diet interaction (χ, χχ $p<0.05$, 0.01), 4-way RM ANOVA. Post-hoc analyses are listed in Tables 3.6-3.8 for body weight, body fat percentage, and fat mass over time.

body fat percentage (p<0.05) and total fat mass (p< 0.05), such that NMS groups generally gained more body fat over time compared to naïve mice; this was particularly evident in control diet mice. Multiple interaction effects on body composition measures were also observed over time: time/exercise on body weight (p<0.05), body fat percentage (p<0.05), and total fat mass (p<0.001), time/diet on body weight (p<0.0001), body fat percentage (p<0.0001), fat free mass (p<0.01), and total fat mass (p<0.0001), time/exercise/diet on body weight (p<0.01), and time/NMS/exercise/diet on body weight (p<0.01) and body fat percentage (p<0.05).

Overall and post-hoc analyses were done on body composition data at each time point and results are listed in Table 3.6 (body weight), Table 3.7 (body fat percentage), and Table 3.8 (total fat mass). In summary, HFS diet had a significant overall impact on increasing body weight starting after only 1-week on the HFS diet (p<0.01) and lasting the duration of the experiment. Post-hoc analyses revealed that NMS-Ex-HFS mice weighed significantly more than NMS-Ex-Control (p<0.05) beginning after only 1-week on HFS diet, while naïve-Sed-HFS weighed significantly more than naïve-Sed-Control (p<0.05) and NMS-Sed-HFS weighed significantly more than NMS-Sed-Control (p<0.01) beginning after 3-weeks on the HFS diet. Naïve-Ex-HFS did not start weighing significantly more than naïve-Ex-Control until 6-weeks on the diet (p<0.01). Diet also had a significant overall effect on increasing body fat percentage beginning after only 1-week on the HFS diet (p<0.0001) and lasting the duration of the experiment. Post-hoc analyses revealed that every HFS group had significantly more body fat percentage compared to their control diet counter parts (p<0.05) after 1-week on the diet with the exception of the naïve-Sed, which became significantly different after 3-weeks on the

Table 3.6: Body weight analyses of Naïve- and NMS- Sed- and Ex- Control and HFS diet groups before (pre) and every 2-3-weeks after introduction of the HFS diet.

		Pre-HFS diet	1-week on diet	3-weeks on diet	6-weeks on diet	9-weeks on diet	11-weeks on diet
Overall analyses							
Effect of exercise		p=0.011	NS	NS	p=0.036	NS	p=0.012
Effect of diet		N/A	p=0.003	p<0.0001	p<0.0001	p<0.0001	p<0.0001
Post-hoc analyses							
Naïve-Ex-HFS vs. NMS-Ex-HFS	Effect of NMS	NS	NS	p=0.034	NS	NS	NS
Naïve-Sed-Control vs. Naïve-Sed-HFS	Effect of diet	N/A	NS	p=0.024	p=0.004	p=0.002	p=0.001
Naïve-Ex-Control vs. Naïve-Ex-HFS	Effect of diet	N/A	NS	NS	p=0.002	p=0.002	p=0.001
NMS-Sed-Control vs. NMS-Sed-HFS	Effect of diet	N/A	NS	p=0.018	p=0.001	p=0.002	p<0.0001
NMS-Ex-Control vs. NMS-Ex-HFS	Effect of diet	N/A	p=0.035	p=0.001	p<0.0001	p<0.0001	p<0.0001

3-way ANOVA & LSD posttest. Significance set at p<0.05. NMS= neonatal maternal separation, Sed= sedentary, Ex= exercised, HFS= western diet, NS= no significance, N/A= not applicable.

Table 3.7: Body fat percentage analyses of Naïve- and NMS- Sed- and Ex- Control and HFS diet groups before (pre) and every 2-3-weeks after introduction of the HFS diet.

		Pre-HFS diet	1-week on diet	3-weeks on diet	6-weeks on diet	9-weeks on diet	11-wks on diet
Overall analyses							
Effect of NMS		*p*=0.017	*p*=0.004	*p*=0.008	*p*=0.009	*p*=0.04	NS
Effect of exercise		*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
Effect of diet		N/A	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001	*p*<0.0001
Stress/Ex interaction			NS	NS	*p*=0.04	*p*=0.033	NS
Ex/diet interaction		N/A	NS	NS	NS	*p*=0.032	*p*=0.044
Post-hoc analyses							
Naïve-Ex-Control vs. NMS-Ex-Control	Effect of NMS	*p*=0.024	NS	NS	*p*=0.02	*p*=0.015	*p*=0.04
Naïve-Ex-HFS vs. NMS-Ex-HFS	Effect of NMS	N/A	*p*=0.003	*p*=0.001	*p*=0.005	NS	NS
Naïve-Sed-Control vs. Naïve-Ex-Control	Effect of exercise	*p*=0.001	*p*=0.002	*p*=0.002	*p*<0.0001	*p*<0.0001	*p*<0.0001
Naïve-Sed HFS vs. Naïve-Ex-HFS	Effect of exercise	N/A	*p*=0.031	*p*=0.011	*p*=0.026	NS	*p*=0.037
NMS-Ex-Control vs. NMS-Sed-Control	Effect of exercise	*p*=0.002	NS	NS	NS	NS	*p*=0.045
Naïve-Sed-Control vs. Naïve-Sed-HFS	Effect of diet	N/A	NS	*p*=0.023	*p*=0.003	*p*=0.004	*p*=0.003
Naïve-Ex-Control vs. Naïve-Ex-	Effect of diet	N/A	*p*=0.014	*p*=0.001	*p*<0.0001	*p*<0.0001	*p*<0.0001

HFS							
NMS-Sed-Control vs. NMS-Sed-HFS	Effect of diet	N/A	p=0.029	p=0.008	p=0.001	p=0.003	p=0.005
NMS-Ex-Control vs. NMS-Ex-HFS	Effect of diet	N/A	p=0.002	p<0.0001	p<0.0001	p<0.0001	p<0.0001

3-way ANOVA, LSD posttest. Significance set at p<0.05. NMS= neonatal maternal separation, Sed= sedentary, Ex= exercised, HFS= western diet, NS= no significance, N/A= not applicable.

Table 3.8: Fat mass analyses of Naïve- and NMS- Sed- and Ex- Control and HFS diet groups before (pre) and every 2-3-weeks after introduction of the HFS diet.

		Pre-HFS diet	1-week on diet	3-weeks on diet	6-weeks on diet	9-weeks on diet	11-wks on diet
Overall analyses							
Effect of NMS		p=0.038	p=0.009	p=0.012	p=0.012	NS	NS
Effect of exercise		p<0.0001	p=0.003	p=0.003	p=0.001	p=0.002	p<0.0001
Effect of diet		N/A	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
Post-hoc analyses							
Naïve-Ex-HFS vs. NMS-Ex-HFS	Effect of NMS	N/A	p=0.006	p=0.001	p=0.002	p=0.027	p=0.025
Naïve-Sed-Control vs. Naïve-Ex-Control	Effect of exercise	p=0.001	p=0.022	p=0.037	p=0.008	p=0.007	p=0.005
Naïve-Sed HFS vs. Naïve-Ex-HFS	Effect of exercise	N/A	NS	p=0.014	p=0.013	p=0.038	p=0.008
NMS-Sed-Control vs. NMS-Ex-Control	Effect of exercise	p=0.002	NS	NS	NS	NS	NS
Naïve-Sed-Control vs. Naïve-Sed-HFS	Effect of diet	N/A	NS	p=0.014	p=0.001	p<0.0001	p<0.0001
Naïve-Ex-Control vs. Naïve-Ex-HFS	Effect of diet	N/A	NS	p=0.013	p<0.0001	p<0.0001	p<0.0001
NMS-Sed-Control vs. NMS-Sed-HFS	Effect of diet	N/A	p=0.031	p=0.006	p<0.0001	p<0.0001	p<0.0001
NMS-Ex-Control vs. NMS-Ex-HFS	Effect of diet	N/A	p=0.004	p<0.0001	p<0.0001	p<0.0001	p<0.0001

3-way ANOVA, LSD posttest. Significance set at p<0.05. NMS= neonatal maternal separation, Sed= sedentary, Ex= exercised, HFS= western diet, NS= no significance, N/A= not applicable

HFS diet (p<0.05). NMS also had an overall impact on body fat percentage starting prior to the introduction of the HFS diet (p<0.05) and lasting through the 11-weeks on the HFS diet. Post-hoc analyses revealed that naïve-Ex-Control had significantly higher body fat percentage than NMS-Ex-Control after 6-weeks on the diet (p<0.05) and Naïve-Ex-HFS had significantly higher body fat percentage than NMS-Ex-HFS after 3-weeks on the diet (p<0.01), but this difference only lasted through 6-weeks on the diet. Exercise had the opposite effect that diet and NMS had on body fat percentage in that it reduced body fat percentage compared to sedentary groups. There was a significant overall effect of exercise starting prior to the introduction of the HFS diet (p<0.0001) and lasting through duration of the experiment. Post-hoc analyses revealed that naïve-Ex-Control mice had significantly lower body fat percentage compared to naïve-Sed-Control mice at the 'Pre' time point (p<0.0001) and this lasted through the duration of the experiment. Naïve-Ex-HFS had significantly lower body fat percentage compared to Naïve-Sed-HFS starting after only 1-week on the HFS diet (p<0.05), while NMS-Ex-Control did not significantly differ from NMS-Sed-Control until 11-weeks on the HFS diet (p<0.05) and NMS-Ex-HFS never significantly differed from NMS-Sed-HFS (Table 3.7). Finally, total fat mass was influenced by diet, NMS, and exercise similarly to body fat percentage (Table 3.8).

Feed efficiency is influenced by exercise, diet, and stress.

Feed efficiency is a measure of an animal's ability to turn energy consumed into weight. It is calculated as (weight gained per pair per week)/(calories consumed per pair per week). An overall effect of exercise (p<0.01), diet (p<0.0001), and a NMS/exercise/diet interaction (p<0.01) was observed on feed efficiency (Figure 3.3A).

Figure 3.3

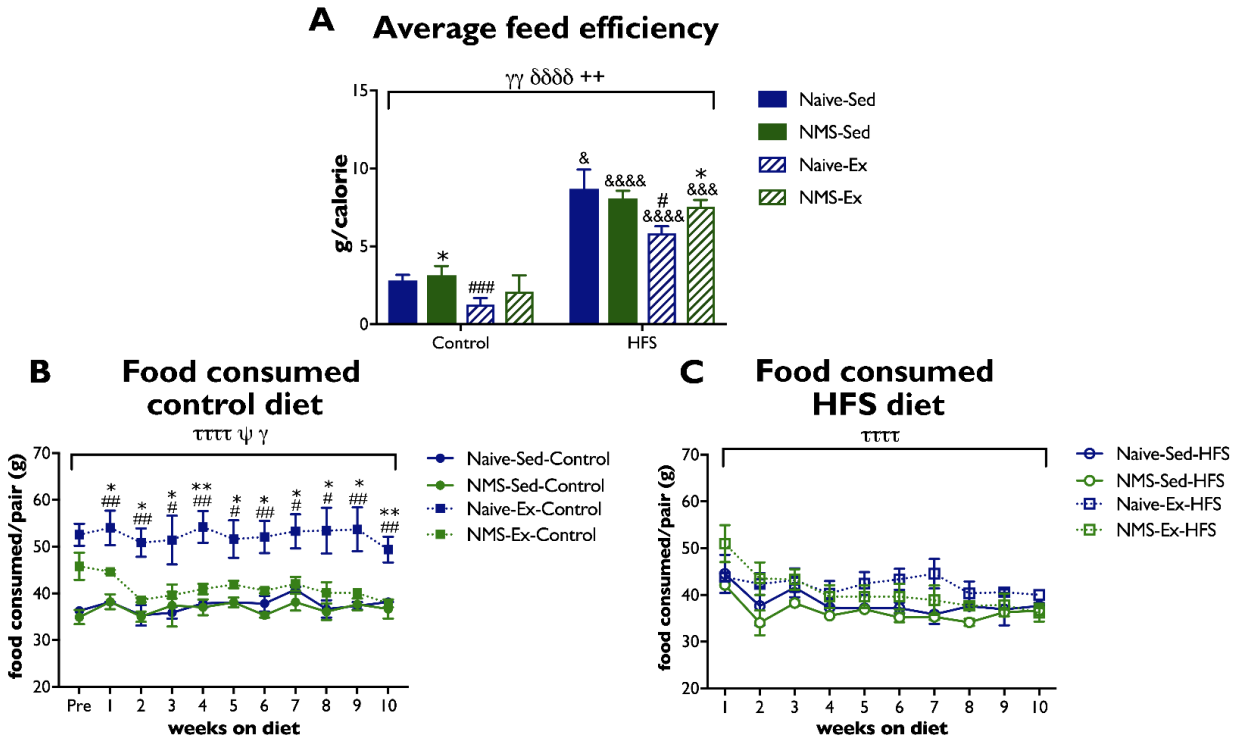
A
Average feed efficiency
γγ δδδδ ++
g/calorie
Control
HFS
Naive-Sed
NMS-Sed
Naive-Ex
NMS-Ex
B
Food consumed
control diet
ττττ ψ γ
food consumed/pair (g)
weeks on diet
Naive-Sed-Control
NMS-Sed-Control
Naive-Ex-Control
NMS-Ex-Control
C
Food consumed
HFS diet
ττττ
food consumed/pair (g)
weeks on diet
Naive-Sed-HFS
NMS-Sed-HFS
Naive-Ex-HFS
NMS-Ex-HFS

Figure 3.3 Weekly food intake was measured and is presented per pair because all mice were pair housed to avoid the stress of single housing. **A)** Feed efficiency represents the ability to convert calories consumed to weight gain. A significant overall effect of exercise, diet, and a NMS/exercise/diet interaction was observed on feed efficiency. NMS-Sed-Control had a greater feed efficiency compared to naïve-Sed-Control, naïve-Ex-Control had a lower feed efficiency compared to naïve-Sed-Control, all HFS diet groups had significantly greater feed efficiency compared to their control counterparts, and naïve-Ex-HFS had a significantly lower feed efficiency compared to naïve-Sed-HFS and NMS-Ex-HFS. **B**) A significant overall effect of time, NMS, and exercise was observed on the amount of control diet consumed over time. Naïve-Ex-Control ate significantly more than naïve-Sed-Control and NMS-Ex-Control every week. **C**) A significant overall effect of time was observed on the amount of HFS food consumed over time. There were no significant differences between groups. Bracket indicates a significant effect of exercise (γ, γγ p<0.05, 0.01), diet (δδδδ p<0.0001), NMS (ψ p<0.05), time (τττττ p<0.0001), or a NMS/exercise/diet interaction (++ p<0.01), 3-way ANOVA (A) or 3-way RM ANOVA (B&C). *, ** p<0.05. 0.01 vs. naïve, #, ##, ### p<0.05, 0.01, 0.001 vs Sed, &, &&&, &&&& p<0.05, 0.001, 0.0001 vs. control diet, LSD posttest.

On the control diet, NMS-Sed mice had a significantly greater feed efficiency compared to naïve-Sed (p<0.05) and naïve-Ex had a significantly lower feed efficiency compared to naïve-Sed (p<0.001). All groups on the HFS diet had significantly greater feed efficiencies compared to their control diet counterparts (p<0.05). Similar to the control diet, naïve-Ex-HFS had a significantly lower feed efficiency compared to Naïve-Sed-HFS. Finally, NMS-Ex-HFS had significantly higher feed efficiency compared to naïve-Ex-HFS (p<0.05).

Stress and exercise affect consumption of control, but not HFS, diet

Food consumption was measured every week. On the control diet, there was an overall significant effect of time (p<0.0001), stress (p<0.05), and exercise (p<0.05; Figure 3.3B). Naïve -Ex-Control mice ate significantly more than naïve -Sed-Control (p<0.05) and NMS-Ex-Control (p<0.05) every week**.** On the HFS diet, there was an overall significant effect of time (p<0.0001), but there were no significant differences between the HFS diet groups (Figure 3.3C)**.**

Running distance is influenced by stress and diet

Exercised mice were pair housed at 4-weeks of age to avoid the extra stress of single housing. Therefore, data is presented as average kilometers ran/day/pair. Over the course of the study, there was a significant overall effect of time (p<0.0001), NMS (p<0.01), and exercise (p<0.05) on running distance (Figure 3.4). Before the introduction of a HFS diet to half of the groups at 16-weeks of age (Pre), NMS mice ran significantly less than naïve mice (p<0.05). After introduction of a HFS diet, both naïve -

Figure 3.4

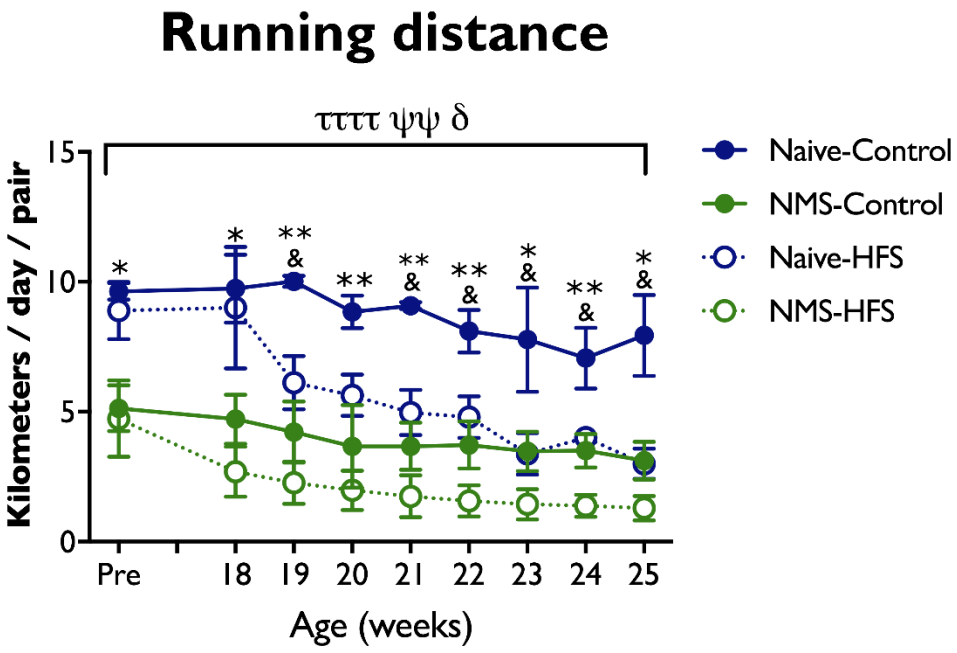
Running distance
τττττ ψψ δ
Kilometers / day / pair
15
10
5
0
Pre
18
19
20
21
22
23
24
25
Age (weeks)
Naive-Control
NMS-Control
Naive-HFS
NMS-HFS

Figure 3.4 At 4-weeks of age, NMS- and naïve mice were divided into sedentary (Sed) and exercised (Ex) groups. Ex groups were pair housed with access to a running wheel in their home cage and total distance ran/day/pair was recorded. At 16-weeks of age, all groups were further divided into control diet and high-fat/high-sucrose (HFS) groups. There was a significant overall effect of time, NMS, and diet on running distance over time. Before the start of the HFS diet (pre) there was a significant effect of NMS on running distance, NMS mice ran significantly less compared to naïve. After the introduction of the HFS diet, both HFS diet groups decreased their running distance. Naïve-Control mice ran significantly more than naïve-HFS and NMS-Control mice throughout the length of the study. Bracket indicates a significant effect of time (τττ $p<.0001$), NMS (ψψ $p<0.01$), or diet (δ $p<0.05$), 3-way RM ANOVA; *, ** $p<0.05$, 0.01 vs. naïve, & $p<0.05$ vs. control diet, Bonferroni posttest.

and NMS-HFS decreased their running distance. Naïve-Control ran significantly more than naïve-HFS (p<0.05) as well as NMS-Control (p<0.05 or 0.01) every week**.**

Exercise and diet have opposite effects on fasting insulin level

At 27-weeks of age, mice were fasted for 6-hours and serum insulin level was measured via blood collected by tail-clip. There was a significant overall effect of exercise (p<0.01) and diet (p<0.0001) on fasting insulin level (Figure 3.5A). Naïve- and NMS-Sed-HFS groups had significantly greater fasting insulin compared to naïve- and NMS-Sed-Control groups (p<0.01 and 0.00001, respectively). Fasting insulin levels of naïve- and NMS-Ex-HFS groups did not differ significantly from naïve- and NMS-Ex-Control groups, but were significantly less than naïve-and NMS-Sed-HFS groups (p<0.01 and 0.001, respectively).

NMS, exercise, and diet influence glucose tolerance

At 27-weeks of age, mice were fasted for 6-hours and a blood draw was taken via tail-clip to determine baseline fasting serum glucose level. Next, an IP glucose injection (1 g/kg) was administered and subsequent blood draws were taken at 15, 30, 60, and 120-minutes after injection to determine glucose tolerance over time. A time course of serum glucose level (Figure 3.5B) and area under the curve (AUC; Figure 3.5C) were analyzed. There was a significant overall effect of time (p<0.0001), NMS (p<0.001), exercise (p<0.0001), diet (p<0.0001), a time/diet interaction (p<0.01), and a time/exercise interaction (p<0.01) on glucose tolerance. 3-way ANOVA and post-hoc analyses for each time point were calculated and results are listed in Table 3.9**.** Briefly, at baseline there was no significant effect of NMS, diet, or exercise on serum glucose

Figure 3.5

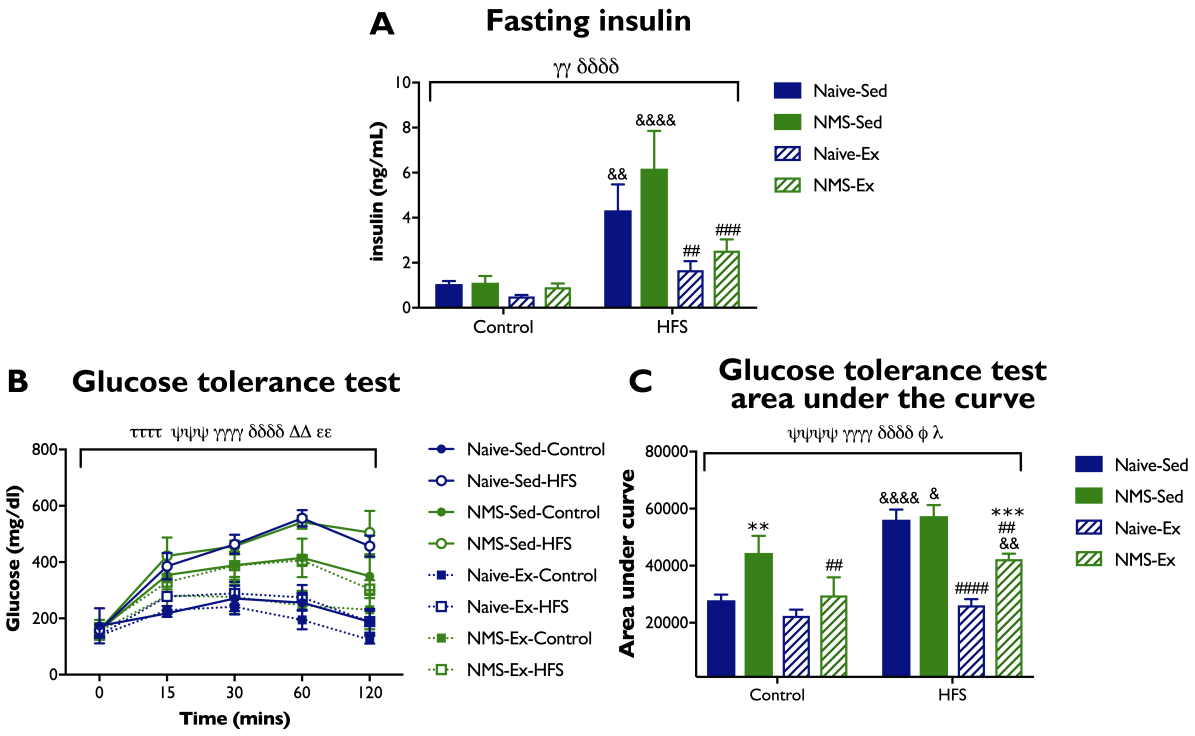
A
Fasting insulin
γγ δδδδ
insulin (ng/mL)
Control
HFS
&&
&&&&
##
###
Naive-Sed
NMS-Sed
Naive-Ex
NMS-Ex
B
Glucose tolerance test
τττττ ψψψ γγγγ δδδδ ΔΔ εε
Glucose (mg/dl)
Time (mins)
Naive-Sed-Control
Naive-Sed-HFS
NMS-Sed-Control
NMS-Sed-HFS
Naive-Ex-Control
Naive-Ex-HFS
NMS-Ex-Control
NMS-Ex-HFS
C
Glucose tolerance test area under the curve
ψψψψ γγγγ δδδδ φ λ
Area under curve
Control
HFS
**
##
&&&& &
####
*** ## &&
Naive-Sed
NMS-Sed
Naive-Ex
NMS-Ex

Figure 3.5 At 27-weeks of age fasting insulin level and glucose tolerance were measured in naïve- and NMS- Ex- and Sed- Control- and high-fat/high-sucrose (HFS) mice. Mice were fasted for 6-hours and fasting insulin level and baseline glucose level were measured. An IP injection of 1g/kg glucose was administered to all mice and subsequent glucose measurements were taken at 15, 30, 60, and 120-minutes after injection. **A**) There was a significant effect of exercise and diet on fasting insulin level. Naïve-Sed-HFS and NMS-Sed-HFS groups had significantly higher insulin level compared to their control counterparts. Naïve-Ex-HFS and NMS-Ex-HFS had significantly lower fasting insulin level compared to their sedentary counterparts. **B**) There was a significant overall effect of time, NMS, exercise, diet, time/diet interaction, and time/exercise interaction on serum glucose levels during the glucose tolerance test (GTT). **C**) There was a significant overall effect of NMS, exercise, diet, diet/exercise interaction, and NMS/diet interaction on the GTT area under the curve (AUC). NMS-Sed-Control had a significantly greater GTT AUC compared to Naïve-Sed-Control and NMS-Ex-Control. Naïve-Sed-HFS, NMS-Sed-HFS, and NMS-Ex-HFS all had significantly greater GTT AUC compared to their control diet counterparts. Naïve-Ex-HFS had a significantly lower GTT AUC compared to Naïve-Sed-HFS and NMS-Ex-HFS. Bracket indicates a significant effect of exercise ($\gamma\gamma$, $\gamma\gamma\gamma\gamma$ p<0.01, 0.0001), diet ($\delta\delta\delta\delta$ p<0.0001), NMS ($\psi\psi\psi$, $\psi\psi\psi\psi$ p<0.001, 0.0001), time ($\tau\tau\tau\tau$ p<0.0001), a time/diet interaction ($\Delta\Delta$ p<0.01), a time/exercise interaction ($\varepsilon\varepsilon$ p<0.01), a diet/exercise interaction (ϕ p<0.05), and a NMS/diet interaction (λ p<0.05), 3-way ANOVA or 4-way RM ANOVA. &, &&, &&&& p<0.05, 0.01, 0.0001 vs. control diet; ##, ###, #### p<0.01, 0.001, 0.0001 vs. Sed; **, *** p<0.01, 0.001 vs. naïve; Fishers LSD post-hoc test.

Table 3.9 Glucose tolerance test

Time point (minutes)		0	15	30	60	120
Overall analyses						
Effect of NMS		NS	p=0.004	NS	p=0.007	p=0.002
Effect of exercise		NS	p=0.018	p=0.004	p<0.0001	p<0.0001
Effect of diet		NS	p=0.001	p=0.002	p<0.0001	p=0.001
Exercise/diet interaction		NS	NS	NS	NS	p=0.025
NMS/exercise/diet interaction		NS	NS	NS	p=0.034	NS
Post-hoc analyses						
Naïve-Sed-Control vs NMS-Sed-Control	Effect of NMS	NS	p=0.01	NS	p=0.012	p=0.028
Naïve-Ex-HFS vs. NMS-Ex-HFS	Effect of NMS	NS	NS	NS	p=0.011	p=0.018
Naïve-Sed-HFS vs. Naïve-Ex-HFS	Effect of exercise	NS	p=0.024	p=0.008	p<0.0001	p<0.0001
Naïve-Sed-Control vs. Naïve-Sed-HFS	Effect of diet	NS	p=0.002	p=0.005	p<0.0001	p=0.001
NMS-Control-Sed vs. NMS-Control-Ex	Effect of exercise	NS	NS	NS	p=0.008	NS
NMS-HFS-Sed vs. NMS-HFS-Ex	Effect of exercise	NS	NS	NS	p=0.018	p=0.004
NMS-Sed-Control vs. NMS-Sed-HFS	Effect of diet	NS	NS	NS	p=0.041	p=0.035
NMS-Ex-Control vs. NMS-Ex-HFS	Effect of diet	NS	NS	NS	p=0.006	NS

Multi-variate ANOVA, LSD posttest. Significance set at p<0.05. NMS= neonatal maternal separation, Sed= sedentary, Ex= exercised, HFS= western diet, NS= no significance.

level. There were also no significant differences between groups. However, following glucose injection there was a significant effect of NMS, exercise, and diet at each time point, with the exception of an effect of NMS at 30-minutes post injection (Table 3.9).

Analysis of the glucose tolerance test (GTT) AUC further supported the results described above. A lower GTT AUC indicates better glucose tolerance, or a greater ability to clear glucose from the blood. There was a significant effect of NMS ($p<0.0001$), exercise ($p<0.0001$), diet ($p<0.0001$), a NMS/diet interaction ($p<0.05$), and an exercise/diet interaction ($p<0.05$) on GTT AUC (Figure 3.5C). On the control diet, NMS-Sed mice had a significantly greater GTT AUC compared to naïve-Sed mice ($p<0.01$) and NMS-Ex ($p<0.01$). On the HFS diet, all groups had a significantly greater GTT AUC compared to their control counterparts ($p<0.05$, 0.01, or 0.0001) with the exception of the Naïve-Ex group. Additionally, Naïve-Ex-HFS had a significantly lower GTT AUC than Naïve-Sed-HFS ($p<0.001$) and NMS-Ex-HFS ($p<0.001$). NMS-Ex-HFS had a significantly lower GTT AUC than NMS-Sed-HFS ($p<0.01$).

NMS, exercise, and diet alter epididymal adipose macrophage and MC population as well as neuroendocrine signaling molecules

At 27-weeks of age, mice were sacrificed, epididymal adipose tissue was removed and immediately frozen in liquid nitrogen, mRNA was extracted, and RT-PCR was used to quantify genes of interest (Figure 3.6). There was a significant effect of diet ($p<0.0001$), exercise ($p<0.01$), and an exercise/diet interaction ($p<0.05$) on mRNA for the general macrophage markers F4/80 and CD68 (Figure 3.6A). There was also a

Figure 3.6

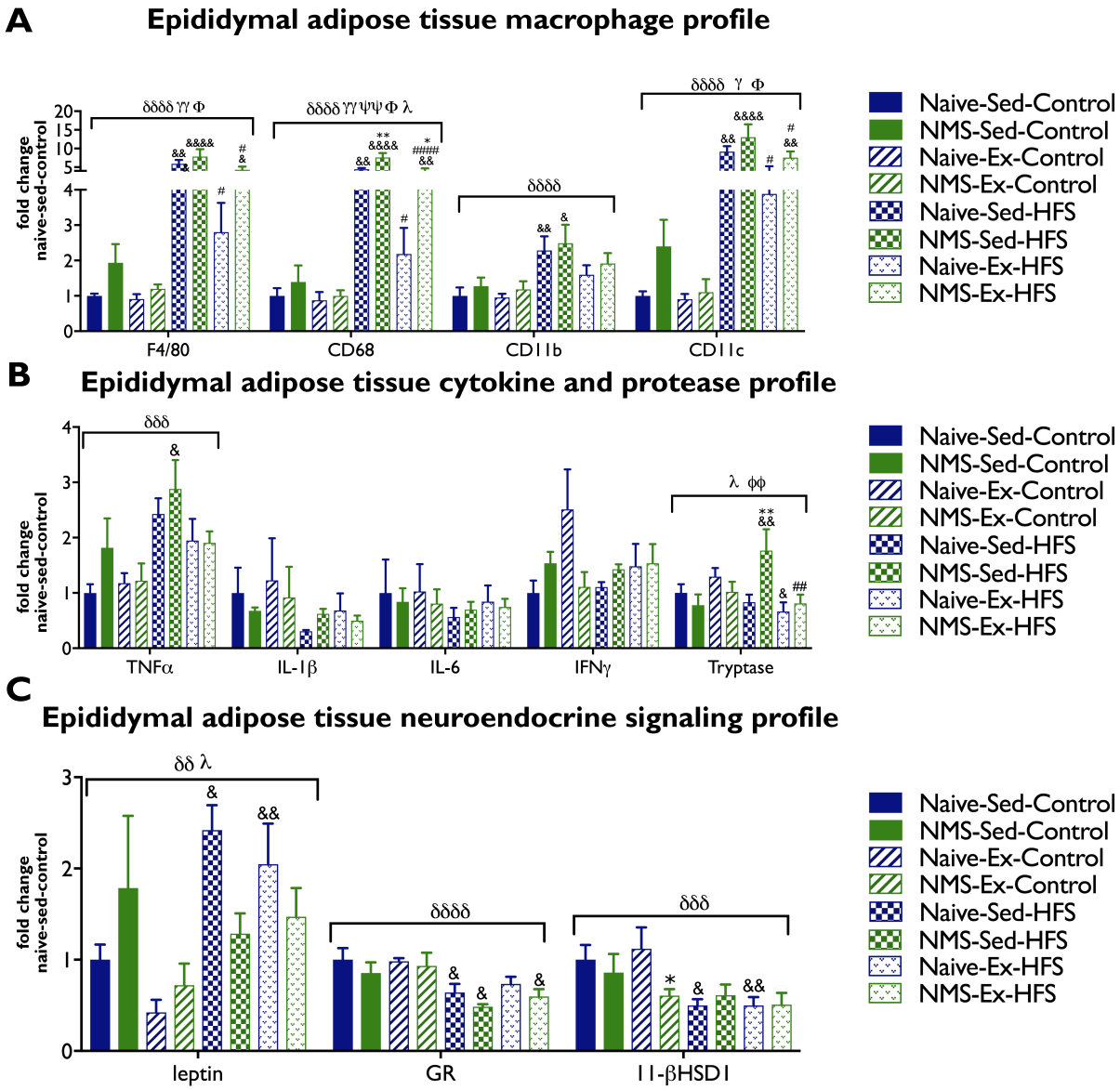
A
Epididymal adipose tissue macrophage profile
fold change naive-sed-control
F4/80
CD68
CD11b
CD11c
B
Epididymal adipose tissue cytokine and protease profile
TNFα
IL-1β
IL-6
IFNγ
Tryptase
C
Epididymal adipose tissue neuroendocrine signaling profile
leptin
GR
11-βHSD1
Naive-Sed-Control
NMS-Sed-Control
Naive-Ex-Control
NMS-Ex-Control
Naive-Sed-HFS
NMS-Sed-HFS
Naive-Ex-HFS
NMS-Ex-HFS

Figure 3.6: RT-PCR was used to analyze mRNA levels in epididymal adipose tissue. **A**) F4/80 and CD68 mRNA was quantified to determine if there were differences in general macrophage markers. There was a significant overall effect of diet, exercise, and an exercise/diet interaction on both F4/80 and CD68 mRNA levels. There was also an effect of NMS and a NMS/diet interaction on CD68 mRNA levels. All HFS groups, with the exception of naïve-Ex-HFS, had significantly higher mRNA levels of F4/80 and CD68 compared to control diet groups. Naïve- and NMS-Ex-HFS groups had significantly lower F4/80 and CD68 mRNA levels compared to naïve-and NMS-Sed-HFS groups. NMS-Sed- and -Ex-HFS had significantly higher CD68 mRNA levels compared to naïve-Sed and -Ex-HFS. Next, CD11b and CD11c mRNA levels were to determine if changes in macrophages were pro-inflammatory or not. There was a significant overall effect of diet on both CD11b and CD11c mRNA levels, as well as an effect of exercise and an exercise/diet interaction on CD11c mRNA levels. Naïve- and NMS-Sed-HFS had significantly higher CD11b and CD11c mRNA levels compared to control diet counterparts. NMS-Ex-HFS had significantly greater CD11c mRNA levels compared to naïve-Ex-Control. Finally, naïve-and NMS-Ex-HFS had significantly lower CD11c mRNA levels compared to Sed-HFS groups. **B**) Various cytokine mRNA were also analyzed to determine the inflammatory status of the epididymal adipose and tryptase to assess putative mast cell infiltration. There was a significant overall effect of diet on TNFα mRNA levels, with NMS-Sed-HFS having significantly higher TNFα mRNA levels compared to NMS-Sed-Control. There were significant NMS/diet and exercise/diet interactions on tryptase mRNA levels, with NMS-Sed-HFS having significantly higher tryptase mRNA levels compared to NMS-Sed-Control, Naïve-Sed-

HFS, and NMS-Ex-HFS. Naïve-Ex-HFS had significantly lower tryptase mRNA levels compared to naïve-Ex-Control. **C**) mRNA levels of leptin, glucocorticoid receptor (GR), and 11β– hydroxysteroid dehydrogenase type 1 (11-βHSD1) due to their roles in neuroendocrine signaling. A significant overall effect of diet was observed on leptin, GR, and 11-βHSD1 mRNA levels. Additionally, there was a NMS/diet interaction on leptin mRNA levels, such that naïve-Sed- and -Ex-HFS mice had significantly higher leptin mRNA levels compared to naïve-Sed- and Ex-control mice. Naïve-and NMS-Sed-HFS and NMS-Ex-HFS had significantly lower GR mRNA levels compared to naïve-and NMS-control and NMS-Ex-Control. Naïve-Sed- and Ex-HFS had significantly lower 11-βHSD1 mRNA levels compared to naïve-Sed and Ex-control and NMS-Ex-Control had significantly lower 11-βHSD1 mRNA levels compared to naïve-Ex-Control. Bracket indicates a significant effect of diet (δδ, δδδ, δδδδ p<0.01, 0.001, 0.0001), exercise (γ, γγ p<0.05, 0.01), NMS (ψψ p<0.01), an exercise/diet interaction (Φ p<0.05), or a NMS/diet interaction (λ p<0.05), 3-way ANOVA; &, &&, &&&& p<0.05, 0.01, 0.0001 vs. control diet; #, ##, ### p<0.05, 0.01, 0.001 vs sedentary; *, ** p<0.05, 0.01 vs. naïve; Fisher's LSD posthoc test.

significant overall effect of NMS (p<0.01) and a NMS/diet interaction (p<0.05) on CD68 mRNA. All HFS diet groups had significantly greater F4/80 and CD68 mRNA compared to control diet groups (p<0.05) with the exception of Naïve-Ex-HFS, which was not significantly different from Naïve-Ex-Control and significantly lower than Naïve-Sed-HFS (p<0.05). NMS-Ex-HFS also had significantly less F4/80 and CD68 mRNA than NMS-Sed-HFS (p<0.05). NMS-Sed-and -Ex-HFS had significantly more CD68 mRNA than Naïve-Sed-and-Ex-HFS groups (p<0.05). CD11b and CD11c epididymal adipose mRNA were also measured to determine the inflammatory status of the macrophages. There was a significant effect of diet on both CD11b and CD11c mRNA (p<0.0001) as well as an effect of exercise (p<0.05) and an exercise/diet interaction (p<0.05) on CD11c mRNA. Naïve- and NMS-Sed-HFS had significantly greater CD11b and CD11c mRNA compared to Naïve-and- NMS-Sed-Control groups (p<0.05). CD11c mRNA levels in HFS groups increased 5-15-fold relative to the Naïve-Sed-Control group while CD11b mRNA levels in HFS groups only increased 2-3 fold. NMS-Ex-HFS also had significantly greater CD11c mRNA levels than NMS-Ex-Control (p<0.01). Finally, Naïve-and NMS-Ex-HFS had significantly lower CD11c mRNA levels than Naïve-and-NMS-Ex-HFS (p<0.05).

Multiple cytokines in the epididymal adipose were measured, including tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1-β), interleukin 6 (IL-6), and interferon gamma (IFNγ; Figure 3.6B). Only TNFα was significantly impacted with a significant overall effect of diet (p<0.001) and NMS-Sed-HFS had a significantly greater mRNA level compared to NMS-Sed-Control (p<0.05)**.** As a means to evaluated MC infiltration, the level of tryptase mRNA in epididymal adipose was measured. There was

a significant overall effect of exercise (p<0.05) and an exercise/diet interaction (p<0.01) on tryptase mRNA levels, with NMS-Sed-HFS mice having significantly higher tryptase mRNA levels than Naïve-Sed-HFS (p<0.01), NMS-Ex-HFS (p<0.01), and NMS-Sed-Control (p<0.01) mice. Naïve-Ex-HFS mice had significantly lower tryptase mRNA levels compared to Naïve-Ex-Control mice (p<0.05).

To determine whether neuroendocrine signaling changes were occurring in epididymal adipose due to diet, exercise, and stress, mRNA levels of leptin, glucocorticoid receptor (GR), and 11 β-hydroxysteroid dehydrogenase type 1 (11-βHSD1; Figure 3.6C) were measured. There was a significant effect of diet (p<0.01) and exercise (p<0.05) on leptin mRNA levels. Naïve-Sed- and -Ex-HFS had significantly higher leptin mRNA levels compared to naïve-Sed- and -Ex-Control groups (p<0.05). There was a significant effect of diet on both GR and 11-βHSD1 mRNA levels (p<0.001). Naïve-Sed-, NMS-Sed-, and naïve-Ex-HFS had significantly lower GR mRNA levels and naïve-Sed-and -Ex-HFS had significantly lower 11-βHSD1 mRNA levels compared to their control counterparts (p<0.05). NMS-Ex-Control had significantly lower 11-βHSD1 mRNA levels compared to naïve-Ex-Control (p<0.05).

3.5 Discussion

Obesity-related metabolic syndrome is a serious illness that is highly prevalent in today's society. In humans, environmental factors including early life stress (160-162), poor diet (178, 289), and low activity level (400-402) increase one's risk to develop this disorder. Many pre-clinical studies have investigated the influence that HFS diet (411-413, 416) and exercise (228, 404, 420) have on the development of obesity-related

metabolic syndrome, while only a few have investigated the role that early life stress plays (163, 164). To my knowledge, this is the first study to combine early life stress, exercise, and diet to evaluate outcomes related to this disorder.

I first found that chow-fed adult female and male NMS-Sed mice have altered body composition compared to naïve-Sed and exercised mice (Tables 3.3 & 3.4). Studies have shown that female mice are not as susceptible to diet-induced weight gain as male mice (303). Our observations confirm this and are described in Chapter 4 of this dissertation. Therefore, male NMS- and Naïve- Ex- and -Sed mice were used in a HFS diet study to see if early life stress and exercise influenced the development of diet-induced obesity-related metabolic syndrome. All mice on the HFS diet gained a significant amount of weight throughout the study, compared to mice on the control diet (Table 3.5). NMS-Ex-HFS mice gained significantly more weight compared to NMS-Ex-Control after only 1-week on the HFS diet while NMS-Sed-HFS and naïve-Sed-HFS weighed significantly more than their control diet counterparts after 3-weeks on the HFS diet. The last group to gain a significant amount of weight was the naïve-Ex-HFS group at 6-weeks on the diet (Figure 3.2A & Table 3.6). These results suggest that exercise was more beneficial in preventing weight gain in the naïve-HFS mice compared to NMS-HFS mice. This is further exemplified in body fat percentage where there was a significant overall effect of NMS, generally causing an increase in this measure over time compared to naïve mice (Figure 3.2b). There were significant differences between naïve-Ex-Control vs. NMS-Ex-Control groups as well as naïve-Ex-HFS vs. NMS-Ex-HFS groups at multiple time points where the NMS groups had significantly more body fat percentage then naïve mice (Table 3.7) despite consuming the same amount of HFS

diet (Figure 3.3C*)*. Similar to weight gain, all groups on the HFS diet gained significant amounts of body fat, however the naïve-Ex-HFS gained the least amount (Table 3.5). This was also true for the naïve-Ex group on the control diet (Table 3.5), again, suggesting that naïve mice gained greater benefit from exercise compared to NMS mice. Interestingly, naïve-Ex mice also ate significantly more control diet compared to the other groups (Figure 3.3B*)*, but they demonstrated appropriate energy balance by running more than NMS-Ex mice (Figure 3.4).

The reduced running distance, and thereby energy expenditure, by NMS mice could partially underlie their increased weight and body fat gain compared to naïve mice. However, differences in feed efficiency, which is the ratio of weight gained/ energy consumed, suggest that basal differences in metabolism are also at play. NMS-Sed-Control mice had significantly higher feed efficiency than naïve-Sed-Control mice, indicating that NMS increases potential weight gain, even in the absence of diet or exercise interventions. Exercise significantly lowered feed efficiency in both control- and HFS-fed naïve groups when compared to sedentary counterparts. In contrast, exercise did not significantly impact feed efficiency in NMS mice on either a control or HFS diet. Again, the low running distance of the NMS, particularly those on HFS diet, definitely contributes to the moderate impact on feed efficiency. Future studies can incorporate treadmill running or capping daily distances to determine the impact of NMS in mice with equivalent aerobic output.

Glucose intolerance is one symptom of metabolic syndrome and a hallmark of prediabetes (167). Following a bolus glucose injection, NMS-Sed-Control mice were significantly less efficient at clearing glucose from circulation compared to Naïve-Sed-

Control, indicating they were glucose intolerant. Exercise significantly improved glucose clearance of control-fed NMS mice, but serum glucose levels remained comparatively higher than that of naïve-Ex-Control. Exercise and HFS diet differentially affected NMS and naïve mice, with NMS-Ex-HFS having lower GTT than NMS-Sed-HFS, but higher than naïve-Ex-HFS and NMS-Ex-control. The only HFS-fed group that did not develop glucose intolerance was the naïve-Ex group. (Figure 3.5C). While it was not altogether surprising that the groups that gained significant amounts of weight and body fat became glucose intolerant, it was intriguing was that the control-fed NMS mice were more glucose intolerant than naïve-control. This finding supports the association between early life stress in humans and the development of obesity and related metabolic disorders in adulthood (6, 160-162).

Increased fasting insulin level is another sign of insulin resistance, which is also a symptom of metabolic syndrome (167). Again, significant effects of diet and exercise were observed in this study. HFS diet significantly increased fasting insulin level in both sedentary naïve and NMS mice, but this was reversed by exercise (Figure 3.5a). These data imply that a HFS diet can induce both glucose intolerance and insulin resistance, which can be partially prevented by voluntary exercise. Furthermore, the NMS-Sed-HFS group had the highest fasting insulin level (Figure 3.5A), suggesting that a combination of early life stress, sedentary caging, and HFS diet generated the most robust measures of metabolic syndrome.

White adipose tissue distribution and composition is important in predicting the development of obesity-related diseases because it plays a key role in regulating systemic metabolic function and inflammation (168). Visceral fat accumulation is

associated with a greater risk of metabolic dysfunction (169) and leads to chronic low-grade inflammation (170). This low-grade inflammation is associated with increased macrophage and MC infiltration as well as the increased prevalence of crown-like structures (CLS) and has been demonstrated in both obese mice and humans (171, 172, 174, 421). CLSs are apoptotic adipocytes surrounded by pro-inflammatory macrophages. They are formed when there is a hypoxic environment, such as when adipose tissue growth outpaces angiogenesis and their accumulation eventually creates fibrotic tissue. RT-PCR was used to analyze the macrophage profile of epididymal adipose tissue. These analyses revealed an effect of diet and exercise on mRNA levels of the macrophage markers F4/80 and CD68 and an effect of NMS on CD68 mRNA levels. Diet and NMS caused an increase in these macrophage markers while exercise prevented this effect, particularly in naïve-HFS mice. The NMS-Sed-Control group also appeared to have more F4/80 and CD68 mRNA compared to naïve- or Ex-Control mice, however it did not reach significance (Figure 3.6a).

Classically macrophages have been divided into two groups: M1, recruited pro-inflammatory macrophages, which are $F4/80^{+}CD11c^{+}$, or M2 resident macrophages, which are $F4/80^{+}CD11b^{+}$ (422). While this dichotomy is largely an over simplification and there exists a spectrum of macrophage phenotypes (423), I was interested in determining the basic inflammatory state of the macrophages in our epididymal adipose tissue. There was a significant effect of diet on both CD11b and CD11c mRNA, but HFS diet increased the pro-inflammatory CD11c expression 5-15-fold and the non-inflammatory CD11b expression only 2-3-fold, when compared to naïve-Sed-control levels (Figure 3.6A). This indicated that the increase in macrophages in epididymal

adipose tissue was mostly of the M1 pro-inflammatory type. Naïve- and NMS-HFS-Ex groups had significantly lower CD11c mRNA levels compared to naïve- and NMS-HFS-Sed groups, indicating that exercise influenced the inflammatory status of the epididymal adipose tissue in a positive way. Similar to the F4/80 and CD68 results, NMS-Sed-Control had greater CD11c mRNA levels compared to naïve or exercised groups, but it did not reach significance (Figure 3.6A).

To further investigate the inflammatory status of our mice, mRNA levels of multiple cytokines in the epididymal adipose tissue were measured. A significant overall effect of diet was observed on TNFα mRNA levels, where the NMS-Sed-HFS group had the greatest increase of TNFα mRNA and NMS-Sed-Control had a greater increase of TNFα mRNA compared to naïve or Ex groups (Figure 3.6B). This is a mechanistically significant finding because TNFα affects insulin sensitivity. Using a targeted null mutation for the gene encoding TNFα and two of its receptors, Uysal et al. (424) found that in diet-induced obese and *ob/ob* genetically modified obese mice, the mice with the null mutation for TNFα had significantly improved insulin sensitivity. The TNFα deficient mice had lower levels of circulating free fatty acids and did not have obesity-related reductions in insulin receptor signaling in muscle and fat tissues, suggesting that TNFα works through several mechanisms. Interestingly, the group with the highest TNFα in epididymal adipose, NMS-Sed-HFS, also had the highest fasting insulin level, suggesting that insulin resistance could be at least partially driven by increased visceral adipose TNFα.

Gurung et al., (173) recently showed that subcutaneous adipose tissue of individuals with metabolic syndrome had a 2.5-fold increase in MCs compared to control subjects. This increase in MCs was positively correlated with markers of fibrosis and inflammation. Obese subjects have also been shown to have significantly higher serum tryptase levels (175). Furthermore, high-fat diet-induced obese mice display increased MCs in visceral adipose tissue (421). Here, RT-PCR was used to quantify the amount of tryptase mRNA in the epididymal adipose to determine putative MC infiltration. Overall, there were significant NMS/diet and exercise/diet interactions. By far, the highest level of tryptase mRNA was found in NMS-Sed-HFS adipose, which was reduced by exercise (Figure 3.6B). These results imply that, much like with glucose intolerance and insulin levels, the combination of early life stress, sedentary caging, and a HFS diet had a cumulative effect on increasing epididymal adipose MC infiltration.

Despite the association of a greater number of MCs in obese humans and rodents, the role of MCs in the development of metabolic syndrome is conflicting. In support of MCs being involved in the development of metabolic syndrome, Liu et al., (175) used two MC-deficient genetic mouse models, $Kit^{W\text{-}sh/W\text{-}sh}$ (C57Bl/6) and Kit^{W/W^v}(WBB6F1/J), as well as wildtype mice treated with two different MC stabilizers, disodium cormoglycate (DSCG) and ketotifen, and found that, when fed a “Western diet” (20% fat, 45.2% carbohydrate, 22.6% protein; 12-13-weeks), these mice gained significantly less body weight than wildtype control mice on the same diet. MC-deficient mice also displayed higher glucose tolerance and insulin sensitivity compared to control mice on this Western diet. Interestingly, switching mice from a Western diet to a control diet and simultaneously treating them with a MC stabilizer resulted in greater

improvement in weight and glucose tolerance compared switching to a control diet alone. However, Gutierrez et al., (425) argue that it was the *Kit* deficiency, not the MC deficiency in the $Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{W/Wv}$ mice, that caused the resistance to high-fat diet induced weight gain, glucose intolerance, and insulin insensitivity. This group used a *Kit*-independent C57Bl/6 $Cpa3^{Cre/+}$MC deficient mouse model. On a high-fat diet (60% kcal fat, 20% kcal protein, 20% kcal carbohydrate; 16-weeks), these mice displayed similar weight as C57Bl/6 $Cpa3^{+/+}$ mice that were not MC deficient. Both groups of mice also had similar glucose tolerance and fasting insulin levels. Finally, they used DSCG to inhibit MC degranulation, similar to the dosage used by Liu et al (2009), but they found that this had no effect on weight gain in high-fat died fed mice with or without MCs. A major difference between these two studies was the percent of fat in the diets. Liu et al. (175) used a diet with 20% fat, while the Guiterrez et al., (425) diet contained 60% fat. The lengths of the diets also differed, 12-13-weeks vs. 16-weeks. While these differences may not explain all of the dissimilarities between the two studies, it raises the point that consistency between high-fat diet studies is necessary to appropriately compare results.

Although there are arguments regarding the necessity of MCs for the development of obesity-related metabolic syndrome, they do play a clear role in altering the inflammatory state of adipose tissue. They are a source of TNFα (426) as well as other cytokines including macrophage colony stimulating factor (427), which causes hematopoietic stem cells to differentiate into macrophages. MCs also have been shown to express mRNA and protein for NGF and release NGF upon activation (428), which could then cause the maturation of more MC progenitors. Therefore, even if MCs are

not required for the development of obesity-related metabolic syndrome, they could contribute to its pathogenesis. In this study, data indicated that although HFS diet and stress alone did not increase MC tryptase in epididymal adipose tissue, the combination of these two factors did. Additionally, exercise prevented this significant increase (Figure 3.6B). Our laboratory has previously shown that MC degranulation is increased in urogenital organs (97, 98) and dura mater (Chapter 2) of NMS-Sed mice and that exercise prevents or attenuates this effect. Therefore, I hypothesize that NMS-induced dysfunction of the HPA axis leads to an increase in systemic CRF release and a subsequent widespread increase in MC infiltration and degranulation. The metabolic stress of a HFS diet could also cause MC activation and elicit further increases in adipose tissue MCs and infiltrating pro-inflammatory macrophage populations, eventually contributing to the development of obesity-related metabolic syndrome.

To further study the role that the HPA axis might play in the development of HFS diet-induced obesity-related metabolic syndrome, GR mRNA levels in epididymal adipose tissue was measured because increased circulating GCs have been associated with the development of obesity, particularly in the visceral region (399). In adipose tissue, GCs promote differentiation of pre-adipocytes into mature adipocytes and increase lipoprotein lipase activity, which can increase the amount of adipose tissue and subsequently cause weight gain (429). Clinical studies in obese patients have shown simultaneous increases in GR and 11β-HSD1 (85, 86), which converts inactive GCs to their active form (430). 11β-HSD1 is also increased in adipose tissue of genetically obese rodent models, such as the obese Zucker rat (431), which is homozygous for a mutation in the leptin receptor gene. Furthermore, overexpression of 11β-HSD1 in

mouse adipose tissue leads to the development of visceral obesity and metabolic syndrome (87). However, Morton et al., (432) studied the effects of 2-and 18-weeks of a high-fat diet on 11β-HSD1 in adipose tissue of C57Bl/6J and A/J mice. They found that both short- and long-term high-fat diet caused a decrease in adipose 11β-HSD1, especially in the obesity-resistant A/J mice. Both strains also became obese on the long-term diet and the C57Bl/6J mice were hyperinsulinemic. High-fat diet-induced reduction in adipose 11β-HSD1 has also been demonstrated in Wistar rats, but it was transient and disappeared after 20-weeks on the diet (433). I measured 11β-HSD1 mRNA in epididymal adipose tissue after 11-weeks on a HFS diet and found a significant overall effect of diet reducing 11β-HSD1 epididymal adipose mRNA. 11β-HSD1 was reduced the most in naïve-Sed-HFS and Naïve-Ex-HFS (Figure 3.6C). These results paired with the studies described above imply that reduction in 11β-HSD1 only occurs in diet-induced obesity in rodents, not genetically obese models. In line with HFS diet causing a reduction in 11β-HSD1 mRNA, there was also an effect of HFS diet reducing the amount of GR mRNA in epididymal adipose tissue. This diet-induced reduction reached significance in naïve-Sed-HFS, NMS-Sed-HFS, and NMS-Ex-HFS groups (Figure 3.6C). A down regulation of 11β-HSD1 and GR could be an adaptive mechanism to attempt to circumvent the detrimental metabolic consequences of increased GC signaling. This adaptive mechanism appears to function better in our naïve-HFS mice as their 11β-HSD1 mRNA levels were significantly reduced while NMS-HFS mice 11β-HSD1 mRNA levels did not reach significance (Figure 3.6C).

White adipose tissue is a complex endocrine organ that secretes adipokines, including leptin (434). Leptin communicates with its receptor in the hypothalamus to regulate food intake and energy expenditure by shutting down hunger signals (435). This circuitry is not fully programmed at birth making it susceptible to the influence of environmental factors (429). Circulating leptin usually peaks during development from P4-P16 in rodents (436) and this peak is important for proper formation of the hypothalamic feeding circuitry (435). Early life stress has been shown to reduce leptin levels early in life (437, 438), which could lead to altered programming of the leptin-hypothalamic circuit and long-term changes in hunger signals and energy expenditure. When leptin mRNA in the epididymal adipose tissue was measured in this study, there was a significant overall effect of diet as well as a NMS/diet interaction. On the HFS diet, Naïve-Sed and -Ex groups had significantly higher leptin mRNA compared to their control diet counterparts. However, NMS-Sed and -Ex-HFS groups were not significantly different than groups on the control diet (Figure 3.6C). A HFS diet is expected to increase leptin levels, so that hunger signals are decreased and energy expenditure is increased to compensate for the extra energy that is being consumed. Our naïve-HFS groups showed an appropriate increase in leptin levels, however our NMS-HFS mice did not (Figure 3.6C). This suggests a NMS-induced alteration in leptin signaling. Increased leptin levels should not be present in mice on a control diet. However, NMS-Sed-Control mice had a much higher leptin mRNA level compared to naïve-Sed-control or NMS-Ex-Control (Figure 3.6c). This suggests that this group may be leptin-resistant, meaning that leptin is not as effective in suppressing food intake. It has been hypothesized that a hyperactive HPA axis, which leads to the elevation of GC

concentrations, is involved in leptin resistance in obesity (439). Our NMS protocol involves separating mice from P1- P21, which includes the important window of development of the leptin-hypothalamic feeding circuitry (435, 436). Therefore, proper programming of both the HPA axis and leptin signaling could be affected by NMS and have long-term consequences on metabolism.

In summary, NMS, exercise, and diet all appear to influence factors related to metabolic syndrome. This was first demonstrated by the finding that chow-fed NMS-Sed female and male mice have altered body compositions compared to naïve and Ex mice and was further exemplified by our long-term HFS diet study. The NMS-Sed-HFS group had the highest fasting insulin level, the greatest amount of TNFα, pro-inflammatory macrophage marker CD11c, and MC tryptase mRNA in epididymal adipose tissue suggesting this group was more prone to visceral inflammation and development of insulin resistance compared to the other groups. NMS-Sed- and -Ex-HFS groups did not demonstrate a HFS diet induced increase in leptin levels, indicating they had alterations in the leptin-signaling pathway. Our results also implied that our NMS-Sed-Control mice were more susceptible to developing symptoms of metabolic syndrome compared to naïve- or Ex-control mice. NMS-Sed-Control mice had increased TNFα, pro-inflammatory macrophage marker CD11c, and leptin mRNA levels compared to other groups on the control diet. This suggests they are more prone to visceral inflammation and are leptin resistant. This group also had an increased GTT AUC, indicating they are less glucose tolerant. Finally, they had a greater feed efficiency, so they were better able to turn calories consumed into weight. Overall, exercise influenced measurements of obesity-related metabolic syndrome in both naïve and NMS mice but appeared to

have a stronger effect in naïve mice compared to NMS in some of our measurements. For example, exercise prevented the increase in body fat over time to a greater extent in naïve-Control and-HFS mice compared to NMS-Control and -HFS mice. It was also more beneficial in naïve-HFS mice in preventing a HFS diet-induced increase in the GTT AUC, where naïve-Ex-HFS had a significantly lower GTT AUC compared to NMS-Ex-HFS mice. However, NMS-Ex-HFS GTT AUC was significantly lower than NMS-Sed-HFS, implying that NMS mice still received some benefit from exercise. Evidence of exercise benefiting NMS and naïve mice equally was that both naïve- and NMS-Ex-HFS groups had a significantly lower fasting insulin level compared to Sed-HFS groups. Exercise also prevented the NMS and HFS induced increase in epididymal tryptase mRNA. However, naïve-Ex-HFS groups were more protected from significant changes in pro-inflammatory macrophage infiltrates compared to all of the other HFS groups, which had significantly more CD11c epididymal adipose mRNA compared to their control diet counter parts. The increased benefit of exercise in naïve mice could be due to the fact that naïve mice ran significantly more than NMS mice. I hypothesize that these differences could be due to changes in reward pathways. Lovallo (2013) explains that early life stress in humans can lead to altered dopaminergic signaling (440), which influences motivated behaviors (441), including physical activity. Future studies in our laboratory will investigate if NMS mice have alterations in the mesolimbic dopamine system. This could cause them to be less motivated to run compared to naïve mice, due to running being less rewarding to them.

Chapter IV: The effect of an anti-inflammatory diet intervention on early life stress-induced co-morbid disorders in mice

4.1 Abstract

Individuals with functional pain disorders such as irritable bowel syndrome, interstitial cystitis/painful bladder syndrome, migraine, and fibromyalgia often present with symptoms of or are diagnosed with more than one disorder. Furthermore, these patients with co-morbid pain disorders are also more likely to present with mood disorders, including depression and anxiety, as well as with obesity-related metabolic syndrome. Although the exact cause of these co-morbid disorders is not fully established, a common underlying factor in many of these patients is a dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response, plays a role in the perception of pain, and impacts downstream metabolic functions. The HPA axis is not fully developed when children are born and therefore is susceptible to incorrect programming caused by early life perturbations. Early life stress is associated with improper programming of the HPA axis and the subsequent development of co-morbid chronic pain, mood, and obesity-related metabolic syndrome in adulthood. These disorders are all associated with a chronic state of low-grade inflammation, which suggests that a reduction in inflammation could be a suitable therapeutic intervention for these patients. In support of this, humans that consume an anti-inflammatory diet exhibit a widespread reduction in inflammation and this type of diet is beneficial in the treatment of symptoms related to obesity-related metabolic syndrome and certain chronic pain conditions. However, how an anti-inflammatory diet influences the development of early life stress-induced co-morbid disorders is unknown. In this study, I investigate if an anti-inflammatory diet (AID) can be used as a non-pharmacological therapeutic intervention for the prevention of putative early life stress-induced co-morbid

disorders in female and male mice. To do this, I used a mouse model of early life stress, neonatal maternal separation (NMS), to assess the development of widespread pain and obesity-related metabolic syndrome compared to non-stressed (naïve) mice and if AID could prevent the predicted development of these early life stress-induced co-morbid disorders. I compared mice consuming AID to mice consuming a non-anti-inflammatory diet (NAID), which had the same composition as AID but without the added anti-inflammatory components, and to mice on a control diet, which was similar to a standard rodent chow diet. I found complex sex, stress, and diet interactions in most of our measures. Specifically, female and male NMS-AID mice were more susceptible to weight and body fat percentage gain compared to mice on the other diets. Female Naïve- and NMS-AID mice were also more glucose intolerant compared to NAID and control mice but male NMS-AID were not, despite them displaying increased susceptibility to weight and body fat gain. One finding that was consistent across groups was that AID was beneficial in the prevention of the development of stress and/or diet-induced perigenital sensitivity in female and male mice and hind paw sensitivity in female mice. Taken together, these data indicate that sex, stress, and diet are all important factors that play a role in the development of co-morbid chronic pain and obesity-related metabolic syndrome. Furthermore, although AID is able to treat pain disorders in humans and prevent the development of pain-like behaviors in our mice, studying a diet that has metabolic benefits in humans might not translate appropriately to the dietary and metabolic needs of rodents.

4.2 Introduction

A recent report found that in the year 2016, 50- million adults in the United States suffered from chronic pain and 20- million from high-impact chronic pain, or pain that limited life or work activities most days during the past 6- months. This report also found that chronic pain is more prevalent in women and older adults (442). Individuals with chronic pain often suffer from co-morbid pain disorders, such as irritable bowel syndrome, chronic prostatitis, vulvodynia, interstitial cystitis/painful bladder syndrome, fibromyalgia, and migraine, as well as mood disorders and obesity-related metabolic syndrome (443-449). Obesity is another chronic disorder that is highly prevalent in the United States and unfortunately the incidence of obesity is rapidly increasing (385). Future predictions indicate that about 42% of the US population will be obese by 2030 and this will cost an additional $549.5 billion each year for medical expenditures (450).

Obesity is generally considered a fundamental pre-requisite for the development of metabolic syndrome (165), which is diagnosed when an individual suffers from 3 of the following 5 criteria: central obesity, insulin resistance, hypertension, high triglycerides, and low HDL-cholesterol (167). However, there is a subset of individuals that are metabolically obese but normal weight (166). Obesity is also a risk factor for the development of chronic pain in weight bearing joints (451) and is associated with a number of chronic pain disorders, including fibromyalgia, headache, abdominal and pelvic pain, and chronic neuropathic pain (447). Fibromyalgia is characterized by widespread pain and tenderness, fatigue, and disability. The most recent criteria for diagnosing fibromyalgia emphasizes the importance of using generalized widespread pain criteria, that “fibromyalgia remains a valid construct irrespective of other

diagnoses", and that the fibromyalgia symptom scale should be used (452). Loevinger et al., (453) studied a sample of 109 adult premenopausal women with fibromyalgia and 46 healthy women as controls and found that those with fibromyalgia were 5.6 times more likely to have metabolic syndrome then healthy controls. Additionally, the incidence of fibromyalgia was also associated with each of the metabolic syndrome criteria that were measured, including greater waist circumference, higher blood pressure, serum triglycerides, and total and LDL cholesterol. Other studies support an association between fibromyalgia and obesity-related metabolic syndrome as well (454, 455) and patients with fibromyalgia are often diagnosed with other chronic pain disorders, such as irritable bowel syndrome, interstitial cystitis/painful bladder syndrome, and headache (446).

These data highlight that there is a high incidence of co-morbidity in individuals with chronic pain and obesity-related metabolic syndrome. Although the exact cause of these co-morbid disorders is not fully understood, a common underlying factor in many of these patients is a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis (3-7).

HPA axis and early life stress

The HPA axis is a neuroendocrine system that regulates the stress response and plays a role in the perception of pain (9, 10). When the HPA axis is functioning appropriately, a stressful event causes the hypothalamus to release corticotropin-releasing factor (CRF), which signals the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). ACTH signals the adrenal cortex to release glucocorticoids (GCs; cortisol in humans and corticosterone in rodents) that have

downstream metabolic effects including glucose and fat metabolism, control of the cardiovascular system, and regulation of the immune response (15-17). A negative feedback loop is established to turn off activation of the HPA axis by suppressing the production of CRF and ACTH upon cessation of the initial stressor (18, 19). Limbic structures including the hippocampus, amygdala, and prefrontal cortex, assist in resetting the HPA axis following a stressful event, as well as regulate its tone (Figure 1.1). The hippocampus and prefrontal cortex usually dampen the HPA axis response while the amygdala usually increases it (16, 25).

The HPA axis is not fully developed when children are born and therefore is susceptible to incorrect programming caused by early life perturbations, such as stress (13). Early life stress is associated with improper development of the HPA axis and the subsequent development of co-morbid chronic pain, mood, and metabolic disorders in adulthood (3-7). The Adverse Childhood Experiences (ACE) study reveals evidence of the severe consequences of early life stress. This study evaluated the prevalence of early life stress in a cohort of over 9,500 individuals and found that half reported at least one ACE and one-fourth reported two or more ACEs (323). From this original cohort of individuals, over 50 studies have been published investigating the long-term effects of early life stress. These studies demonstrate that early life stress can lead to the development of a number of chronic diseases, including chronic pain in the form of migraine (324), and a lower health-related quality of life (456). They also found that individuals that had four or more ACEs had a 1.4- 1.6 fold increase in physical inactivity and severe obesity (323). Other groups have found that childhood neglect and maltreatment are two forms of early life stress that are associated with the development

of obesity in adulthood (160-162). Additionally, HPA axis hyperactivity is seen in patients with visceral obesity (395, 396). Finally, a large study of 3,000 individuals with chronic pain evaluated the association between a history of abuse, including abuse early in life, and how they ranked on the Fibromyalgia Scale using the 2011 Fibromyalgia Survey Criteria and a Symptom Severity Scale. They found that the patients that had a history of abuse had greater pain severity, depression, anxiety, worse physical functioning, and higher scores on the Fibromyalgia Survey Criteria, also referred to as greater "fibromyalgianess", compared to those without a history of abuse (457). Data from these studies indicate that experiences early in life can have long-term consequences resulting in the development of chronic pain and/or obesity in adulthood. Understanding why this association exists and how we can treat these disorders is extremely important.

Neonatal maternal separation (NMS)

To study the many consequences of early life stress, our laboratory uses a mouse model known as NMS. This stress paradigm involves separating pups as a whole litter from the dam for 3- hours/day starting from postnatal day 1 (P1) to P21. Previously our lab has shown that NMS in mice results in the development of symptoms similar to those experienced by chronic pelvic pain patients, including urogenital sensitivity, increased mast cell (MC) degranulation in urogenital organs, and altered limbic control of the HPA axis (96-98). NMS mice also show increased MC infiltrate or MC degranulation in the dura mater, a tissue important in the pathophysiology of migraine (Chapter 2) and weigh more and have increased body fat percentage compared to naïve mice on a standard chow diet (Chapter 3). Our laboratory has also

demonstrated that exercise in the form of voluntary wheel running is a therapeutic intervention that prevents or attenuates many NMS-induced effects (100, 229)(Chapters 2 & 3). Patients with chronic pain and/or obesity-related metabolic syndrome often have trouble completing prescribed exercise regimes. Therefore, our goal is to determine if diet can be used as an alternative non-pharmacological therapeutic intervention. Specifically, in this study I am investigating if an anti-inflammatory diet (AID) can be used to prevent the predicted development of early life stress induced widespread pain and obesity-related metabolic syndrome.

AID

Many chronic pain, mood, and metabolic disorders are associated with a chronic state of low-grade inflammation (255, 256, 268). Therefore, an AID is thought to be a good dietary intervention for patients that suffer from these disorders (269). In humans, an AID is high in fruits, vegetables, lean or plant based protein, whole grains, high fiber, and healthy fats such as omega-3 fatty acids (FAs) (270). In this study, the AID used was developed from the AID used in Totsch et. al., (282). It is made of 20% protein, 45% carbohydrate with 0% sucrose, and 35% fat. The fat source is soybean and flaxseed oil, which are high in omega-3 FAs. It is made on a hi-maize 260-starch background, which is a high fiber resistant starch that aids in maintaining healthy blood sugar levels. AID also includes epigallocatechin gallate (EGCG), sulforaphane, resveratrol, curcumin, and ginseng (Table 4.1), all of which have anti-inflammatory properties. Furthermore, all of these anti-inflammatory ingredients are beneficial in treating symptoms of obesity-related metabolic syndrome, pain, and

Table 4.1 Diet compositions

	AID		Control		NAID	
Product #	**D17072401**		**D17072402**		**D17072403**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	22	19	19	19	22	19
Carbohydrate	51	45	69	71	51	45
Fat	18	35	4	10	18	35
Total		*100*		*100*		*100*
kcal/gm	4.5		3.9		4.5	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	218	*872*	218	*872*	218	*872*
L-Cystine	1.8	*7*	1.8	*7*	1.8	*7*
Corn Starch	0	*0*	609.5	*2438*	0	*0*
Hi-Maize 260	337.019	*1348*	0	*0*	337.019	*1348*
Maltodextrin 10	170	*680*	150	*600*	170	*680*
Sucrose	0	*0*	40	*160*	0	*0*
Cellulose, BW200	50	*0*	50	*0*	50	*0*
Soybean Oil	171.5	*1544*	41.5	*374*	171.5	*1544*
Flaxseed Oil	8.5	*77*	8.5	*77*	8.5	*77*
t-Butylhydroquinone	0.34	*0*	0.34	*0*	0.34	*0*
Mineral Mix S10022M	35	*0*	35	*0*	35	*0*
Vitamin Mix V10037	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2.5	*0*	2.5	*0*	2.5	*0*
EGCG (80%)	0.15	*0*	0	0	0	*0*
Sulforaphane (90%)	0.00067	*0*	0	0	0	*0*
Resveratrol	0.0006	*0*	0	0	0	*0*
Curcumin (65%)	0.154	*0*	0	0	0	*0*
Ginseng	0.06	*0*	0	*0*	0	*0*
FD&C Dye, Yellow #5	0.05	*0*	0	*0*	0.025	*0*
FD&C Dye, Red #40	0	*0*	0.05	*0*	0.025	*0*
Total	**1005.07**	**4567**	**1167.19**	**4567**	**1004.71**	**4567**

interact with the HPA axis to prevent the development of stress-induced depression-like behaviors in rodents as described below (Summarized in Table 4.2).

EGCG is the most abundant polyphenol found in green tea and is known for its antioxidant properties (458). In humans, a diet that is supplemented with polyphenols aids in preventing the development of obesity, metabolic syndrome, and type 2 diabetes (459). The beneficial effect of EGCG on the development of obesity-related metabolic syndrome is also seen in pre-clinical research. For example, male C57Bl/6 mice fed a high-fat diet supplemented with EGCG (3200 mg/kg diet) for 16-weeks had lower adipose tissue, lower fasting blood glucose and cholesterol compared to mice fed a high-fat diet with no EGCG (460). Pre-clinical studies have also shown EGCG's therapeutic properties for the treatment of pain. Chronic constriction injury (CCI) in rats leads to decreased mechanical and heat pain thresholds and increased pro-inflammatory cytokines in the spinal dorsal horn. However, after intrathecal injection of EGCG (1 mg/kg) starting 1- day before and continuing 3- days after CCI, pro-inflammatory cytokines were reduced, the anti-inflammatory cytokine IL-10 was significantly increased, and pain thresholds were increased (461). Finally, EGCG influences the HPA axis. Lee et al., (462) used a single prolonged stress paradigm that caused cognitive deficits and decreased hippocampal brain-derived neurotrophic factor (BDNF) in rats. However, rats that received IP injections of EGCG (25 mg/kg) following the stressful event had improved cognition and did not show alteration in hippocampal BDNF.

Sulforaphane is found in cruciferous vegetables such as broccoli, cauliflower, and Brussel sprouts and has antioxidant, anti-inflammatory, and neuroprotective effects

Table 4.2 Therapeutic benefits of anti-inflammatory ingredients in pre-clinical studies

Anti-inflammatory ingredient	Disorder treated		
	Obesity-related metabolic syndrome	**Pain**	**Stress-induced depression-like behavior and HPA axis dysfunction**
EGCG	High-fat (HF) diet-induced obesity (460)	Chronic constriction injury (CCI) (461)	Single prolonged stress (462)
Sulforaphane	HF diet-induced obesity (463)	CCI (464)	Chronic mild stress(465)
Resveratrol	HF diet-induced obesity (466)	Streptozotocin – induced diabetic neuropathy (467)	Exogenous corticosterone administration (468)
Curcumin	HF diet-induced obesity & ob/ob mice (469)	CCI (470)	Chronic unpredictable stress (471)
Ginseng	HF diet-induced obesity (472)	Paw capsaicin injection (473)	Chronic unpredictable stress (474)

(475, 476). Furthermore, it prevents the differentiation of pre-adipocytes into adipocytes *in vitro* (477), which is important in the development of obesity. C57Bl/6N mice fed a high-fat diet supplemented with sulforaphane (1000 mg/kg diet) for 6-weeks had lower feed efficiency, body weight, visceral adipose tissue, and fat accumulation in the liver compared to mice on a high-fat diet without sulforaphane (463). Furthermore, IP administered sulforaphane (0.1-100 mg/kg) for 7- days prevented the development of CCI-induced mechanical and thermal hypersensitivity and increased pro-inflammatory cytokine levels seen in CCI mice not treated with sulforaphane (464). Finally, sulforaphane prevents stress-induced changes in mood disorders through alteration of the HPA axis. Wu et. al., (465) used a chronic mild stress paradigm in mice to induce depressive and anxiety like behaviors. However, mice that received repeated IP injections of sulforaphane (10 mg/kg) did not display stress-induced behaviors. Sulforaphane-treated mice had increased sucrose preference, increased immobility time in a forced swim test, and increased time in the center of an open field test compared to mice that did not receive treatment. They also demonstrated that sulforaphane administration inhibited the stress-induced increase in serum ACTH and corticosterone.

Resveratrol is a natural polyphenol and phytoalexin that is produced by several plants and fruits in response to injury, including grapes, apples, raspberries, blueberries, pistachios, plums, and peanuts and has anti-inflammatory and antioxidant effects (478). 150 mg/day of resveratrol benefited obese men by reducing resting metabolic rate and decreasing circulating inflammatory markers, fatty acids, and glycerol compared to obese men that received a placebo. Resveratrol-treated men also had enhanced muscle mitochondrial respiration (479). Benefits of resveratrol on symptoms of

metabolic syndrome were also seen in C57Bl/6J mice fed a high-fat diet supplemented with resveratrol for 20-weeks (200 g/kg diet). These mice had reduced hepatic steatosis and adipose tissue macrophages, and were more insulin sensitive compared to mice fed a high-fat diet without resveratrol supplementation. Resveratrol supplementation also improved high-fat diet-induced memory impairments; the authors suggest this is likely due to reduced inflammation in the hippocampus (466). Additionally, resveratrol reduces pain-like symptoms in mice. For example, in a mouse model of streptozotocin-induced diabetic neuropathy, mice develop higher glucose levels, tail and paw heat hyperalgesia, and increased circulating TNFα. However, resveratrol treatment (5, 10, and 20mg/kg) daily for 4-weeks attenuated thermal hyperalgesia, decreased circulating TNFα levels, and whole brain nitric oxide in a dose dependent manner (467). Finally, resveratrol can prevent the development of mood disorders through alterations of the HPA axis. This was demonstrated in rats that were administered exogenous corticosterone and subsequently developed depression-like symptoms, such as anhedonia in sucrose preference test, and a decrease in hippocampal BDNF. However, when Swiss albino mice were treated with resveratrol (80g/kg) before corticosterone injection for 3-weeks, these effects were prevented (468).

Curcumin is the most bioactive polyphenol in the spice turmeric and has anti-inflammatory, anti-oxidant, and anti-angiogenesis properties (480). In both high-fat diet-induced C57Bl/6J mice and ob/ob genetically obese mice, dietary curcumin supplementation (300 mg/kg) improved glucose tolerance and insulin sensitivity, as well as reduced adipose tissue macrophage infiltration (469). Clinical trials demonstrated that humans treated with curcumin and piperine, which enhances the bioavailability of

curcumin, have decreased circulating triglyceride and inflammatory cytokine levels but no changes in body weight or body fat (481, 482). Clinical data also reveal that curcumin (1,500 mg/day for 4-weeks) used for treatment of knee osteoarthritis patients is just as effective as ibuprofen treatment, but curcumin has less reported gastrointestinal problems (181). Pre-clinical work investigating the influence of curcumin on pain demonstrates that in a rat model of CCI, curcumin (100mg/kg) starting the day after the operation and lasting 14-days prevents the development of thermal and mechanical paw hypersensitivity (470). Additionally, in humans with major depressive disorder, 500 mg twice daily for 8-weeks of curcumin was more effective than placebo in improving depressive symptoms (483). Finally, curcumin can treat depression-like symptoms through alterations in the HPA axis. This was observed in a 20-day chronic unpredictable stress paradigm in Sprague-Dawley rats that resulted in a deficit in escape behaviors in a shuttle box test, which is used to evaluate depression-like behavior in rodents, as well as elevated serum corticosterone and decreased hippocampal glucocorticoid receptor and BDNF mRNA. However, 21-day administration of curcumin at 5 or 10 mg/kg reversed these stress-induced behavioral and molecular effects (471).

Ginseng is a medicinal herb and its root has been used for thousands of years in Asia because of its anti-inflammatory and anti-oxidant properties. The major pharmacological compounds in ginseng are ginsenosides but it also contains flavonoids, alkaloids, lignans, and vitamins (484). Ginseng extract (4g x 2 times a day) taken for 8-weeks by obese adult women significantly reduced body weight and body mass index (485). Interestingly, this study showed that the gut microbiota differed in

those that significantly lost weight and those that did not. Positive effects from ginseng supplementation on weight and pain are also demonstrated in pre-clinical studies. For example, mice that were fed a high-fat diet supplemented with wild ginseng ethanol extract (250 and 500 mg/kg) had lower body weight and smaller adipocytes compared to mice on a non-supplemented high-fat diet (472). The mice that received ginseng also had lower fasting glucose levels and improved insulin sensitivity. Furthermore, ginsenoside (50mg/kg) inhibited the development of pain-like behaviors following capsaicin injection into the hind paw, including biting or licking of the paw (473). Finally, ginseng interacts with the HPA axis and treats depression-like behaviors in male C57BL/6J mice treated with chronic unpredictable stress for 8-weeks. Stressed mice developed depression-like responses during forced swim and tail suspension tests and had elevated serum corticosterone. However, when mice were treated IP 10 mL/kg ginsenoside during the last 14- days of the stressor, the depression-like behaviors and increased serum corticosterone did not develop. Additionally, ginsenoside up-regulated hippocampal BDNF mRNA in mice that underwent chronic mild stress (474).

Dietary fat source can also influence inflammation. Omega-6 FAs are considered pro-inflammatory, while omega-3 FAs are considered anti-inflammatory (270) and it is the ratio of the two that determine inflammatory status. Increased omega-3 FAs have been linked to long-term health benefits such as improved weight management and cardiovascular, immune, and neuronal function (486) and increased omega-6 FAs is linked to an increased risk of developing obesity (487). A study investigating the effect of omega-3 FA supplementation in high-fat diet-induced obese mice showed that including omega-3 FAs in the diet prevented increased macrophage infiltration in

adipose tissue normally seen in a high-fat diet not supplemented with omega-3 FAs (284). Interestingly, body weight was higher in the mice whose diet was supplemented by omega-3 FAs but their plasma triglyceride concentrations were significantly lower. The ratio of FAs also influences pain in patients. For example, an increase in omega 3-FA consumption paired with a reduction in omega-6 FAs reduced headache pain and improved quality of life in a study of chronic headache patients (179). Additionally, Goldberg and Katz (180) found that adding omega-3 FAs to the diet of patients with rheumatoid arthritis, as well as joint pain associated with inflammatory bowel diseases and dysmenorrhea, reduced joint pain intensity, number of painful joints, and NSAID consumption for the treatment of joint pain. The AID used in this study is made of 35% fat that comes from flaxseed and soybean oils (Table 4.1), which are considered a healthy fat source because they contain a greater omega-3 to omega-6 FA ratio (488).

Finally, the amount of dietary added sugar is also an important factor to consider in diet studies. A diet high in added sugar, usually fructose-containing sugars such as high fructose corn syrup or sucrose, is associated with the development of metabolic syndrome, cardiovascular disease, and type 2 diabetes (489). Added sugar contributes to the positive energy balance associated with obesity and the subsequent development of metabolic syndrome. The detrimental consequences of added sugar were demonstrated in a clinical study that compared the effects of consuming glucose- or fructose- sweetened beverages for 10-weeks. They found that although subjects gained the same amount of weight, those that consumed fructose-sweetened beverages had increased liver lipogenesis, dyslipidemia, and visceral adipose tissue as well as decreased insulin sensitivity compared to those that consumed glucose-sweetened

beverages (490). The AID used in this study has 0% added sucrose in an attempt to avoid the negative effects of added sugar (Table 4.1).

Sex differences

Women are overrepresented in many chronic pain disorders and healthy women are more sensitive to experimentally induced pain compared to healthy men (491, 492). Additionally, females display higher levels of basal circulating corticosterone compared to males (298) and sex differences have been found in animal responses to stress (297, 493, 494). Finally, obesity is more prevalent among women (386), while type 2 diabetes mellitus is diagnosed at a lower age and body mass index in men (387). However, pre-clinical studies suggest that female mice are resistant to diet-induced weight gain and other metabolic alterations compared to males (303, 414). Due to the high incidence of sex differences in these disorders, both female and male mice were used in this study.

Significance

Co-morbid pain, mood, and obesity-related metabolic syndrome are all highly prevalent in the United States and many individuals that suffer from these co-morbid disorders have experienced early life stress. Unfortunately, most pharmacological therapeutic treatments for these disorders are not universally successful or have dangerous side effects. Therefore, studying appropriate treatments to prevent the development of stress-induced chronic disorders is important. Anti-inflammatory components are successful in the treatment of obesity-related metabolic syndrome, certain chronic pain disorders, and also influence the HPA axis in rodents (Table 4.2). In this study AID (Table 4.1) is used as a therapeutic intervention for putative early life

stress-induced co-morbid disorders. To my knowledge, this is the first study to investigate the influence of AID in NMS female and male mice and evaluate sex, stress, and diet interactions.

4.3 Methods

Neonatal maternal separation: Pregnant C57Bl/6 dams at 14-16- day gestation were ordered from Charles River and housed in the Department of Laboratory Animal Resources at the University of Kansas Medical Center. Litters were divided equally into NMS and naïve groups. NMS pups were removed as whole litters from their home cage for 180- minutes (11am-2pm) daily beginning at postnatal day 1 (P1) until P21. During separation, pups were placed in a clean glass beaker with bedding from their home cage. The beaker was placed in an incubator maintained at 33°C and 50% humidity. Naïve mice remained undisturbed in their home cage except for normal animal husbandry. All mice were weaned on P22 and pair-housed with same-sex litter mates and *ad libitum* access to water and a control diet composed of 20% kcal protein, 70% kcal carbohydrate (3.5% sucrose) and 10% kcal cat (Research Diets, Inc. New Brunswick, NJ; Table 4.1).

Anti-inflammatory diet (AID): At 4-weeks of age, female and male naïve and NMS mice were further divided. Half remained on the control diet and half were switched to AID, which was composed of 20% kcal protein, 45% kcal carbohydrate (0% sucrose), and 35% kcal fat with a Hi-Maize 260 starch source and added anti-inflammatory components: EGCG, sulforaphane, resveratrol, curcumin, and ginseng (Research Diets, Inc. New Brunswick, NJ); Table 4.1). Food intake was measured weekly.

Non-anti-inflammatory diet (NAID): At 4-weeks of age in a different cohort, female and male naïve and NMS mice were further divided. Half remained on the control diet and half were switched to NAID, which was composed of 20% kcal protein, 45% kcal carbohydrate (0% sucrose), and 35% kcal fat with a Hi-Maize 260 starch source (Research Diets, Inc. New Brunswick, NJ; Table 4.1). Food intake was measured weekly.

Feed efficiency: Food consumption per pair and weight gain were measured every week. Feed efficiency was then calculated as weight gained/calories consumed per pair of mice per week. An average feed efficiency was quantified from weekly feed efficiency throughout the course of the experiment.

Body composition analysis: Mice were weighed and placed in an EchoMRI 2015 to measure lean mass and fat mass. Total weight, percent body fat, and free fat mass were quantified.

Perigenital mechanical sensitivity: Perigenital mechanical withdrawal threshold was assessed every 4-weeks starting after 4-weeks on the AID or NAID diets. For 2-days prior to the test day, mice were acclimated to a sound proof room for 30-minutes and then placed into individual clear plastic chambers (11 x 5 x 3.5 cm) on a wire mesh screen elevated 55cm above a table for 30-minutes. On the test day, mice were also acclimated to the sound proof for 30-minutes and then placed on the table for 30-minutes. The up-down method was performed to test mechanical sensitivity using a standard set of Semmes-Weinstein monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.31, 4.74 g; Stoelting, Wood Dale, IL) (372, 495). Beginning with the 3.22 g monofilament,

mice received a single application to either the scrotum or perivaginal area. A negative response was followed by the next larger filament and a positive response (considered a brisk jerk or licking the affected area) was followed by the next smaller filament. The experimenter continued to move up or down the series, depending on the previously elicited response, for an additional four applications after the first positive response was observed for a minimum of five or a maximum of nine total monofilament applications. The value in log units of the final monofilament applied in the trial series was used to calculate 50% g threshold for each mouse (372).

Hind paw mechanical sensitivity: Hind paw sensitivity was assessed every 4-weeks starting after 5-weeks on the AID or NAID diets. On the test day, mice were acclimated to a sound proof room for 30- minutes and then placed into individual clear plastic chambers (11 x 5 x 3.5 cm) on a wire mesh screen elevated 55cm above a table for 30-minutes. An electronic von Frey device (IITC Life Science Inc. Woodland Hills, CA) was used to measure hind paw withdrawal threshold. A filament was applied to the hind paw and the force that elicited a withdrawal was recorded from the electronic device. Each mouse was tested 6-times. The highest and the lowest value of each mouse were excluded. Therefore, an average of 4-measurements/mouse was quantified.

Nestlet behavior: After 16-17-weeks on the AID or NAID diets, a nestlet building assay was carried out. One hour before the start of the dark phase, mice were individually placed in a clean cage with 3g-nestlet material. Pictures were taken of the nestlets the next morning and un-torn nestlet material was weighed. Nestlet score was assigned using a nestlet rating scale ranging from 1-5 (496).

Fasting insulin: After 18-19-weeks on the AID or NAID diets, fasting insulin level was measured. Following a 6- hour fast, blood was collected via tail-clip, placed on ice for 1-hour, and centrifuged at 10,000 rpm for 10- minutes. Serum was collected and frozen until analyses using an insulin *ELISA* kit (American Laboratory Products Company, Salem, NH).

Glucose Tolerance Test: After 18-19-weeks on the AID or NAID diets, a glucose tolerance test was carried out. Following a 6-hour fast, mice were given an IP injection of glucose at 1 g/kg body weight. Blood glucose levels (glucose diagnostic reagents, Sigma) were measured via tail clip immediately before glucose injection and 15, 30, 60, and 120- minutes thereafter.

Corticosterone level: At After 18-19-weeks on the AID or NAID diets, mice were sacrificed during the early half of the light cycle (0800-1100- hours) and trunk blood was collected, placed on ice for 1-hour, and centrifuged at 10,000 rpm for 10-minutes. Serum was removed and frozen until analysis using a corticosterone ELISA kit (American Laboratory Products Company, Salem, NH).

Statistical analyses: Comparisons were made between control groups from the AID and NAID cohorts based on sex and NMS status and no significant differences were observed for body weight, body fat, food intake, feed efficiency, fasting insulin, serum glucose levels, nestlet building behavior, and serum corticosterone; therefore, the control groups were combined for statistical comparisons. Calculations of the measurements described above, with the exceptions of perigenital and hind paw sensitivity, were made in Excel (Microsoft, Redmond, WA) and statistical analyses were

performed using GraphPad Prism (GraphPad, La Jolla, CA) or IBM SPSS Statistics (IBM Corporation, Armonk, NY). Differences between groups were determined by 2- or 3-way ANOVA or 3-or 4- way RM ANOVA and Bonferroni posttest. Statistical significance was set at $p<0.05$. For hind paw sensitivity, using Generalized Estimating Equation (GEE) framework, the following model was created for each diet/sex: $Hindpaw_{ij} = \alpha_0 + \alpha_1 week_{ij} + \alpha_2 week_{ij}^2 + \alpha_4 stress_{ij} + \varepsilon_{ij}$ and for perigenital sensitivity the following model was created for each diet/sex: $\log(Perigenital_{ij}) = \alpha_0 + \alpha_1 week_{ij} + \alpha_2 week_{ij}^2 + \alpha_4 stress_{ij} + \varepsilon_{ij}$. To fit these models, a Markov structure was employed for the working correlation matrix. For posttest analyses, each subgroup was compared in terms of area under the curve.

4.4 Results

Female and male NMS mice on the AID diet are more susceptible to weight gain over time compared to other groups.

Body weight was measured weekly throughout the course of the experiment in female and male mice after the introduction of the AID or NAID diets (Figure 4.1). There was a significant overall effect of time ($p<0.0001$), diet ($p<0.01$), NMS ($p<0.01$), and a time/diet interaction ($p<0.01$) for female body weight (Figure 4.1A). Female NMS-AID mice weighed significantly more than female NMS-control mice at 7, 9, and 14-18-weeks on their respective diets ($p<0.05$). Female NMS-AID mice also weighed significantly more than naïve-AID mice at 14-18-weeks on their diets ($p<0.05$). In male

Figure 4.1

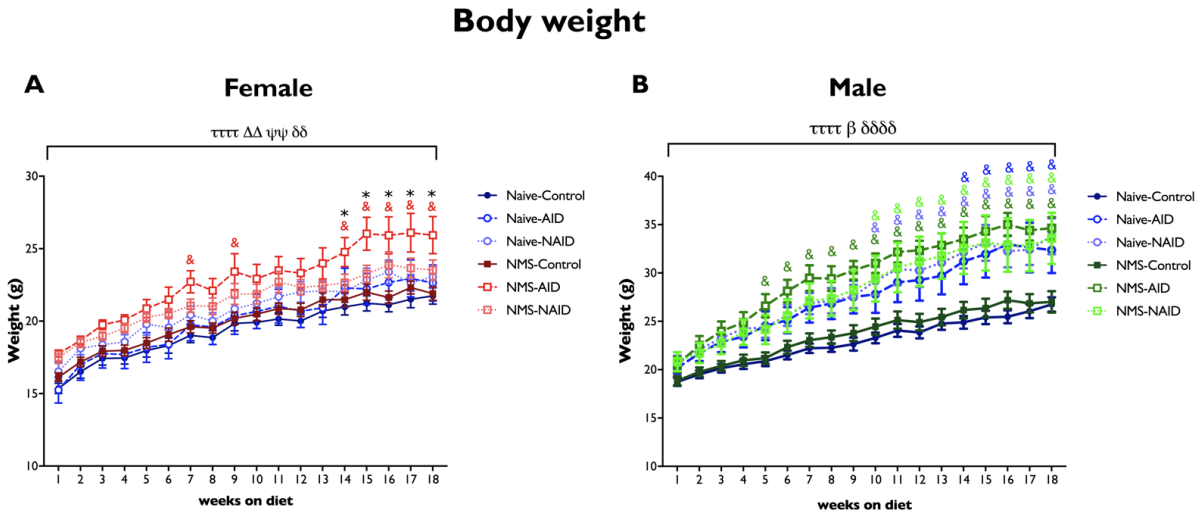
Body weight
A
Female
B
Male
Weight (g)
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID

Figure 4.1 Body weight was measured weekly in female and male mice on a control, AID, or NAID diet. **A)** In female mice there was a significant overall effect of time, NMS, diet, and a time/diet interaction. At weeks 7, 9, and 14-18 NMS-AID weighed significantly more than NMS-Control and at weeks 14-18 NMS-AID weighed significantly more than NMS-NAID. **B)** In male mice there was a significant overall effect of time, diet, and a NMS/time interaction. Starting at 5-weeks on the diet and lasting through the duration of the experiment, NMS-AID mice weighed significantly more than NMS-Control mice, starting after 10-weeks on the diet and lasting through the duration of the experiment NMS-NAID mice weighed significantly more than NMS-control mice and naïve-NAID mice weighed significantly more than naïve-control mice, and starting at 14-weeks on the diet naïve-AID mice weighed significantly more than naïve-control mice. In an overall analysis of female and male body weight over time, there was a significant effect of time ($p<0.0001$), NMS ($p<0.05$), a sex/time interaction ($p<0.0001$), a diet/time interaction ($p<0.0001$), a sex/diet interaction ($p<0.01$), and a sex/diet/time interaction ($p<0.001$), 4-way RM ANOVA. Bracket indicates a significant effect of time (τττττ $p<0.00001$), NMS (ψψ $p<0.01$), diet (δδδδ $p<0.0001$), a time/stress interaction (β $p<0.05$), or a time/diet interaction (Δ $p<0.01$); 3-way RM ANOVA. & $p<0.05$ vs. control diet, * $p<0.05$ vs. naïve; Bonferroni posttest.

mice, there was a significant overall effect of time (p<0.0001), diet (p<0.0001), and a NMS/time interaction (p<0.05) on body weight (Figure 4.1B). Starting at 5-weeks on the diets and lasting through the duration of the experiment, male NMS-AID mice weighed significantly more than male NMS-control mice (p<0.05). Starting at 10-weeks on the diets and lasting through the duration of the experiment, male NMS-NAID weighed significantly more than male Naïve-NAID (p<0.05) and male naïve-NAID weighed significantly more than male naïve-Control (p<0.05). Starting at 14-weeks on the diets, male naïve-AID started weighing significantly more than male naïve-Control (p<0.05). In an overall analysis of female and male body weight over time, there was a significant effect of time (p<0.0001), NMS (p<0.05), a sex/time interaction (p<0.0001), a diet/time interaction (p<0.0001), a sex/diet interaction (p<0.01), and a sex/diet/time interaction (p<0.001). Analyses of body weight at sacrifice are listed in Tables 4.3 (female) and 4.4 (male).

Female and male NMS mice on the AID diet are more susceptible to body fat percentage gain over time compared to other groups.

Body composition was assessed using EchoMRI every 2-4-weeks starting 4-weeks after the introduction of the AID or NAID diets and body fat percentage was quantified (Figure 4.2). In female mice, there was a significant overall effect of time (p<0.0001), diet (p<0.0001), and a time/diet interaction (p<0.0001) on body fat percentage (Figure 4.2A). At every time point that was measured, female NMS-AID mice had a significantly higher body fat percentage compared to female NMS-control mice (p<0.05) and at 4, 8, 16, and 18-weeks on the diets, female NMS-AID had a

Table 4.3 Female body composition at sacrifice

Variable	Overall analyses	Naïve-Control	Naïve-AID	Naïve-NAID	NMS-Control	NMS-AID	NMS-NAID
Body weight (g)	δδδδ ψ	21.138 ± 0.430	24.067 ± 1.459	24.6 ± 0.937 **&**	22.436 ± 0.534	27.067 ± 1.413 **&&**	24.688 ± 0.940
Body fat percentage (%)	δδδδ	15.237 ± 0.977	22.855 ± 2.743	19.863 ± 1.993	15.985 ± 1.242	26.661 ± 3.620 **&**	19.210 ± 1.709

Data are expressed as mean ± S.E.M. Significant effect of diet, δδδδ $p<0.0001$, or NMS ψ $p<0.05$, 2-way ANOVA. &, && $p<0.05$, 0.01 vs. control diet, Bonferroni posttest.

Table 4.3 Male body composition at sacrifice

Variable	Overall analyses	Naïve-Control	Naïve-AID	Naïve-NAID	NMS-Control	NMS-AID	NMS-NAID
Body weight (g)	δδδδ	26.092 ± 0.591	32.367 ± 2.369	33.388 ± 1.786 **&**	26.771 ± 0.905	34.633 ± 1.148 **&**	33.650 ± 2.908 **&**
Body fat percentage (%)	δδδδ	18.769 ± 0.937	27.064 ± 4.386	30.006 ± 2.464 **&**	20. 698 ± 1.961	33.772 ± 1.324 **&**	27.850 ± 3.507

Data are shown as mean ± S.E.M. Significant effect of diet δδδδ $p<0.0001$, 2-way ANOVA. & $p<0.05$ vs. control diet, Bonferroni posttest.

Figure 4.2

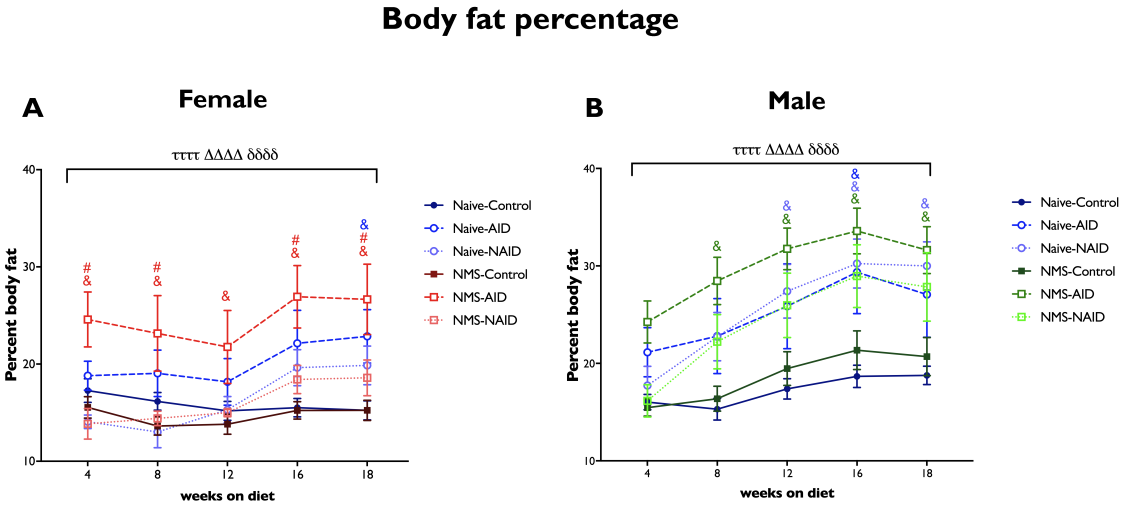
Body fat percentage
A
Female
B
Male
τττ ΔΔΔΔ δδδδ
Percent body fat
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID

Figure 4.2 Beginning 4-weeks after the start of the AID or NAID diets, body composition was assessed using EchoMRI and body fat percentage was quantified in female and male mice. **A)** In female mice there was a significant overall effect of time, diet, and a time/diet interaction. Female NMS-AID mice had a significantly greater body fat percentage compared to female NMS-control mice at every time point that body composition was measured. Female NMS-AID mice had a significantly higher body fat percentage compared to female NMS-NAID mice at the 16- and 18- week measurements and female naïve-AID mice had a significantly greater body fat percentage compared to female naïve-control mice at the 18- week measurement. **B)** In male mice there was a significant overall effect of time, diet, and a time/diet interaction on body fat percentage. Male NMS-AID mice had a significantly higher body fat percentage compared to male NMS-control mice starting at the 8- week measurement and lasting the duration of the experiment. Male naïve-NAID mice had a significantly higher body fat percentage compared to male naïve-control mice starting at the 12-week measurement and male naïve-AID mice had a significantly higher body fat percentage compared to male naïve-control mice at the 16-week measurement, which lasted the duration of the experiment. In an overall analysis of female and male body fat percentage over time there was a significant effect of time (p<0.0001), diet (p<0.0001), sex (p<0.05), a NMS/diet interaction (p<0.05), a diet/sex interaction (p<0.05), a diet/time interaction (p<0.0001), a sex/time interaction (p<0.0001), and a sex/diet/time interaction (p<0.0001), 4-way RM ANOVA. Bracket indicates a significant effect of time ($\tau\tau\tau\tau$ p<0.0001), diet ($\delta\delta\delta\delta$ p<0.0001), or a time/diet interaction ($\Delta\Delta\Delta\Delta$ p<0.0001); 3-way RM ANOVA. & p<0.05 vs. control, # p<0.05 vs. NAID; Bonferroni posttest.

significantly higher body fat percentage compared to female NMS-NAID mice (p<0.05). At 18-weeks on the diets, female naïve-AID mice had a significantly higher body fat percentage compared to female naïve-control mice (p<0.05). In male mice, there was a significant overall effect of time (p<0.0001), diet (p<0.0001), and a time/diet interaction (p<0.0001) on body fat percentage (Figure 4.2B). At 8, 12, 16, and 18-weeks on the diets, male NMS-AID had a significantly higher body fat percentage compared to male naïve-AID mice (p<0.05). At 12, 16, and 18-weeks on the diets male NMS-NAID mice had a significantly higher body fat percentage compared to male NMS-control mice (p<0.05). At 16-weeks on the diets, male naïve-AID mice had a significantly higher body fat percentage compared to male naïve-control mice (p<0.05). In an overall analysis of female and male body fat percentage over time there was a significant effect of time (p<0.0001), diet (p<0.0001), sex (p<0.05), a NMS/diet interaction (p<0.05), a diet/sex interaction (p<0.05), a diet/time interaction (p<0.0001), a sex/time interaction (p<0.0001), and a sex/diet/time interaction (p<0.05). Analyses of body fat percentage at sacrifice are listed in Tables 4.3 (female) and 4.4 (male).

Sex influences food intake while diet influences food feed efficiency

Food intake was measured weekly and is reported as food consumed per pair due to the fact that mice were pair housed to avoid the added stress of single housing (Figure 4.3). In female mice, there was a significant effect of time on food intake (p<0.05; Figure 4.3A) with no significant differences between groups in post-hoc analysis. In male mice, there was a significant effect of time (p<0.0001) and diet (p<0.01) on food intake (Figure 4.3C). After 1-week on the diets, male naïve-AID mice ate significantly more than male naïve-control mice (p<0.05) and after 5-weeks on the

Figure 4.3

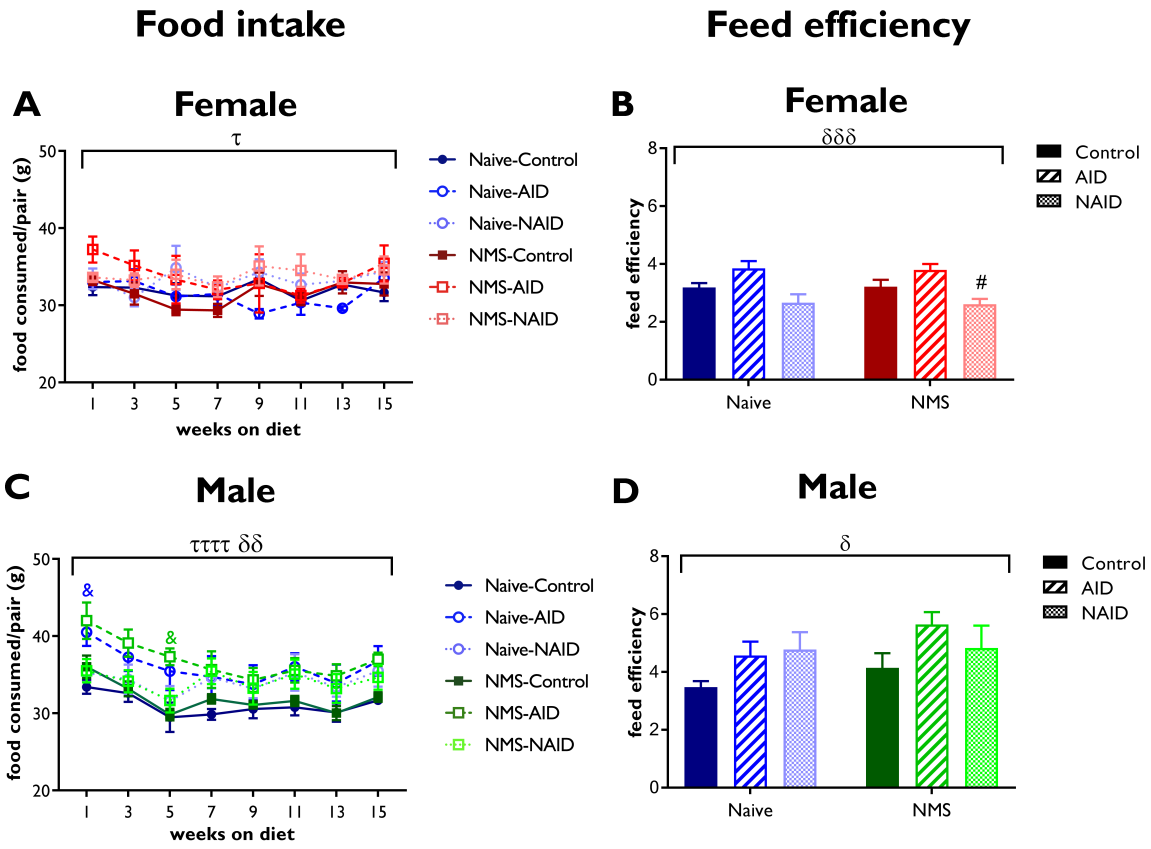
Food intake
Feed efficiency
A
Female
τ
food consumed/pair (g)
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID
B
Female
δδδ
feed efficiency
#
Naive
NMS
Control
AID
NAID
C
Male
ττττ δδ
&
D
Male
δ

Figure 4.3 Food intake is reported as food consumed per pair because mice were pair housed to avoid the extra stress of single housing. Feed efficiency is calculated as weight gained/calories consumed per week. An average feed efficiency was quantified from weekly feed efficiency throughout the course of the experiment. In female mice, there was a significant effect of time on food intake (**A**) and of diet on feed efficiency (**B**). Female NMS-NAID mice had a significantly lower feed efficiency compared to NMS-AID mice. **C**) In male mice, there was a significant overall effect of time and diet on food intake. After 1- week on the diets, naïve-AID mice ate significantly more than naïve-control mice and, at 5- weeks on the diets, NMS-AID mice ate significantly more than NMS-control mice. **D**) There was also a significant effect of diet on feed efficiency in male mice. In an overall analysis of female and male feed efficiency, there was a significant overall effect of diet ($p<0.01$), sex ($p<0.0001$), and a diet/sex interaction ($p<0.05$), 3-way ANOVA. Bracket indicates a significant effect of time (τ, $\tau\tau\tau\tau$ $p<0.05$, 0.0001) or diet (δ, $\delta\delta$, $\delta\delta\delta$ $p<0.05$, 0.01, 0.001), A, C: 3-way RM ANOVA, B, C: 2-way ANOVA; # $p<0.05$ vs. AID, & $p<0.05$ vs. control, Bonferroni posttest.

diets, NMS-AID ate significantly more than NMS-control mice (p<0.05). Feed efficiency is calculated as weight gained/calories consumed per week. An average feed efficiency was quantified from weekly feed efficiency throughout the course of the experiment in female and male mice (Figure 4.3). In female mice, there was a significant overall effect of diet on average feed efficiency (p<0.0001; Figure 4.3B). Female NMS-NAID mice had a significantly lower feed efficiency compared to female NMS-AID mice (p<0.05). In male mice, there was a significant overall effect of diet on average feed efficiency (p<0.05; Figure 4.3D). In an overall analysis of female and male feed efficiency, there was a significant overall effect of diet (p<0.01), sex (p<0.0001), and a diet/sex interaction (p<0.05).

Male naïve-AID and –NAID mice are insulin resistant compared to male naïve-control mice, while male NMS mice nor female mice do not differ in their fasting insulin levels.

After 18- 19- weeks on the diets, mice were fasted for 6-hours and blood was drawn via tail-clip. Serum was collected and an insulin *ELISA* was performed (Figure 4.4). In female mice, there were no significant effects of NMS or diet or differences between groups for fasting insulin level (Figure 4.4A). In male mice, naïve-AID and -NAID groups had a significantly higher fasting insulin level compared to naïve-control mice (p<0.05; Figure 4.4B). In an overall analysis of female and male fasting insulin level there was a significant effect of sex, with males having much higher fasting insulin levels than female (p<0.0001).

Female naïve- and NMS-AID and male naïve- AID and NAID mice are more glucose intolerant compared to their control diet counterparts.

Figure 4.4

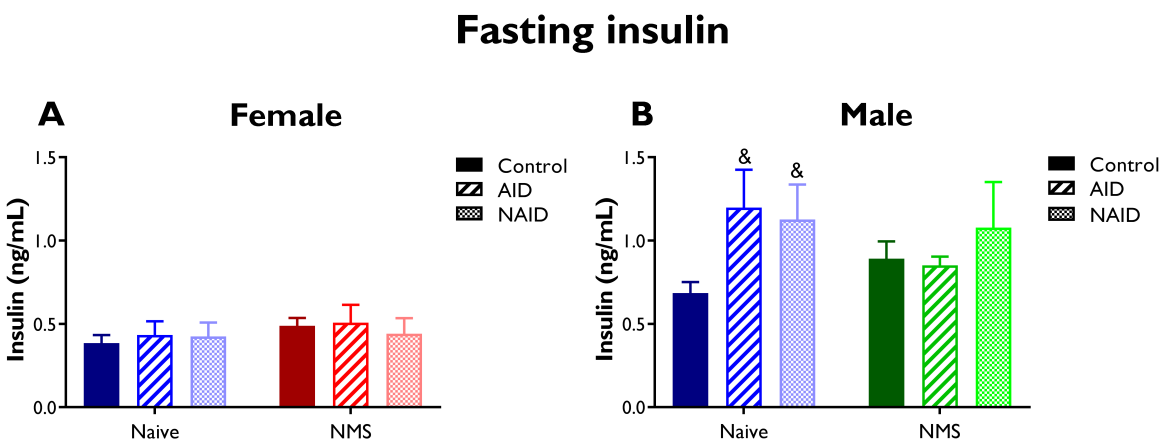
Fasting insulin
A
Female
Insulin (ng/mL)
1.5
1.0
0.5
0.0
Naive
NMS
Control
AID
NAID
B
Male
&
&
Insulin (ng/mL)
1.5
1.0
0.5
0.0
Naive
NMS
Control
AID
NAID

Figure 4.4: After 18-19- weeks on the AID or NAID diets, mice were fasted for 6- hours, blood was collected, and serum insulin level was measured in female and male mice. **A)** In female mice, there was no significant effect of diet or stress on fasting serum insulin level. **B)** In males, naïve-AID and -NAID mice had significantly higher serum fasting insulin level compared to male naïve-control mice. There was a significant overall effect of sex on fasting insulin level ($p<0.0001$; 3-way ANOVA). 2-way ANOVA; & $p<0.05$ vs. control diet, Bonferroni posttest.

After 18-19 weeks on the diets a glucose tolerance test (GTT) was performed. After a 6- hour fast, blood was drawn via tail-clip to determine a baseline fasting glucose level. Then an IP injection of 1g/kg body weight glucose was administered and subsequent blood draws were taken at 15, 30, and 60, and 120- minutes after injection. In female mice, there was a significant effect of time ($p<0.0001$), diet ($p<0.01$), a time/diet interaction ($p<0.05$), and a NMS/diet/time interaction ($p<0.05$) on serum glucose levels (Figure 4.5A). Female NMS-AID mice had a significantly higher blood glucose level compared to female NMS-control mice 15, 30, and 60- minutes after glucose injection ($p<0.05$). Female NMS-AID mice had a significantly higher blood glucose level compared to female NMS-NAID mice ($p<0.05$) and female naïve-AID mice had a significantly higher blood glucose level compared to female naïve-control mice ($p<0.05$) 30- minutes after glucose injection. GTT area under the curve (AUC) was also quantified and analyzed. There was a significant effect of diet on female GTT AUC ($p<0.0001$; Figure 4.5B). Female naïve- and NMS-AID mice had a significantly greater GTT AUC compared to their control diet counterparts ($p<0.05$) and female NMS-NAID mice had a significantly greater GTT AUC compared to female NMS-control mice ($p<0.05$). In male mice, there was a significant overall effect of time ($p<0.0001$) and diet ($p<0.0001$) on glucose tolerance over time (Figure 4.5 C). Male naïve-NAID mice had a significantly greater blood glucose level compared to male naïve-control mice 30- and 60- minutes after glucose injection ($p<0.05$). There was a significant effect of diet on male GTT AUC ($p<0.0001$; Figure 4.5D). Male naïve-AID ($p<0.05$) and -NAID mice ($p<0.01$) had a significantly greater GTT AUC compared to male naïve-control mice. In an overall analysis of female and male GTT there was a significant effect of time

Figure 4.5

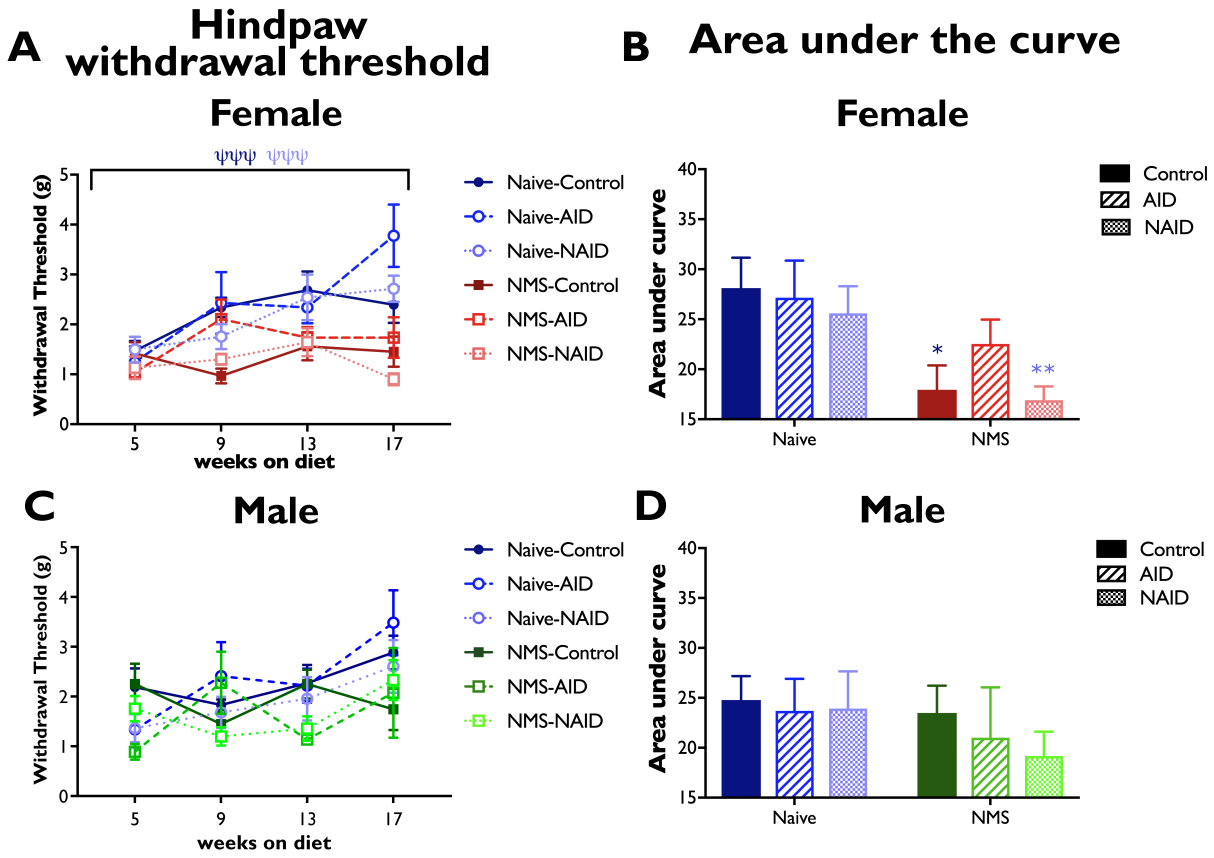
A
Hindpaw withdrawal threshold
Female
ψψψ ψψψ
Withdrawal Threshold (g)
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID
B
Area under the curve
Female
Area under curve
Naive
NMS
Control
AID
NAID
C
Male
D
Male

Figure 4.5: At 18-19 weeks on the AID or NAID diets, a glucose tolerance test (GTT) was performed in female and male mice. After a 6- hour fast, a baseline blood draw was taken, followed by an IP injection of 1g/kg body weight glucose and blood draws were taken 15, 30, 60, and 120- minutes after injection. Serum was collected and glucose level was determined. **A)** In female mice there was a significant effect of time, diet, a time/diet interaction, and a time/NMS/diet interaction. Female NMS-AID mice had a significantly greater glucose level compared to female NMS-control mice at 15, 30, and 60- minutes after glucose injection. Female NMS-AID mice had a significantly greater serum glucose level compared to female NMS-NAID mice and female naïve-AID mice had a significantly greater serum glucose level compared to female naïve-control mice 30- minutes after glucose injection. **B)** There was a significant effect of diet on female GTT AUC. Female naïve- and NMS-AID mice had a significantly greater GTT AUC compared to female naïve-and NMS-control mice, respectively. Female NMS-NAID mice had a significantly greater GTT AUC compared to female NMS-control mice. **C)** In male mice there was a significant effect of time and diet on GTT. At 30- and 60- minutes after glucose injection, male naïve-NAID mice had a significantly higher serum glucose level compared to male naïve-control mice. **D)** There was a significant effect of diet on male GTT AUC. Male naïve-AID and -NAID mice had a significantly greater GTT AUC compared to male naïve-control mice. In an overall analysis of female and male GTT there was a significant effect of time ($p<0.0001$), diet ($p<0.0001$), sex ($p<0.0001$), a sex/time interaction ($p<0.0001$), and a diet/time interaction ($p<0.0001$), 4-way RM ANOVA. In an overall analysis of female and male GTT AUC there was a significant effect of sex ($p<0.0001$), and diet ($p<0.0001$), 3-way ANOVA. Bracket indicates a

significant effect of time (ττττ p<0.0001), diet (δδ, δδδδ p<0.01, 0.0001), a time/diet interaction (Δ p<0.01), or a time/NMS/diet interaction (Ω p<0.05); A, C: 3-way RM ANOVA, B, D: 2-way ANOVA; &, &&, &&&& p<0.05, 0.01, 0.0001 vs. control diet, # p<0.05 vs. NAID, Bonferroni posttest.

(p<0.0001), diet (p<0.0001), sex (p<0.0001), a sex/time interaction (p<0.0001), and a diet/time interaction (p<0.0001). In an overall analysis of female and male GTT AUC there was a significant effect of sex (p<0.0001), and diet (p<0.0001).

AID prevents the development of NMS- or diet-induced perigenital sensitivity in female and male mice

Perigenital mechanical sensitivity was measured every 4- weeks beginning 4-weeks after the start of the AID or NAID diets using von Frey monofilaments and the up-down method. AUC was analyzed to determine differences in withdrawal thresholds over the course of the experiment (Figure 4.6). In females, naïve-AID mice had a significantly greater perigenital withdrawal threshold AUC compared to naïve-NAID mice (p<0.001) and NMS-AID mice had significantly greater withdrawal threshold AUC compared to NMS-control (p<0.05) and NMS-NAID (p<0.0001) mice (Figure 4.6B). In male mice on the NAID diet there was an overall effect of stress (p<0.01; Figure 4.6C), where naïve-NAID mice had significantly greater withdrawal AUC over time compared to NMS-NAID mice (p<0.01; Figure 4.6D). Additionally, male naïve-AID mice had a significantly greater perigenital withdrawal threshold AUC compared to male naïve-control (p<0.01) and naïve-NAID mice (p<0.0001) and male NMS-AID mice had significantly greater perigenital withdrawal threshold AUC compared to NMS-control (p<0.05) and NMS-NAID (p<0.0001) mice. Finally, male NMS-control mice had a significantly greater perigenital withdrawal threshold over time compared to male NMS-NAID mice (p<0.01).

Figure 4.6

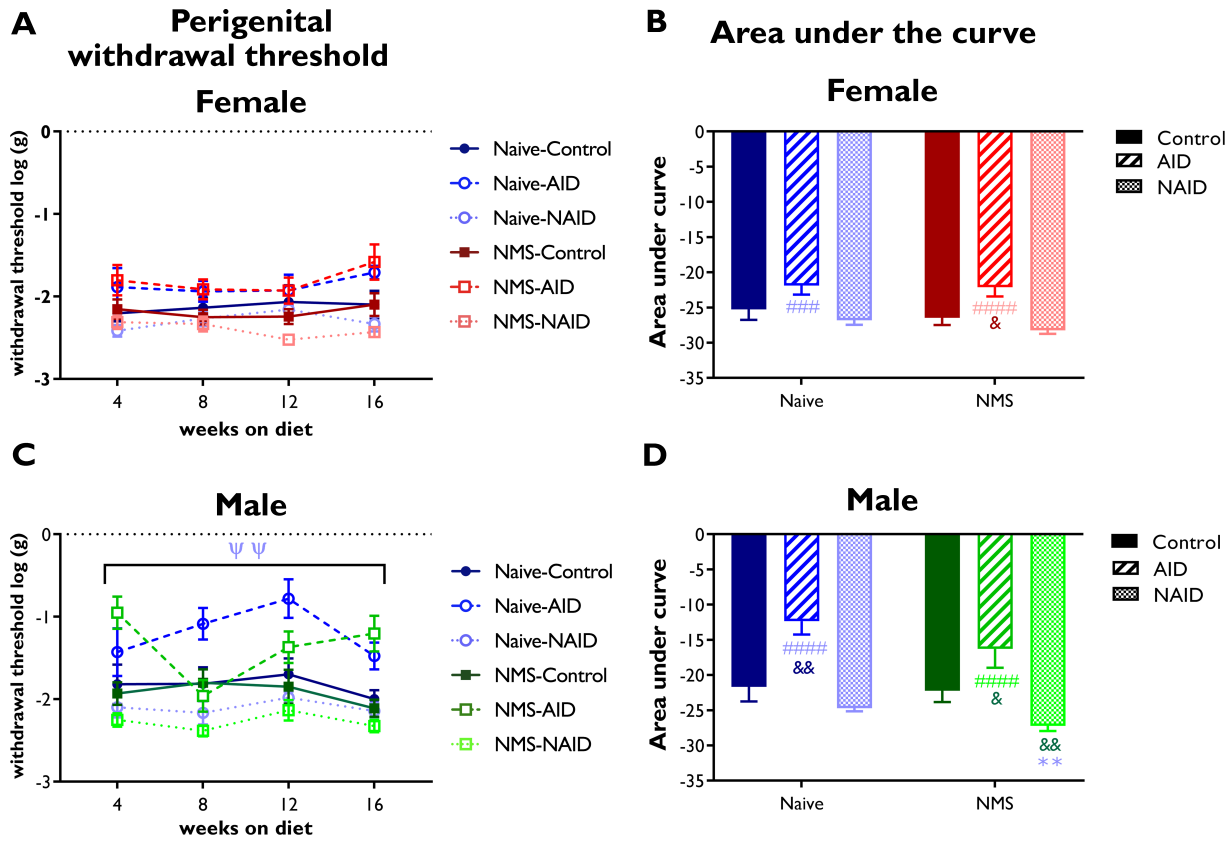
A
Perigenital withdrawal threshold
Female
withdrawal threshold log (g)
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID
B
Area under the curve
Female
Area under curve
Naive
NMS
Control
AID
NAID
C
Male
withdrawal threshold log (g)
weeks on diet
D
Male
Area under curve
Naive
NMS
Control
AID
NAID

Figure 4.6 Perigenital mechanical withdrawal threshold was measured using von Frey monofilaments and the up-down method every 4- weeks beginning 4- weeks after the start of the AID or NAID. AUC was analyzed to determine differences in withdrawal thresholds over the course of the experiment. In females, naïve-AID mice had a significantly greater perigenital withdrawal threshold AUC compared to female naïve-NAID mice (**B**). Additionally, female NMS-AID mice had a significantly greater withdrawal threshold AUC compared to female NMS-control and NMS-NAID mice. In male mice on the NAID diet there was an overall significant effect of stress on perigenital mechanical withdrawal threshold over time (**C**). Male naïve-NAID mice had a significantly greater perigenital withdrawal threshold AUC compared to male NMS-NAID mice (**D**). Additionally, male naïve-AID mice had a significantly greater perigenital withdrawal threshold AUC compared to male naïve-control and naïve-NAID mice and male NMS-AID mice had significantly greater perigenital withdrawal threshold AUC compared to male NMS-control and NMS-NAID mice. Finally, male NMS-control mice had a significantly greater perigenital withdrawal threshold AUC compared to male NMS-NAID mice. Bracket indicates a significant effect of NMS for male NAID mice (ψψ $p<0.01$), ###, #### $p<0.001$, 0.0001 vs. NAID &, && $p<0.05$, 0.01 vs. control diet, ** $p<0.01$ vs. naive. Generalized estimating equation framework and Markov structure.

AID prevents the development of NMS- induced hind paw sensitivity in female mice, while male mice do not differ in their hind paw sensitivity.

Hind paw mechanical sensitivity was measured every 4- weeks starting after 5-weeks on the AID and NAID diets using an electronic von Frey instrument. AUC was analyzed to determine differences in withdrawal thresholds over the course of the experiment. In female mice, there was an overall effect of stress on hind paw mechanical withdrawal threshold in mice on the control (p<0.001) and NAID diets (p<0.0001; Figure 4.7A). Female naïve-control mice had a significantly greater hind paw withdrawal threshold AUC compared to female NMS-control mice (p<0.05) and female naïve-NAID mice had a significantly greater hind paw withdrawal threshold AUC compared to female NMS-NAID mice (p<0.01; Figure 4.7B). There were no significant differences in female-AID mice or in male mice (Figure 4.7 C&D).

Nestlet building behavior is not influenced by sex, NMS, or diet.

After 16-17- weeks on the AID or NAID diets, nestlet building behavior was assessed. There were no significant effects of NMS or diet in female or male mice in nestlet building behavior. There were also no significant differences between groups and no significant effect of sex (Figure 4.8).

Significant sex/diet interactions alter serum corticosterone level.

After 18-19- weeks on the AID or NAID diets mice were sacrificed between 0800-1100 hours and trunk blood was collected. In female mice, there was a significant effect of diet on serum corticosterone level (p<0.05; Figure 4.9A). Female NMS-AID and -

Figure 4.7

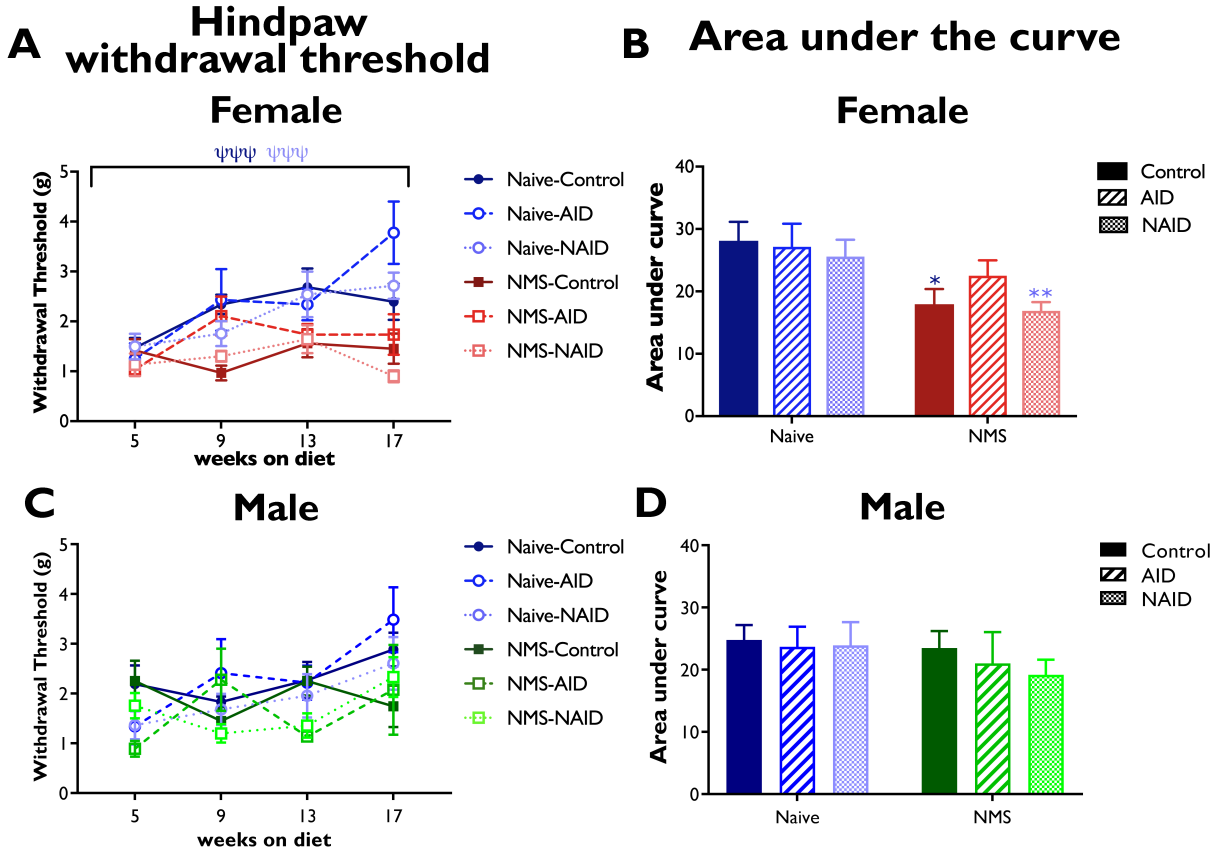
A
Hindpaw withdrawal threshold
Female
ψψψ ψψψ
Withdrawal Threshold (g)
weeks on diet
Naive-Control
Naive-AID
Naive-NAID
NMS-Control
NMS-AID
NMS-NAID
B
Area under the curve
Female
Area under curve
Naive
NMS
Control
AID
NAID
C
Male
D
Male

Figure 4.7: Hind paw mechanical withdrawal threshold was measured every 4-weeks using an electronic von Frey instrument beginning after 5-weeks on the AID or NAID. The AUC was analyzed to determine differences in withdrawal thresholds over the course of the experiment. In female mice, there was an overall effect of stress in mice on the control and NAID diets (**A**). Female naïve-control mice had a significantly greater hind paw withdrawal threshold AUC compared to female NMS-control mice and female naïve-NAID mice had significantly greater hind paw withdrawal threshold AUC compared to female NMS-NAID mice (**B**). There were no significant differences in hind paw mechanical withdrawal threshold in male mice (**C** & **D**). Bracket indicates a significant effect of NMS for female mice on the control diet and NAID (ψψψ $p<0.001$); *, ** $p<0.05$, 0.01vs. naïve. generalized estimating equation framework and Markov structure.

Figure 4.8

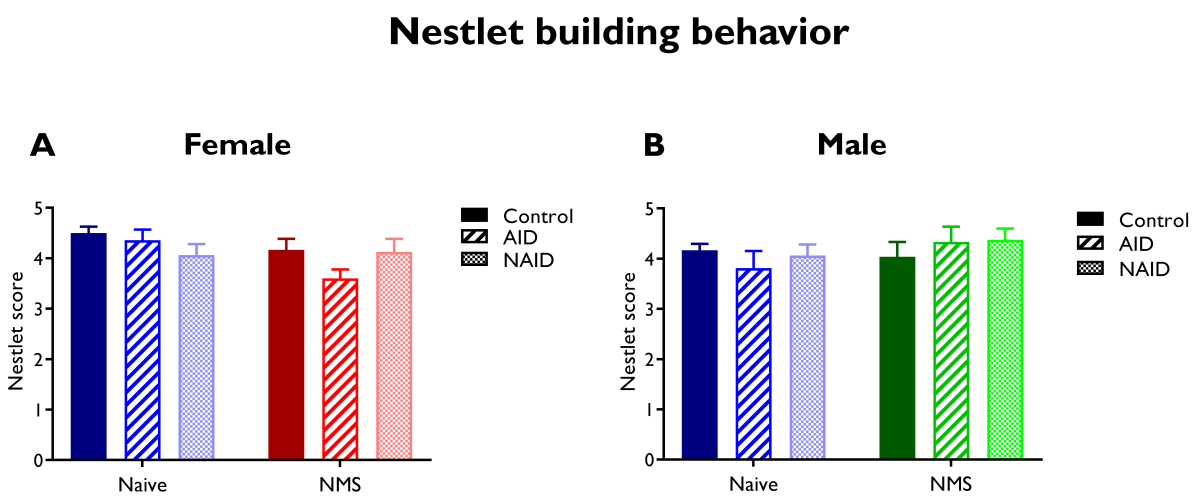
Nestlet building behavior
A
Female
B
Male
Nestlet score
0
1
2
3
4
5
Naive
NMS
Control
AID
NAID

Figure 4.8 After 16-17- weeks on the AID or NAID diets, nestlet building behavior was assessed in female and male mice. There were no significant effects of stress or diet on nestlet building behavior in female (**A**) or male (**B**) mice; 2-way ANOVA and Bonferroni posttest. There was also no significant effect of sex on nestlet building behavior 3-way ANOVA.

Figure 4.9

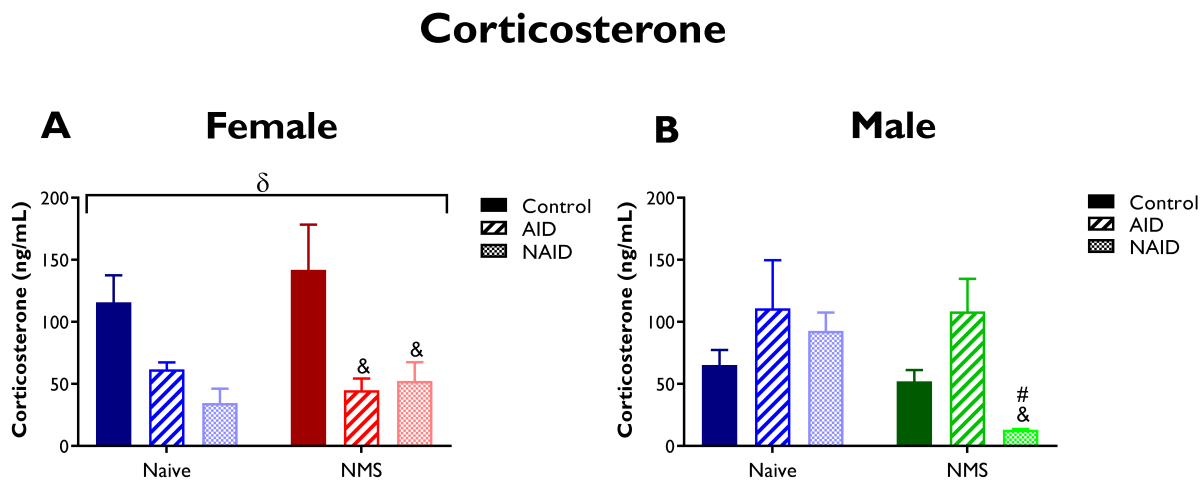
Corticosterone
A
Female
B
Male
Corticosterone (ng/mL)
200
150
100
50
0
δ
&
&
#
&
Naive
NMS
Control
AID
NAID

Figure 4.9 After 18-19- weeks on the AID or NAID diets, female and male mice were sacrificed and trunk blood was collected. Serum corticosterone level was then determined. **A)** In female mice, there was a significant overall effect of diet on serum corticosterone level. Female NMS-AID and -NAID mice had significantly lower serum corticosterone compared to female NMS-control mice. **B)** In males, NMS-NAID mice had significantly lower serum corticosterone compared to NMS-control and -AID mice. Bracket indicates a significant effect of diet (δ $p<0.05$); 2-way ANOVA. & $p<0.05$ vs. control diet, # $p<0.05$ vs. AID; Bonferroni posttest.

NAID mice had significantly lower serum corticosterone compared to female naïve-control ($p<0.05$) mice. In males, NMS-NAID mice had significantly lower serum corticosterone level compared to NMS-control and -AID mice ($p<0.05$; Figure 4.9B). In an overall analysis of female and male serum corticosterone level there was a significant overall sex/diet interaction ($p<0.01$).

4.5 Discussion

Chronic pain (442) and obesity-related metabolic syndrome (385) are two conditions that are highly prevalent in the United States. These disorders are often experienced co-morbidly and with mood disorders such as depression and anxiety (443-449). A common underlying factor in many patients with these disorders is a dysfunctional HPA axis (3-7), which regulates the stress response, influences the perception in pain, and has downstream metabolic effects (15-17). Incorrect programming of the HPA axis can occur when an individual experiences stressful events early in life because this axis is not fully developed at birth (13). Early life stress has been associated with the development of chronic pain, mood, and obesity-related metabolic syndrome in adulthood (3-7). Our laboratory has previously demonstrated that NMS, a mouse model of early life stress, elicits the development of symptoms similar to those experienced by chronic urogenital pain and migraine patients (96-98, 125) (Chapter 2). We have also demonstrated that older NMS mice weigh more and have increased body fat percentage compared to naïve mice on a standard chow diet and that NMS mice are more susceptible to some factors related to high-fat/high-sucrose diet-induced obesity-related metabolic syndrome (Chapter 3). Furthermore, we have shown that exercise is able to prevent or attenuate the development of these

chronic pain and metabolic symptoms (100, 229) (Chapters 2 & 3). However, because many individuals with chronic pain and/or obesity-related metabolic syndrome are not able or are unwilling to complete exercise recommendations, this study investigates if a dietary intervention might prevent the development of NMS-induced symptoms.

Many chronic pain, mood, and metabolic disorders are associated with a chronic state of low-grade inflammation (255, 256, 268) and an AID or diet supplemented with anti-inflammatory substances are beneficial in attenuating or preventing the development of obesity-related metabolic syndrome and pain in humans (181, 459, 477, 479, 481, 482) and rodents (Table 4.2). These diets have also been shown to interact with the HPA axis and prevent the development of depression-like behavior in rodents (Table 4.2). Therefore, I investigated if AID would influence early life stress-induced co-morbid widespread pain, obesity related-metabolic syndrome, and depression-like behavior. Additionally, because of the sex differences that exist in the incidence of chronic pain, metabolic syndrome, and responses to stress (297, 298, 386, 387, 491-494), both female and male mice were used in this study. I hypothesized that mice consuming AID would be protected from developing NMS-induced widespread pain, obesity-related metabolic syndrome, and depression-like behavior compared to mice on a non-anti-inflammatory diet (NAID) or control diet, which was similar to a standard rodent chow diet. My results demonstrate sex differences in stress, metabolic, and pain measurements. Additionally, I found complex diet, sex, and stress interactions.

Weight was measured every week and body fat percentage was measured every 4- weeks. Male mice weighed more and had greater body fat percentage compared to female mice. When each sex was analyzed separately, the groups that were the most

susceptible to weight and body fat gain were female and male NMS-AID groups (Figures 4.1 and 4.2). This was surprising because the AID and NAID both had 35% kcal fat content compared to the control diet's 10% kcal fat control (Table 4.1). Therefore, although weight gain was expected in mice consuming either AID or NAID, it was expected to happen at the same rate. The finding that NMS-AID females were susceptible to diet-induced weight and body fat gain was particularly intriguing because female mice are thought to be resistant to diet-induced development of obesity-related metabolic syndrome (303, 414), which was seen in female NMS-NAID mice. Male NMS-NAID mice were also delayed in their weight gain compared to NMS-AID. Thus, something about the added anti-inflammatory components enhanced weight gain in NMS mice specifically. Finally, male naïve-AID mice were less susceptible to weight gain compared to male naïve-NAID mice as hypothesized.

Food intake and feed efficiency were also analyzed in this study to see if an increase in either of these measures could explain the NMS-AID group's increased susceptibility to gains in weight and body fat percentage. Feed efficiency is a measure of an animal's ability to turn energy consumed into weight and a greater feed efficiency is indicative of a greater ability to put on weight from calories consumed. Similar to weight and body fat percentage, males generally had greater feed efficiency and ate more at the beginning of the study compared to females (Figure 4.3*).* Female food intake did not significantly differ between groups (Figure 4.3A*)* but there was a significant effect of diet on feed efficiency. Female mice consuming AID had greater feed efficiency compared to the other groups and NMS-AID feed efficiency was significantly greater than NMS-NAID (Figure 4.3B*).* Increased feed efficiency in the

female AID mice partially explains why female NMS-AID gained significantly more weight and body fat percentage compared to female NMS-NAID. However, this does not explain why female NMS-AID was more susceptible to died-induced weight and body fat gain compared to female naïve-AID mice. In males, there was a significant overall effect of diet on food intake (Figure 4.3C*)* and feed efficiency, where both the AID and NAID diets had greater feed efficiency compared to mice on the control diet (Figure 4.3D*).* Male NMS- and naïve-AID both ate more at the beginning of the study compared to other groups until 7-weeks on the diets. However, the amount that they ate was similar throughout the study and NMS-AID mice weighed significantly more than NMS-Control after only 5-weeks on the diet but naïve-AID did not start weighing significantly more than naïve-Control until 14-weeks on the diets. Similarly, NMS-AID had a significantly higher body fat percentage compared to NMS-Control at the 8-week measurement but naïve-AID did not reach significance until the 16-week measurement. Therefore, an increase in food intake cannot completely account for NMS-AID mice being more susceptible to weight and body fat percentage gain compared to naïve-AID mice, but it does explain why they were more susceptible compared to NMS-NAID mice. Additionally, although it did not reach statistical significance, male NMS-AID had the greatest feed efficiency out of all the groups (Figure 4.3D), which could partially explain the findings that they were the most susceptible to weight and body fat gain.

After 18-19- weeks on the diets fasting insulin level was measured and glucose tolerance test area under the curve (GTT AUC) was analyzed to assess other diagnostic criteria of obesity-related metabolic syndrome. A higher fasting insulin level suggests that an animal is more insulin resistant and a greater GTT AUC is indicative of

lower glucose tolerance. Male mice had higher fasting insulin level and greater GTT AUC compared to female mice (Figures 4.4 & 4.5*)*. There were no significant differences between the fasting insulin levels of female groups (Figure 4.4A*)*. However, in males, naïve-AID and-NAID had significantly greater fasting insulin level compared to male naïve-Control mice. Interestingly, NMS-AID and -NAID mice did not have significantly different fasting insulin levels compared to male NMS-Control mice (Figure 4.4B). There was a significant overall effect of diet on GTT AUC in both female and male mice (Figure 4.5B & D*)*. In females, naïve- and NMS-AID and NMS-NAID had a significantly greater GTT AUC compared to their control diet counter parts (Figure 4.5B*)*. In males, similar to their fasting insulin levels, naïve-AID and -NAID had significantly greater GTT AUC compared to naïve-Control and but NMS-AID and -NAID did not significantly differ from NMS-Control (Figure 4.5D). Insulin resistance and glucose intolerance are often seen in patients with obesity-related metabolic syndrome (167). Therefore, the finding that female naïve- and NMS-AID mice had greater GTT AUC is in line with them having higher body fat percentages compared to control mice at the time of these measures. Likewise, male naïve-AID and -NAID mice displayed these same trends. However, although male NMS-AID mice weighed more and had increased body fat percentage compared to NMS-Control mice, they did not display significantly higher fasting insulin level or greater GTT AUC compared to NMS-control mice. This suggests that although AID had a detrimental effect on male NMS body composition, it was protective towards other factors associated with obesity-related metabolic syndrome. These data are interesting because obesity usually leads to the development of other symptoms associated with metabolic syndrome (165), including

insulin resistance and glucose intolerance. Additional symptoms of metabolic syndrome include hypertension, high triglycerides, and low HDL-cholesterol (167). Future studies in our laboratory will investigate if AID influenced circulating triglyceride and cholesterol levels differently in our groups of mice. It could be that although NMS-AID mice were susceptible to changes in body composition, their other metabolic functions were not altered. This would be in line with clinical trials demonstrating that individuals with obesity-related metabolic syndrome treated with curcumin have decreased circulating triglyceride and inflammatory cytokine levels but no changes in body weight or body fat (481, 482).

To investigate potential NMS-induced co-morbid obesity-related metabolic syndrome and chronic pain as well as the effect of AID on the development of these disorders, perigenital and hind paw mechanical sensitivity was measured every 4-weeks throughout the course of the experiment. These two measures were taken because our laboratory has previously demonstrated that NMS causes perigenital sensitivity (96-98) and hind paw sensitivity represents widespread hypersensitivity, similar to what is seen in fibromyalgia patients (452). When the AUC of these pain-like responses over time was analyzed, in female mice, AID significantly improved perigenital withdrawal thresholds (Figure 4.6A) and prevented NMS-induced development of hind paw hypersensitivity (Figure 4.7A). In male mice, AID prevented the development of diet and stress induced-perigenital sensitivity (Figure 4.6B) but there were no significant differences in hind paw sensitivity between any of the groups (Figure 4.7B).

These data suggest that female mice are more susceptible to widespread sensitivity compared to male mice. Male AID and control mice had higher perigenital withdrawal thresholds compared to female AID and control mice and female NAID and control mice displayed stress-induced hind paw hypersensitivity while male mice did not. It is generally accepted that sex differences exist in the incidence of chronic pain disorders as well as in laboratory-evoked pain responses. Females are over represented in most pain conditions that present in both sexes including fibromyalgia, migraine, temporomandibular disorder, irritable bowel syndrome, and interstitial cystitis/painful bladder syndrome, while males suffer more frequently from cluster headache and post-traumatic stress headache (497). Additionally, females exhibit increased sensitivity to evoked painful stimuli compared to males (491, 492). The mechanisms behind sex differences in pain are attributed to a complex interaction between biological and psychosocial mechanisms. Although psychosocial mechanisms cannot be directly measured in rodents, biological mechanisms can. Pre-clinical work has revealed that pain processing is a complex circuitry that involves the peripheral and central nervous systems and both inhibitory and facilitatory mechanisms. Sex hormones are known to influence this pathway at multiple levels through complicated interactions (498). Further support for sex hormones playing a role in chronic pain is that certain disorders, including migraine and temporomandibular disorder, peak in prevalence during the reproductive years (499). While I have not investigated differences in sex hormone levels in these mice or what mechanism might be behind the sex differences seen in the pain-like behaviors, it is clear that these results are in line with other studies that demonstrate sex differences in results from pre-clinical and clinical pain research.

Patients with chronic pain and obesity-related metabolic syndrome are often diagnosed with depression (448, 449) and the anti-inflammatory components in the AID used in this study all interact with the HPA axis and prevent the development of stress-induced depressive like symptoms in rodents (Table 4.2). Therefore, a nestlet building assay (496) was used as a natural, non-evoked method to assess depression-like behavior in this study. Wild and laboratory female and male mice build nests when they have access to appropriate nest building material (500) and poor nest building is thought to be a sign of poor wellbeing (501) or depression-like behavior. Our lab has found that mice subjected to chronic foot-shock stress have significantly lower nestlet scores compared to those that do not experience food-shock (502). Therefore, I hypothesized that NMS mice would also show lower nestlet building scores, suggesting greater depression-like behavior compared to naïve mice. Additionally, I hypothesized that AID mice would not exhibit stress-induced depression-like behavior. However, there were not significant differences in nestlet building behavior in female or male mice overall or between the different groups (Figure 4.8). The lack of differences in this measure of depression-like behavior could be due to the method that we used. Other studies that have evaluated stress induced depression-like behavior and the role of anti-inflammatory components have used various methods such as the forced swim test (465, 487) or the sucrose preference test as a measure of anhedonia (465, 468). Therefore, different results might have been observed if a different method to measure depression-like behavior had been used.

The last measure in this study was serum corticosterone level to investigate if there were differences in HPA axis output in these mice. Corticosterone in rodents and

cortisol in humans are GCs released by the adrenal glands in a circadian rhythm as well as upon activation of the HPA axis (15-17). In naïve laboratory rodents, corticosterone peaks in the early evening just before the start of the dark cycle and is lowest at the beginning of the light cycle (383). However, the normal circadian rhythm of corticosterone can be altered by stress (383) and early life stress in particular disrupts the appropriate programming of the HPA axis (13), which could result in changes in GC output. Furthermore, sex differences are seen in adult stress responses in rodents that have experienced early life stress (493, 494, 503). In line with this, female NMS-Control mice had increased serum corticosterone compared to male NMS-Control mice. Additionally, an overall analysis revealed a significant sex/diet interaction on serum corticosterone level. In female mice, there was an overall significant effect of diet, where AID and NAID mice had a lower corticosterone level compared to control mice, which reached significance in NMS mice (Figure 4.9A). In male mice, naïve-AID and -NAID had greater corticosterone level compared to naïve-control and NMS-AID had higher corticosterone level compared to NMS-control, but NMS-NAID had a significantly lower corticosterone level compared to NMS-control and NMS-AID (Figure 4.9B*)*.

Little is published about long-term dietary effects on the HPA axis. However, it is known that high-fat diet fed rodents have decreased adipose tissue 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (432, 433)(Chapter 3), which converts inactive cortisol and corticosterone into their active forms (430). This could subsequently decrease the amount of circulating corticosterone. While AID and NAID had only a moderately high percent of dietary fat (35%), this could explain why circulating corticosterone was decreased in female AID and NAID mice. Conversely, it does not

explain why male AID and NAID mice, with the exception of NMS-NAID, had higher circulating corticosterone levels. Additionally, the anti-inflammatory ingredients in AID have all been shown to interact with the HPA axis and either prevent a stress-induced increase in serum corticosterone (465, 471, 474) or decrease in hippocampal BDNF (462, 468, 471, 474), which is a limbic structure that usually dampens activation of the HPA axis (16, 25). However, in female NMS mice there were not significant differences in AID and NAID corticosterone level. It could be that because my stress paradigm was early in life and the HPA axis was already altered by the time AID was administered, such that it was too late to prevent the stress-induced effects on corticosterone. This stress paradigm is different than other studies described that use adult stress paradigms (Table 4.2), in which animals should have an appropriately-functioning HPA axis. In male NMS mice, AID did prevent the development of the hypocortisolism displayed by NMS-NAID males. Studies in humans have shown both hypercortisolism (104, 105) and hypocortisolism (9, 106) in adults that report a history of childhood abuse or stress. Hypocortisolism is postulated to come about as a compensatory response to a preceding period of hypercortisolism and excessive glucocorticoid release (102). Excess or deficient glucocorticoids are both detrimental to appropriate functioning of pain, metabolic, and immune systems as discussed in Chapter 1. This could explain why male NMS-NAID had lower perigenital withdrawal threshold compared to naïve-NAID and NMS-control and -AID.

Taken together, these data suggest that an AID has differential therapeutic benefits for male vs. female and naïve vs. NMS mice. Interestingly, no group benefited in every measure. Male naïve-AID mice were protected from significant weight and

body-fat percentage gain as well as the development of perigenital sensitivity over time compared to naïve-NAID mice but they were susceptible to developing insulin resistance and glucose intolerance compared to naïve-control mice. Male NMS-AID mice were susceptible to increased weight and body fat percentage compared to NMS-NAID mice, but they were protected from developing perigenital sensitivity over time compared to NMS-NAID mice as well as insulin resistance and glucose intolerance compared to NMS-control mice. Female naïve-AID mice were protected from weight and body-fat percentage gain as well as the development of perigenital sensitivity compared to naïve-NAID mice, but they were susceptible to glucose intolerance compared to naïve-control and –NAID mice. Finally, female NMS-AID mice were susceptible to weight and body fat percentage gain compared to NMS-NAID mice and were glucose intolerant compared to NMS-control and -NAID, but were protected from stress-induced hind paw sensitivity and diet-induced perigenital sensitivity.

The differences in these measurements demonstrate complex sex, diet, and stress interactions that influence pain, metabolic, and stress pathways. It was surprising that the AID and NAID had different metabolic effects depending on sex and stress condition. Both diets had the same proportions and sources of protein, carbohydrates, and fat. The only difference between the two diets was that the NAID did not have the anti-inflammatory additives (Table 4.1). Other groups have demonstrated the metabolic benefits of adding anti-inflammatory components to a high-fat diet. However, these studies only used naïve male mice and had quantities of anti-inflammatory additives that were 2-1000 times the amount used in this study (Table 4.2). The present study was unique in that it used different stress conditions, both female and male mice, and the

amount of anti-inflammatory components were based on quantities that are more realistically attainable in the human diet (504). Additionally, other studies use diets with beef tallow and lard as their fat source, which are high in omega-6 FAs, while the fat source for our laboratory's AID and NAID is soybean and flaxseed oils, which are high in omega-3 FAs. Although omega-3 FAs are beneficial in humans in regards to weight loss and metabolic function (179, 180, 486), in mice the addition of omega-3 FAs to a high-fat diet actually caused an increase in body weight but decreased macrophage infiltration and plasma triglyceride concentration compared to mice fed a high-fat diet without added omega-3 FAs (284). I did not measure macrophage infiltration in the adipose tissue of my mice to determine their inflammatory status. However, it would be interesting to see if this varied in the different sex/stress/diet groups and if so, if these variations could help explain my results.

Studying a diet that has metabolic benefits in humans might not translate completely to rodents because humans and rodents have different digestive systems and nutrient needs. Although the general anatomy of mouse and human digestive systems are similar, there are some differences that allow these two species to extract different nutrients from the diet. For example, humans have a greater small to large intestine length and surface area ratio compared to mice and mice have a large cecum that allows them to ferment plant materials (505). Mice and humans also differ in the bacterial species found in their gut microbiome, which is made up of microbial organisms with properties that contribute to an animal's metabolic capacity and help to breakdown dietary components that animals otherwise would not be able to (506). One study found that 85% of the gut bacteria found in mice are not found in humans (507).

These differences in digestive capability could be why humans get a metabolic benefit from an anti-inflammatory diet with smaller quantities of anti-inflammatory components compared to studies in rodents that generally use much higher quantities of anti-inflammatory components to see positive metabolic results.

The stress-induced differences in this study could also have been influenced by differences in the microbiomes of stressed and non-stressed mice. The gut microbiota communicates with the brain in a bidirectional manner, referred to as the brain-gut axis, and plays a role in neural, endocrine, immune, and metabolic pathways (508, 509). Sudo et al., (510) demonstrated that the gut microbiome is important in the appropriate development of the HPA axis and stress response in adulthood. They showed that germ-free mice had higher ACTH and corticosterone levels after restraint-stress in adulthood compared to specific-pathogen free mice. Additionally, they found that a hyper-responsive HPA axis in germ free mice could be attenuated by gut transplantation with specific pathogen free mice early in development but not at a later stage. In a different study, Mahoney et al., (511) showed that NMS rats had visceral hypersensitivity, increased corticosterone level, increased immune response after challenge with lipopolysaccharide, and significantly different gut microbiota compared to their non-separated counterparts. The gut microbiome is important to digest food appropriately and if this system is altered, such as by early life stress, animals are not able to appropriately process the foods that they eat. Sex differences also exist in the composition of the gut microbiota. In humans, puberty has been shown to initiate the start of sexually dimorphic changes in the gut microbiome that continue to change until adulthood (512). In rodents, testosterone appears to be particularly important in the

development of gut microbial sex differences. This was demonstrated in a study that used castration to reduce testosterone levels, which prevented the development of sex differences in gut microbiota of adult mice (512). Finally, obesity in humans and rodents has been shown to be associated with an abundance of Firmicutes and deficiency in Bacteroidetes species (507, 513). Authors from these studies suggest that guts that are colonized with this ratio of bacterial species are better equipped to take energy from the diet and turn it into fat. Turnbaugh et al., (514) showed that when germ free mice were colonized with microbiota from obese mice they had significantly greater body fat compared to germ free mice colonized with microbiota from lean mice. It is plausible that NMS and/or sex hormones in the mice in the present study altered the gut microbiota in a way that made it possible for different groups to use different nutrients in the AID and NAID. It would be interesting to analyze the gut microbiota in these mice to see if differences emerge that could explain these results.

Despite differences in the metabolic benefits of AID, one consistent finding in this study was that AID prevented the development of widespread diet or stress-induced hypersensitivity in male and female mice. This is an exciting finding because many pharmacological agents have been studied for treating widespread pain, which is seen in fibromyalgia patients, but none have proved to be universally successful. Shapiro et al., (515) demonstrated that a behavioral weight loss program, which consisted of education about diet and physical activity, led to weight loss and an improvement in fibromyalgia symptoms. The results from the present study suggest that an anti-inflammatory diet can prevent the development of pain independent of weight or body fat percentage, at least in rodents. Totsch et al., (2018) created the AID that was used

in this study and found similar results. In their study, they compared the influence of AID to a high-fat/high-sucrose “standard American diet (SAD)” and a control diet. Their major findings were that male AID mice weighed less and had less fat mass compared to male SAD mice but weighed more and had greater fat mass compared to male control mice. But, this effect of AID increasing weight and body fat was not seen in females. Additionally, both male and female AID mice were more glucose intolerant compared to control mice. Finally, AID shortened recovery time after an injection in the hind paw of the inflammatory substance Complete Freund’s Adjuvant in both male and female mice compared to mice on the SAD but not compared to mice on the control diet, as measured by mechanical withdrawal threshold (282). Although there are differences in the experimental design of the present study compared to Totsch et al., (2018), both studies found that although AID caused metabolic differences, it was beneficial for the treatment of pain.

In conclusion, I found complex sex, diet, and stress interactions that influence pain, metabolic, and stress pathways in mice. Although I attempted to study a diet that would confer metabolic benefits to humans (AID), it appears that these metabolic benefits may not translate appropriately in mice. This could be due to species differences in metabolic and dietary needs, or because of different digestion capabilities. However, a promising finding in this study was that AID did prevent the development of stress and/or diet-induced widespread pain in mice. This suggests that an AID could be a good treatment option for humans with early life stress-induced chronic pain disorders. Future work in our laboratory will evaluate other symptoms of metabolic syndrome in these mice including circulating triglyceride and cholesterol

levels. We will also determine the inflammatory status of these mice by assessing circulating cytokines and adipose tissue macrophage infiltration. I hypothesize that these data will help explain some of the differences I saw in my groups of mice.

Chapter V: Discussion

Although acute stress is crucial for survival, stress can become detrimental when experienced in the long-term, especially early in life such as when children are subjected to maltreatment, neglect, physical or emotional abuse, or witness parental discord. Early life stress is associated with the development of co-morbid chronic pain, such as migraine, fibromyalgia, and chronic pelvic pain, mood, and obesity-related metabolic syndrome in adulthood (3-7). This has partially been attributed to improper function of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response, plays a role in pain perception, and has downstream metabolic effects (9, 10, 17). Our laboratory has previously used a mouse model of early life stress known as neonatal maternal separation (NMS), to study chronic urogenital pain disorders in female and male mice (96-98). Additionally, we have used exercise in the form of voluntary wheel running as a therapeutic intervention (100, 229). Expanding our knowledge of the many consequences of early life stress, as well as investigating non-pharmacological interventions for early life stress-induced disorders, has been the purpose of the studies described in this dissertation. Specifically, I investigated if NMS in mice could also be used as a model for susceptibility to evoked migraine (chapter 2) and obesity-related metabolic syndrome (chapter 3) and if exercise (chapters 2 and 3) or an anti-inflammatory diet (chapter 4) could be used as therapeutic interventions for the treatment of these putative stress-induced disorders.

This research is significant because pre-clinical models investigating early life stress induced migraine and obesity-related metabolic syndrome are lacking. Millions of Americans currently suffer from migraine (304) and obesity-related metabolic syndrome (385) and although not all migraineurs or individuals with obesity-related metabolic

syndrome have a history of early life stress, many do and the lack of pre-clinical models replicating early life stress-induced disorders leaves gaps in our knowledge. Additionally, the rate of childhood maltreatment is rising (12), which suggests that the incidence of early life stress-induced disorders will rise as well. Establishing a pre-clinical model that naturally replicates human disorders is the first step in understanding the mechanisms underlying the association between early life stress and the subsequent development of co-morbid disorders. My overall goal is to use translational models so that I can have a positive impact on human health.

5.1 NMS in female mice alters susceptibility to evoked migraine

Migraine is a complex neurological disorder that is difficult to study in rodents. In clinical research, one can ask an individual if they are experiencing migraine symptoms, whereas in pre-clinical studies, the researcher has to evaluate animal behavior and infer if they are suffering from migraine-like symptoms. This task is made more difficult by the fact that rodents are prey animals and therefore do not readily show pain-like symptoms. However, because of the high clinical incidence of migraine in chronic urogenital pain patients (8) and because migraine is associated with early life stress (323-325), I was interested in studying if NMS mice were more susceptible to evoked migraine compared to naïve mice and if exercise could be use a therapeutic intervention for the treatment of migraine-like symptoms (Chapter 2).

It is generally accepted that the dura mater is an important tissue involved in migraine pathophysiology (309). It is populated with mast cells (MC) (300) and innervated by the ophthalmic division of the trigeminal nerve (313). When MCs become

activated, they release vasoactive, nociceptive, and inflammatory molecules (e.g. histamine, bradykinin, serotonin, CGRP, SP) (44) and they can expand their population during immune and inflammatory responses by increasing recruitment, survival, and maturation of MC precursors in the area (319). Additionally, MCs are increased in migraineurs (39) and histamine levels, a major component of MC granules released upon activation, are higher in the serum of migraineurs during migraine attacks (153). Evaluation of MC characteristics in the dura mater in female and male NMS- and naïve-Sedentary (Sed) and -Exercised (Ex) mice revealed that female NMS-Sed mice had significantly greater dural MC degranulation compared to female naïve-Sed and this effect was attenuated by exercise. Additionally, male NMS-Sed mice had significantly more dural MC compared to male naïve-Sed and this effect was also attenuated by exercise. These data suggested that because NMS mice had altered dural MC characteristics, they might be more susceptible to evoked migraine-like behaviors. The subsequent evoked-migraine studies were continued in female mice because migraine affects three times more females then males (305, 306).

A commonly used method to evoke migraine involves a surgically implanted cannula to administer noxious solutions onto the dura mater (329-331). While this method is successful in evoking migraine-like symptoms, it is time consuming and might alter some of the natural behaviors exhibited by rodents. In Chapter 2 of this dissertation, I used a novel non-surgical method (339) to administer an inflammatory soup (IS) to the dura mater and subsequently evaluated mouse grimace score (MGS) and widespread allodynia, or increased sensitivity to normally non-painful stimuli. MGS is a tool used to indicate if an animal is experiencing ‘pain’ (353) and widespread

allodynia is a symptom commonly experienced during a migraine attack in humans (307). Results indicated that compared to groups that had saline applied to their dura mater, groups that had IS applied had significantly lower forepaw mechanical withdrawal thresholds, suggesting they had wide spread hypersensitivity. This implied that the dural application method was working, however there were not differences between naïve- and NMS-Sed and -Ex groups. I also tried a 'two-hit' experiment where we applied saline or IS to the dura and then 3- days later applied IS to all mice. I chose to explore this method because it has been shown that one migraine-like event can lead to meningeal sensitization, or priming, to subsequent events (378). However, this study did not demonstrate consistent results.

Next, an intraperitoneal (IP) nitroglycerin (NTG) injection was used to attempt to evoke migraine-like behaviors, including MGS and hind paw mechanical withdrawal threshold. NTG injection is another method commonly used to evoke migraine-like symptoms in rodents (340, 345, 346) and also evokes migraines in human migraineurs (344). Compared to groups that received an IP saline injection, all of the groups that received an IP NTG injection had significantly increased MGS and hind paw sensitivity was decreased in NMS-Sed-NTG mice compared to the other NTG groups. Finally, photophobia, or sensitivity to light, was tested, which is one symptom that meets the diagnostic criteria of migraine according to the International Classification of Headache Disorders (381). Following IP NTG injection, NMS-Sed mice significantly decreased their time spent in the light compared to baseline while the other groups did not. This suggests that NMS-Sed mice were more photophobic following NTG injection compared to the other groups.

While these results demonstrate that female NMS-Sed mice might be more susceptible to NTG evoked migraine, there are still questions regarding the mechanism behind this. Female NMS-Sed have increased dural MC degranulation, therefore I believe that this plays a role in their susceptibility to NTG evoked migraine. However, I have not evaluated activation in other tissues that are important in migraine pathophysiology, including the trigeminal ganglia (TG) and trigeminal nucleus caudalis (TGN) (310, 311). Markovics et al., (345) measured c-Fos expressing cells in the TG and TGN following IP NTG injection and demonstrated significant neuronal activation in both tissues. Furthermore, Ramachandran et al., (516) demonstrated a significant increase in the expression of phosphorylated extracellular signal-related kinases (pERK) in the dura mater, TGN, and TG after NTG infusion. Based on my behavioral results, I hypothesize that NMS-Sed mice will display greater NTG induced activation in the TG and TGN compared to naïve and Ex mice. Future studies will test this hypothesis.

A surprising finding from this migraine work was that exercise consistently increased MGS in naïve and NMS mice following dural application of saline or IS as well as in mice that did not receive an application of any solutions. It is unclear why I saw this effect of exercise. MGS is a spontaneous pain-like behavior and simply evaluating MGS suggests that Ex mice are experiencing more pain than Sed mice. However, Ex mice did not display greater evoked pain behavior in the measurements of paw withdrawal threshold or increased photophobic behavior. It could be that Ex mice are more prone to sleeping, due to excess energy expenditure, throughout their dark cycle and their eyes appear more closed during the testing period. Only 2 of the facial

behaviors of the original 5 that make up the MGS were used, orbital tightening and ear position. The other 3 facial behaviors are check bulge, nose bulge, and whisker position (353). However, the MGS was created in white CD-1 mice and these behaviors proved to be very difficult to evaluate in a black mouse. It is possible that if all 5 of the facial behaviors were included in the assessment of MGS, there could have been different conclusions. However, an automated 'eye squint' assay has also been developed that highly correlates with MGS (380), suggesting that eye squint alone is predictive of what all 5 MGS behaviors will reveal. Another factor that could have influenced the MGS data is the isoflurane anesthesia that administered prior to MGS testing, even in those mice that did not have any solution applied to their dura. Miller et al., (517) measured MGS in DBA/2 mice before and 30- minutes after isoflurane anesthesia and found a significant increase in MGS following anesthesia. Although MGS was measured 1- hour after isoflurane administration in my studies, it is possible that Ex mice were not able to recover as quickly from the isoflurane and therefore exhibited a higher MGS. To my knowledge, this study is the first to use MGS in Ex mice and my findings bring into question the accuracy of the MGS if it is used in conditions other than in the naïve sedentary CD-1 mice that it was constructed from. In support of this, Miller and Leach (518) found sex and strain differences in baseline MGS measurements of C57BL/6, C3H/He, CD-1, and BALB/c mice. This differs from the original report of MGS, which did not find statistically significant sex differences in their mice (353).

Taken together, the data in this study indicate that both the method used to evoke migraine and the subsequent behaviors that are evaluated are critical when designing a migraine study in mice. These results demonstrate that female NMS-Sed

mice have greater dural MC degranulation and are more susceptible to NTG-evoked hind paw sensitivity and photophobia compared to NMS-Ex or naïve mice. Despite the strong association between early life stress and the development of migraine in adulthood (323-325), I could not find any other studies in the literature that evaluate early life stress as a factor in the development of evoked migraine-like behaviors in rodents. Further, although exercise improves migraine frequency and intensity, as well as stress level in human migraineurs (364, 365), it appears that these studies are the first to investigate exercise in rodents as a therapeutic intervention for evoked migraine-like behaviors. This work is important because many pharmacological treatments are not universally successful and have unwanted off-target side effects. Voluntary exercise provides a much safer intervention, if carried out appropriately. By establishing NMS as a model to study early life stress-induced susceptibility to evoked-migraine like behaviors, as well as exercise as a therapeutic intervention for these behaviors, future work can now focus on the mechanisms behind these findings and work to translate them to human migraineurs that experienced early life stress.

5.2 Lifestyle factors alter metabolic function in male mice

Early life stress in humans not only increases an individual's susceptibility to developing chronic pain disorders, it also increases the susceptibility to developing obesity (160-162), which is generally considered a pre-requisite for the development of obesity-related metabolic syndrome. This syndrome is diagnosed when an individual suffers from 3 of the following 5 criteria: central obesity, insulin resistance, hypertension, high triglycerides, and low HDL-cholesterol (167). Additionally, obesity-related metabolic syndrome and chronic pain are often experienced co-morbidly (447, 453).

Therefore, Chapter 3 of this dissertation expands our studies from early life stress-induced chronic pain to early life stress-induced obesity-related metabolic syndrome. I first used our laboratory's mouse model of early life stress, NMS, to study the body composition of naïve- and NMS-Sed and -Ex mice. I found that on a standard chow diet, adult female and male NMS-Sed mice had altered body composition compared to adult female and male naïve-Sed or -Ex mice (Tables 3.3 & 3.4*)*.

A sedentary lifestyle (400-402) and a diet with a high proportion of saturated fat and refined sugars (HFS), referred to as a "Western diet" (286, 287), are both significant environmental risk factors associated with the development of obesity and related chronic diseases. Unfortunately, HFS diet consumption has grown due to its convenience and lower cost compared to healthier foods such as fruits and vegetables (288). Sedentary behavior is also highly prevalent in the United States, where most individuals do not meet the recommended requirements for daily physical activity (403). With this information in mind, I was interested in studying if lifestyle factors, including diet and exercise, influence NMS and naïve mice differently in the development of obesity-related metabolic syndrome. Therefore, naïve-and NMS- Sed and -Ex male mice were exposed to a HFS or control diet. This resulted in 8 groups of male mice: naïve-Sed-Control, naïve-Sed-HFS, naïve-Ex-Control, naïve-Ex-HFS, NMS-Sed-Control, NMS-Sed-HFS, NMS-Ex-Control, and NMS-Ex-HFS. Male mice were used for this study because other groups have demonstrated that female mice are resistant to diet-induced obesity-related metabolic syndrome (303, 414).

I found that although all HFS groups developed characteristics similar to symptoms of metabolic syndrome including increased weight, body fat percentage, and

fat mass (Figure 3.2), as well as insulin resistance and glucose intolerance (Figure 3.5) compared to control diet groups, exercise was fairly protective in some of these measures, especially in naïve-Ex-HFS mice. These results suggest that naïve mice gain greater benefit from exercise than NMS mice. However, this could be due to the fact that naïve mice run significantly more than NMS mice before and after the introduction of a HFS diet (Figure 3.4). Early life stress in humans leads to altered dopaminergic signaling (440), which influences motivated behaviors (441). Voluntary wheel running in rodents is considered rewarding, as most rodents will choose to run when provided a running wheel (231). Therefore, future studies in our laboratory investigate if NMS alters reward pathways, such as the mesocorticolimbic dopamine system, that could potentially explain why NMS mice run significantly less than naïve mice.

White adipose tissue distribution and composition is important in predicting the development of obesity-related diseases because it plays a key role in regulating systemic metabolic function and inflammation (168). Visceral fat accumulation is associated with a greater risk of metabolic dysfunction (169) and leads to chronic low-grade inflammation (170). Therefore, macrophage, MC tryptase, and pro-inflammatory cytokine mRNA were measured in epididymal adipose tissue to assess the inflammatory status of visceral adipose in the mice. I found that the adipose tissue of HFS mice, especially that of NMS-Sed-HFS mice, had significantly greater pro-inflammatory macrophage mRNA levels and significantly increased mRNA levels of the pro-inflammatory cytokine TNFα. Naïve-Ex-HFS mice were more protected from these pro-inflammatory changes than NMS-Ex-HFS mice, similar to this group being more protected from the other metabolic changes. NMS-Sed-HFS mice also had significantly

more MC tryptase compared to the other groups. MCs are filled with granules that contain tryptase, histamine, heparin, as well as other proteases and cytokines (44). Upon activation they release these factors and cause an inflammatory response (45, 46). Their role in the development of obesity-related metabolic syndrome is controversial. Obese individuals with metabolic syndrome have increased MCs in subcutaneous adipose tissue compared to control subjects (173) and a certain MC deficient mouse model is protected from the development of diet-induced obesity (175), while another model is not (425). However, it is clear that regardless of whether they contribute directly to the development of obesity-related metabolic syndrome, they do contribute to the pro-inflammatory environment associated with visceral obesity. In previous studies, our laboratory has found that female and male NMS-Sed mice have increased MCs and MC degranulation in urogenital organs (96-98) and dura mater (Chapter 2) and that exercise attenuates this effect (100, 229) (Chapter 2). Therefore, it appears that NMS-Sed mice have widespread increases in MCs and MC degranulation, which could be one factor linking the development of co-morbid chronic pain and obesity-related metabolic syndrome.

Glucocorticoid receptor (GR) and 11 β-hydroxysteroid dehydrogenase type 1 (11-βHSD1) mRNA levels were also measured in epididymal adipose. Glucocorticoids (GCs) are the final output of the HPA axis (15-17) and they bind to GRs (68), which are found throughout the body (67). In adipose tissue, GCs promote the differentiation of pre-adipocytes to mature adipocytes (429) and 11-βHSD1 converts inactive GCs to their active form (430). Obese humans (85, 86) and genetic (87, 431) rodent models of obesity have increased levels of adipose tissue GR and 11-βHSD1. However, other

groups have found that a HFS diet actually causes a decrease in adipose tissue GR and 11-βHSD1 (432, 433). In line with these results, in this study a HFS diet resulted in an overall decrease in GR and 11-βHSD1 mRNA levels in epididymal adipose tissue (Figure 3.6). I posit this may represent a compensatory mechanism to attenuate the detrimental metabolic consequences of increased GC signaling.

Finally, leptin mRNA in epididymal adipose tissue was measured because it is an important adipokine that communicates with its receptor in the hypothalamus to regulate food intake and energy expenditure by shutting down hunger signals (435). I hypothesized that HFS mice would have increased leptin levels, which should down regulate hunger signals to compensate for the extra energy that is being consumed. I indeed found that naïve-Sed- and -Ex-HFS mice had significantly higher leptin levels compared to naïve-Sed- and Ex-Control mice. However, NMS-Ex- and Sed-HFS mice did not, suggesting an alteration in the leptin-hypothalamic feeding circuit. This circuitry is not fully programmed at birth (429), which makes it susceptible to the influence of environmental factors that cause long-term changes in hunger signals and energy expenditure. Therefore, it is likely that early life stress not only alters the HPA axis, but other hypothalamic-driven axes involved in metabolism, as well, and together these alterations could potentially underlie the association between early life stress and the subsequent development of obesity-related metabolic syndrome in adulthood.

An interesting finding in this study is that male NMS-Sed-Control mice were more glucose intolerant than naïve-Sed-Control mice (Figure 3.5) and had a greater amount of pro-inflammatory macrophage, TNFα, and leptin mRNA in their epididymal adipose tissue (Figure 3.6). Mice on a control diet are not expected to have altered leptin

signaling. Therefore, an increased leptin level in this dietary condition suggests that NMS-Sed-Control mice are leptin resistant. Together, these data imply that at a basal level, without any sort of lifestyle change (e.g. exercise or diet), male NMS mice have altered metabolic functioning compared to male naïve mice. This supports human data indicating that early life stress in humans is associated with the development of obesity-related metabolic syndrome (160-162).

In conclusion, these data demonstrate that NMS in mice models early life stress-induced alterations in metabolic function and that exercise is a beneficial therapeutic option for treating some symptoms of stress- and diet-induced obesity-related metabolic syndrome. To my knowledge, this is the first study to combine early life stress, exercise, and HFS diet to investigate the complex environmental interactions that contribute to the development of obesity-related metabolic syndrome. My goal was to replicate common environmental factors that are associated with an increased incidence of this syndrome in humans. In this way, my results are more translatable than other studies that do not take all lifestyle factors into consideration. Now that we have a mouse model to study early life stress-induced obesity-related metabolic syndrome, future work can focus on investigating the specific mechanism(s) underlying this association. It is clear that many pathways are altered by NMS, including the HPA axis, reward pathways, and the leptin-hypothalamic feeding circuit. Our laboratory plans to study how these pathways interact and lead to the development of such a debilitating syndrome. Finally, future work in our laboratory will evaluate the effect, if any, of a HFS diet in female NMS- and naïve- Sed- and Ex- mice to determine if there are sex differences in the development of early life stress- and HFS- induced obesity-related metabolic syndrome.

5.3 Stress and sex differences in response to an anti-inflammatory diet

Many chronic pain, mood, and metabolic disorders are associated with a chronic state of low-grade inflammation (255, 256, 268). Therefore, a diet high in anti-inflammatory components is recommended as a good dietary intervention for these patients (269). An anti-inflammatory diet (AID), which is similar to the Mediterranean diet, is high in fruits, vegetables, lean protein or plant based protein, whole grains, high fiber, and healthy fats such as omega-3 fatty acids (FAs) (270). AID improves many of the symptoms of metabolic syndrome and reduces certain forms of chronic pain in humans. For example, patients with metabolic syndrome that consumed a Mediterranean diet for 2-years showed decreased weight, gained cardiovascular benefits, and increased insulin sensitivity compared to the non-intervention group (279). This form of diet has also been shown to reduce the development of diabetes by around 20% (210). Although somewhat different than AID, a vegetarian diet with increased antioxidant and anti-inflammatory properties has positive results on pain and quality of life in rheumatic and fibromyalgia patients (519, 520). Furthermore, Kuptniratsaikul et al., (181) compared the treatment of knee osteoarthritis patients with curcumin, the main active ingredient in turmeric that has anti-inflammatory properties, versus treatment with ibuprofen and found that both treatments were effective in reducing knee pain but individuals taking curcumin reported fewer gastrointestinal problems.

Studies in rodents have also investigated the influence of anti-inflammatory components for the treatment of high-fat diet-induced obesity-related metabolic syndrome (460, 463, 466, 469, 472), chronic pain (461, 464, 467, 473), and stress-induced depression-like behavior (462, 465, 468, 471, 474) (Summarized in Table 4.2).

However, these studies focus on the therapeutic effect of one anti-inflammatory component in quantities that are not likely attainable in the human diet. In my study, I used an AID that was composed of 20% protein, 45% carbohydrates, with 0% coming from sucrose, and 35% fat from flaxseed and soybean oils, which are high in omega-3 FAs and considered healthy fats. This diet also had added anti-inflammatory ingredients in quantities attainable in the human diet (504) including epigallocatechin gallate (EGCG), sulforaphane, resveratrol, curcumin, and ginseng (Table 4.1*)*. In female and male NMS and naïve mice, perigenital and hind paw sensitivity were measured to evaluate chronic widespread pain, body weight, body fat percentage, fasting insulin level, and glucose tolerance to evaluate obesity-related metabolic syndrome, nestlet building to evaluate depression-like behavior, and serum corticosterone level to evaluate differences in HPA axis output. I compared these measures in mice on the AID to mice on a non-inflammatory diet (NAID) and a control diet. NAID had the same composition as AID but without the added anti-inflammatory components and the control diet was similar to a standard laboratory rodent chow diet and was composed of 20% protein, 70% carbohydrates, and 10% fat (Table 4.1*)*.

Complex sex, diet, and stress interactions that influenced metabolic, pain, and stress outcomes were revealed. These data indicate that AID had differential therapeutic benefits for male vs. female and naïve vs. NMS mice and that no group benefited in every measure. AID and NAID both have a higher fat content compared to the control diet (35% vs. 10%). Therefore, increased weight gain on these two diets was expected but I hypothesized that weight gain from the two diets would happen at the same rate or NAID mice would be more susceptible to weight gain compared to AID.

While this was true in naïve male mice, the opposite effect in NMS female and male mice was seen. These groups were actually more susceptible to weight and body fat gain on the AID (Figures 4.1 & 4.2). In males, this could be partially attributed to them eating more at the beginning of the study (Figure 4.3) compared to the other groups, but I do not believe this was the sole cause of their weight gain due do the fact that male naïve-AID mice also ate more at the beginning of the study but they did not weigh significantly more or have a higher body fat percentage than naïve-control mice until at the very end of the study. Additionally, female NMS-AID mice did not eat significantly more compared to the other groups (Figure 4.3), but they still gained significantly more weight and body fat.

Another unexpected finding was that female naïve- and NMS-AID mice were more glucose intolerant compared to female naïve- and NMS-control mice (Figure 4.5B). This result was surprising as neither AID nor NAID had added sucrose, so mice on either of these diets were not accustomed to processing this form of carbohydrate. However, female mice on the AID diet were more glucose intolerant than female mice on the NAID diet, whose glucose tolerance did not significantly differ from mice on the control diet. This suggests that there is another factor causing the female AID mice to become more glucose intolerant. Interestingly, despite the fact that male NMS-AID mice were more susceptible to weight and body fat gain, they had similar fasting insulin level and glucose tolerance compared to NMS-Control mice but male naïve-AID and –NAID had higher fasting insulin level and were more glucose intolerant compared to naïve-control (Figures 4.4B and 4.5D). In humans, obesity is usually a pre-requisite for the development of the other symptoms of metabolic syndrome (165), including insulin

resistance and glucose intolerance. However, this might not be the case in male NMS-AID mice. Data suggests that while AID has negative consequences in regard to body composition in NMS mice, it was protective in the development of other metabolic syndrome symptoms in male NMS mice. This is similar to a subset of humans that are classified as ‘metabolically normal, healthy obese’ (OBMN), who seem to be protected from obesity-related metabolic syndrome despite being overweight or obese. Unlike other obese individuals with metabolic syndrome, OBMN individuals usually become obese during childhood but do not develop insulin resistance, high triglycerides, and low HDL- cholesterol (521). Karelis et al., (522) studied this phenomenon in a group of obese postmenopausal women and reported that OBMN women had a better inflammatory profile compared to women with obesity-related metabolic syndrome. Specifically, they had 92.7% less C-reactive protein levels. The authors suggest that this could be one reason OBMN are protected from developing the other symptoms of metabolic syndrome.

I have so far analyzed only a few of the known factors that constitute obesity-related metabolic syndrome in my mice, including body fat percentage, fasting insulin level, and glucose tolerance. Future work in our laboratory will evaluate if sex, NMS, and/or diet influenced other symptoms of this syndrome including circulating triglyceride and cholesterol levels. These data will help us determine if these mice actually suffer from obesity-related metabolic syndrome, or only a subset of the characteristics. Future work in our laboratory will also evaluate the inflammatory status of the visceral adipose tissue in these mice. White adipose tissue distribution and composition is important in predicting the development of obesity-related diseases because it plays a key role in

regulating systemic metabolic function and inflammation (168). Visceral fat accumulation is associated with a greater risk of metabolic dysfunction (169) and leads to chronic low-grade inflammation (170). In Chapter 3 of this dissertation, I found that male NMS-Sedentary mice on a control or high-fat/high-sucrose (HFS) diet were particularly susceptible to pro-inflammatory changes in their visceral adipose tissue compare to NMS-Ex and naïve mice (Figure 3.6). It will be interesting to see if AID prevented stress- and diet-induced adipose tissue changes in these mice and if this can help explain my complex results.

To study the development of co-morbid obesity-related metabolic syndrome and chronic pain, hind paw and perigenital sensitivity were measured over time. I hypothesized that mice consuming NAID would gain weight and subsequently develop widespread sensitivity, similar to observations in patients with fibromyalgia that are often also diagnosed with obesity-related metabolic syndrome (453-455). I also postulated that NMS mice would be particularly susceptible to the development of widespread pain on both the control and NAID diets, because our laboratory has previously demonstrated NMS-induced perigenital sensitivity (96-98), and that AID would prevent diet- and stress-induced development of wide spread hypersensitivity. Remarkably, despite the sex/stress/diet differences in my metabolic measures, I found that AID was consistently beneficial in preventing the development of diet- and/or stress-induced perigenital sensitivity in female and male mice and hind paw sensitivity in female mice. This is an exciting finding because many pharmacological agents have been studied for the treatment of widespread pain but none have proved to be universally successful. Although clinical studies reveal that fibromyalgia symptoms can be improved with

weight loss (515), this study suggests that AID can prevent the development of stress- and diet-induced pain independent of weight or body fat percentage, at least in rodents.

The inflammatory response influences both peripheral and central nervous systems and a long-term increase in inflammation can lead to the development of acute and chronic pain (256). As explained in section 5.1, MCs are involved in the inflammatory response and when they become activated such as by endogenous neuropeptides, they release vasoactive, nociceptive, and inflammatory molecules (44). MCs are increased in a number of chronic pain disorders including irritable bowel syndrome (37), interstitial cystitis/painful bladder syndrome (38), migraine (39), and fibromyalgia (40). They reside close to sensory nerve endings (47-49), including small unmyelinated (C-fiber) or thinly myelinated (Aδ-fiber) peptidergic neurons that predominately make up peripheral sensory innervation. These fibers express receptors involved in nociception, including transient receptor potential vanilloid 1 (TRPV1), transient receptor potential ankyrin 1 (TRPA1), and protease-activated receptor 2 (PAR2) that respond to noxious thermal, chemical, and mechanical stimuli (51-53) and are co-expressed (58-60). Tryptase is one peptide released upon MC activation. This peptide is known to activate the PAR2 receptor, which subsequently sensitizes TRPV1 and TRPA1 receptors (55-57). When these nociceptors become activated, they can generate an action potential that signals pain in the central nervous system. They also perform an efferent function by releasing inflammatory molecules including substance P (SP) and calcitonin gene related peptide (CGRP) in the peripheral tissue (63-66). SP and CGRP activate MCs, so this process can cause a loop of activation that can eventually lead to chronic pain. Although I did not evaluate MCs in this study, previous

work in our lab demonstrates that NMS mice have increased MC and/or increased MC activation in urogenital organs compared to naïve mice (96-98). I also found that NMS-Sedentary-HFS diet mice have increased tryptase in their adipose tissue (Figure 3.6). Future work in our laboratory will evaluate MC characteristics in the urogenital organs and adipose tissue to establish if AID prevented NMS- and/or high-fat diet-induced increases in MCs and MC activation. If AID is able to prevent the inflammatory loop that can lead to the development of chronic pain this could explain why there was reduced hypersensitivity in mice consuming AID.

In conclusion, in this study I found complex sex, diet, and stress interactions that influence pain, metabolic, and stress pathways in mice. Although I attempted to study a diet that would confer metabolic benefits to humans (AID), it appears that these metabolic benefits may not translate appropriately in mice. This could be due to species differences in metabolic and dietary needs, or because of different digestion capabilities. Despite this finding, these results are intriguing and lead to new questions in our laboratory regarding why stressed mice respond differently to diet interventions compared to non-stressed mice. A promising finding in this study was that AID prevented the development of stress- and/or diet-induced widespread pain, regardless of the metabolic state of the mice. This suggests that AID could be a good treatment option for humans with early life stress-induced chronic pain disorders. Future work in our laboratory will evaluate other symptoms of metabolic syndrome, including circulating triglyceride and cholesterol levels. We will also determine the inflammatory status of these mice by assessing circulating cytokines, adipose tissue macrophage infiltration,

and MC characteristics in urogenital and adipose tissues. I hypothesize that these data will help explain some of the differences in these groups of mice.

5.4 Overall conclusions

The rate of childhood maltreatment is very high (12, 323) and many studies have demonstrated an association between adverse childhood events and the development of co-morbid chronic pain, mood, and obesity-related metabolic syndrome in adulthood (3-7). Therefore, establishing pre-clinical models to study the many consequences of early life stress is extremely important. This work will help us determine the mechanisms that underlie this association and aid in the development of appropriate therapeutic interventions for these early life stress-induced disorders. This dissertation used a mouse model of early life stress, NMS, to expand our studies and provide evidence that this model is also more susceptible to evoked migraine-like behaviors, obesity-related metabolic syndrome, and diet-induced metabolic changes. I also show that exercise is a therapeutic intervention that can prevent the development of early life stress-induced susceptibility to evoked-migraine or certain symptoms associated with obesity-related metabolic syndrome. Additionally, although the presumed metabolic benefits of an AID do not translate completely to rodents, I obtained preliminary evidence that AID may be a good therapeutic intervention for the prevention of early life stress- or high-fat diet-induced widespread hypersensitivity. While it is well accepted that early life stress alters the HPA axis and this has consequences in stress, pain, and metabolic systems, there are other mechanisms underlying the development of these disorders that are not as well established. My data suggest that NMS also alters reward, metabolic, and/or digestive pathways and that sex differences exist in many of these

pathways. Studying the interactions of the pathways that are altered by NMS is key if we want to understand how we can prevent the development of or treat early life stress induced disorders.

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