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NEONATAL MATERNAL SEPARATION IN MICE AS A PRE-CLINICAL MODEL FOR
FEMALE CHRONIC PELVIC PAIN

By

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Submitted to the graduate degree program in Neuroscience and the Graduate Faculty
of the University of Kansas in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

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NEONATAL MATERNAL SEPARATION IN MICE AS A PRE-CLINICAL MODEL FOR
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Abstract

Chronic pelvic pain is currently estimated to impact 9.2 million women in the United States. As many as half of these women will have comorbid conditions, such as chronic pain in multiple pelvic organs, mood disorder, and/or somatic functional pain disorders, which are often of unknown etiology and complicate already less-than-optimal treatment strategies. Functional pain patients are more likely than the general population to report histories of early life adverse events, including abuse, neglect, and trauma. Exposure to these early life stressors can have a profound lifelong impact on neurodevelopment, behavior, and the neuroendocrine response to stress by influencing the hypothalamic-pituitary-adrenal (HPA) axis, the tone of which is set during this critical period of development. Using an animal model of early life stress, this project evaluated changes in behavior and urogenital hypersensitivity in adulthood, assessed alterations in the regulation of the HPA axis, and investigated voluntary exercise as a potential therapeutic intervention. This project was the first of its kind to study the impact of early life stress on adult behavior relevant to pain and associated comorbidities in female mice. Specifically, molecular approaches evaluated how early life stress influenced long-term changes in the expression of genes that regulate the HPA axis. The impact of early life stress on the neuroendocrine response to acute stress in adulthood was evaluated by characterizing HPA axis output and the downstream immune response to stress in the pelvic organs. Finally, this project provided novel preclinical model of exercise as an intervention for early life stress-induced urogenital pain and associated comorbid behaviors. Together, this work provides insight into how early life stress alters the function of the nervous system, impacts the neuroimmune profile of the female

genitourinary tract, and whether exercise, an easily translatable intervention method, can attenuate the impact of early life adverse events.

Dedication

This dissertation is dedicated to my loving husband, Bobby Pierce. His calm encouragement, understanding and sacrifice have given me the courage and means to strive for success.

Acknowledgements

Mine has been an interesting path that brought me to completing this dissertation. The wisdom of a plethora of individuals spanning the entirety of my education was instrumental in guiding me along such a path. Although I wish I could acknowledge them all, I will only mention a few here. To the unnamed, they will never know how important they were to me, but I am immensely grateful for their influences nonetheless.

First and foremost, I want to acknowledge and thank my research mentor, Dr. Julie Christianson. From my first day in graduate school, I was told that the selection of a mentor was probably the most important decision I could make in my career. I could never appreciate the wisdom of such advice until now. She transformed an unrecognizable clump of a fledgling scientist into a well-molded independent scientist. Her patience in teaching scientific principles and bench work was remarkable given my relative lack of experience at the bench. She graciously encouraged attendance at scientific conferences, including those abroad, and advocated on my behalf to introduce me to prominent scientists in the field. As a successful woman in science, she has also been a role model in addition to a mentor. I am thicker-skinned than I once was and I now accept that the best way to learn is by making mistakes. Her teaching, patience, forgiveness, and understanding have meant more to me than she can know: for that, and so much more, thank-you.

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Finally, to my husband, Bobby Pierce, you carried me through the hardest times of graduate school and celebrated with me during all the joyous occasions. You are truly the partner every wife dreams about. The joy during the years we have been together will only be surpassed by that to be experienced in our future. Thank you.

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Chapter 1

Introduction to Dissertation

1.1 Chronic Pelvic Pain

In the United States alone, 9.2 million women aged 18-50 years are estimated to currently suffer from chronic pelvic pain (CPP), which is defined as non-cyclical pain of 6 months duration or longer in the lower abdomen or reproductive organs. In the context of this definition, 14.7% of women of reproductive age have reported CPP at some point in their lives (1). Notably, more than half of these women reported the cause of their CPP was unknown (1). In the United States, total annual costs for out of pocket expenses related to CPP are estimated to be \$2.8 billion and annual indirect costs from lost productivity due to missing work are estimated to be \$555.3 million (1).

Functional pain disorders affecting the genitourinary system are complex, multifactorial syndromes of unknown etiology and are frequently associated with CPP. Chronic pain disorders of idiopathic origin present frustrating cases in clinical practice especially when diagnostic workup is disproportionate to the severity of symptoms. When studied by organ involvement, half of CPP syndromes are related to the genitourinary systems. Urogenital laparoscopic and biopsy findings remain relatively unremarkable in proportion to symptom severity (2). As a result, urogenital functional pain disorders are diagnosed symptomatically, in exclusion of other apparent findings, where pain itself is the primary symptom. CPP is therefore a descriptive term for the ongoing spontaneous or evoked pain associated with such diagnoses as interstitial cystitis/painful bladder syndrome (IC/PBS), vulvodynia, and irritable bowel syndrome (IBS): syndromes that severely impact quality of life and disability. CPP affecting the urogenital organs and common comorbidities are the primary focus of this dissertation and will be further described in the following sections.

1.1.1 Interstitial cystitis/Painful bladder syndrome

Estimations indicate 11% of women and 5% of men meet the high sensitivity definition of IC/PBS (3-5). Direct medical costs for individual IC/PBS patients total approximately \$4000/year (6), indicating that, at minimum, \$20-40 billion dollars are spent each year in the United States to treat IC/PBS alone. However, these statistics do not represent the full burden of this disease. For instance, comorbidity with other CPP disorders is common (1, 7-10). Up to 93% of women with IC/PBS have pelvic pain and more than 50% have vulvar pain (11). Other commonly reported comorbidities include fibromyalgia, allergies, IBS, and mood disorder (12-16), creating a greater negative impact on quality of life and complicating already less-than-optimal treatment strategies (13, 17-22).

Clinically, IC/PBS is defined as pelvic pain or discomfort lasting for at least 6 weeks in duration that is perceived to be related to the urinary bladder. Classification of IC/PBS must also include at least one additional urological symptom that is non-attributable to known causes of bladder pain, such as infection or organic disease (23) (24, 25). Like many idiopathic pain disorders, IC/PBS is polysyndromic, that is patients may exhibit one of multiple subtypes generally classified as an ulcerative or non-ulcerative presentation (26). Classically, a diagnosis of interstitial cystitis referred to observable findings on cystoscopic examinations: namely, glomerulations, overt histopathology, or Hunner's ulcers (24, 25); however, the patient population was far more heterogeneous than the strict criteria originally defined. Therefore, diagnoses today are primarily attributed symptomatically where IC/PBS subtypes may be differentiated by additional features, clinical presentation, or history (27). The only FDA

approved treatments for IC/PBS are oral pentosan polysulfate sodium, whose mechanism of action is unknown, and intravesicular installation of dimethyl sulfoxide; off-label treatment options include oral tricyclic antidepressants or antihistamines and intravesicular instillation of heparin or lidocaine (28-30).

Reflecting the broad spectrum of clinical subtypes, the pathophysiology underlying IC/PBS is likely multifactorial. A wide array of etiologies has been explored in IC/PBS that, when taken together, proposes a cyclical cascade of events involving neurogenic inflammation, a hyper-responsive immune system, and urothelial lining dysfunction that collectively results in chronic pain. The initial insult that triggers IC/PBS pathophysiology is less clear. As IC/PBS is primarily an adult-onset disorder, identification of the prodrome will need to be a priority in order to halt the circular nature of the early-pathophysiology. To that end, a number of urine biomarkers associated with IC/PBS, such as interleukin-6 (IL-6) (31-34), histamine (31), and nerve growth factor (NGF) (35, 36), have been identified.

1.1.2. Vulvodynia

The International Society for the Study of Vulvovaginal Disease (ISSVD) defines vulvodynia as “vulvar discomfort, most often described as a burning pain without relevant visible findings or a specific, clinically identifiable, neurologic disorder” (37). Patients with vulvodynia may present with a variety of symptom characteristics. The pain associated with localized and generalized vulvodynia is further distinguished between provoked or unprovoked pain, or a mix of both while symptom onset of these subtypes may arise suddenly or gradually (38). Despite the variability in clinical presentation, allodynia of the vulva, vestibule, or vaginal canal are hallmark symptoms

of vulvodynia and together are qualitatively described as a burning, stinging, or itching sensation (39, 40). Diagnosis is primarily made by cotton-swab examination of the vulva as a means to reproduce severe pain or discomfort (38). As such, in the absence of other clinically identifiable organic disease, vulvodynia is a diagnosis of exclusion. Observed erythema is minor and biopsy of the vulva is pathologically unremarkable; therefore, the diagnosis of vulvodynia more accurately reflects symptomatology as an idiopathic pain disorder rather than a disease process. Vulvodynia has been shown to be significantly associated with a diminished quality of life and increased rates of comorbid diagnoses of fibromyalgia, IBS, and depression (17).

At any given time, vulvodynia affects 4% of women in the United States. Lifetime cumulative evidence predicts 16% of women among the general population will have vulvodynia at some point in their lives (41, 42). Conservative estimates suggest the national costs associated with vulvodynia range \$31-72 billion annually (43). Similar to idiopathic pain disorders in general, vulvodynia is proposed to be of multifactorial nature. Given the elusive etiology, managing symptoms associated with vulvodynia is the primary focus in the literature. The magnitude of treatment options span non-specific lifestyle interventions, topical or oral medications, injections, physical therapy, and surgical procedures (44). Recently, epidemiological studies have found predispositions between the onset of vulvodynia as triggered by a traumatic life event or a history of early life stress (45). Antidepressant (46-48) and anticonvulsant (49, 50) medication have been shown to alleviate symptoms associated with vulvodynia, and, as a first-line treatment, suggests a psychosomatic pathophysiologic component to the disease process; however, other molecular mechanisms are at play.

1.1.3. Comorbidities

Up to 50% of women diagnosed with CPP experience symptoms from more than one disorder (22). While clinical features such as dysfunctions in the immune system, central nervous system, and peripheral nervous system are common characteristics across CPP syndromes (1, 7), the underlying etiology bridging these syndromes remains to be elucidated. While CPP frequently affects more than one pelvic organ, formal diagnosis of a secondary disorder is not necessarily reflective of actual symptom patterns. For instance, 88% of sexually active women with CPP and no formal gynecological diagnosis reported that symptom exacerbation due to sexual intercourse was the greatest interference to their quality of life and the chief cause of their health distress (1). Accordingly, vaginal pain is also reported in 52% of women diagnosed with IC/PBS (51). Unfortunately, neither the underlying etiology of CPP syndromes nor and the associated comorbidities involving the genitourinary systems has yet to be discerned.

Patients with chronic pain involving pelvic or genital dysfunction are less likely to seek treatment and, for those that do, the combined apparent lack of organ pathology with comorbid mood disorder prompts clinicians to approach these patients as exhibiting a psychological, rather than a physiological, disturbance. As evidence of this diagnostic pattern, women with functional pelvic pain disorders are more likely to be given a primary diagnosis of anxiety or depression; however, treatment with antidepressants, the most common pharmacological intervention for this group, does not necessarily relieve their pain (52). Vulvodynia, in particular, has been associated with other chronic pain disorders of the pelvic organs including IC/PBS and increased rates of mood

disorders such as depression or anxiety (53). Conversely, odds of vulvodynia are more likely among women with antecedent mood disorders (54) suggesting higher levels of chronic stress among these patients serves to increase symptom severity (55). Indeed, vulvodynia patients demonstrate a blunted serum cortisol cycle that manifests in a variety of emotional and physiological indicators (56).

In addition to presenting with symptomology in adjacent organs, CPP patients are also more likely to present with somatic functional pain disorders affecting more discreet locations such as migraine, fibromyalgia, or temporomandibular joint disease. This disparate localization of affected regions, combined with comorbid mood disorder, has led scientists and clinicians to investigate central sensitization or altered pain processing as potential etiologies.

1.2 Hypothalamic-pituitary-adrenal gland axis

One area of research that has come under recent increasing investigation involves the links between stress and pain physiology. The influence of stress on the modulation of disease has been studied for many years. In particular, chronic exposure to stress can raise blood pressure, increase the risk of heart attack and stroke, slow wound healing, increase vulnerability to anxiety, and depress the immune response (57). Differential effects can occur depending on the nature of the stressor, i.e. physiological or psychological, and in what stage of life the stress is experienced. It is during the earliest stages of life that chronic stress exerts profound life-long molecular, cellular, and neurophysiological changes. By impacting neurodevelopment and the response to acute stress in adulthood, early life stress can diminish health, increase susceptibility to disease, and impact premature mortality (58). Unfortunately, social

trends regarding childhood maltreatment, poverty, and witnessing substance abuse in the home are increasing in the United States (59). In fact, the national rate of maltreatment in 2011, reported primarily from professional sources such as primary care physicians and investigated by Child Protective Services, was 27.4 per 1,000 children in the national population, a trend which had steadily increased over the previous 5 years (60). In 2011, this resulted in the confirmation of 676,569 unique cases of child abuse and neglect in the United States alone, and 27.1% of victims were less than two years of age (60).

In particular, stress experienced during critical periods of development exert lifelong impacts on the health of that individual by altering the programming of the body's physiological stress response system: the hypothalamic-pituitary-adrenal gland (HPA) axis. The HPA axis is responsible for initiating and coordinating the physiological response to acute or prolonged stress (61). Ultimately, this results in a systemic response designed to facilitate the physiological need to endure, fight, or flee from a perceived threat and eventually return to homeostasis (62). Systemic effects of HPA axis activation include shifting energy balance from anaerobic to aerobic metabolism, increasing water reabsorption in the kidney, and systemic immunosuppression (63). Due to its potency, altered programming of HPA axis regulation impacts central nervous system architecture and the peripheral neuroendocrine response to stress (64).

Under stressful conditions, parvocellular cells within the paraventricular nucleus (PVN) of the hypothalamus are activated via noradrenergic, adrenergic, and peptidergic innervation from brainstem nuclei to release corticotropin-releasing factor (CRF) into the median eminence. CRF is then transported through the hypophyseal portal system to

stimulate the release of adrenocorticotrophic hormone (ACTH) from corticotrophs in the anterior pituitary. ACTH is released into systemic circulation and stimulates the release of glucocorticoids, cortisol in humans and corticosterone in rodents, and mineralocorticoid from cells in the zona fasciculata and zona glomerulosa of the adrenal cortex, respectively (65-67). Basal levels of glucocorticoid release follow diurnal rhythms, with serum concentrations peaking at the beginning of the active period (68). Glucocorticoids bind one of two nuclear receptors. The first is the high-affinity mineralocorticoid receptor (MR) responsible for maintaining the diurnal tone of the HPA axis (69). The second is the low-affinity glucocorticoid receptor (GR), which is bound during surges in glucocorticoids (69). Both MR and GR are highly expressed in hippocampal neurons that send glutamatergic input to GABAergic PVN relays within the hypothalamus, activation of which shuts down the HPA axis at the cessation of a psychogenic stress response (70-72). This process of HPA axis negative feedback from limbic circuitry is programmed during postnatal development during the stress hyporesponsive period (73-77), which, in rodents, is driven by maternally-maintained buffering of neonatal HPA axis activation through physical presence of the dam (78). Activity of MR and GR are relatively slow-acting and effect long-term changes in gene transcription (79). Fast-acting, nongenomic effects of glucocorticoids have also been observed at the membrane (80, 81). While the receptor involved has not been identified, intracellular signaling cascades resulting in endocannabinoid synthesis have been observed upon glucocorticoid binding. These endogenous cannabinoid ligands modulate synaptic plasticity through retrograde action on presynaptic neurotransmitter release. In the case of parvocellular corticotrophs of the hypothalamus,

endocannabinoid activity reduces presynaptic glutamate release and thereby facilitates rapid negative feedback (80). In the hippocampal neurons, fast-acting effects of glucocorticoids stimulate a putative $G_{i/o}$ signaling cascade that ultimately results in the recruitment and retention of N-Methyl-D-aspartic acid (NMDA)-receptors at the cell surface (82).

HPA axis modulation is also coordinated by two G-protein coupled receptors (CRF₁ and CRF₂) that bind CRF and three related endogenous ligands: urocortin (Ucn) 1-3. CRF preferentially binds CRF₁, Ucn1 binds CRF₁ and CRF₂ with equal affinity, and Ucn2 and Ucn3 preferentially bind CRF₂ (83-85). CRF₁ and CRF₂ generally evoke opposing effects, causing activation and suppression of the HPA axis, respectively. This has been evidenced in rodent studies where activation of CRF₁ increases anxiety-like behaviors in rodents and pharmacological antagonism or genetic deletion of CRF₁ has been shown to be anxiolytic, whereas pharmacological blockade or genetic deletion of CRF₂ is anxiogenic (86-88).

Limbic structures work in conjunction with one another to maintain homeostatic regulation of the HPA axis. The PVN is heavily innervated by GABAergic interneurons that themselves are responsive to limbic activity (89). Under normal circumstances, limbic structures such as the amygdala and hippocampus activate and inhibit the HPA axis, respectively, through modulation of the local inhibitory tone (65, 90). The central nucleus of the amygdala (CeA) is well recognized as a key integrator of stress-related behaviors such as fear responses (91). Under conditions of chronic stress, however, production of CRF is increased in the CeA, consequently enhancing CRF production in the PVN and glucocorticoid release (92). In the hippocampus, chronic stress can result

Figure 1.1

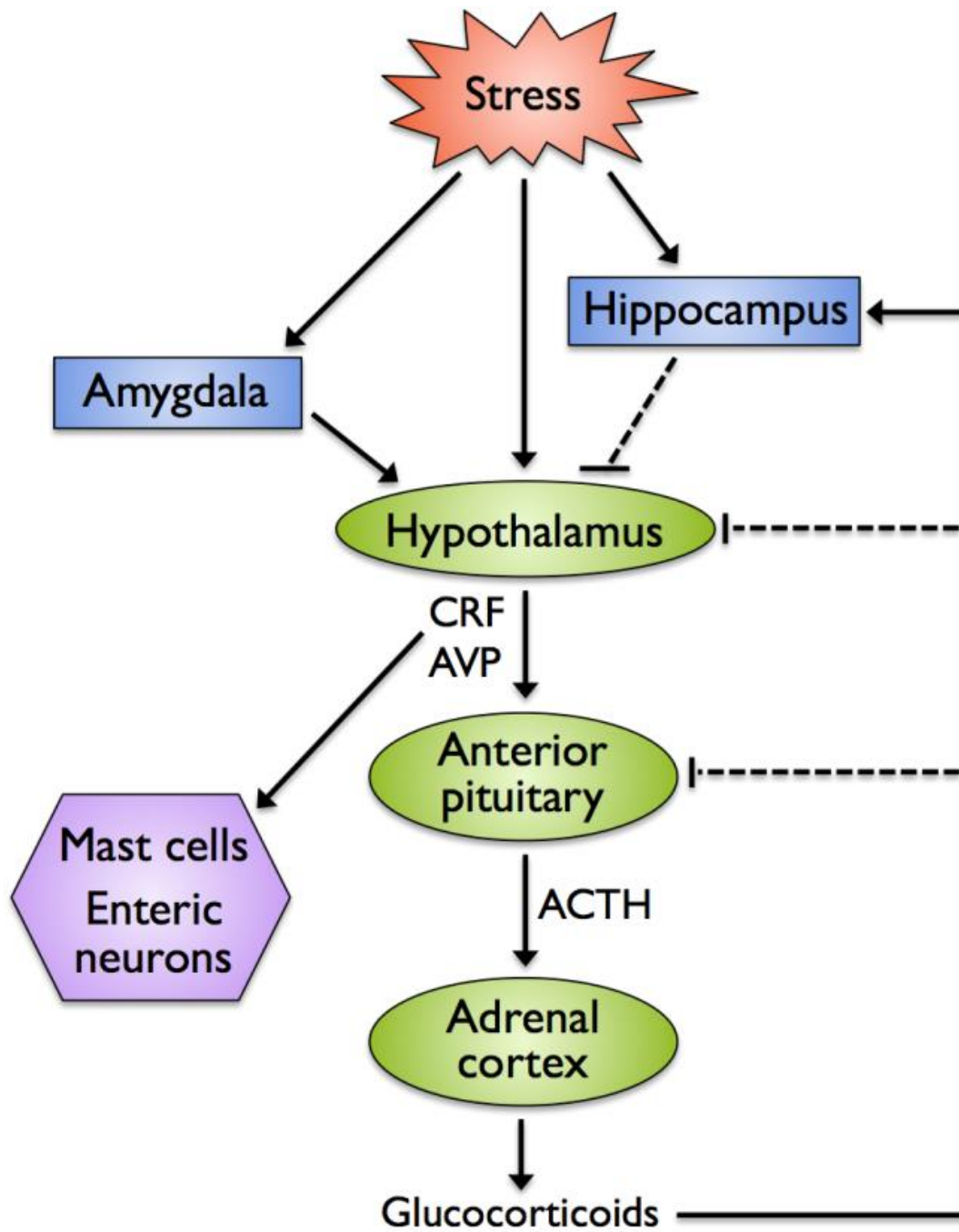


Figure 1.1 Schematic diagram of downstream activation and limbic control of the hypothalamic-pituitary-adrenal (HPA) axis. Acute stress induces activation of the parvocellular neurons of the paraventricular neurons (PVN) of the hypothalamus and synthesis and release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) into the hypophysial portal system. Both CRF and AVP will reach the anterior pituitary and synergistically promote the release of adrenocorticotrophic hormone (ACTH), which will act at the adrenal cortex to produce glucocorticoids (cortisol in humans; corticosterone in rodents). Glucocorticoids act in a negative feedback loop at the hypothalamus and anterior pituitary to cease production of CRF and ACTH, respectively, as well as at higher limbic structures, including the amygdala and hippocampus, which respectively activate and inhibit the hypothalamus. Peripheral release of CRF also acts on mast cells and enteric neurons.

Figure 1.2

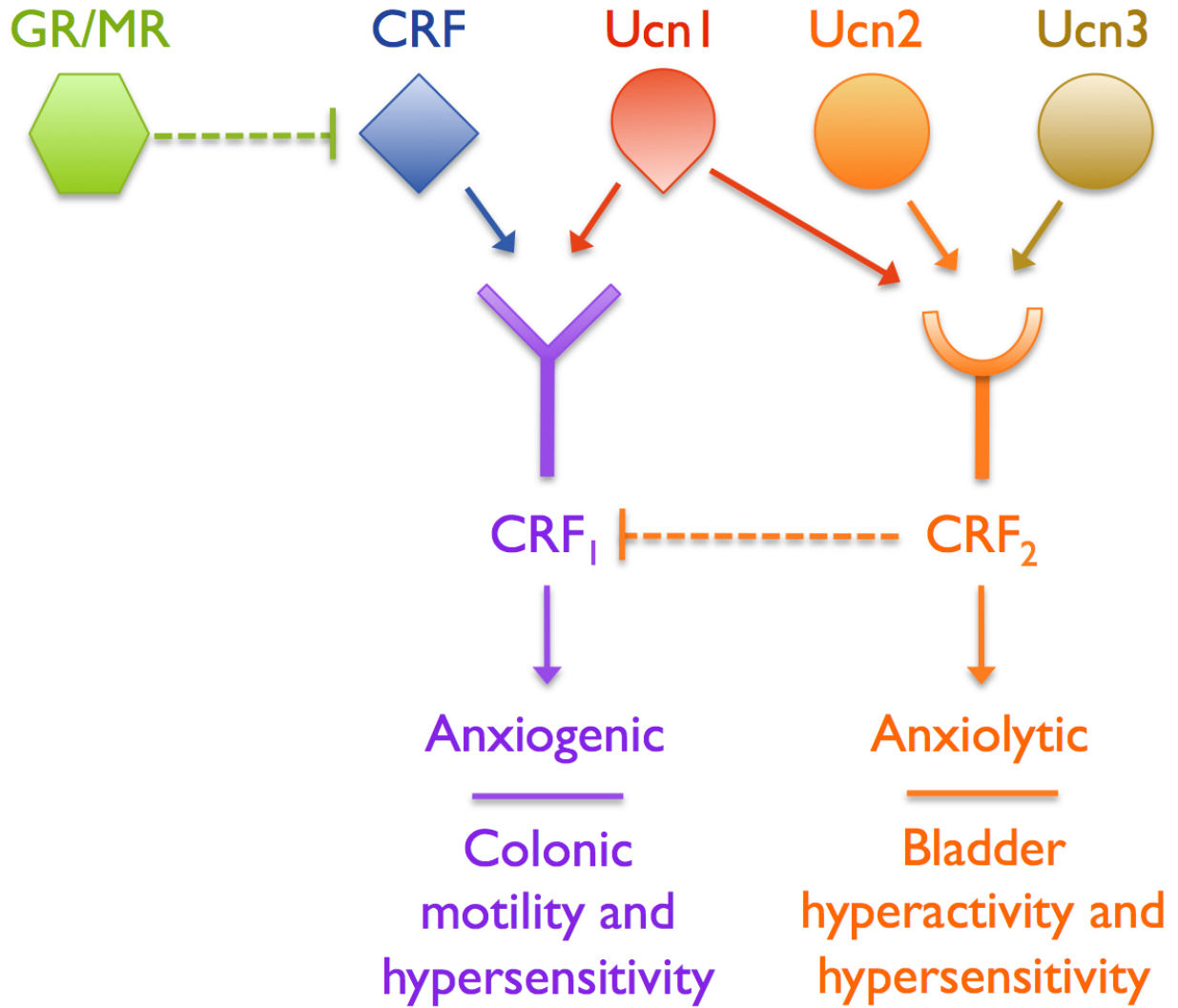


Figure 1.2 Schematic diagram of the binding scheme and cross-talk between corticotrophin-releasing factor (CRF) and glucocorticoid receptors. CRF and the related Urocortins (Ucn) 1–3 act via a two-receptor system to regulate the hypothalamic-pituitary-adrenal gland axis. Activation of CRF receptor 1 (CRF₁) occurs via CRF or Ucn1 and activation of CRF₂ occurs via all three Ucns. Activation of CRF₁ has been shown to increase anxiety-like behaviors in rodents as well as increase colonic motility and hypersensitivity. In contrast, activation of CRF₂ has largely been shown to be anxiolytic and can increase urinary bladder hyperactivity and hypersensitivity. Activation of CRF₂ has also been shown to inhibit CRF₁ and activation of either glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) can inhibit CRF production and signaling.

in decreased expression of GR or increased resistance to GR binding (93), thereby diminishing inhibitory activity onto the HPA axis and subsequently enhancing glucocorticoid release.

1.3 Mast cells

In addition to CRF systems, one commonality linking chronic stress to multiple categories of peripheral pathology is the infiltration and activation of mast cells. Part of the innate immune response, mast cells are primarily resident in tissues interfacing with the environment: the integument, meninges, and respiratory-, gastrointestinal-, and genitourinary tracts (94-96). Hematopoietic progenitors of mast cells develop in the bone marrow, are lightly or non-granulated, and express the stem cell factor (SCF) receptor c-kit and the IgE receptor FcεRI (97). A complex network of signaling and transcription factors regulates the recruitment of immature mast cells to the peripheral tissue where they will take up residence and undergo maturation. Once mature, mast cells are generally considered to be terminally differentiated and do not undergo cell division (98), therefore increases in mast cell infiltration in the periphery, associated with inflammation or disease, is attributed to active recruitment and maturation of continuously released mast cell progenitors from the bone marrow (99, 100). Two subtypes of mast cell populations have been described in mouse (101) and human (102): mucosal mast cells and connective tissue mast cells. While each subtype responds to different compounds, and differs in size and granular content, both exhibit similar histochemical properties, express the SCF receptor, and are derived from the same population of embryonic stem cell progenitors (103). Levels of mast cells may also increase in non-barrier tissues such as kidney upon injury or inflammation (104).

Ample evidence exists for the role of mast cells in driving neurogenic inflammation and visceral afferent sensitization in mucosal diseases such as IBS and asthma (105, 106). In addition, mast cell stabilizing agents such as sodium chromoglycates are used to treat such diseases. Mechanistically, mast cell stabilizers are thought to inhibit IgE-directed mediator release from human mast cells (107), but their specific mode of action remains unclear. Due to their safety profile, they are widely prescribed as treatment for asthma, rhinitis, and other wheezing disorders as an alternative to corticosteroids (108, 109). Mast cell stabilizers also have therapeutic efficacy in conditions that feature non-allergen directed mast cell activation, such as exercise-induced bronchoconstriction (110-114). Furthermore, in animal models of CPP, mast cell stabilizers have been shown to prevent, but not reverse, stress and inflammation-induced colorectal hypersensitivity (115-117).

Mast cells express both functional CRF receptors and CRF mRNA and, accordingly, can respond to and release CRF and Ucn1, as well as increase receptor expression following stimulation with lipopolysaccharide (LPS) administration (118, 119). Mast cell-dependent vascular permeability in the skin is mediated by CRF₁, and is prevented by antalarmin, a specific CRF₁ antagonist (120). As such, mast cells have dual functions: as a sensory cell, it responds to environmental cues, and, as an effector cell, it releases biologically-active mediators such as cytokines, tryptase, proteases, and CRF itself (119, 121-123). Mast cell paracrine functions involve degranulated tryptase and histamine binding to protease activated receptors (PAR) and histamine receptors, respectively, on nearby visceral afferents, which can drive stress-mediated sensitization, a process shown to contribute to chronic functional pain (124, 125). This

nerve-mast cell axis and ensuing neuropeptide release from peripheral termini (126) is perpetuated by the chemoattraction of mast cells to antidromic release of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP) (127, 128). In turn, CGRP has been shown to increase expression of tumor necrosis factor (TNF)- α by mast cells (129).

Given the molecular evidence and the close anatomical approximation between mast cells and peripheral nerves (130), bidirectional neuroimmune signaling likely contributes to propagation of painful stimuli. As such, the critical role of mast cells in the development and maintenance of CPP continues to be investigated in both clinical and preclinical settings. Sensory innervation of the viscera is primarily provided by unmyelinated (C-fiber) or thinly myelinated (A δ -fiber) peptidergic neurons (131-133). A number of receptors expressed by these afferents, including transient receptor potential vanilloid 1 (TRPV1) (134-136), transient receptor potential ankyrin 1 (TRPA1) (137, 138), and PAR2 (139-141), transmit noxious stimuli and are necessary for the development of inflammatory-induced hyperalgesia. Tryptase, a major component of mast cell granules, can bind to and activate PAR2, which has been shown to sensitize TRPV1 and TRPA1 *in vivo* (139, 142). Interestingly, stress ligands such as CRF and Ucn are expressed in the bladder (143) and mediate neuroimmune dysfunction in animal models of cystitis (144, 145). Visceral signaling of the physiological stress response results in phenomena of heightened neuro-immune interactions and visceral hypersensitivity in animal models of IBS (146-149), vulvodynia (150), and IC/PBS (144, 151-153).

1.4 Inflammation

Numerous inflammatory mechanisms contribute to the spectrum of CPP and comorbid mood disorders. The extent of rote inflammatory infiltrate observed in CPP syndromes is generally below histological levels of detection as no overt pathophysiological observations are made upon pelvic organ biopsy samples from CPP patients (2). Yet proinflammatory molecules such as the cytokines interleukin-6 (IL6) and TNF- α , among other biomarkers, are elevated in urine or serum of CPP patients (31-36, 154, 155). The role of cytokines as contributors to hyperalgesia and allodynia is well delineated in injury and inflammatory models (156, 157). The normally low levels of cytokines secreted by human smooth muscle cells, such as the detrusor in the urinary bladder, are enhanced by exposure to inflammatory mediators (158). Other mediators of inflammation include neurotrophins, protons, prostaglandins, and bradykinin and are likewise implicated in the direct sensitization of nociceptive afferents leading to persistent pain (159).

Numerous studies support the pro-nociceptive effect of NGF (160), which was first described following efforts undertaken by Rita Levi-Montalcini (161). The biological action of NGF is mediated through the high-affinity tyrosine receptor kinase A (TrkA) and low-affinity pan-neurotrophin receptor 75 (p75). Local injection of NGF induces hyperalgesia or allodynia that lasts up to 49 days (162-164). NGF also exerts proinflammatory effects by acting on cells of the immune system (165, 166), particularly mast cells (167-169). Selective overexpression of NGF in the bladder can result in hyperreflexia, hyperinnervation, and increased mast cell infiltrates (170). Hyperexcitability of bladder afferents has also been reported following intrathecal administration of NGF (171). In multiple studies, cyclophosphamide-induced bladder

inflammation has been shown to increase activity in signaling pathways known to be downstream of TrkA, including phosphorylation of Erk1/2 in bladder afferents (172, 173). Erk1/2 are serine/threonine kinases phosphorylated downstream of the Ras-Raf-MEK signaling cascade required for subsequent enzyme activation (174). Several studies have shown a potential analgesic effect of Erk1/2 antagonists. For instance, the severity of inflammation and hyperalgesia induced by experimental cystitis was attenuated with an agonist of the cannabinoid receptor 2 (CB2) that was Erk1/2-activity mediated (175, 176). Furthermore, behavioral indicators associated with experimental cystitis could be attenuated with systemically-administered NGF neutralizing antiserum or intravesicular infusion of a nonspecific Trk receptor antagonist (177) and highlights a dominant role for this neurotrophin with direct relevance to IC/PBS patients (178).

Biopsy samples from vulvodynia patients have found increases in the pro-inflammatory cytokines IL-1 and TNF- α (179, 180), both of which have been shown to be produced by activated mast cells (181). Increased activity of IL-1 β is associated with diminished activity of the associated IL-1 receptor antagonist (IL-1RA), which is otherwise decreased in vulvodynia patients (182), and polymorphisms in allele 2 of the *IL-1RA* gene are more prevalent in patients with chronic vulvar pain (179, 183, 184). Moreover, inflammatory challenge results in a blunted IL-1RA response in vulvodynia patients *in vivo* (182) and increased IL-1 β among tissues derived from vulvodynia patients (185). There also exists strong evidence to link cytokine-induced activation of the HPA axis, particularly IL-1 β [For review: (186)]. Stress-induced elevations in IL-1 β increases HPA axis activity that is blocked with pre-treatment of IL-1RA (187). This

suggests that peripheral cytokines can influence central perceptions of pain routed through limbic and suprahypothalamic brain regions.

Another important immunoregulatory cytokine is IL10, typically considered to be a molecule with anti-inflammatory properties and serves to limit tissue damage and restore homeostasis (188, 189). Proinflammatory stimuli stimulate the secretion of IL10 from a variety of cell types of the innate and adaptive immune systems. For instance, secretion of IL10 from macrophages leads to downregulation of its own expression in an autocrine fashion (190). IL10 deficient mice develop inflammatory bowel disease that surprisingly is exacerbated in mast-cell deficient double knock-out mice (191). Conversely, excess IL10 can be detrimental to the system by hindering the host response to pathogenic presence and preventing resolution of tissue damage.

1.5 Exercise

The lack of feasible treatment options for chronic pain patients has led investigators to evaluate the efficacy of alternative treatment strategies. Human studies reveal that regular exercise leads to improvements in symptom severity among fibromyalgia patients and those with other types of chronic muscle pain (192). For instance, land or water based aerobic exercise and strength-based exercises improved aerobic physical fitness, physical function, and depression among fibromyalgia patients (193, 194). Exercise has also been shown to improve symptom scores of depression and anxiety (195-197). Although CPP does not involve overt musculoskeletal dysfunction, patients with IBS also benefit from exercise in randomized controlled trials (198-203). Although women with dysmenorrhea and premenstrual symptoms benefit from exercise (204, 205), the favorable effects of exercise for women with non-cyclical

urogenital pain have not been tested. For instance, survey analysis of IC/PBS patients revealed exercise as a helpful therapy among other complementary and alternative therapies, but randomized controlled trials have not been conducted to definitively support this finding (206).

Exercise has been shown to induce numerous beneficial changes in the neuroendocrine-immune system axis by altering the systemic cytokine profile in the periphery. For instance, it was discovered in the last ten years that skeletal muscle is itself a large endocrine organ. Skeletal muscle-derived cytokines are secreted during muscle contraction and these “myokines” have autocrine, paracrine, and endocrine effects (207). The first myokine discovered was IL6, which increases in plasma concentration with increasing duration and intensity of exercise that is not associated with muscle damage (154, 207-209). Serum concentrations of IL6 may increase 100-fold during sustained exercise, and levels decline sharply following the cessation of exercise. A surge in a classically pro-inflammatory cytokine is a somewhat conflicting idea, because IL6 is typically associated with chronic inflammation and immune activation with detrimental health effects (210). However, a transient increase in IL6 during exercise without a subsequent surge in TNF- α appears to have anti-inflammatory, rather than pro-inflammatory, effects. Peripheral administration of cytokines will also impact the central nervous system (211). Mechanisms by which peripheral cytokines influence synaptic development, neurogenesis, and brain circuitry are of enormous interest because administration of cytokines in dosages and time-courses that mimic acute activation of the innate immune system induces protective behavioral repertoires responsible for sustaining the organism during threats (212-215).

However, administration of cytokines that mimics chronic exposure is associated with chronic inflammation, neuropsychiatric symptoms, and neurogenic inflammation (216-218) and LPS-induced increase in circulating cytokines has been shown to directly activate the HPA axis and induce anhedonic-behaviors (219).

One way in which cytokines influence brain function is by interfering with the phosphorylation of tyrosine receptor kinase B (TrkB), the receptor for brain-derived neurotrophic factor (BDNF) (220). The TrkB signal is transduced via intracellular signaling cascades including stimulation of PI3K-Akt, PLC γ , and Erk pathways (221). These pathways control many aspects of cell physiology, including the promotion of growth and survival, plasticity, and differentiation. Expression of BDNF in the hippocampus is upregulated by neuronal activity secondary to exercise or cognitive challenges (222, 223). The mechanism by which hippocampal BDNF potentiates glutamatergic input to inhibitory interneurons in the hypothalamus is dependent on both TrkB and N-methyl-d-Aspartate receptors (NMDAR) signaling (224) and therein regulates negative feedback upon the HPA axis.

There is additional triangular cross-talk between skeletal muscle (acting as an endocrine organ), the hypothalamus, and the immune system (208). Exercise increases mRNA expression of neurotrophic factors important in synaptic plasticity, neurogenesis, and neuronal structure in the central nervous system (225). Systemically, exercise induces the secretion of myokines that exert broad effects on chronic low-levels of inflammation such as those observed in CPP syndromes (208, 226). Overall, while many mechanisms may be involved, extensive evidence suggests that exercise improves cytokine profiles reflective of chronic, low-level inflammation and

influences brain neurogenesis, plasticity, and behavior, mechanisms implicated as contributing to depression and CPP in human patients.

1.6 Neonatal Maternal Separation

Subsets of CPP patients are more likely to report histories of early life adverse events that in turn increase the risk of developing a CPP syndrome later in life (227, 228). The rodent model of neonatal maternal separation (NMS) provides an opportunity to study the effects of early life adverse events upon the structure and function of the nervous system by modeling chronic stress during the critical period of postnatal neurodevelopment. In fact, chronic stress experienced during early neurodevelopment has been proposed to contribute to functional pain disorders (57), yet the relationships between female urogenital pain and other comorbid somatic and behavioral conditions have largely gone unstudied in rodent models. Animal models of NMS have demonstrated increased colorectal hypersensitivity, aberrant responses to future stress, and increased susceptibility to infection or experimental colitis in adulthood (146, 229, 230). While this model of early life stress is well established in rats, it has not been used to study the effect on urogenital hypersensitivity in either rats or mice. Physiological and behavioral changes persisting to adulthood result from NMS during the first few weeks of life. This section will briefly describe the different NMS paradigms and the resultant molecular and behavioral changes in adulthood.

In the 1950's, Seymour Levine catalyzed the study of the field in which neonatal stimulation impacted the developing organism (231). Neonatal rat pups were subjected to stimuli such as 3-minutes of isolation, stroking, starvation, and shock, which resulted in altered responses to stressful stimuli later in life (232-237). It was during these early

studies that he proposed the idea of critical periods of development: when neonatal manipulation occurs during a specific window of postnatal development it manifests long-lasting effects (238). Over the following decades, differing experimental manipulations, nature of the insults, and duration of the condition across the critical window resulted in differential outcomes. From this body of knowledge emerged insights into the mechanisms behind resilience and vulnerability. Discrepancies between animals, strain, and sex have also been investigated.

Rodent NMS involves the physical separation of the pups from the presence of the dam on a scale from minutes to hours to days. Typically, the less severe models of deprivation (daily 15 minute separations) result in behaviors of resiliency: reduced anxiety-like behaviors, reduced CORT, and enhanced negative feedback onto the HPA axis (239, 240). Exposure to prolonged periods of separation, on the other hand, generally increases output of the HPA axis resulting in enhanced duration of ACTH and CORT release following a stressful event (64, 241-243) and vulnerability to anxiety-like behaviors, fearfulness, and susceptibility to ethanol preference (244-246). Significant effects of strain were observed in prepulse inhibition and startle-reflex (247) but not anxiety-like behaviors (248). One study by Millstein et al. (248) found the effect of mouse strain only impacted the behavior of the dam during NMS but not anxiety-like and depression-like behaviors exhibited by the pups in adulthood. While no studies have evaluated the effect of strain differences on visceral hyperalgesia following early life stress, the C57Bl/6 strain has already been shown to be an acceptable strain with which to evaluate stressful manipulation during the postnatal window (149, 230, 243, 249) and is the strain utilized in the present study.

Interestingly, there appears to be a propensity for sex discrepancies for behavioral alterations in models of rodent stress (250-255). Anhedonia, increased immobility during forced swim test, and decreased exploration of the open arms in the elevated plus maze, all behaviors thought to be reflective of depression or anxiety, were observed in male Sprague-Dawley rats following NMS (256). Romeo et al., (243) reported sex differences in adult mice following NMS where males exhibited increased and females exhibited decreased anxiety-like behavior. In contrast, Aisa et al., (241) reported increased immobility during the forced swim test in female rats following NMS but no change in anxiety-like behaviors. Taken together, the prevalence of chronic pain and affective disorders in women and the evidence of a sex-divergent manifestation of early life adverse events, indicates a need to evaluate adult behaviors in the NMS model.

1.7 Study Significance

The goal of this study is to evaluate pain modulatory mechanisms involved in female urogenital hypersensitivity resulting from early life stress. The central hypothesis of this proposal is that NMS reduces negative feedback onto the HPA axis and drives urogenital hypersensitivity and depressive behaviors in female mice, which can be attenuated by exercise. The following series of experiments determined the underlying mechanisms mediating these changes in behavior. Together, these investigations define the neuroendocrine changes responsible for urogenital hypersensitivity and altered behavior consequential to early life stress.

The first study in this dissertation tested the hypothesis that NMS produces long-lasting vaginal hypersensitivity, somatic allodynia, and anxiety-like behaviors in female

mice. Neonatal stress or injury in rodents produces a phenotype in adulthood similar to what is seen in IBS patients, such as visceral hyperalgesia, increased firing rates of dorsal horn neurons responding to colonic distension, and increased expression of TRP channel subtypes in sensory neurons that innervate the colon (146-149). Furthermore, patients diagnosed with IBS often present with secondary pain disorders affecting the pelvic viscera such as IC/PBS and vulvodinia or diffuse somatic pain syndromes such as fibromyalgia (257, 258). Experiments in this study evaluated the effect of NMS on the sensitivity of the vagina and hindpaw, open-field exploratory behavior, and expression of genes involved in negative feedback onto the HPA axis.

The second study in this dissertation determined the effect of neonatal and adult stress on bladder hypersensitivity and dysfunction, limbic regulation of the HPA axis, and downstream neurogenic inflammation of the bladder. Urogenital pain patients commonly report stress-related symptom onset or exacerbation of dormant symptoms, as well as difficulty coping with stressful situations and comorbid depression and/or anxiety (7, 15, 17, 259-263). Patients who suffered early life adverse events commonly display altered functional output of the HPA axis (76, 264-266), which is largely regulated by feedback from higher limbic structures via CRF and glucocorticoid receptors (65, 90). Experiments in this study evaluated the impact of NMS and water avoidance stress (WAS) on bladder and colorectal sensitivity and dysfunction, mast cell activity and evidence of neurogenic inflammation in the pelvic organs, and HPA axis output and regulation.

The final study in this dissertation evaluated the potential of exercise as an intervention for the visceral hypersensitivity and associated behaviors in NMS mice.

Exercise has been shown to confer protective benefits for neurological disorders such as Parkinson's disease, Alzheimer's disease, stroke, and traumatic brain injury (267-269). Clinically, exercise reduces the effects of depression and anxiety and associated perceptions of pain (199, 270, 271). Voluntary exercise in the post-weaning period has been shown to attenuate neuromolecular and behavioral changes associated with a dysfunctional HPA axis implicated in NMS-induced visceral hypersensitivity (272). This study evaluated visceral hypersensitivity, anhedonia behaviors, mast cell degranulation in the bladder, and hippocampal regulation of the HPA axis in sedentary and exercised NMS mice.

Women with idiopathic, chronic pelvic pain affecting the urogenital organs have a diminished quality of life, loss of productivity, and poor health outcomes. This project is therefore significant because it provides the first rodent model to demonstrate the impact of early life stress on female urogenital physiology, pain, and dysfunction, as well as novel preclinical evidence of voluntary exercise as a potent interventional method. Enhancing our understanding of these frustrating and debilitating conditions provides a framework by which to fully appreciate the lifelong impact of early life adverse events, evaluate future therapeutic options, and potentially treat a population of patients suffering from chronic pain.

Chapter 2

Vaginal hypersensitivity and hypothalamic-pituitary-adrenal axis dysfunction as a result of neonatal maternal separation in female mice

2.1 Abstract

Early life stress can permanently alter functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response and influences the perception of pain. Chronic pelvic pain patients commonly report having experienced childhood neglect or abuse, which increases the likelihood of presenting with comorbid chronic pain and/or mood disorders. Animal models of neonatal stress commonly display enhanced anxiety-like behaviors, colorectal hypersensitivity, and disruption of proper neuro-immune interactions in adulthood. Here, we tested the hypothesis that early life stress impacts vaginal sensitivity by exposing mice to neonatal maternal separation (NMS) for 3hr/day during the first two (NMS14) or three (NMS21) postnatal weeks. As adults, female mice underwent vaginal balloon distension (VBD), which was also considered an acute stress. Before or after VBD, mice were assessed for anxiety-like behavior, hindpaw sensitivity, and changes in gene and protein expression related to HPA axis function and regulation. NMS21 mice displayed significantly increased vaginal sensitivity compared to naïve mice, as well as significantly reduced anxiety-like behavior at baseline, which was heightened following VBD. NMS21 mice exhibited significant thermal and mechanical hindpaw hypersensitivity at baseline and following VBD. NMS14 mice displayed no change in anxiety-like behavior and only exhibited significantly increased hindpaw mechanical and thermal sensitivity following VBD. Centrally, a significant decrease in negative regulation of the HPA axis was observed in the hypothalamus and hippocampus of NMS21 mice. Peripherally, NMS and VBD

affected the expression of inflammatory mediators in the vagina and bladder.

Corticotropin releasing factor (CRF) receptor and transient receptor potential (TRP) channel protein expression was also significantly, and differentially, affected in vagina, bladder, and colon by both NMS and VBD. Together these data indicate that NMS affects both central and peripheral aspects of the HPA axis, which may drive changes in vaginal sensitivity and the development of comorbid chronic pain and mood disorders.

2.2 Introduction

Growth and development during the formative years, beginning prenatally and lasting until age 5, occurs at a pace exceeding that of any subsequent stage of life (273). As a result, the nervous system is remarkably pliant to nurturing and adverse events during this period of development (274). Psychological stressors, such as childhood neglect or abuse; low socioeconomic status; and witnessing domestic violence, parental discord, or crime in the home, have profound lifelong impacts on behavior and also serve as risk factors for functional pain disorders later in life (275-278). Accordingly, a significant subset of patients with functional pelvic pain disorders, including irritable bowel syndrome (IBS), interstitial cystitis/painful bladder syndrome (IC/PBS), and vulvodynia, report a history of adverse childhood events such as abuse or neglect (279-281). Subsets of these patients report stress-related symptom onset or increased severity, have difficulty coping with stressful situations, and suffer from depression and/or anxiety (7, 17, 282).

The comorbidity between mood disorders and chronic pelvic pain is due, in part, to altered functioning of corticotropin-releasing factor (CRF)-responsive regions of the brain, including the hypothalamic-pituitary-adrenal (HPA) axis, which regulates stress response and influences the perception of pain (227, 283, 284). Under stressful conditions, CRF is secreted from the paraventricular nucleus (PVN) of the hypothalamus and induces the systemic release of adrenocorticotrophic hormone

(ACTH) from the anterior pituitary corticotrophs. ACTH then stimulates glucocorticoid (cortisol in humans and corticosterone in rodents), synthesis and secretion from the adrenal cortex (65, 90). Activity of the HPA axis is largely regulated by positive and negative feedback within the hypothalamus and from higher limbic structures, including the amygdala and hippocampus, which stimulate and inhibit CRF release, respectively (65, 90). Glucocorticoid-driven negative feedback occurs through two receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which are relatively slow-acting and effect long-term changes in gene transcription (79). The two CRF receptors, CRF₁ and CRF₂, bind CRF and the related Urocortins (Ucn1-3) with varying affinity and work in opposition to one another to facilitate and depress activation of the HPA axis, respectively (285).

Neonatal maternal separation (NMS) in rodents has been used for several decades as a model of early life stress that significantly affects functioning of the HPA axis. NMS has generally been shown to increase the output of the HPA axis, evidenced as enhanced anxiety-like behaviors and prolonged release of ACTH and corticosterone following a stressful event (64, 241-243). CRF production in both the PVN and the central nucleus of the amygdala (CeA) (241, 242, 286), as well as altered hypothalamic and limbic CRF receptor and GR expression (64, 241, 242, 287), have been reported in NMS rats. Peripheral CRF release, in response to chronic stress, has been shown to promote neurogenic inflammation through mast cell degranulation and increased cytokine expression and neuropeptide release (288). Mast cell infiltration and hypertrophy of sensory innervation have been reported in biopsies from patients with IBS (124, 289, 290), IC (291, 292), and vulvodynia (293, 294). Involvement of transient

receptor potential vanilloid 1 (TRPV1), the capsaicin receptor necessary for the development of inflammatory hyperalgesia (136, 295), has also been shown to contribute toward acute stress-induced colonic hypersensitivity in NMS rats (116). Together these studies suggest that NMS increases the activity of the HPA axis resulting in central and peripheral changes underlying increased anxiety-like behaviors and visceral sensitivity and neurogenic inflammation.

Stress or direct organ irritation/inflammation during early development has been shown to permanently enhance sensitivity and nociceptive signaling within the gastrointestinal (147, 149, 296-298) and urinary (299, 300) systems of rodents, therefore, we hypothesized that early life stress would also increase sensitivity within the reproductive system concordant with impaired regulation of the HPA axis. Previous studies of early life stress in mice have largely performed NMS only during the stress hyporesponsive state (postnatal day [P] 1-14) (301), and subsequently reported a mild anxiety-like phenotype (248), or a decrease in behavioral anxiety (243, 302, 303). In the current study, we compared adult female mice that underwent NMS during the first two weeks of life to those that underwent NMS during the entire preweaning period (P1-21), to determine if stress following the stress hyporesponsive state, yet still during final synaptic maturation within the limbic system (304), would result in a more pronounced phenotype. To assess vulnerability to adult stress exposure, mice were tested for anxiety-like behavior and hindpaw sensitivity, both prior to and following vaginal balloon distension (VBD), which quantified vaginal sensitivity and was considered an acute stress exposure. Protein and mRNA expression of mediators involved in regulating the HPA axis both centrally and peripherally, as well as protein expression of TRPV1, and

the related TRPA1, within the vagina, bladder, and colon were measured to determine how early life and adult stress may influence comorbid pelvic pain disorders.

2.3 Materials and methods

Animals

Experiments were performed on female C57Bl/6 mice (Charles River, Wilmington, MA) born and housed in the Research Support Facility at the University of Kansas Medical Center. Mice were housed on a 12-hour light cycle from 600 to 1800 hours and received water and food ad libitum. All research performed conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals in accordance with the guidelines specified by the University of Kansas Medical Center Animal Care and Use Protocols.

Neonatal maternal separation

Beginning on postnatal day 1 (P1, date of birth was considered P0), pups were removed daily from their home cages for 180 minutes (1100 to 1400 hours) and placed as a litter, with a small amount of home bedding material, into a clean glass beaker and held at 34°C and 50% humidity. Each litter was weighed *en masse* prior to and at the end of the separation period. NMS14 mice underwent daily separation from P1 through P14 and then remained undisturbed, with the exception of routine animal husbandry, in their home cages until weaning at P22. NMS21 mice underwent daily separation from P1 through P21 and were weaned at P22. Naïve mice were born in-house and remained undisturbed, with the exception of daily weighing and routine animal husbandry, in their home cages until weaning at P22. Three separate cohorts of

NMS21 mice and two separate cohorts of NMS14 mice were used in this study. Each cohort of NMS14 and NMS21 mice was compared to a corresponding naïve group of mice that were born, housed, and weaned during the same time frame to avoid potential complications arising from variations in prenatal shipping conditions, housing environment, and investigator handling.

Experimental design

All naïve, NMS14, and NMS21 mice were subjected to vaginal balloon distension (VBD) as adults (between 9-36 weeks of age, Table 2.1). VBD was considered a stressor in these experiments, as colorectal distension (CRD) significantly elevated serum corticosterone levels in a separate cohort of naïve female mice (761.6 ± 83.7 ng/ml) compared to age-matched non-distended naïve female mice (236.8 ± 106 ng/ml; $p < 0.05$, Mann-Whitney test, $n=5$). Either prior to (Baseline group) or following VBD (post-VBD group), mice underwent open field, thermal analgesiometer, and von Frey monofilament testing, as described in Table 2.1. With the exception of naïve mice in the NMS14 cohort, separate groups of mice were used for baseline and post-VBD behavioral measurements. All mRNA and protein analysis was performed on tissue from the same cohort of naïve and NMS21 mice that was euthanized a week after VBD testing or from age-matched non-VBD exposed mice.

Behavioral analysis

All mice underwent a 30-minute acclimatization period within the testing room for at least one day prior to each behavioral test. For both thermal and mechanical hindpaw

sensitivity testing, the mice were allowed to acclimate to the apparatus for 30 minutes prior to testing and the experimenter was blinded to the group status of the mice.

Table 2.1 Age of mice at experimental time points.

	Open Field	Thermal Hindpaw	Mechanical Hindpaw	Vaginal Balloon Distension	Open Field	Thermal Hindpaw	Mechanical Hindpaw
NMS14							
Baseline*	6	9	10	12-13			
Post-VBD*				12-13	15	15.5	17
VBD only				9-10			
NMS21							
Baseline	6	6.5	7	9			
Post-VBD				9-11	12	14	14.5
VBD only				30-36			

Table 2.1 NMS14, NMS21, and corresponding naïve mice underwent behavioral or physiological testing at the above noted ages (in weeks). Mice were assessed for anxiety-like behaviors using an open field (OF) actimeter and/or thermal and mechanical hindpaw sensitivity either before (Baseline) or after (post-VBD) vaginal balloon distension (VBD). With the exception of the naïve mice in the NMS14 cohort, separate groups of mice were used for baseline and post-VBD measurements. All mRNA and protein expression analysis utilized tissue from naïve and NMS21 mice exposed to OF and/or hindpaw measurements prior to VBD and euthanized one week following VBD or from naïve and NMS21 mice born and euthanized at the same time, but without exposure to VBD.

Open field testing:

Activity in NMS14 (baseline: n=5, post-VBD: n=8), NMS21 (baseline: n=8, post-VBD: n=8) and naïve (NMS14 cohort, baseline: n=6, post-VBD: n=8; NMS21 cohort, baseline: n=7, post-VBD: n=7) mice was measured using a Force Plate Actimeter (BASi, San Diego, CA), which consists of a rigid, low-mass horizontal plate (44cm×44cm) coupled to high sensitivity force transducers on each corner. A Plexiglas enclosure rests a few millimeters above the plate to create a transparent enclosure, all of which rests within a light and sound-attenuated box. Animals were individually placed into the middle of the testing arena and allowed to move freely for 10 minutes. During this time, the software recorded the distance traveled and position of the mouse. The total distance traveled and percent of time spent in the perimeter (outermost 8.25cm; increased time spent in the perimeter is indicative of anxiety (305)) was calculated and binned and the data from the second 5 min bin is reported here.

Thermal analgesiometer testing:

NMS14 (baseline: n=5, post-VBD: n=8), NMS21 (baseline: n=8, post-VBD: n=8) and naïve (NMS14 cohort, baseline: n=6, post-VBD: n=8; NMS21 cohort, baseline: n=8, post-VBD: n=8) mice were placed in individual clear plastic chambers (11×5×3.5cm) on the 30°C heated glass surface of a thermal analgesiometer (UARDG; Department of

Anesthesiology, University of California San Diego, La Jolla, CA). A high intensity light (4.25 Amperes) was directed at the plantar aspect of the hindpaw and the latency to withdrawal from the stimulus was automatically recorded within 0.01 second. Alternating hindpaws were tested for a total of three times per side with a minimum of 5 minutes between applications. The stimulus terminated automatically at 20 seconds to avoid tissue damage. Individual responses were averaged together/mouse and group means were determined as previously described (306).

Von Frey monofilament testing:

NMS14 (baseline: n=4, post-VBD: n=8), NMS21 (baseline: n=8, post-VBD: n=8) and naïve (NMS14 cohort, baseline: n=6, post-VBD: n=7; NMS21 cohort, baseline: n=8, post-VBD: n=8) mice were placed into individual clear plastic chambers (11×5×3.5cm) on a wire mesh screen elevated 55 cm above a table. The up-down method was performed to test mechanical sensitivity using a standard set of von Frey monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.08, 4.31, 4.74g; Stoelting, Wood Dale, IL)(307). Beginning with the 3.22g monofilament, mice received a single application to the plantar surface of the right hindpaw. A negative response was followed by the next larger filament and a positive response (considered a brisk withdrawal of the paw) was followed by the next smaller gram 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 von Frey monofilament applied in the trial series was used to calculate a 50% g threshold for each mouse and group means were determined as previously described (308).

Electromyographic electrode implantation and vaginal balloon distension

The visceromotor response (VMR) to vaginal balloon distension (VBD) was evaluated in adult (≥ 9 weeks) NMS14 ($n=19$) and NMS21 ($n=19$) mice, along with corresponding naïve cohorts ($n=13$ and 20 , respectively). Electrode implantation was performed as previously described (249). Under inhaled isoflurane (4% induction, 2.5% maintenance) and aseptic conditions, the bare ends of two Teflon-coated stainless steel wires (3mm; Grass Technologies, West Warwick, RI) were inserted into the right lateral abdominal musculature, secured via 5-0 prolene sutures, tunneled subcutaneously to a small incision made in the nape of the neck and externalized for access during testing. Skin incisions were closed using 5-0 silk suture. Following recovery from anesthesia, mice were housed singly and allowed to recover for a minimum of 4 days before undergoing testing.

To facilitate balloon insertion and obtain proper restraint during VBD, mice were briefly sedated with inhaled isoflurane and a custom-made latex balloon (1cm in length) was inserted into the vagina and secured to the base of the tail with tape. The mouse was then placed into a Broome-style rodent restraint (Kent Scientific, Torrington, CT), the free ends of the electrode wires were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA), and the mice were allowed to recover from anesthesia for 30 minutes. VBD was produced by inflating the balloon with air from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO) and a separate pressure monitor (World Precision Instruments, Sarasota, FL) was used to regulate the pressure inside of the balloon. Each pressure (40, 60, 80, 100 and 120mmHg) was applied three times for 20

seconds with a 4-minute rest period in between. A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow through the system. Electromyographic (EMG) activity was amplified, filtered and recorded on a personal computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) for off-line analysis. VMR was quantified by measuring the area under the curve for the entire distension period divided by the duration of the distension and expressed as a percent of baseline activity (10s prior to VBD).

mRNA extraction and qRT-PCR

Mice were overdosed with inhaled isoflurane (>5%) and transcardially perfused with ice cold 0.9% saline. Brains were removed and frozen on dry ice. Hypothalamus, hippocampus, and amygdala were dissected, immediately snap frozen in liquid nitrogen, and stored at -80°C. The entire length of the vagina (not including the cervix), urinary bladder, and 1.5cm distal segment of the colon were removed, bisected longitudinally (to facilitate both mRNA and protein [see below] analysis), snap frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated from dissected tissues using Trizol reagent (Ambion, Austin, TX) and RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and cDNA was synthesized from total RNA (0.63 µg) 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 20µM primers (Integrated DNA

Technologies, Coralville, IA) listed in Table 2.2. GAPDH was used as a control gene for brain tissues and β -actin was used as a control for vagina, bladder, and colon.

Western blot

Table 2.2 Primers used for real-time PCR analysis

Gene	Forward (5' – 3')	Reverse (3' – 5')
CRF	CCTCAGCCGGTTCTGATCC	GCGGAGGAAGTATTCTTCACCC
Ucn2	ACCCGTGTCATACTCTCCCTG	CAGCCTTGTAACGAGCCTG
CRF ₁	CCCTGCCTTTTTCTACGGTGT	TTCCCGGTAGCCATTGTTTGT
CRF ₂	CCTGTGGACACTTTTGGAGCA	TGTTGCAGTAGGTGTAGGGAC
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
MR	GAAAGGCGCTGGAGTCAAGT	CCATGTAGCTGTTCTCATTGGT
IL10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
IL6	CTGCCAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
TNF α	ATGGGCTTTCCGAATTCAC	GAGGCAACCTGACCACTCTC
Art	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
NGF	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT
β -actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT

Total protein was isolated from approximately 50 mg of snap-frozen vagina, bladder, and colon using Cell Extraction Buffer (Invitrogen, Grand Island, NY) containing Halt protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA) and Na_3VO_4 (Sigma, St. Louis, MO). Protein concentrations were determined using a D_C protein assay (ThermoFisher). Samples were reduced by heating to 95°C for 5 minutes in the presence of 2-mercaptoethanol, subjected to SDS-PAGE (Criterion 4% to 12% Bis-Tris gels; Bio-Rad, Hercules, CA), and transferred to Nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) by Criterion Blotter wet transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature in 5% milk in Tris-buffered saline with Tween-20 (TBST) and incubated overnight at 4°C with antisera to CRF_1 (1:500; Millipore, Billerica, MA), CRF_2 (1:800; Millipore), TRPV1 (1:1000; Alomone Labs, Jerusalem, Israel), or TRPA1 (1:1000; Aviva Systems Biology, San Diego, CA) and GAPDH (1:2000; Cell Signaling Technology, Danvers, MA) diluted in 5% milk in TBST. Membranes were then washed with TBST and incubated for 1 hour with anti-rabbit secondary antibody (1:10,000; Cell Signaling, Danvers, MA). Densitometry was performed using Quantity One 4.6.9 software (Bio-Rad).

Statistics

Calculations were made using Microsoft Excel and statistical analysis was performed using Student's t-test, and 1-way or 2-way (with or without repeated measures) analysis of variance (ANOVA) followed by Fisher's least significant

difference (LSD) or Bonferroni's posttest (IBM SPSS Statistics, IBM Corporation, Armonk, NY; GraphPad Prism, GraphPad Software, La Jolla, CA), as denoted in the manuscript. All data are expressed as mean \pm SEM. A p value of less than 0.05 was considered significant.

2.4 Results

NMS for 21 days impacts vaginal sensitivity

The effect of NMS and length of separation on vaginal sensitivity was determined by measuring the VMR during VBD in adult female NMS14, NMS21, and corresponding naïve mice. Similar to VMR during colorectal distension (149), the EMG activity of the abdominal musculature significantly increased in response to greater intraballoon pressure applied within the vagina (Figure 2.1A-B). NMS14 mice did not display significantly increased vaginal sensitivity compared to naïve counterparts (Figure 2.1C). In contrast, NMS21 mice had significantly greater VMR over the entire distension series than their naïve counterparts and at every individual pressure beyond the lowest in posthoc comparison (Figure 2.1D).

NMS and VBD affect anxiety-like behaviors

To determine the effect of NMS and length of separation on anxiety-like behavior, female NMS14, NMS21 and corresponding naïve mice were subjected to open field testing on a force plate actimeter to measure exploratory behavior either prior to or following VBD. NMS14 mice did not differ significantly from naïve mice with regards to time spent in the perimeter of the open field, either prior to or following VBD (Figure 2.2A). In contrast, NMS21 mice displayed significantly reduced anxiety-like behaviors

at baseline, measured as less time spent in the perimeter of the open field (Figure 2.2B). Following VBD, NMS21 mice spent significantly more time in the perimeter of the

Figure 2.1

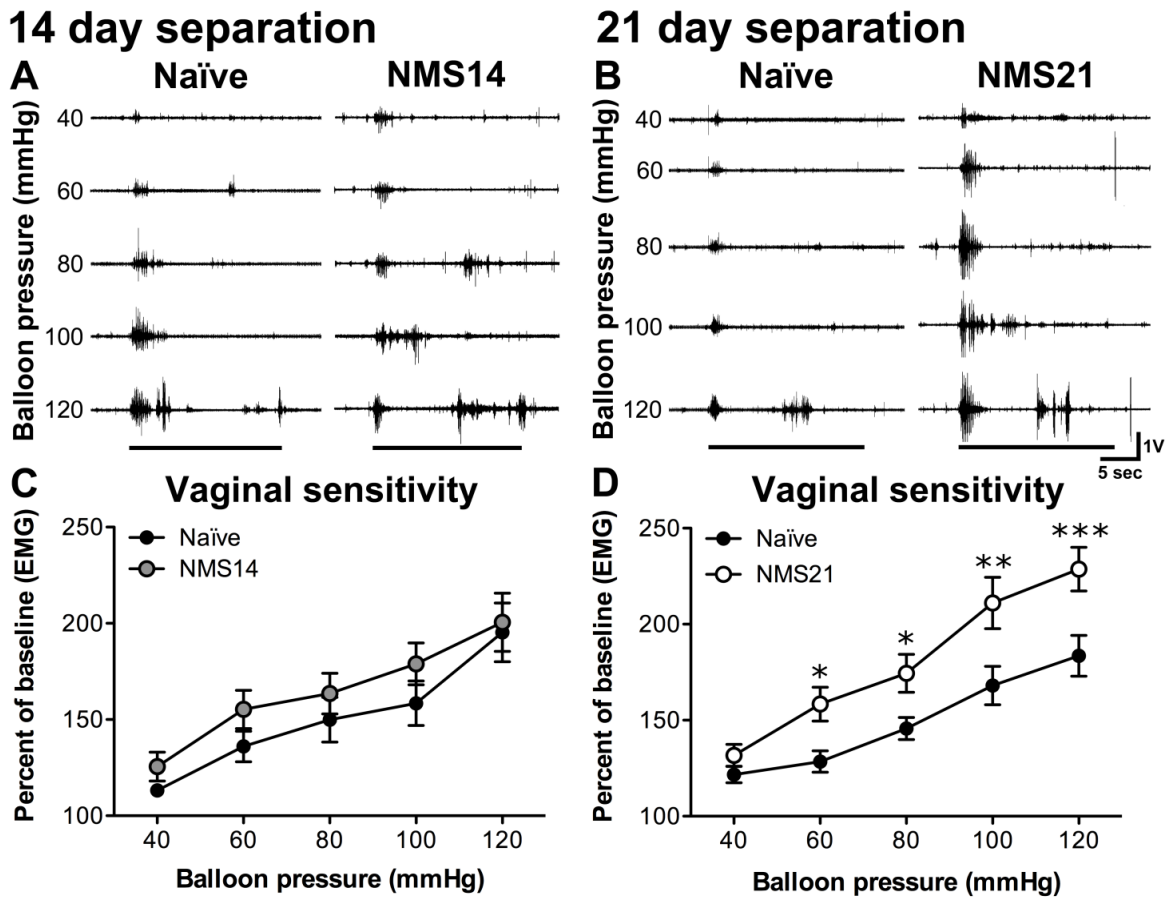


Figure 2.1 Vaginal sensitivity was significantly affected by neonatal maternal separation (NMS). The visceromotor response (VMR) of NMS14 (**A**), NMS21 (**B**), and corresponding naïve mice was measured by recording the electromyographic (EMG) activity of the abdominal musculature during graded balloon distension of the vagina (VBD). **C**) No significant difference in VMR was determined between NMS14 and naïve mice ($p > 0.05$). **D**) NMS21 mice had a significantly higher VMR than naïve mice over the entire distension series ($p < 0.01$), as well as at every pressure above the lowest applied. *, **, *** $p < 0.05, 0.01, 0.001$, two-way RM ANOVA and Fisher's LSD.

Figure 2.2.

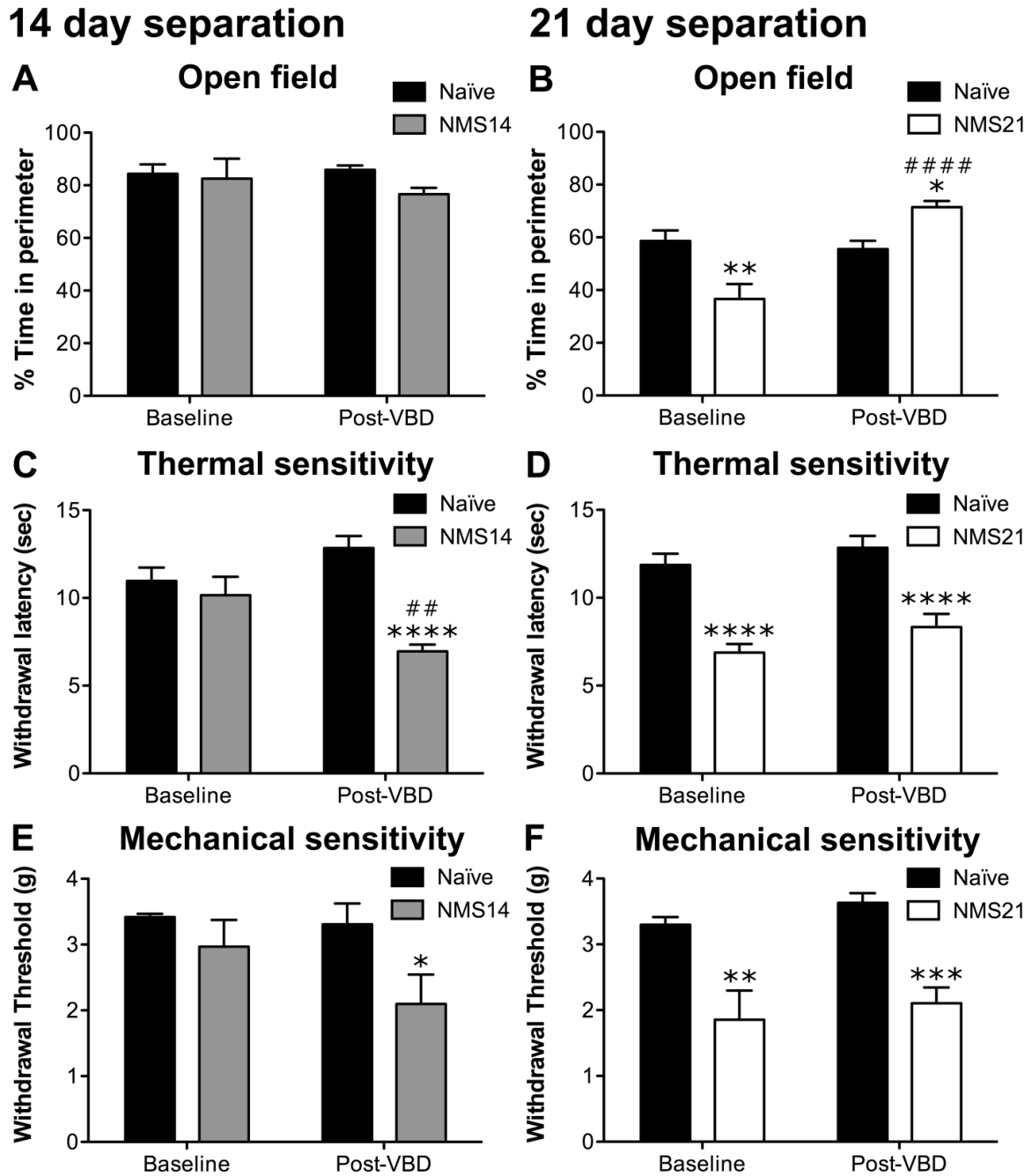


Figure 2.2 Anxiety-like behavior and hindpaw sensitivity were dose-dependently affected by neonatal maternal separation (NMS) and vaginal balloon distension (VBD). The percent of time spent in the perimeter of an open field actimeter was recorded as a measure of behavioral anxiety in NMS14 (A), NMS21 (B), and corresponding naïve mice, both prior to (Baseline) and following VBD (Post-VBD). **A)** No significant effect of NMS or VBD on exploratory behavior was observed within the NMS14 group. **B)** NMS21 mice displayed a significant decrease in the percent of time spent in the perimeter of the open field at baseline compared to naïve mice. Following VBD, NMS21 mice spent a significantly larger percentage of their time in the perimeter compared to both post-VBD naïve mice and baseline NMS21 mice. **C)** The withdrawal latency of the hindpaw to a thermal stimulus was significantly shorter in NMS14 mice only following VBD, compared to both post-VBD naïve and NMS14 baseline measurements. **D)** NMS21 mice displayed a significantly shorter withdrawal latency to thermal stimulation of the hindpaw than naïve mice, both at baseline and post-VBD. **E)** The withdrawal threshold of the hindpaw to a mechanical stimulus was significantly lower in NMS14 mice only following VBD, compared to post-VBD naïve mice. **F)** NMS21 mice also displayed a significantly lower withdrawal threshold to mechanical stimulation of the hindpaw than naïve mice, both at baseline and post-VBD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. naïve, ## $p < 0.01$, ##### $p < 0.0001$ vs. baseline; two-way ANOVA and Bonferroni posttest.

open field than naïve mice or baseline NMS21 measurements, indicating an increase in anxiety-like behaviors (Figure 2.2B).

Length of NMS affects hindpaw sensitivity

The impact of NMS and length of separation on hindpaw thermal and mechanical sensitivity was determined either prior to or following VBD. At baseline, NMS14 mice showed no significant difference in thermal or mechanical hindpaw sensitivity compared to naïve counterparts (Figure 2.2C, E). Following VBD, NMS14 mice displayed significantly reduced thermal withdrawal latencies and mechanical withdrawal thresholds, compared to naïve counterparts (Figure 2.2C, E), indicative of hypersensitivity. In comparison, NMS21 mice had significantly lower thermal withdrawal latencies and mechanical withdrawal thresholds both at baseline and following VBD, compared to naïve counterparts (Figure 2.2D, F). VBD had no significant effect on naïve thermal (Figure 2.2C-D) or mechanical (Figure 2.2E-F) hindpaw sensitivity in either cohort.

NMS and VBD disrupted regulatory gene expression of the HPA axis

To determine the impact of NMS and VBD on gene expression within central structures involved in the regulation and output of the HPA axis, we used RT-PCR to measure CRF, Ucn2, CRF₁, CRF₂, GR, and MR mRNA levels in the hypothalamus, amygdala, and hippocampus of naïve and NMS21 mice with and without exposure to VBD. In the hypothalamus, NMS had a significant effect on increasing CRF and GR mRNA levels, as well as inducing a trend towards increased Ucn2 and CRF₁ mRNA levels (Figure 2.3A). NMS, as well as VBD, significantly decreased the level of CRF₂

Figure 2.3

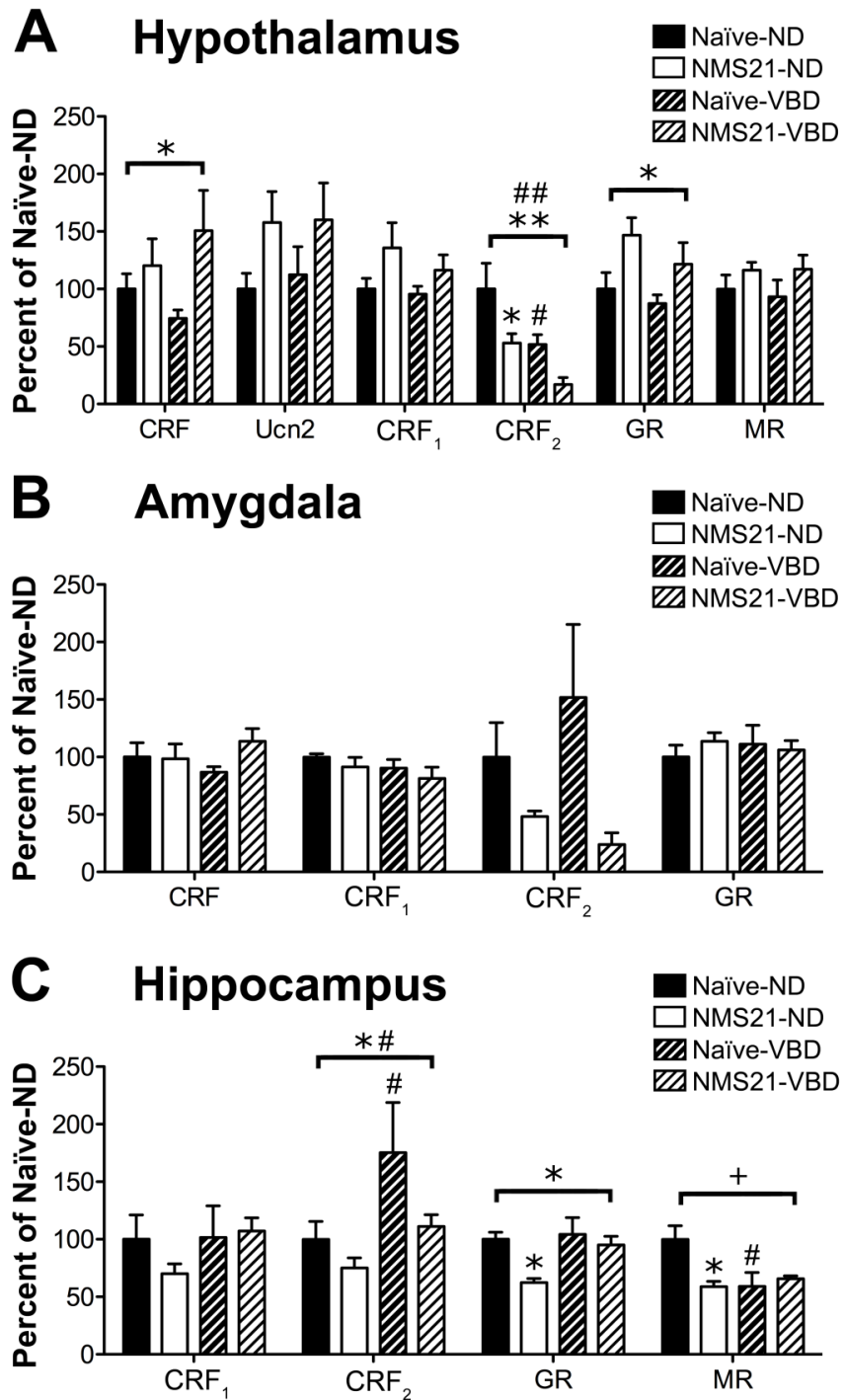


Figure 2.3 Neonatal maternal separation (NMS) and vaginal balloon distension (VBD) significantly impacted negative regulatory feedback onto the hypothalamic-pituitary-adrenal (HPA) axis. Gene expression was measured by RT-PCR in the hypothalamus **(A)**, amygdala **(B)**, and hippocampus **(C)** of naïve and NMS21 mice that either did not (Naïve-ND and NMS21-ND) or did undergo VBD (Naïve-VBD and NMS21-VBD). **A)** In the hypothalamus, NMS significantly increased CRF and GR mRNA expression and induced a trend towards increased Ucn2 and CRF₁ mRNA expression. NMS and VBD both significantly decreased CRF₂ mRNA expression with NMS21-ND and naïve-VBD expressing significantly less CRF₂ mRNA than naïve-ND. **B)** NMS caused a trend towards decreased CRF₂ mRNA expression in the amygdala ($p = 0.051$). **C)** In the hippocampus, NMS significantly decreased the mRNA expression of CRF₂ and GR with NMS21-ND expressing significantly less GR mRNA than naïve-ND, whereas VBD significantly increased CRF₂ mRNA expression with naïve-VBD expressing significantly more CRF₂ mRNA naïve-ND. NMS and VBD had a significant interaction effect on decreasing MR mRNA expression in the hippocampus with NMS21-ND and naïve-VBD both expressing significantly less MR than naïve-ND. Housekeeping gene: β -actin. Brackets indicate significant impact of NMS (*, ** $p < 0.05, 0.01$), VBD (^{#,##} $p < 0.05, 0.01$) or an NMS/VBD interaction (⁺ $p < 0.05$), two-way ANOVA; * $p < 0.05$ vs. naïve, # $p < 0.05$ vs. baseline, Bonferroni posttest.

mRNA in the hypothalamus, with non-distended NMS21 mice and post-VBD naïve mice both expressing significantly less CRF₂ mRNA than non-distended naïve mice (Figure 2.3A). In the amygdala, only CRF₂ was affected by NMS with a trend towards decreased expression (Figure 2.3B). In the hippocampus, NMS significantly decreased the mRNA levels of CRF₂ and GR (Figure 2.3C). In opposition to the effect of VBD on CRF₂ in the hypothalamus, VBD had a significant impact on increasing CRF₂ mRNA levels in the hippocampus, particularly in naïve mice (Figure 2.3C). NMS and VBD had a significant interaction effect on MR mRNA levels in the hippocampus, with non-distended NMS21 mice and post-VBD naïve mice both expressing significantly less MR mRNA than non-distended naïve mice (Figure 2.3C).

NMS and VBD impact adult vaginal inflammatory mediator, CRF receptor, and TRP channel expression patterns

We performed RT-PCR to determine mRNA levels of inflammatory mediators shown to be affected by NMS (309-311) and Western blotting to determine the potential contribution of peripheral CRF receptor and TRP channel protein expression on vaginal sensitivity following NMS and/or VBD. NMS alone did not significantly impact inflammatory mediator expression within the vagina. However, NMS and VBD had a significant interaction effect on both IL10 and TNF α mRNA levels, with post-VBD NMS21 mice expressing significantly higher levels of both genes than post-VBD naïve mice (Figure 2.4A). VBD was shown to significantly impact Artemin (Art) mRNA levels, with post-VBD NMS21 mice expressing significantly higher levels of Art mRNA than non-distended NMS21 mice. NMS and VBD also had a significant interaction effect on CRF₁ protein expression in the vagina, with post-VBD NMS21 mice expressing

Figure 2.4

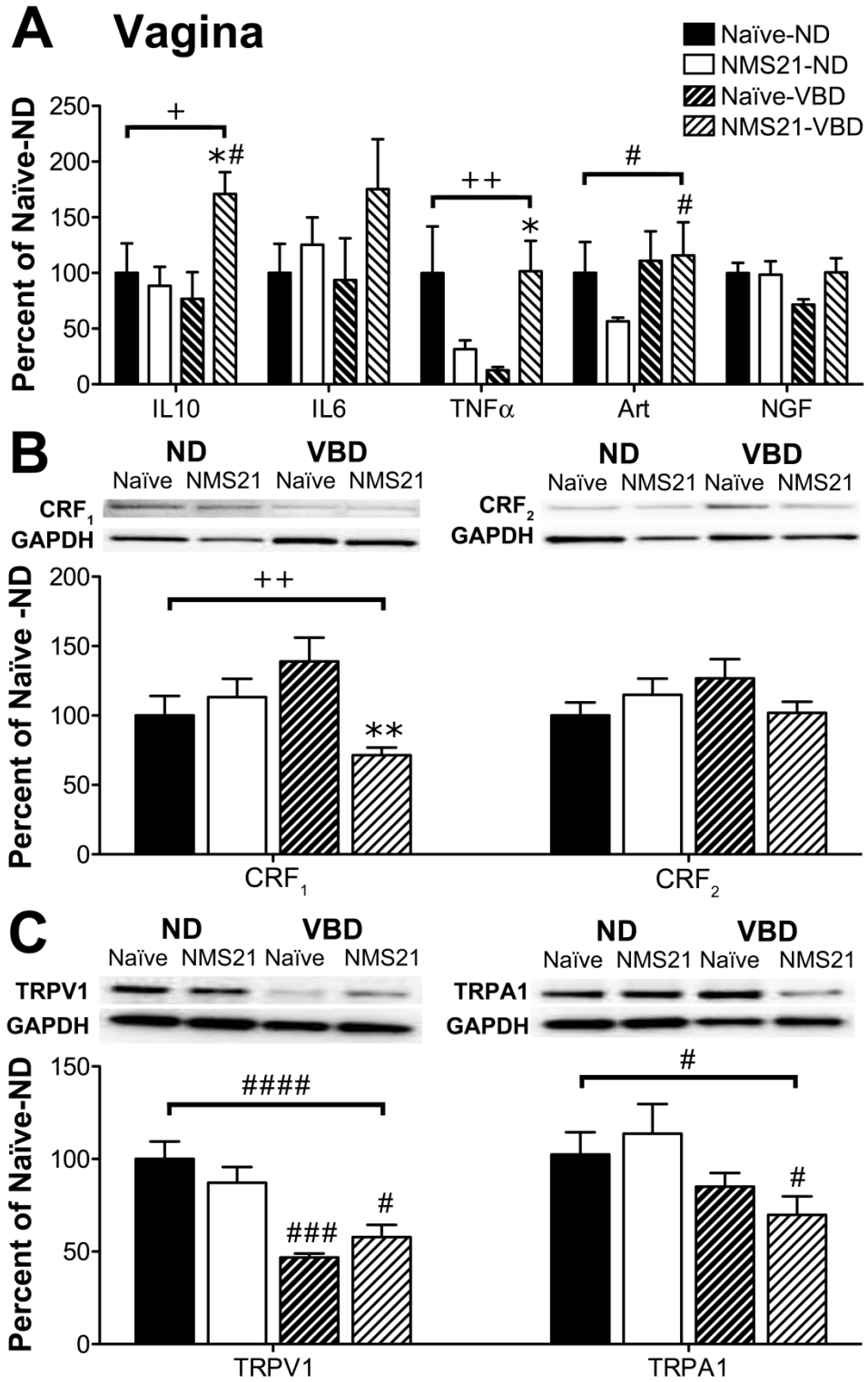


Figure 2.4 Neonatal maternal separation (NMS) and vaginal balloon distension (VBD) significantly disrupted gene and protein expression within the vagina. RT-PCR (**A**) and Western blot (**B-C**) was performed on vagina from naïve and NMS21 mice that either did not (Naïve-ND and NMS21-ND) or did undergo VBD (Naïve-VBD and NMS21-VBD).

A) NMS and VBD had a significant interaction effect on IL10 mRNA expression with NMS21-VBD expressing significantly more IL10 mRNA than NMS21-ND or naïve-VBD. NMS and VBD also had a significant interaction effect on TNF α mRNA expression with NMS21-VBD expressing significantly more TNF α mRNA than naïve-VBD. VBD significantly increased Artemin (Art) mRNA expression with NMS21-VBD expression significantly more Art than NMS21-ND. IL6 and nerve growth factor (NGF) transcripts were not affected by either NMS or VBD. **B)** Representative Western blots are shown for CRF₁, CRF₂, and corresponding GAPDH protein expression with bands at 55, 47, and 35kD, respectively. NMS and VBD had a significant interaction effect on CRF₁ protein expression in the vagina with NMS21-VBD expressing significantly less CRF₁ protein than naïve-VBD. CRF₂ protein expression in the vagina was unaffected by NMS or VBD. **C)** Representative Western blots are shown for TRPV1, TRPA1, and corresponding GAPDH protein expression with bands at 85, 127, and 35kD, respectively. VBD significantly decreased the protein expression of both TRPV1 and TRPA1 in the vagina. Naïve-VBD and NMS21-VBD both expressed significantly less TRPV1 than their non-distended counterparts. NMS21-VBD also expressed significantly less TRPA1 protein than NMS21-ND. Housekeeping gene: GAPDH (**A**). Brackets indicate significant impact of VBD (^{#,####} $p < 0.05, 0.0001$) or an NMS/VBD interaction

(^{+,++} $p < 0.05, 0.01$), two-way ANOVA; *, ** $p < 0.05, 0.01$ vs. naïve, ^{#,###} $p < 0.05, 0.001$ vs. baseline, Bonferroni posttest.

significantly less CRF₁ protein than post-VBD naïve mice (Figure 2.4B). Despite being significantly altered in central structures, the protein expression of CRF₂ was not altered by either NMS or VBD in the vagina (Figure 2.4B). NMS also did not affect protein expression of TRPV1 or TRPA1 in the vagina; however VBD significantly decreased the expression of both proteins compared to non-distended counterparts (Figure 2.4C).

Influence of NMS and VBD on inflammatory mediator, CRF receptor, and TRP channel expression patterns in the bladder and colon

Up to 50% of women with chronic pelvic pain experience symptoms from more than one disorder (7, 13, 17-22). To determine how NMS and VBD impact neighboring viscera, which have been shown to be affected by early adverse events (147, 149, 296, 300), we performed RT-PCR to measure mRNA levels of inflammatory mediators and Western blotting to assess CRF receptor and TRP channel protein expression in the bladder and colon. In the bladder, NMS significantly increased IL10 and NGF mRNA levels and VBD significantly decreased IL6 mRNA (Figure 2.5A). In the colon, only a trend towards NMS-induced increased IL10 mRNA levels was observed. Peripheral CRF receptor expression was differentially affected in the bladder and colon by NMS and VBD (Figure 2.6A). In the bladder, CRF₁ protein expression was significantly increased by VBD with post-VBD NMS21 bladder expressing significantly more CRF₁ than non-distended NMS bladder (Figure 2.5B). CRF₁ protein expression was not impacted by NMS or VBD in the colon; however NMS and VBD had a significant interaction effect on CRF₂ in the colon with non-distended NMS21 expressing significantly more CRF₂ protein than non-distended naïve or post-VBD NMS21 (Figure 2.6B). As in vagina, TRPV1 protein expression was significantly decreased post-VBD in

Figure 2.5

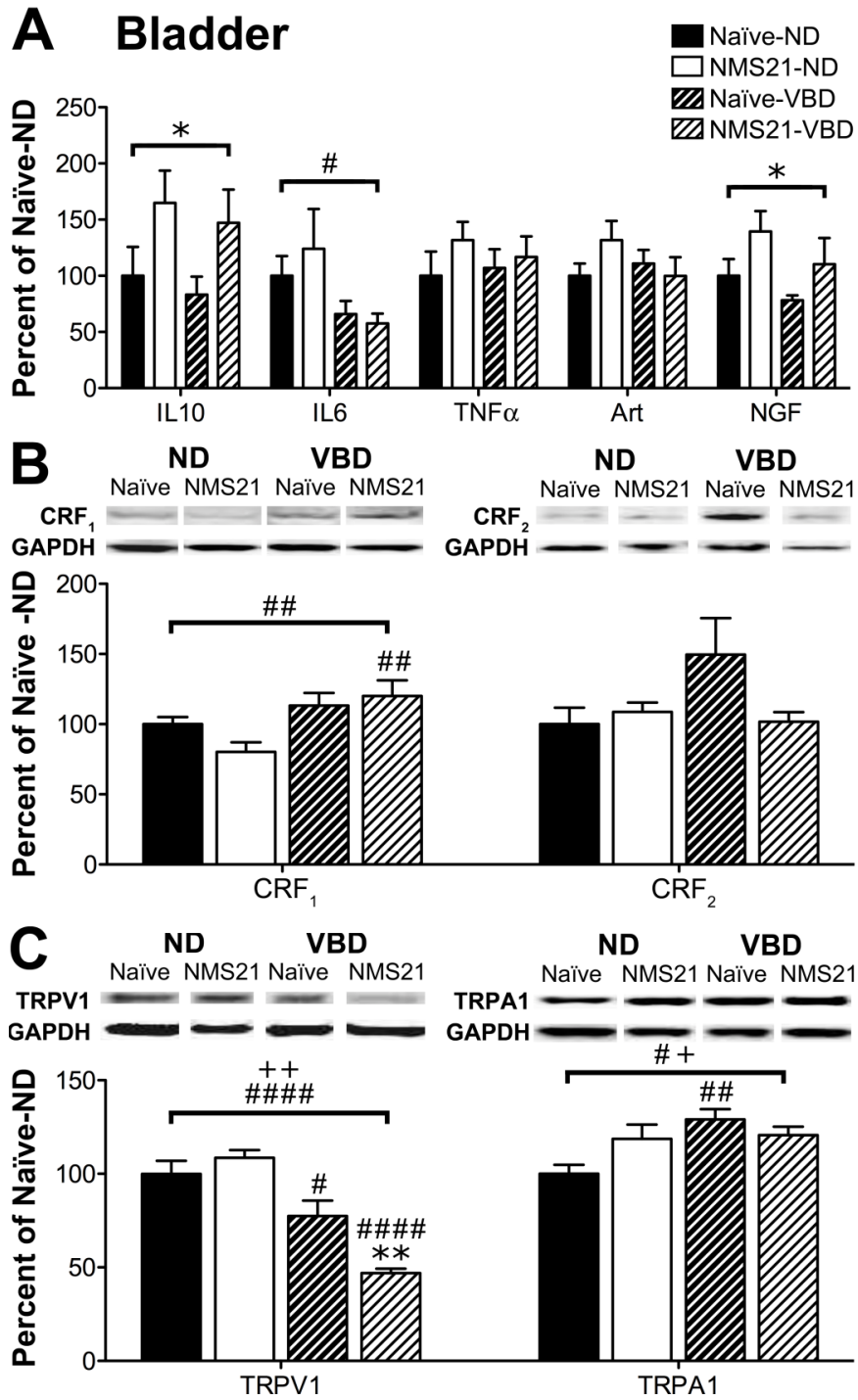


Figure 2.5 Neonatal maternal separation (NMS) and vaginal balloon distension (VBD) significantly disrupted gene and protein expression within the bladder. RT-PCR **(A)** and Western blot **(B-C)** was performed on bladder from naïve and NMS21 mice that either did not (Naïve-ND and NMS21-ND) or did undergo VBD (Naïve-VBD and NMS21-VBD).

A) NMS significantly increased both IL10 and nerve growth factor (NGF) mRNA expression. VBD significantly decreased IL6 mRNA expression. Neither NMS nor VBD had a significant effect on TNF α or Artemin (Art) mRNA expression in the bladder. **B)** Representative Western blots are shown for CRF₁, CRF₂, and corresponding GAPDH protein expression with bands at 55, 47, and 35kD, respectively. VBD significantly increased CRF₁ protein expression in the bladder with NMS21-VBD expressing significantly more CRF₁ protein than NMS21-ND. CRF₂ protein expression in the bladder was not significantly affected by either NMS or VBD. **C)** Representative Western blots are shown for TRPV1, TRPA1, and corresponding GAPDH protein expression with bands at 85, 127, and 35kD, respectively. TRPV1 protein expression in the bladder was significantly affected by VBD alone and by an interaction effect of NMS and VBD. NMS21-VBD bladder expressed significantly less TRPV1 than either NMS21-ND or naïve-VBD bladder and naïve-VBD expressed significantly less TRPV1 than naïve-ND bladder. In contrast, TRPA1 protein expression was significantly increased by VBD and there was also a significant interaction effect of NMS and VBD. Naïve-VBD bladder expressed significantly more TRPA1 than naïve21-ND bladder. Housekeeping gene: GAPDH **(A)**. Brackets indicate significant impact of NMS (* $p < 0.05$), VBD (#,##,#### $p < 0.05, 0.01, 0.0001$) or an NMS/VBD interaction (+,++ $p < 0.05, 0.01$), two-way

ANOVA; ** $p < 0.01$ vs. naïve, #,##,#### $p < 0.05, 0.01, 0.0001$ vs. baseline, Bonferroni posttest.

Figure 2.6

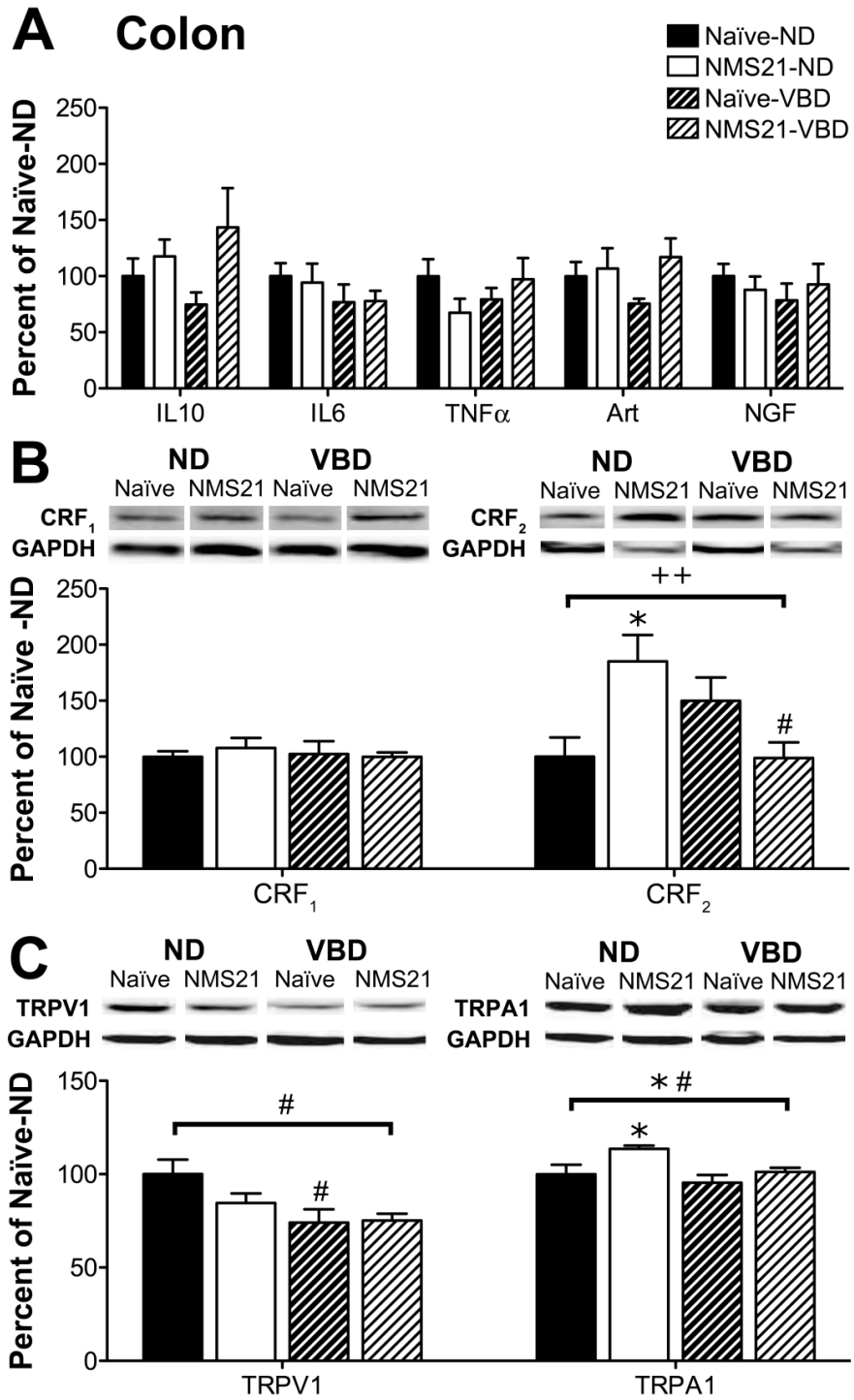


Figure 2.6 Neonatal maternal separation (NMS) and vaginal balloon distension (VBD) significantly disrupted protein expression within the colon. RT-PCR (**A**) and Western blot (**B-C**) was performed on colon from naïve and NMS21 mice that either did not (Naïve-ND and NMS21-ND) or did undergo VBD (Naïve-VBD and NMS21-VBD). **A**) NMS only had a moderate effect on mRNA expression in the colon, with a trend towards increased IL10 mRNA expression in NMS21 colon. Neither NMS nor VBD had a significant effect on IL6, TNF α , Artemin (Art), or nerve growth factor (NGF) mRNA expression in the colon. **B**) Representative Western blots are shown for CRF₁, CRF₂, and corresponding GAPDH protein expression with bands at 55, 47, and 35kD, respectively. NMS and VBD had a significant interaction effect on CRF₂ protein expression in the colon with NMS21-ND expressing higher CRF₂ protein than both naïve-ND and NMS21-VBD colon. CRF₁ protein expression in the colon was not significantly affected by either NMS or VBD. **C**) Representative Western blots are shown for TRPV1, TRPA1, and corresponding GAPDH protein expression with bands at 85, 127, and 35kD, respectively. Similar to its effects on vagina and bladder, VBD significantly decreased TRPV1 protein expression in the colon with naïve-VBD expressing significantly less TRPV1 than naïve-ND colon. NMS and VBD both separately induced a significant increase in TRPA1 protein expression in the colon. NMS21-ND colon expressed significantly more TRPA1 protein than naïve-ND colon. Housekeeping gene: GAPDH (**A**). Brackets indicate significant impact of NMS ($*p < 0.05$), VBD ($^{\#}p < 0.05$) or an NMS/VBD interaction ($^{++}p < 0.01$), two-way ANOVA; $*p < 0.05$ vs. naïve, $^{\#}p < 0.05$ vs. baseline, Bonferroni posttest.

both bladder and colon (Figure 2.5C and 2.6C). An additional significant interaction effect of NMS and VBD was observed on TRPV1 expression in the bladder with NMS21 post-VBD bladder expressing significantly less TRPV1 than naïve post-VBD bladder (Figure 2.5C). TRPA1 protein expression was differentially affected by NMS and VBD in bladder and colon. In the bladder, a significant effect of VBD and a significant interaction effect of NMS and VBD on TRPA1 protein expression was observed with naïve post-VBD bladder expressing significantly more TRPA1 protein than non-distended naïve bladder (Figure 2.5C). In the colon, NMS significantly increased TRPA1 protein expression, while VBD significantly decreased TRPA1 protein expression (Figure 2.6C).

2.5 Discussion

Patients suffering from chronic pelvic pain syndromes, particularly IBS, IC/PBS, and vulvodynia, commonly report that stress initiates or exacerbates existing symptoms (7, 17, 282). A history of early adverse events increases the likelihood of diagnosis of one or more of these pain syndromes, as well as comorbidity with depression and/or anxiety (275-278). The current study demonstrates that early life stress has a dose response-like effect on vaginal and hindpaw sensitivity and anxiety-like behaviors in adult female mice. Changes in mRNA and protein expression related to the regulation and output of the HPA axis may underlie these behavioral outcomes.

Clinical evidence suggests a strong link between child abuse or neglect and the development of vulvodynia in adulthood (21, 278, 312-314). A recent study reported that patients with provoked vestibulodynia have a lower vaginal distension threshold than control patients, suggesting that vulvodynia may be associated with vaginal

allodynia (315). In the current study, mice that experienced the greatest amount of neonatal stress – in the form of NMS for 21 days, displayed robust vaginal allodynia by generating a significantly enhanced VMR at every intraballoon pressure greater than the lowest applied. This response differs markedly from what we (149) and others (298, 299, 316) have observed following neonatal irritation or inflammation in the pelvic viscera where VMR was generally increased only at the highest applied pressures. An earlier study of colorectal sensitivity in NMS rats reported an increase in VMR at both low and high intraballoon pressures following acute water avoidance stress (146), which has been shown to impact visceral sensitivity (317, 318). The difference in outcomes between NMS and neonatal visceral irritation/inflammation likely involves recruitment of higher structures involved in regulating the HPA axis. The lack of effect of NMS for 14 days on vaginal sensitivity was striking considering a recent publication by Maloney et al., (319), that demonstrated increased VMR during colorectal distension in male mice that underwent maternal separation combined with unpredictable maternal stress from P1-14. It is likely that variances in the neonatal stress paradigm used, organs of interest, and the sex of the mice that were studied underlie the observed differences between the two studies.

To our knowledge, this is the first report of measuring VMR to quantify physiological responses to graded distension of the vagina in conscious mice. Previous studies by Berkley, et al., have measured escape behaviors in awake rats (320), as well as VMR in anesthetized rats (321), in response to VBD, and demonstrated significant effects of estrous cycle (322) and cyst burden and innervation (323, 324) in an experimental model of endometriosis. Miranda et al. (325), also reported an estrous

cycle effect on VMR during colorectal distension in awake rats that received intravesicular zymosan as neonates to induce cystitis. Importantly, both studies reported that the effect of the experimental treatment was large enough to negate the estrous cycle effect. Based on these observations and the unknown effect of collecting vaginal smears on vaginal sensitivity in NMS mice, the estrous cycle of the mice in this study was unknown.

The observations of the impact of NMS on hindpaw sensitivity are novel, as previous studies have shown either no impact (326) or a decrease (327) in thermal hindpaw sensitivity in adult female NMS rats. No literature could be found assessing thermal hindpaw sensitivity in mice or mechanical hindpaw thresholds in either rodent species following NMS. Viscero-somatic convergence between the hindpaw and bladder or colon has been previously demonstrated following inflammation of either organ (328, 329) or the hindpaw (328) of adult rodents. Neonatal irritation of the colon (149), bladder (300) or vagina (unpublished observation, manuscript in preparation), has been shown to have differential effects on hindpaw sensitivity, suggesting that the nature, location, and timing of an early adverse event can dramatically affect the outcome of considered “secondary” symptomology. Vaginal and/or cervical stimulation has previously been shown to be antinociceptive and decrease hindpaw sensitivity and/or completely block hindpaw withdrawal from noxious stimuli (330). However, these measurements were taken during vaginal/cervical stimulation and not as a consequence of the stress generated during the visceral stimulation, therefore cervical stimulation likely did not influence the observations in the current study.

Stressful events experienced early in life can greatly increase the likelihood of developing anxiety- or depression-like symptoms during adulthood (230, 331). NMS in rats has generally been shown to increase anxiety-like behaviors and the duration of ACTH and corticosterone release following a stressful event (64, 241, 242), whereas NMS in mice has been shown to generate a mild anxiety-like phenotype (248), or decrease behavioral anxiety (302, 303, 332). In the current study, we observed no change in anxiety-like behavior in NMS14 mice and a decrease in anxiety-like behavior in NMS21 mice at baseline, which was reversed following VBD. There was an observable difference in anxiety-like behavior between the two naïve groups that corresponded to the NMS14 and NMS21 mice. Considering that corresponding naïve mice were weighed daily at the time of separation, the additional week of brief daily separations experienced by the NMS21 naïve group may have contributed to this difference, as brief daily separations have been shown to reduce anxiety-like behaviors (333). This observation illustrates the importance of testing corresponding naïve and NMS groups to control for differences in environmental stressors that may influence behavioral outcomes. Changes in gene expression within the hypothalamus of NMS mice, both at baseline and following VBD, suggest that increased HPA output, resulting from diminished negative feedback, may underlie the increase in anxiety-like behaviors. NMS simultaneously increased CRF and decreased CRF₂ mRNA levels. The trend towards an increase in Ucn2 mRNA in NMS21 mice may be a compensatory action, as VBD also decreased CRF₂ mRNA in naïve mice, but did not show a corresponding increase in Ucn2. GR was also increased in NMS21 hypothalamus, which could be a compensatory response to decreased hippocampal GR expression (334) or GR

resistance (93). Up-regulation of both CRF₁ protein expression (287) and binding (242) in the hypothalamus of NMS rats has been previously reported and supports our findings here.

The greater impact of the three week-long NMS period on all behavioral and physiological measurements in the current study, compared to the standard two week-long NMS period, may be due to disruption of proper limbic structure maturation. The peak period of hippocampal neurogenesis overlaps with the stress hyporesponsive period (P1-14) (335); however, mature firing patterns do not emerge until the third postnatal week (304). Limbic structures have been shown to influence pain and anxiety through HPA- and non-HPA-mediated mechanisms. Systemic and local corticosterone production increases CRF expression in the CeA of the amygdala through a GR-dependent mechanism (92). The level of CRF expression within the amygdala has been shown to mediate pain effects, which can be blocked by CRF₁ antagonist treatment, but not CRF₂ antagonist (336). This observation was supported by earlier work demonstrating that basal CRF₁ signaling contributes to pain-related synaptic facilitation and CRF₂ exerts a latent inhibitory influence (337). Observations in the current study support this earlier work, as a trend toward decreased CRF₂ mRNA expression was detected in amygdala of NMS mice, suggesting that a loss of inhibition within the amygdala could be contributing towards the increase in vaginal sensitivity and anxiety-like behaviors. Hippocampal inhibition of the HPA axis can be compromised by chronic stress or long-term high dose corticosteroid treatment, resulting in decreased GR expression (65). Observations in the current study support this mechanism, as NMS significantly decreased both GR and MR protein expression in the hippocampus,

potentially compromising hippocampal inhibition of the HPA axis. Acute adult stress, in the form of VBD, also significantly impacted mRNA levels in the hippocampus, specifically increasing CRF₂ and decreasing MR levels. The increase in CRF₂ mRNA levels was likely compensatory in response to either heightened HPA output or decreased hippocampal MR levels following VBD. The lack of a similar increase in CRF₂ in NMS21 hippocampus suggests potential disruption in proper limbic response following acute stress. A prolonged decrease in hippocampal MR expression has been previously reported to occur following a single sustained acute stress in rats and has been theorized to contribute to post-traumatic stress disorder (338).

Up to 50% of women with chronic pelvic pain experience symptoms from more than one disorder, creating a greater negative impact on quality of life and complicating already less-than-optimal treatment strategies (7, 13, 17-22). A potential role for CRF in comorbidity has been established for psychological and chronic pain disorders. A significant correlation has been observed between IBS and depression/anxiety in patients with high cortisone levels (339). Epidemiological studies have also found a link between anxiety and voiding disorders (340); and IC/PBS patients with concomitant comorbid diagnoses of fibromyalgia, chronic fatigue syndrome, or rheumatoid arthritis had a higher mean afternoon cortisol level and increased pain during bladder filling than IC/PBS patients with no additional diagnoses (341). Downstream propagation of neurogenic inflammation resulting from dysregulation of the HPA axis has been proposed as a possible underlying mechanism for numerous functional pain syndromes (288). In the current study, we observed significant effects of NMS, VBD, and interaction effects of both on all three visceral structures. Examination of mRNA levels of genes

previously shown to be affected by NMS or involved in mediating pelvic hypersensitivity (309-311, 342, 343) revealed possible disruption of downstream HPA axis effects. For example, NMS and VBD had a significant interaction effect on IL10 and TNF α mRNA levels in the vagina, which resulted in a significant increase in NMS21 vagina post-VBD, but not in naïve vagina. The lack of baseline changes in NMS21 vagina suggests that the response to acute stress in these animals is altered, compared to that in naïve mice, producing an altered downstream local cytokine response to VBD. This observation is supported by the significant interaction effect of NMS and VBD on CRF $_1$ protein expression in the vagina, where naïve vagina showed a trend toward increased CRF $_1$ protein expression, which was significantly lower in NMS21 vagina, suggesting dysregulation of peripheral CRF receptor expression. Cytokine and growth factor mRNA levels were also significantly altered by NMS and VBD, separately, in the bladder. With the exception of NMS producing a significant increase in NGF mRNA levels, the impact of NMS on IL10 levels and VBD on IL6 levels was largely anti-inflammatory, suggesting that these interventions may result in lowered bladder sensitivity. Spinally-administered CRF $_2$ antagonist was shown to reverse stress-induced bladder sensitivity in the rat, whereas CRF $_1$ antagonist had no effect (144), however, the role of peripheral CRF receptors in bladder sensitivity has not yet been established. Future studies will determine whether the increase in CRF $_1$ protein expression in the bladder inhibits or drives bladder sensitivity following acute stress. The expression pattern of CRF $_2$ in the colon was similar to that reported by O'Malley et al., (344), which showed a baseline increase in CRF $_2$ protein expression in NMS rats that was reversed by open field stress. However, they also reported a similar effect on CRF $_1$ protein expression, which was not

evident in our study; however, the species studied, length of NMS, and nature of the adult stressor likely contributes to this discrepancy. It is unclear whether the VBD effect on expression changes resulted from stress or from a physiological response to organ distension. Due to the close proximity of the investigated pelvic organs, it is possible that pressure exerted onto the bladder and colon during VBD could have impacted gene expression within these organs, as well. Regardless, these data support that NMS and VBD have effects on the vagina, as well as on the immediately adjacent viscera.

The TRP family of receptors has been shown to contribute towards acute inflammatory colorectal hypersensitivity (135, 138, 345), as well as to colonic hypersensitivity resulting from neonatal colon insult (149, 298). Local upregulation of TRPA1 and/or TRPV1 protein expression has also been reported following acute inflammation of the colon (346, 347) and bladder (348). In non-distended mice, we only observed a significant increase in TRPA1 protein expression in NMS colon. However, VBD had a significant effect on TRPV1 and TRPA1 protein expression in every tissue type examined. VBD significantly decreased TRPV1 protein expression in naïve and NMS vagina, bladder and colon; and NMS and VBD had a significant interaction effect on decreasing TRPV1 expression in bladder. VBD had a similar effect on TRPA1 protein expression in the vagina and colon; however, VBD significantly increased TRPA1 protein expression in the bladder, most prominently in naïve bladder. Although, bladder and colon sensitivity were not assayed in the current study, it is likely that TRP channel expression did not contribute towards the observed vaginal hypersensitivity in the NMS21 mice. It is striking that distension of an adjacent organ would significantly decrease TRPV1 protein expression in both bladder and colon. Future studies assaying

less invasive forms of stress will address whether this and the aforementioned expression changes were due to potential physical deformation of the tissue or if they were truly stress-mediated.

2.6 Conclusion

This study provides evidence that early life stress in female mice has significant effects on vaginal sensitivity, anxiety-like behaviors, and central and peripheral expression of mediators involved in the regulation and output of the HPA axis. Exposure to neonatal stress had a dose response-like effect on most behavioral outcomes, which were further enhanced by exposure to adult stress in the form of VBD. Together with previous studies on the effect of NMS on psychological disturbances and pelvic organ sensitivity, the findings here demonstrate that neonatal stress in females can impact adult pain processing associated not only with the bladder and colon, but also with the reproductive tract and may provide a potential mechanism for the development of comorbid chronic pelvic pain syndromes.

Chapter 3

Urinary bladder-specific increase in sensitivity and dysregulation of the hypothalamic-pituitary-adrenal axis following early life and adult stress in female mice.

3.1 Abstract

Early adverse events, such as childhood trauma, neglect, or abuse have been shown to increase the incidence of interstitial cystitis/painful bladder syndrome (IC/PBS) in adulthood, a syndrome that is nine times more common in women than in men. Despite high clinical relevance and reports of stress-related symptom exacerbation, animal models investigating the contribution of early life stress to female urological pain are lacking. We examined the impact of neonatal maternal separation (NMS), a model of early life stress, on bladder sensitivity both prior to, and following, water avoidance stress (WAS) in adult female mice. C57Bl/6 mice were born in-house and were either separated as litters from their dams for 3 hours/day from postnatal day 1-21 (NMS mice) or remained undisturbed (naïve mice). Bladder mast cell degranulation, as well as protein and mRNA expression related to neuroimmune interactions, was evaluated using toluidine blue staining, Western blot, and RT-PCR, respectively. Compared to naïve mice, NMS mice demonstrated bladder hypersensitivity that was robustly exacerbated by WAS. Protein expression of protease activated receptor-2 (PAR2) and transient receptor potential ankyrin 1 (TRPA1), receptors involved in facilitating neurogenic inflammation through mast cell proteases, were increased in the bladder of NMS mice. Consequently, WAS increased bladder mast cell degranulation in NMS mice as well as mRNA expression of cytokines and growth factors involved in facilitating inflammation or neuroimmune interactions. In brain regions that regulate the HPA axis, altered expression of receptors or stress ligands suggested diminished negative feedback and glucocorticoid resistance that resulted in increased corticosterone secretion in NMS animals. Mechanistically, mast cell-mediated neurogenic

inflammation downstream of a dysregulated HPA axis could be driving bladder hypersensitivity and predispose NMS mice to an aberrant response to acute stress. Together, this project identifies a novel impact of early life stress on adult female urological pain.

3.2 Introduction

Stress can have dichotomous effects on pain signaling. In instances of acute stress the effect can be to diminish the perception of pain, termed stress-induced hypoalgesia (318). However, when stress becomes chronic, increased circulation of glucocorticoids and dysregulation of higher systems controlling the stress response pathway can initiate or increase the perception of pain (349). Patients with chronic pelvic pain commonly report stress-related symptom onset or increased severity, have difficulty coping with stressful situations, and many suffer from depression, anxiety, and panic disorder (7, 13, 17). Comorbidity among chronic pelvic pain syndromes and mood disorders has been associated with altered functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response and influences the perception of pain (65, 350). Exposure to early life stress or trauma is a significant risk factor for developing HPA axis abnormalities and associated chronic pain syndromes (265, 266). As such, a significant subset of patients with chronic pelvic pain disorders report having experienced adverse childhood events such as abuse or neglect (279-281).

Regulation of the HPA axis occurs both within the hypothalamus, which initiates the stress response by secreting corticotropin-releasing factor (CRF), and from higher limbic structures, including the hippocampus (65). This regulation largely occurs through activation of the CRF receptors, which work in opposition of one another to drive (CRF₁) and dampen (CRF₂) HPA axis output (351), as well as by glucocorticoid-driven feedback through glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (79). Downstream of chronic HPA axis activation, neurogenic inflammation may be promoted through mast cell degranulation and associated sensitization of transient

receptor potential ankyrin 1 (TRPA1) (126, 139, 142) resulting in altered cytokine profiles (352) and continuous cell signaling of the neuroimmune axis in the viscera (353). In fact, biopsy findings support evidence of increased mast cell degranulation within the involved pelvic organ among patients diagnosed with irritable bowel syndrome (IBS) (124, 289, 290), vulvodynia (293, 294), or interstitial cystitis/painful bladder syndrome (IC/PBS) (291, 292). Rodent models of neonatal stress display disruption of proper feedback onto the HPA axis, resulting in visceral hyperalgesia, permanent changes in central and peripheral pain processing, and increased peripheral expression of inflammatory mediators (reviewed in (354)). The effect of early life stress has been well described in the gastrointestinal system (146, 230, 265, 311, 355) and data presented in chapter 2 provide evidence of altered sensitivity within the reproductive system (150); however, despite extensive characterization of the long-term effect of postnatal bladder inflammation (299, 300, 316, 356-358), the impact of early life stress on urinary bladder sensitivity and function has largely gone unstudied particularly in female models.

Using a model of neonatal maternal separation (NMS), we investigated the impact of early life stress on gene expression in the hypothalamus and hippocampus and downstream pelvic organ sensitivity and function both prior-to, and following, an acute water avoidance stress (WAS) in adulthood. To understand whether our findings were pelvic organ-specific or due to a global change in pain processing, we also evaluated colorectal sensitivity as a function of the same stressful conditions. Mast cell degranulation and infiltration was quantified, while mRNA expression of cytokines, growth factors and protein expression of the tryptase receptor PAR2 and TRPA1 were

evaluated in the pelvic organs. Finally, expression of factors regulating the HPA axis from the hypothalamus and hippocampus were measured in NMS and naïve animals at baseline and following WAS. The central hypothesis of this study is that NMS results in altered bladder function and increased sensitivity and enhances susceptibility to stress-induced behavioral changes in adult female mice. Together, these results increase the understanding of how early life stress predisposes an individual to developing bladder pain syndromes during adulthood.

3.3 Materials and methods

Animals

Experiments were performed on female C57Bl/6 mice (Charles River, Wilmington, MA) born and housed in the Research Support Facility at the University of Kansas Medical Center at the indicated ages in Table 3.1. Mice were housed on a 12-h light cycle from 600 to 1800h and received water and food ad libitum. All research performed conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals in accordance with the guidelines specified by the University of Kansas Medical Center Animal Care and Use Protocols.

Neonatal Maternal Separation

All mice used in this study were born in house from pregnant dams (Charles River, Wilmington, MA) delivered to the animal facility during the last week of gestation. Day of birth was designated as postnatal day (P) 0 and from P1 until P21 individual

Table 3.1 Age of mice at experimental time points

	Baseline	WAS	1d post-WAS	8d post-WAS
Behavioral testing				
Micturition	11	11	11	
CRD	14			
UBD	16	17	17	18
CRD	21	22	22	23
Euthanasia				
mRNA, protein, CORT		8		9
mRNA, protein, CORT		11	12	
mRNA, protein, CORT	12			
Mast cells		10	11	
Mast cells	28			

Table 3.1 NMS and corresponding naïve mice underwent behavioral testing at the above ages noted in weeks. Each row represents different cohorts of animals that were exposed to the stated behavioral test (micturition analysis, urinary bladder distension [UBD], and colorectal distension [CRD]) or euthanized for tissue collection. Tissue utilized for mRNA/protein expression, corticosterone (CORT) concentration, and the mast cell degranulation assay were taken from naïve and NMS mice that had not previously undergone behavioral testing.

litters were removed daily and placed *en masse* into clean glass beakers containing a small amount of home cage bedding to maintain scent. Pups were held at 34°C and 50% humidity from 1100 to 1400 hours. Fresh gloves were rubbed with home cage bedding before handling each litter to avoid rejection by the dam. Corresponding naïve mice were born, housed, and weaned during the same time frame to avoid potential complications arising from variations in prenatal shipping conditions, housing environment, and normal husbandry procedures. All mice were weaned on P22.

Water avoidance stress

Water avoidance stress (WAS) was performed for one hour, within the first six hours of the light cycle. Mice were placed individually on a round platform (5 cm diameter) centrally affixed to the bottom of a container (36 cm length x 31 cm width x 27 cm height) filled with room temperature tap water up to 1 cm below the top of the platform.

Urinary bladder distension

Under inhaled isoflurane (4% induction, 2% maintenance), the bare ends of two Teflon-coated stainless steel electrode wires (0.003" diameter; Grass Technologies, West Warwick, RI) were acutely implanted into the left and right abdominal musculature using a 26-gauge needle. A 24-gauge angiocatheter was inserted intravesically via the urethra and secured in place with tape. Anesthesia was lowered until hindlimb reflexes, but not escape behaviors, were present (approximately 1% isoflurane). A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow from a compressed nitrogen tank equipped with a dual-

stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO) and a separate pressure monitor (World Precision Instruments, Sarasota, FL) was used to regulate the pressure within the bladder. Following three 60mmHg distensions to establish stable responses, each pressure (15, 30, 45, 60mmHg) was applied in triplicate for 20 seconds with a 2-minute rest period in between. Electromyographic (EMG) activity was amplified, filtered, and recorded using Spike 2 software (Cambridge Electronic Design, Cambridge, UK) on a personal computer and analyzed off-line. The visceromotor response (VMR) was quantified by measuring the area under the curve of the entire distension period divided by the duration of the distension and expressed as a percent of baseline activity.

Colorectal distension

Electrode implantation was performed as previously described (249). Under inhaled isoflurane (4% induction, 2% maintenance) and aseptic conditions, the bare ends of two Teflon-coated stainless steel electrode wires (0.003" diameter; Grass Technologies) were surgically implanted into the right abdominal musculature, secured via 5-0 prolene sutures, tunneled subcutaneously to a small incision made in the nape of the neck, and externalized for access during testing. Skin incisions were closed using 5-0 silk suture. Following recovery from anesthesia, mice were housed singly and allowed to recover for a minimum of 4 days before undergoing testing.

To facilitate balloon insertion and maintain proper restraint during testing, mice were briefly sedated with inhaled isoflurane and a custom-made polyethylene plastic balloon (length, 1.5 cm; diameter, 0.8 cm) was inserted into the distal colon, 0.5cm past the anal verge, and secured to the base of the tail with tape. The mouse was then

placed into a Broome-style rodent restraint (Kent Scientific, Torrington, CT), the free ends of the electrode wires were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA), and the mice were allowed to recover from anesthesia for 30 minutes. The balloon was inflated with air from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator (Matheson-Linweld) and a separate pressure monitor (World Precision Instruments) was used to regulate the pressure inside the balloon. Each pressure (15, 30, 45, 60, 75mmHg) was applied in triplicate for 20 seconds with a 4-minute rest period in between. A custom-made distension control device (The University of Iowa Medical Instruments) was used to control the gas flow through the system. The EMG activity was amplified, filtered, and recorded off-line as described for UBD.

Micturition analysis

Mice were gavaged with 300 μ L drinking water and immediately placed on a piece of filter paper and covered by a clear plexiglass container (36 cm length x 20 cm width x 13 cm height) for 1 h. The number and size of urine spots were measured using Image J following visualization with ultraviolet light. Micturition frequency and total fecal output were determined as the total number of individual urine spots or fecal pellets, respectively. Total urine output was determined by quantifying the total area of urine spots during the testing period.

Mast cell infiltration and degranulation

Urinary bladder and distal colon were separately mounted in Tissue-Tek OCT mounting media (Sakura Finetek, Torrance, CA) and cut transversely into thin sections

(7 μm) using a cryostat. Slides were stained for 10 minutes with a 1% toluidine blue solution acidified with 1M HCl to a pH less than 1.0, dried overnight, washed and coverslipped with 1xPBS for analysis. Using light microscopy (Nikon eclipse 90i, Nikon Instruments, Inc., Melville, NY), digital images were captured (QIClick digital CCD Camera, QImaging, Surrey, BC, Canada) and the total number of non-degranulated (NG) mast cells (dense metachromasia with no or faint nuclear outline and/or no granular extrusion around the cell) and degranulated (DG) mast cells (less intense metachromasia and obvious clear outline of the nucleus and/or free granules within the cytoplasm) were counted in at least 8 separate sections spanning the length of each tissue. The percentage of DG to total mast cells was calculated according to the following equation for each tissue/mouse: $(\text{DG mast cells}/\text{Total mast cells}) \times 100$

mRNA extraction and qRT-PCR

Mice were overdosed with inhaled isoflurane (>5%) and, following decapitation, whole brains were removed and frozen on dry ice. Hypothalamus and hippocampus were dissected, immediately snap frozen in liquid nitrogen, and stored at -80°C . The urinary bladder and distal 1.5cm segment of the colon were also removed and subsequently bisected longitudinally (to facilitate both mRNA and protein [see below] analysis), snap frozen in liquid nitrogen, and stored at -80°C . Total mRNA was isolated using Trizol reagent (Ambion, Austin, TX) and RNeasy micro kit (Quiagen, Valencia, CA), as per the manufacturer's instructions. Sample concentration and purity was determined using a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and cDNA was synthesized from total RNA (0.5 μg) using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative, real-time PCR amplification was

performed using 0.2 µg of total cDNA (from the 0.5 µg reverse-transcribed cDNA) and SsoAdvanced SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX manager 3.1 real time PCR system with indicated 20µM primers (Integrated DNA Technologies, Coralville, IA) listed in Table 3.2. Samples were run in triplicate and negative control reactions were run with each amplification series with β-actin (bladder and colon) or GAPDH (brain) as the housekeeping gene. To reduce variability among efficiency due to fluctuations in baseline fluorescence, the raw (i.e. non-baseline corrected) PCR data was imported to the LinRegPCR software (version 2012.3) (359-361) and PCR efficiency values were derived for each individual sample by fitting a regression line to a subset of data points within the sample's log-linear phase. Threshold cycle (Ct) values were subtracted from that of the selected housekeeping gene and the percentage of fold change over naïve controls was calculated using the Pfaffl method (362).

Protein analysis

Total protein was isolated using Cell Extraction Buffer containing Halt protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham MA) and Na₃VO₄. Protein concentrations were determined using a D_c protein assay (ThermoFisher Scientific). Samples were reduced by heating to 95°C for 5 minutes in the presence of 2-mercaptoethanol, subjected to SDS-PAGE (Criterion 4% to 12% Bis-Tris gels; Bio-Rad Laboratories), and transferred to Nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) by Criterion Blotter wet transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature in 5% milk in Tris-buffered saline with Tween-20 then incubated overnight at 4°C with CRF₁ (1:500), CRF₂ (1:800), PAR2

Table 3.2 Primers used for real-time PCR analysis

Gene	Forward (5' – 3')	Reverse (3' – 5')
IL6	CTGCCAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
IL10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
SCF	CCCTGAAGACTCGGGCTTA	CAATTACAAGCGAAATGAGAGCC
NGF	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
CRF	CCTCAGCCGGTTCTGATCC	GCGGAGGAAGTATTCTTCACCC
BDNF	CAGGTTTCGAGAGGTCTGACGA	CGCGTCCTTATGGTTTTCTTCG
CRF ₁	CCCTGCCTTTTTCTACGGTGT	TTCCCGGTAGCCATTGTTTGT
CRF ₂	CCTGTGGACACTTTTGGAGCA	TGTTGCAGTAGGTGTAGGGAC
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
MR	GAAAGGCGCTGGAGTCAAGT	CCATGTAGCTGTTCTCATTGGT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT
β -actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT

(1:1000), and TRPA1 (1:1000) antisera. Membranes were washed with Tris-buffered saline with Tween-20 and incubated for 1 hour with anti-rabbit secondary antibody (1:10,000). Densitometry was performed using Quantity One 4.6.9 software (Bio-Rad Laboratories).

Statistics

Calculations were performed on Microsoft Excel and statistical analysis was performed using two-way (with or without repeated measures) analysis of variance (ANOVA) followed by Bonferroni's posttest (GraphPad Prism 6, GraphPad Software, Inc, La Jolla, CA) as denoted in the results/figures. All data are expressed as mean \pm SEM. A *p* value of less than 0.05 was considered significant.

Vaginal lavage

The perineum was dried from urine and 100 μ l of phosphate buffered saline (PBS) was gently expelled into the vaginal canal 3 times with a transfer pipette without penetrating the vaginal orifice. The collected fluid was placed into a single well of a 96-well plate and immediately examined with light microscopy. The stage of estrous cycle was recorded by determining the ratios of the following cell types: nucleated epithelial cells, cornified epithelial cells, and leukocytes.

3.4 Results

Neonatal and adult stress alter HPA axis output and regulation

Previous studies in our laboratory have determined that NMS impacts adult anxiety-like behaviors and vaginal sensitivity with associated molecular changes in the HPA axis and pelvic viscera of female mice (Chapter 2; (150)). The purpose of the

current study was to determine the impact of acute adult stress exposure on measurements of HPA axis output and regulation and pelvic organ sensitivity in the same model of female NMS mice.

To determine changes in the output of the HPA axis, resting serum corticosterone (CORT) levels were measured in naïve and NMS mice at baseline and 1 and 8d post-WAS exposure. At baseline, serum CORT was significantly elevated in NMS mice compared to naïve (Figure 3.1A). Exposure to WAS significantly depressed serum CORT in naïve mice only at 1d post-WAS, as it was returned to baseline levels at 8d post-WAS (Figure 3.1A). Serum CORT was also significantly reduced in NMS mice at 1d post-WAS and remained significantly lower than baseline NMS measurements at 8d post-WAS (Figure 3.1A).

Real-time PCR was performed to determine if changes in mRNA expression in the hypothalamus may be underlying the differential CORT serum levels in naïve and NMS mice. The mRNA level of hypothalamic CRF was not significantly different between naïve and NMS mice at baseline or following WAS (Figure 3.1B). Likewise, levels of CRF₁ mRNA were not affected by NMS or WAS (Figure 3.1C). However, WAS significantly increased the mRNA levels of CRF₂, GR, and MR in the hypothalamus of both naïve and NMS mice, particularly at 8d post-WAS (Figure 3.1D-F).

To determine potential changes in limbic regulation of the HPA axis, mRNA levels of the same genes were measured in the hippocampus. Unlike in the hypothalamus, WAS exposure significantly reduced the mRNA level of CRF₁, particularly at 8d post-WAS in NMS mice (Figure 3.2A). Exposure to WAS significantly increased CRF₂ mRNA levels in the hippocampus, particularly at 8d post-WAS (Figure

Figure 3.1

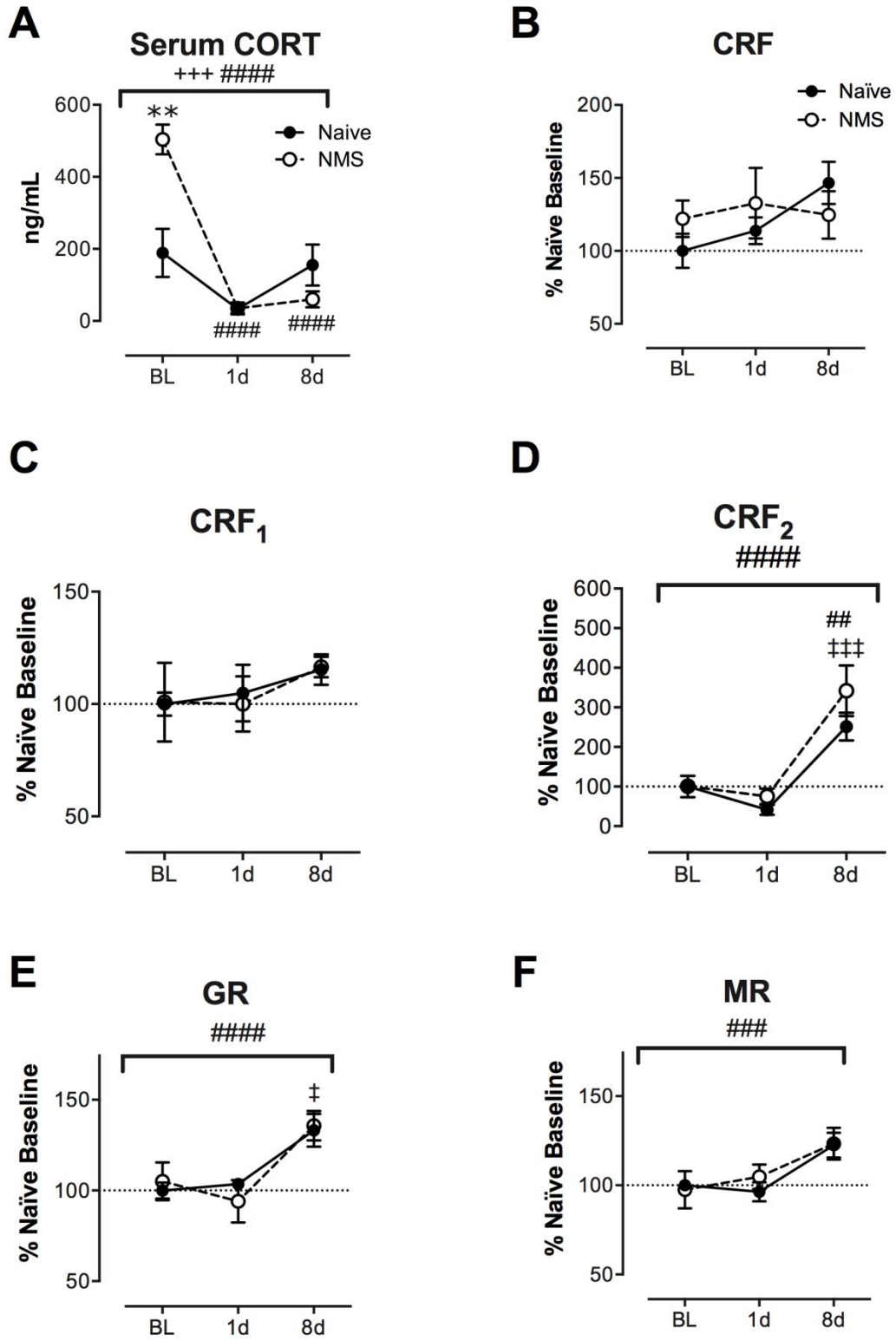


Figure 3.1 To evaluate HPA axis output, serum corticosterone (CORT) and mRNA expression of receptors involved in HPA axis regulation in the hypothalamus were evaluated in response to neonatal maternal separation (NMS) and water avoidance stress (WAS). **A)** Serum CORT was significantly elevated in NMS mice compared to naïve mice at baseline (BL) and was significantly reduced at 1 and 8d post-WAS compared to BL levels. No significant effect of NMS or WAS was observed on hypothalamic mRNA levels of corticotropin-releasing factor (CRF, **B**) or its receptor, CRF₁ (**C**). Exposure to WAS significantly increased mRNA levels of CRF₂ (**D**), glucocorticoid receptor (GR, **E**), and mineralocorticoid receptor (MR, **F**) in both NMS and naïve hypothalamus. Brackets indicate a significant effect of WAS (###, #### $p < 0.001, 0.0001$) or NMS/WAS interaction (++ $p < 0.01$), two-way ANOVA; * $p < 0.05$ vs. naïve; #, ## $p < 0.05, 0.01$ vs. BL; †, ‡ $p < 0.05, 0.001$ vs. 1d post-WAS; Tukey's posttest.

Figure 3.2

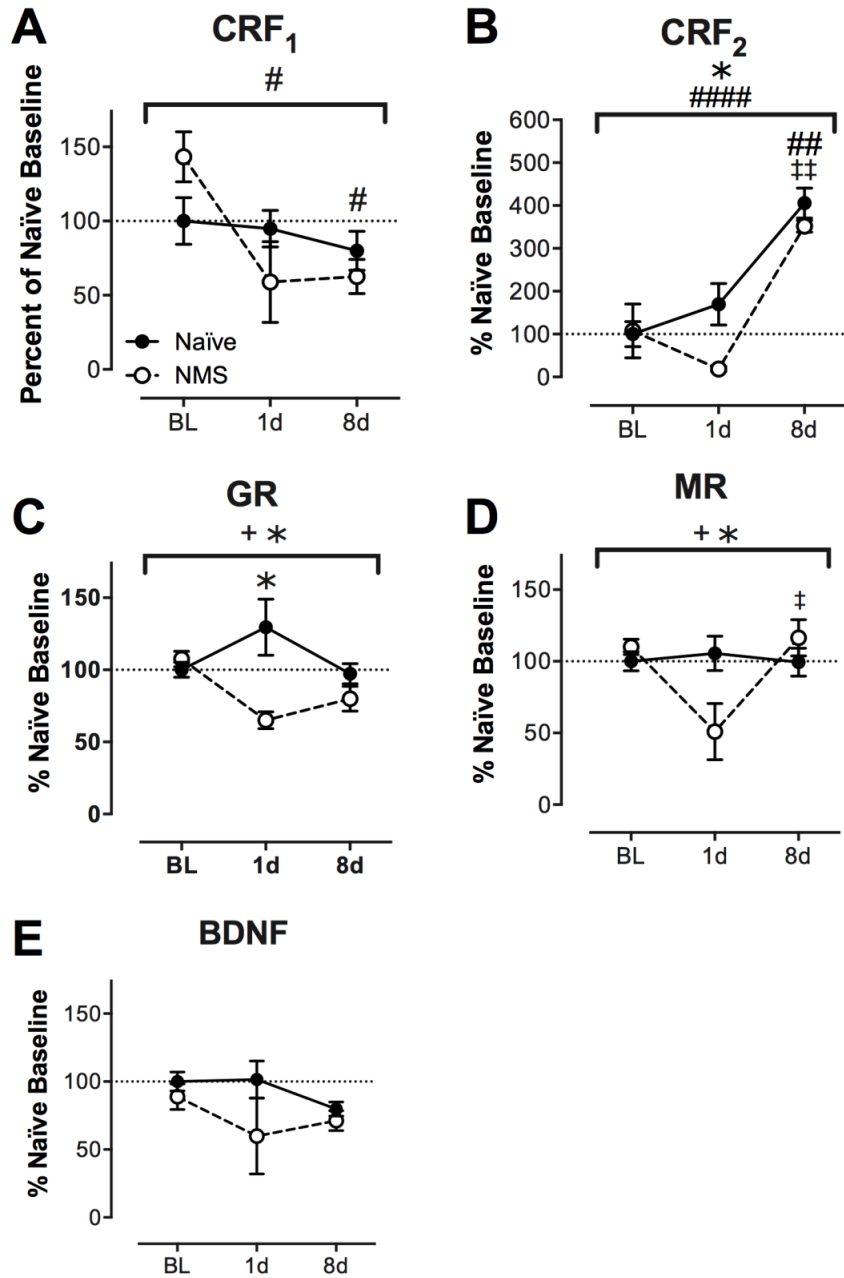


Figure 3.2 Hippocampal mRNA levels of receptors involved in negative regulation of the HPA axis were evaluated in response to neonatal maternal separation (NMS) and water avoidance stress (WAS). **A)** Exposure to WAS significantly decreased the hippocampal mRNA level of corticotropin-releasing factor receptor 1 (CRF₁) overall, as well as in NMS mice at 8d post-WAS compared to baseline (BL) levels. **B)** NMS similarly decreased overall hippocampal mRNA levels of CRF receptor 2 (CRF₂), however, WAS exposure significantly increased CRF₂ mRNA levels in both NMS and naïve hippocampus compared to their respective BL and 1d post-WAS levels. **C)** The hippocampal mRNA levels of glucocorticoid (GR) and mineralocorticoid receptor (MR) were similarly affected by NMS and an NMS/WAS interaction, resulting in a transient decrease in expression at 1d post-WAS in the NMS mice and a return to BL levels at 8d post-WAS. **E)** Despite a slight reduction at 1d post-WAS in NMS mice, no significant effects of NMS or WAS were observed on the hippocampal mRNA levels of brain-derived neurotrophic factor (BDNF). Brackets indicate a significant effect of NMS (* $p < 0.05$), WAS (#, #### $p < 0.05$, 0.0001), or NMS/WAS interaction (+ $p < 0.05$), two-way ANOVA; * $p < 0.05$ vs. naïve; #, ##, ### $p < 0.05$, 0.01, 0.001 vs. BL; †, ††, ††† $p < 0.05$, , 0.01, 0.001 vs. 1d post-WAS; Tukey's posttest.

3.2B); whereas NMS had an overall effect of significantly decreasing hippocampal CRF₂ mRNA levels (Figure 3.2B). Hippocampal GR and MR mRNA levels were significantly impacted by NMS and the combined effect of NMS and WAS, resulting in a transient decrease in expression in NMS mice at 1d post-WAS and returning to baseline levels at 8d post-WAS (Figure 3.2C-D). The mRNA levels of brain-derived neurotrophic factor (BDNF) were also measured in hippocampus, however no significant impacts of NMS or WAS were observed, despite a trend towards decreased BDNF mRNA at 1d post-WAS in NMS mice (Figure 3.2E).

Neonatal and adult stress exposure impact peripheral cytokine and NGF mRNA expression

Real-time PCR was also used to determine the impact of NMS and WAS on peripheral gene expression of cytokines and neurotrophins that are associated with dysfunction of the HPA axis (124, 363, 364). The mRNA level of interleukin-6 (IL6) was significantly impacted by a NMS/WAS interaction effect in the bladder (Figure 3.3A) and by NMS alone in the colon (Figure 3.3E). The mRNA level of interleukin-10 (IL10) was differentially impacted by NMS and WAS in the two organs, as NMS and WAS both significantly increased IL10 expression in the bladder (Figure 3.3B), yet had a significant interaction effect on reducing IL10 expression in the colon (Figure 3.3F). Stem cell factor (SCF), a mast cell signaling cytokine and growth factor, was significantly increased by WAS in bladder, particularly at the 8d post-WAS time point (Figure 3.3C), but no effects of NMS or WAS were observed in colon (Figure 3.3G). Nerve growth factor (NGF) mRNA levels were significantly impacted by WAS and a NMS/WAS interaction effect in both bladder (Figure 3.3D) and colon (Figure 3.3H), but with

Figure 3.3

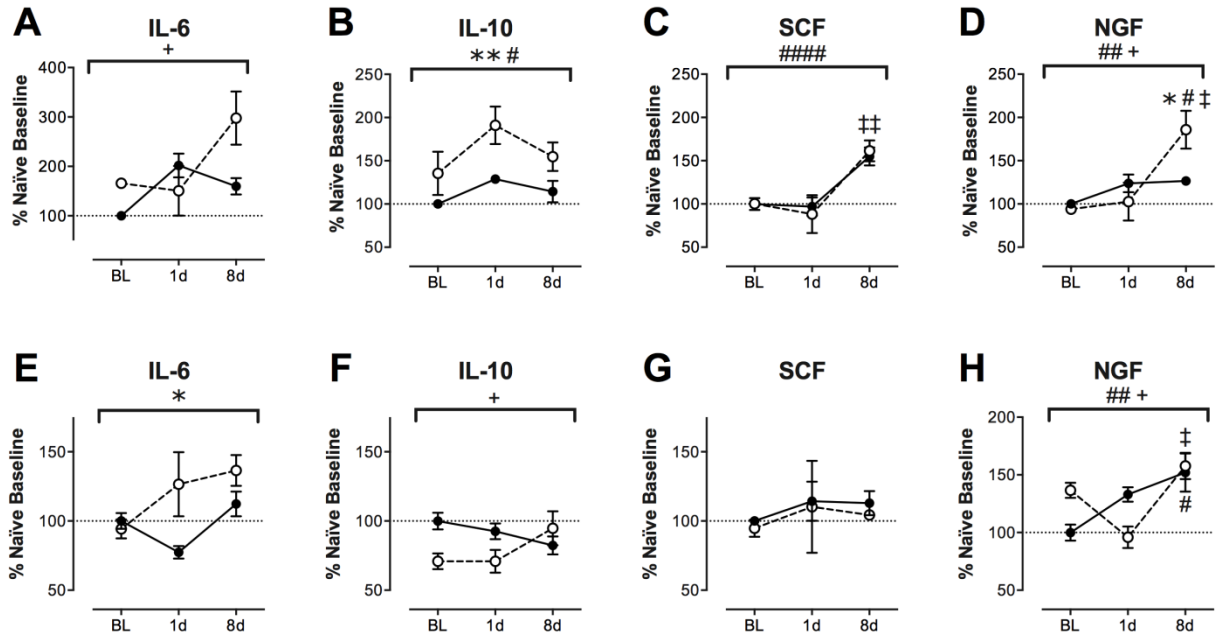


Figure 3.3 The mRNA levels of cytokines and growth factors involved in neurogenic inflammation were measured in the bladder (**A-D**) and colon (**E-H**) to determine alterations in downstream activation of the HPA axis in response to neonatal maternal separation (NMS) and water avoidance stress (WAS). **A**) A significant interaction effect of NMS and WAS was observed on mRNA levels of interleukin (IL) 6 in the bladder. Both NMS and WAS separately and significantly increased IL-10 mRNA levels in the bladder (**B**), whereas WAS alone or a NMS/WAS interaction significantly increased stem cell factor (SCF, **C**) and nerve growth factor (NGF, **D**) mRNA levels in the bladder, respectively. The mRNA level of IL6 in the colon was significantly increased in NMS mice overall (**E**). A significant NMS/WAS interaction effect was observed for both IL-10 (**F**) and NGF (**H**) mRNA levels in the colon, with NGF mRNA levels being significantly elevated at 8d post-WAS in both naïve and NMS colon. The mRNA levels of SCF were unaffected by NMS or WAS in the colon. Brackets indicate a significant effect of NMS (*, ** $p < 0.05, 0.01$), WAS (#, ##, #### $p < 0.05, 0.01, 0.0001$), or NMS/WAS interaction (+ $p < 0.05$), two-way ANOVA; # $p < 0.05$ vs. BL; †, †† $p < 0.05, 0.01$ vs. 1d post-WAS; Tukey's posttest.

differential patterns in the NMS mice: WAS significantly increased NGF expression in NMS bladder at 8d post-WAS (Figure 3.3D) and transiently decreased expression in NMS colon at 1d post-WAS (Figure 3.3H).

Neonatal and adult stress exposure differentially increase urinary bladder and colorectal sensitivity

The visceromotor response (VMR) during urinary bladder distension (UBD) or colorectal distension (CRD) was recorded to evaluate changes in pelvic organ sensitivity following WAS in naïve and NMS mice. In all mice, the VMR during either UBD (Figure 3.4A) or CRD (Figure 3.4C) significantly increased in response to greater intravesicular or balloon pressure, respectively, confirming a physiological response to organ distension (150, 365). At baseline, the VMR of NMS mice during UBD was significantly higher than that of naïve mice over the entire distension series and at the highest pressure applied (Figure 3.4A). In contrast, NMS mice displayed significantly decreased VMR during CRD, both over the entire distension series and at the highest applied pressure (Figure 3.4C). Following exposure to WAS, naïve mice exhibited no change in VMR during UBD at either 1d or 8d post-WAS; however, NMS mice displayed a transient decrease in VMR during UBD at 1d and a significant increase in VMR at 8d post-WAS, when compared to both their 1d post-WAS measurements and their naïve counterparts (Figure 3.4B). Exposure to WAS transiently increased VMR during CRD only in naïve mice at 1d post-WAS, compared to baseline and NMS VMR (Figure 3.4D). The VMR during CRD was unaffected by WAS in NMS mice at both the 1d and 8d time points (Figure 3.4D). No significant correlations between VMR and estrous cycle stage were observed in either NMS or naïve mice (data not shown).

Figure 3.4

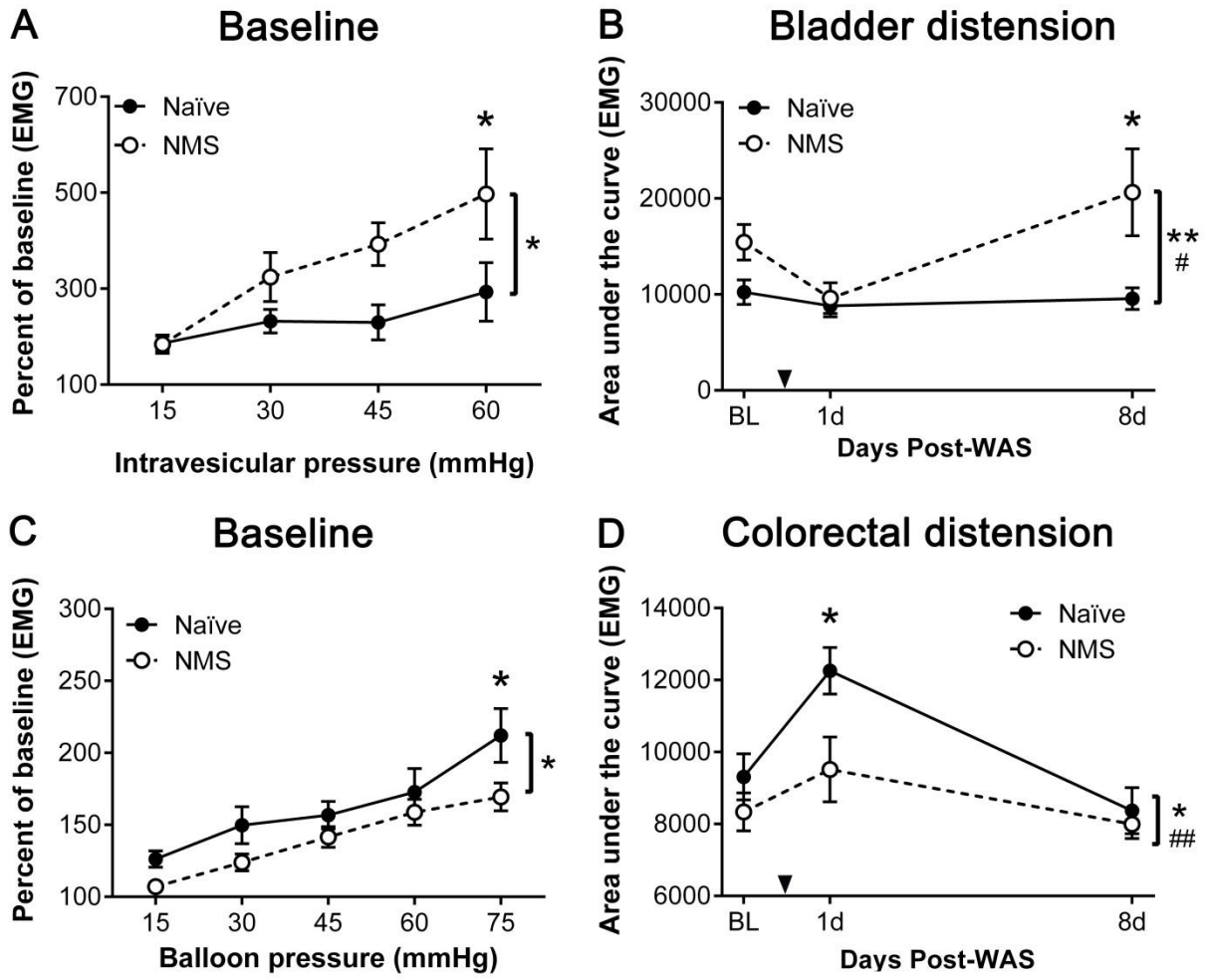


Figure 3.4 The visceromotor response (VMR) during urinary bladder distension (UBD) or colorectal distension (CRD) was measured to determine the impact of neonatal maternal separation (NMS) and water avoidance stress (WAS) on pelvic organ sensitivity. **A)** At baseline (BL), NMS mice displayed significantly greater VMR during UBD across the entire distension series, and at the highest intravesicular pressure, compared to naïve mice. **B)** The area under the curve (AUC) was measured for VMR during UBD at 1d and 8d post-WAS and compared between NMS and naïve mice. This comparison revealed a significant effect of both NMS and WAS on bladder sensitivity, particularly at the 8d post-WAS timepoint when NMS mice displayed an even greater increase in bladder sensitivity as compared to naïve mice, which were largely unaffected by WAS. **C)** At BL, NMS mice had a slight, but significant reduction in VMR during CRD compared to naïve mice, particularly at the highest intraballoon pressure applied. **D)** The AUC was measured for VMR during CRD at 1d and 8d post-WAS and revealed a significant effect of both NMS and WAS on colorectal sensitivity, particularly at the 1d post-WAS timepoint when naïve mice had a significant increase in VMR that was not observed in NMS mice. Brackets indicate a significant effect of NMS (*, ** $p < 0.05, 0.01$) or WAS (#, ## $p < 0.05, 0.01$), two-way RM ANOVA; * $p < 0.05$ vs. naïve, Bonferroni posttest.

Neonatal and adult stress exposure significantly impact urinary bladder output

The functional output of the bladder and colon was characterized in NMS and naïve mice prior-to and following WAS to determine the impact of early life and adult stress on micturition and defecation rates. The number of voidances and total urine output were not different between naïve and NMS mice at baseline (Figure 3.5A-B). However, at 1d post-WAS, both the number of voidances and total urine output were significantly increased in NMS mice, compared to naïve (Figure 3.5A-B). Fecal output was also measured pre- and post-WAS and neither NMS nor WAS had a significant impact (Figure 3.5C). No significant correlations between urine output or voidance frequency and estrous cycle stage were observed in either NMS or naïve mice (data not shown).

Neonatal and adult stress exposure significantly increase mast cell degranulation and protein expression of associated pain-signaling receptors in the bladder

To determine if mast cell activation could be contributing towards the bladder-specific effects observed in NMS mice following WAS, bladder tissue from NMS and naïve mice was processed for mast cell visualization and Western blotting. Mast cells were visualized using acidified toluidine blue and analyzed for state of degranulation (Figure 3.6B-C'). Bladders from NMS mice contained a significantly larger percentage of mast cells exhibiting evidence of degranulation than did bladders from naïve mice (Figure 3.6A). Exposure to WAS had an overall effect of increasing mast cell degranulation, primarily driven by a non-significant increase in the percentage of degranulated mast cells in naïve bladder (Figure 3.6A). Protein expression of the tryptase receptor, protease antigen receptor type 2 (PAR2), and transient receptor

Figure 3.5

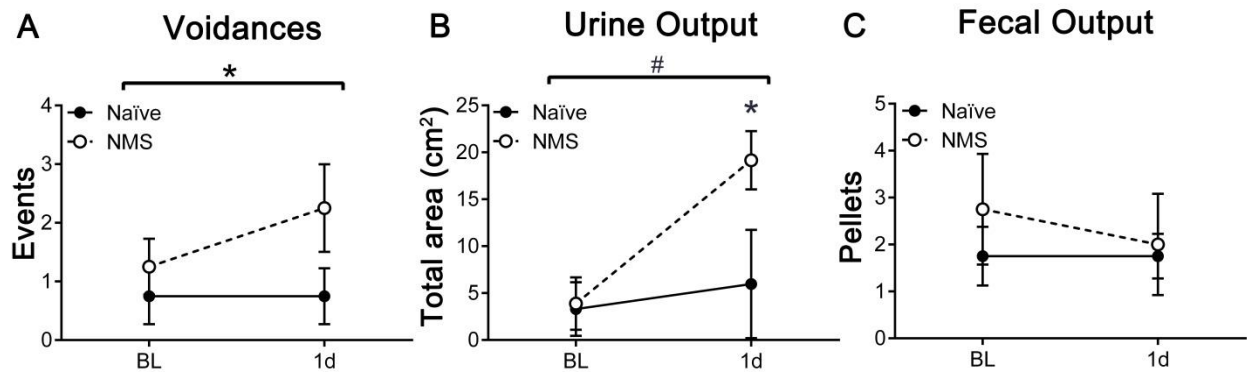


Figure 3.5 Micturition frequency and output, and fecal output, were measured over a 1 h testing period to determine changes in bladder or gastrointestinal function resulting from neonatal maternal separation (NMS) and water avoidance stress (WAS). **A)** Void frequency, measured as the number of voidance events during a 1 h test period, was significantly increased by NMS. **B)** WAS significantly increased total urine output during the 1 h test period, particularly in NMS mice which had a significantly higher output than naïve mice at the 1d post-WAS time point. **C)** Neither NMS nor WAS significantly impacted fecal output. Brackets indicate a significant effect of NMS ($*p < 0.05$) or WAS ($\# p < 0.05$), two-way RM ANOVA; $* p < 0.05$ vs. naïve, Bonferroni posttest.

Figure 3.6

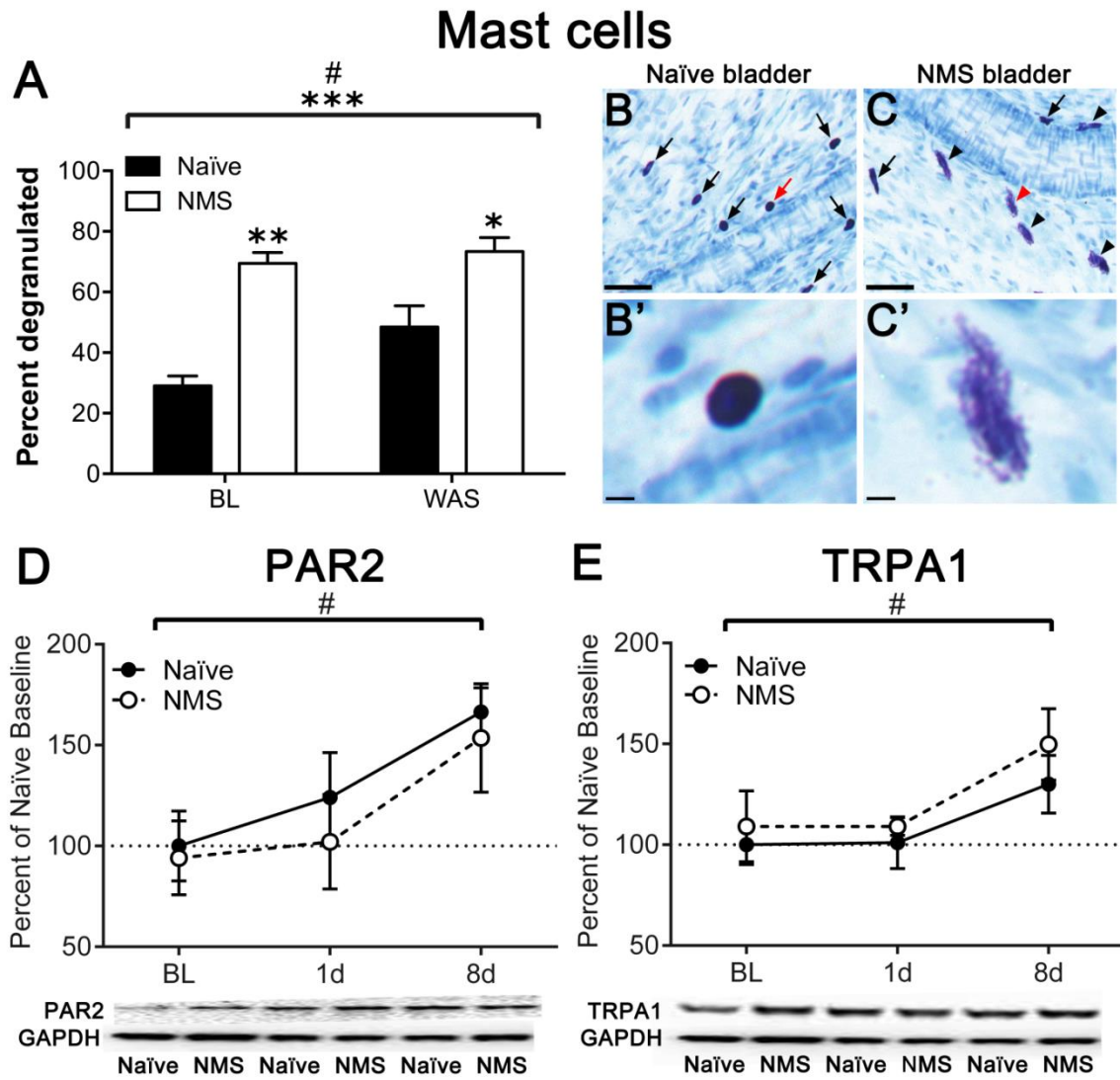


Figure 3.6 The extent of mast cell degranulation and the protein levels of the tryptase receptor, protease-activated receptor 2 (PAR2) and the transient receptor potential ankyrin 1 (TRPA1) were measured in the bladder to determine the potential role of the mast cell-nerve axis in bladder hypersensitivity resulting from neonatal maternal separation (NMS) and water avoidance stress (WAS). Intact (red arrow, **B**, **B'**) and degranulated (red arrowhead, **C**, **C'**) mast cells were visualized in bladders from NMS and naïve mice at baseline (BL) and 1d post-WAS. A significant effect of NMS and WAS was observed on mast cell degranulation, such that NMS bladder had a significantly larger percentage of degranulated mast cells, both at BL and 1d post-WAS (**A**). WAS significantly increased protein levels of both PAR2 (**D**) and TRPA1 (**E**) in the bladder from naïve and NMS mice. Representative Western blots are shown for PAR2, TRPA1, and corresponding GAPDH protein expression with bands at 32, 127, and 35kD, respectively. Brackets indicate a significant effect of NMS (**** $p < 0.0001$) or WAS (# $p < 0.05$), two-way ANOVA; *, ** $p < 0.05$, 0.01 vs. naïve, Tukey's posttest. Scale bars represent 100 μ m (**B**, **C**) and 10 μ m (**B'**, **C'**).

potential ankyrin repeat 1 (TRPA1) was significantly increased following WAS exposure in both naïve and NMS bladder (Figure 3.6F-G).

3.5 Discussion

Patients suffering from chronic pelvic pain syndromes commonly report symptom onset or worsening during times of stress. Experiencing stress or trauma early in life increases the likelihood of developing chronic pelvic pain later in life, as well as comorbid mood disorders (281). Here we have provided the first evidence of early life stress-induced bladder hypersensitivity in mice. Exposure to adult stress further exacerbated urinary bladder sensitivity in NMS mice and, conversely increased colorectal sensitivity in naïve mice. Together with enhanced neuroimmune profiles in the bladder and molecular evidence of diminished HPA axis regulation, these data suggest that NMS alters the response to stress later in life.

In our previous study, we demonstrated that performing NMS throughout the 3 week pre-weaning period resulted in a stronger phenotype than the standard 14 day-long separation period, which we attributed to disruption of proper limbic structure maturation during the third week of life (150). Here, we show that although WAS-induced changes in mRNA levels were observed in both the hypothalamus and hippocampus, only in the latter were NMS-associated changes denoted. Exposure to WAS significantly, and selectively, decreased the mRNA levels of MR and GR only in the NMS hippocampus at the 1d time point and had a longer-lasting effect on CRF₁ and CRF₂ mRNA levels. This is opposite what has previously been reported in rat NMS models following acute stress exposure, however, this is likely due to variations in NMS-duration, species, and the nature of the acute stressor (287). The transient decrease in

mRNA levels returned to naïve-like levels by 8d post-WAS, suggesting that negative regulation from the NMS hippocampus was acutely, but not chronically, impaired following WAS exposure. Interestingly, WAS-induced changes in mRNA levels in the hypothalamus did not differ between naïve and NMS mice, and also were not observed until the 8d post-WAS time point. The observed increase of genes involved in negative regulation of the HPA axis, including CRF₂, GR, and MR, and the delayed increase in expression together suggest that changes in gene expression within the hypothalamus may have been driven by the transiently reduced negative regulation from the hippocampus.

Under normal conditions, acute stress exposure induces long term potentiation (LTP) in the hippocampus, which underlies learning and memory formation and is dependent upon BDNF (366, 367). Exposure to chronic stress disrupts LTP, which can be rapidly reversed by GR antagonist treatment (368). Exposure to acute or chronic stress has been shown to significantly downregulate BDNF mRNA and protein levels in the rodent hippocampus, as has treatment with exogenous glucocorticoids (366). Dexamethasone treatment or genetic overexpression of GR has been shown to increase hippocampal BDNF mRNA or protein levels and, conversely, knockdown of GR decreased BDNF protein content in the hippocampus (369, 370). Our observation of a concurrent decrease in hippocampal GR and BDNF mRNA levels only in the NMS mice at 1d post-WAS, further suggests that the hippocampal response to adult stress is dysregulated following neonatal stress exposure.

Despite no transcriptional evidence of decreased regulation of the HPA axis at baseline, we observed an increase in resting serum CORT concentration in NMS mice

prior to WAS exposure. Following exposure to WAS, serum CORT levels from NMS mice remained lower than those from naïve mice, further suggesting a maladaptive response to adult stress. Genetic deletion of CRF₂ has been shown to increase basal and evoked CORT levels, without affecting the mRNA level of CRF in the hypothalamus (86). Therefore, the observed changes in serum CORT levels in the current study could be driven, in part, by the significant and robust increase in CRF₂ mRNA level in both the hypothalamus and hippocampus. The mechanisms underlying the specific increase in CRF₂, and the significance thereof, will be a focus of future investigation.

Increased colorectal (146, 319, 344, 355) or vaginal (150) hypersensitivity has previously been shown to manifest in rodent models of NMS and this study provides the first evidence of urinary bladder hypersensitivity following NMS in female mice. Baseline bladder hypersensitivity was further exacerbated by exposure to WAS, in a delayed and prolonged fashion, but this increase in urinary bladder hypersensitivity was observed only in the NMS mice and a more immediate increase in colorectal sensitivity was observed only in naïve mice. The difference in VMR at 1d post-WAS, both in terms of NMS- and organ-effect, likely reflects a dysregulated stress-response in NMS mice rather than stress-induced hypoalgesia, considering that naïve mice showed no change in VMR during UBD and an increase in VMR during CRD at the same time point. Likewise, the gene expression changes in the hippocampus of NMS mice at 1d post-WAS point towards decreased regulatory input. Interestingly, the current study revealed an overall negative impact of NMS on colorectal sensitivity, both at baseline and following WAS, which is contradictory to previous studies using a 14d-long NMS protocol in either rats (146, 287, 371) or mice (319) and suggests that a three-week long

NMS protocol in mice may generate a phenotype restricted to the urogenital organs (150).

Subsets of IC/PBS patients that report histories of early life stress such as sexual and physical abuse frequently present with increased voiding and urgency (18, 284, 372), and individuals that experienced multiple types of maltreatment are more likely to develop comorbid pain disorders (373). Furthermore, patients with bladder dysfunction, in the absence of pain, are more likely to report early life abuse than the general population (374). Increased expression of CRF and CRF₂ was observed in both the urothelium and associated innervation of the bladder following cyclophosphamide-induced bladder inflammation in rats (375), and micturition retention has been reported in mice exposed to social defeat stress (376, 377). Single or chronic WAS exposure has been shown to increase micturition rates in female (153) and male (378) rats, but with sex-dichotomous outcomes resulting in decreased and increased void volumes, respectively. In the present study, female NMS mice exhibited increased micturition rate while WAS exposure significantly increased average urine output for each voidance event in this group. Strikingly, neither NMS nor WAS had effects on fecal output, increased rates of which have been reported in animal models of IBS in males, a phenotype sensitive to CRF antagonism (379-382). Here, we report altered micturition, but not defecation, following WAS in NMS females that parallels the heightened urogenital sensitivity in mice that underwent early life stress.

The role of mast cells in driving neurogenic inflammation and visceral afferent sensitization in mucosal diseases such as IBS and asthma is well recognized (105, 106). Degranulated mediators from mast cells, including tryptase and histamine, bind to

protease activated receptors (PAR) on nearby visceral afferents and can drive stress-mediated sensitization, a process thought to contribute to chronic functional pain (124, 125). Increased tryptase, histamine, IL6, and nerve growth factor (NGF) have all been reported in the urine of IC/PBS patients (383). This nerve-mast cell axis is perpetuated by the chemoattraction of mast cells to antidromic release of peptidergic molecules (127, 128), which, in turn, has been shown to increase mast cell cytokine expression (129). Here we observed a significant increase in mast cell degranulation in NMS bladder compared to naïve. Although both naïve and NMS mice had similar increases in the mRNA levels of the mast cell signaling cytokine, SCF, and both IL6 and NGF were specifically increased in NMS bladder at 8d post-WAS indicating greater neuroimmune activation in this tissue. Indeed, mast cell activation and associated pelvic organ hypersensitivity has been shown to be increased by acute stress, a phenotype that can be prevented, but not reversed, by treatment with mast-cell stabilizers (116, 384).

Physiological stress signaling within the viscera results in phenotypes suggesting heightened neuro-immune interactions underlying visceral hypersensitivity in animal models of IBS (146-149), vulvodynia (150), and IC/PBS (144, 151-153). On one hand, CRF₁ has been implicated in WAS-induced colorectal hypersensitivity (385) and gut-barrier dysfunction (318, 386); however, CRF₂ antagonism blocked the release of the proinflammatory cytokine IL6 from cultured cardiomyocytes (387) and expression of IL10 was increased in cytotrophoblast cells by a CRF₂-mediated mechanism (388). Similarly, stress-induced bladder hypersensitivity and vascular permeability have also been shown to be driven by a CRF₂ rather than a CRF₁-mediated mechanism (144,

145). Here, we report expression of IL6 and IL10 were increased by NMS and enhanced by WAS in bladder. Interestingly, mast cells, rather than T-regulatory cells or macrophages are the major source of IL10 in the bladder (389).

Neonatal stress or visceral injury has been shown to alter expression of inflammatory mediators and TRP channels in the pelvic viscera, changes implicated in driving chronic pain phenotypes (126, 139, 142, 147, 149, 300, 386). Bladder sensitivity and urination frequency during hemorrhagic cystitis or spinal cord injury has been shown to be mediated by a TRPA1 mechanism (390, 391). TRPA1 and TRPV1 have been shown to be sensitized *in vivo* by stimulation of PAR receptors from factors such as those released from degranulated mast cells (139, 142) and likely contributes to chronic functional pain (124, 125). Furthermore, early life stress increased TRPA1 protein expression in bladder (150) while experimental cystitis induced a TRPA1-, but not TRPV1-dependent hyperalgesia (392). Expression of mast cell mediators such as histamine and NGF as well as the tryptase receptor are susceptible to stress-induced increases in the bladder (393) that are not observed in mast cell deficient rats (394). Here, we report acute stress increased expression of PAR2 and TRPA1 in the bladder, findings that when combined with the increased activation of mast cells in the bladder, indicate a heightened presence of neuroimmune signaling in NMS mice. Indeed, bladder, but not colon, NGF was upregulated in NMS, but not naïve, mice exposed to WAS.

3.6 Conclusion

This study provides evidence that early life stress in female mice increased susceptibility to stressful events in adulthood. Altered gene expression in the

hypothalamus following WAS exposure, which was precipitated by hippocampal gene changes, suggests that NMS disrupts negative limbic regulation of the HPA axis. This manifested behaviorally in NMS mice as increased bladder sensitivity both at baseline and 8d after WAS exposure, as well as increased functional output from the bladder. Peripheral evidence of neurogenic inflammation in the bladder, including increased mast cell degranulation and pro-inflammatory gene expression, suggest increased downstream activation of the HPA axis may drive the specific urological hypersensitivity. Together with previous studies demonstrating psychological abnormalities and pelvic organ sensitivities associated with NMS, this study provides new insight into mechanisms that contribute to stress-associated symptom onset and exacerbation in a population of patients exposed to early life stress.

Chapter 4

Voluntary exercise can prevent or reverse urinary bladder hypersensitivity and dysfunction in maternally-separated female mice

4.1 Abstract

Urogenital pain patients commonly report having experienced adverse events early in life, which has been shown to disrupt proper functioning of hypothalamic-pituitary-adrenal (HPA) axis, thereby permanently altering stress responses and the perception of pain. Voluntary exercise has been shown to attenuate many of the negative outcomes associated with HPA axis dysfunction; however, the therapeutic potential of exercise for improving urogenital pain and dysfunction or comorbid mood disorders arising from early life stress has not been determined. Therefore, we housed female mice, that did (NMS) or did not (naïve) undergo daily maternal separation from postnatal day 1-21, in cages with free access to running wheels beginning at 4 or 8 weeks of age to determine if exercise could prevent or reverse urogenital hypersensitivity and dysfunction or altered patterns of sucrose intake resulting from early life stress. Sedentary NMS mice displayed significantly increased visceromotor response (VMR) during urinary bladder distension (UBD), had an increased micturition rate, and consumed greater volumes of sucrose compared to sedentary naïve mice. Exercise beginning at 4 or 8 weeks of age significantly diminished VMR, improved micturition events, and further enhanced sucrose intake in NMS mice. Exercise also prevented or reversed the increase in mast cell degranulation and expression of the tryptase receptor, PAR2, in NMS bladder. In the hippocampus, a limbic regulator of the HPA axis, exercise increased expression of brain-derived neurotrophic factor while glucocorticoid receptor remained elevated in NMS. Taken together, these findings provide novel insight on the efficacy of exercise, an easily translatable clinical

intervention, as a potential treatment strategy for chronic urogenital hypersensitivity and neuroimmune dysfunction associated with early life stress.

4.2 Introduction

Voluntary physical activity and exercise training exerts a wide range of metabolic, neuroendocrine, and neurobiological effects. While exercise physiology has been studied for many decades, the full potential of health benefits derived from exercise have not been explored. Specifically, no studies have evaluated the impact of exercise on animal models of stress-induced chronic pelvic pain (CPP). Interstitial cystitis/painful bladder syndrome (IC/PBS) is characterized by chronic pain and urologic dysfunction and associated with altered activity of the hypothalamic-pituitary-adrenal (HPA) axis, which is thought to contribute to comorbid mood disorders (57, 227, 265, 395) and neuroendocrine dysfunction within the viscera (353, 372). The hippocampus in particular is a source of neural circuitry that provides negative feedback onto the HPA axis, which is mediated, in part, through the glucocorticoid receptor (GR) (65, 396). Peripherally, mast cells may become partially or fully degranulated in response to a range of stimuli, including glucocorticoids (397). Tryptase, a major inflammatory mediator released by activated mast cells, binds to and activates the protease activated receptor (PAR) 2, which has been shown to sensitize transient receptor channels vanilloid 1 and ankyrin 1 (TRPV1 and TRPA1) *in vivo* (139, 142), receptors required for the transmission of noxious inflammatory stimuli (134, 137).

Under normal conditions, exposure to acute stress enhances learning and memory formation via brain-derived neurotrophic factor (BDNF)-induced long-term potentiation (LTP) of hippocampal neurons, a process that is mediated by glucocorticoids (366) and is critical for adaptation to stress (398). Chronic stress, on the

other hand, diminishes LTP, decreases neurogenesis, and disrupts crosstalk between GR and the BDNF receptor, tyrosine receptor kinase (Trk)-B, in the hippocampus (72, 368, 399). Administration of exogenous glucocorticoids or dexamethasone challenge has also been shown to diminish BDNF expression and LTP (367); however, neuronal activity secondary to exercise or cognitive challenges can upregulate BDNF expression and associated neurogenesis in the hippocampus (222, 223). Through the interactions of BDNF on its cognate receptor, TrkB with N-methyl-d-Aspartate receptors (NMDAR) and GR, potentiation of glutamatergic input to inhibitory interneurons in the hypothalamus likely influences negative feedback upon the HPA axis (72, 224, 400).

For several decades, neonatal maternal separation (NMS) has been used as a rodent model of early life stress (401-405) and has been shown to confer long-term changes in limbic, reward, and stress circuitry in central brain regions (150, 406, 407) that are also heavily influenced by exercise (225). In fact, voluntary exercise has been shown to normalize hippocampal BDNF and GR gene expression in NMS rats, as well as concordant behavioral responses to acute stress exposure (272). While exercise intervention has been shown to ameliorate some of the negative effects of NMS (272, 406), no study has evaluated the impact of exercise on stress-associated visceral hypersensitivity. Therefore, pairing exercise as an intervention for the negative effects of NMS is not only warranted but also translatable to clinical practice. This project tested the potential for exercise to ameliorate the predominant behavioral changes in female mice following NMS, namely urological hypersensitivity and altered micturition behavior. Broadening the impact of our findings, this project also evaluated behaviors indicative of conditions comorbid with IC/PBS, including mood and somatic pain

disorders, and the potential impact exercise has on these comorbidities. Finally, molecular and cellular changes downstream of neuroendocrine dysfunction were investigated, specifically, expression of GR and BDNF in the hippocampus and mast cell degranulation and PAR2 expression in the bladder and whether these factors were responsive to exercise intervention.

4.3 Materials and methods

Neonatal maternal separation

Litters were born in house from pregnant dams (Charles River, Wilmington, MA) delivered to the laboratory animal facility during the last week of gestation. Day of birth was designated as postnatal day (P) 0 and on P1 through P21 individual litters were removed daily and placed *en masse* into clean glass beakers containing a small amount of home cage bedding to maintain scent. Pups were held at 34°C and 50% humidity throughout the separation time period (generally from 1100 - 1400 hours). The investigator's gloves were changed between litters and fresh gloves were rubbed with home cage bedding before handling. Corresponding naïve mice were born, housed, and weaned during the same time frame as NMS mice to avoid potential complications arising from variations in prenatal shipping conditions, housing environment, and normal husbandry procedures. All mice were weaned on P22.

Exercise

For the early exercise group, four week-old NMS and naïve animals were pair-housed with littermates to minimize the potential effects of social isolation in cages equipped with *ad libitum* access to a stainless steel running wheel for the duration of the

study (Mini Mitter; Bend, OR). Corresponding sedentary NMS and naïve animals remained housed with littermates in standard cages compliant with IACUC enrichment standards. For the late exercise group, baseline behaviors were first established in eight week-old NMS and naïve animals and the mice were then singly-housed in cages equipped with free access to running wheels. Quantification of wheel revolutions was monitored by Vital View Acquisition and Analysis software (Harvard Apparatus, Holliston, MA) and average running distance per week per animal (late exercise) or per pair of animals (early exercise) was calculated on a personal computer off-line.

Vaginal lavage

The vaginal opening was rinsed of urine and 100 µl of phosphate buffered saline (PBS) was gently expelled into the vaginal canal 3 times with a transfer pipette. Care was taken to not penetrate the orifice. The collected fluid was placed into a single well of a 96-well plate and immediately examined with light microscopy. The stage of estrous cycle was recorded by determining the ratios of the following cell types: nucleated epithelial cells, cornified epithelial cells, and leukocytes.

Urinary bladder distension

Under inhaled isoflurane (4% induction, 2% maintenance), the bare ends of two Teflon coated electrodes (0.003" diameter; Grass Technologies, West Warwick, RI) were acutely implanted into the right and left abdominal musculature using a 26-gauge needle and a 24-gauge angiocatheter was inserted intravesically via the urethra and secured via tape. Following catheterization, isoflurane anesthesia was lowered until hindlimb reflexes, but not escape behaviors, were present (approximately 0.9%

isoflurane). A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO) and a separate pressure monitor (World Precision Instruments, Sarasota, FL) was used to regulate the pressure within the bladder. Following three 60mmHg distensions to establish stable responses, each pressure (15, 30, 45, 60mmHg) was applied in triplicate for 20 seconds with a 2-minute rest period in between. Electromyographic (EMG) activity was amplified, filtered, and recorded using Spike 2 software (Cambridge Electronic Design, Cambridge, UK) on a personal computer and analyzed off-line. The visceromotor response (VMR) was quantified by measuring the area under the curve of the entire distension period and expressed as a percent of baseline activity. Immediately following UBD, animals were monitored for estrous cycle stage and returned to their home cages.

Micturition events

Animals were placed on a piece of filter paper and covered by a clear plexiglass container (36 cm length x 20 cm width x 13 cm height) for 1 h duration. The number and size of urine spots were measured using Image J following visualization with ultraviolet light (408). Micturition frequency, average urine output for each micturition event, and total fecal output were analyzed across all groups. All testing took place during the early portion of the light-cycle (before 0900 h) to control for effects of diurnal rhythms of stress hormones. After the testing procedure, animals were monitored for estrous cycle stage and returned to their home cages.

Sucrose preference placement

Mice were individually housed in BioDAQ Liquid Choice Unplugged Allentown cages (Biological Data Acquisition, New Brunswick, NJ) equipped with two Polysulfone BioDAQ drinking bottles. Following a two-day acclimatization period during which both bottles contained standard drinking water, one bottle was filled with 1% sucrose diluted in drinking water and fluid volume levels were recorded and the position of the bottles interchanged daily for a total of two days. The total volume of 1% sucrose consumed, as well as the percentage of 1% sucrose was calculated.

Mast cell degranulation

Prior to sacrifice, vaginal lavage and cytology was performed daily, for at least 4 consecutive days, to determine cycle stage. Upon verification of estrus, mice were transcardially perfused with ice cold 4% paraformaldehyde and whole bladder tissue and dura mater from the supratentorial calvaria were removed, postfixed for 1 hour, and cryoprotected overnight in 30% sucrose at 4°C. Bladders were washed in 1x PBS and flash frozen in ice cold heptane. Bladder cryosections were cut at 10 µm thickness from whole tissue mounted in Tissue-Tek OTC mounting medium (Sakura Finetek, Torrance, CA) using a cryostat. The dura mater was washed in 1x PBS and whole-mounted onto slides and stored at -20°C until staining. Non-serial sections spanning the length of bladder tissue and whole-mounted dura mater were stained with a 1% toluidine blue solution acidified with 1M HCl to a pH less than 1.0, dried overnight, dehydrated with 95% and 100% ethanol-dips, and coverslipped with 1x PBS for analysis. Using light microscopy (Nikon eclipse 90i, Nikon Instruments, Inc., Melville, NY), digital images were captured (QIClick digital CCD Camera, QImaging, Surrey, BC, Canada) and the total number of non-degranulated (NG) mast cells (dense metachromasia with no or

faint nuclear outline and/or no granular extrusion around the cell) and degranulated (DG) mast cells (less intense metachromasia and obvious clear outline of the nucleus and/or free granules within the cytoplasm) were counted in at least 8 separate sections spanning the length of bladder tissue or 10 non-adjacent fields (800 μm^2 per field) across the dura mater. The percentage of DG to total mast cells was calculated for each tissue/mouse and differences between groups were analyzed.

mRNA extraction and qRT-PCR

Vaginal lavage and cytology was performed daily, for at least 4 consecutive days, to determine cycle stage. Upon verification of estrus (approximately 1 week after UBD) mice were deeply anesthetized with inhaled isoflurane (>5%) and, following decapitation, whole brains were removed and frozen on dry ice. Bilateral hippocampi were dissected, immediately snap-frozen in liquid nitrogen, and stored at -80°C . The urinary bladder was removed and bisected longitudinally for both mRNA and protein (see below) analysis, snap-frozen in liquid nitrogen, and stored at -80°C until processing. Total mRNA was isolated using Trizol reagent (Ambion, Austin, TX) and RNeasy micro kit (Quiagen, Valencia, CA) as per manufacturer's instructions. Sample concentration and purity was determined using a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and cDNA was synthesized from total RNA (0.5 μg) using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR amplification was performed using 0.2 μg of total cDNA (from the 0.5 μg reverse-transcribed cDNA) and SsoAdvanced SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX manager 3.1 real time PCR system with indicated 20 μM primers (Integrated DNA Technologies, Coralville, IA) as listed in Table 4.1. Samples were run

Table 4.1 Primers used for real-time PCR analysis

Gene	Forward (5' – 3')	Reverse (3' – 5')
PAR2	GAAACACCCCGCCGTGATTTA	CTCCCCGTAGACCCAGTTG
BDNF	CAGGTTTCGAGAGGTCTGACGA	CGCGTCCTTATGGTTTTCTTCG
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT
β -actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT

in triplicate and negative control reactions were run with each amplification series with β -actin (bladder) or GAPDH (hippocampus) as the housekeeping gene. To reduce variability among efficiency due to fluctuations in baseline fluorescence, the raw (i.e. non-baseline corrected) PCR data was imported to the LinRegPCR software (version 2012.3) (359-361) and PCR efficiency values were derived for each individual sample by fitting a regression line to a subset of data points within the sample's log-linear phase. Threshold cycle (Ct) values were subtracted from that of the selected housekeeping gene and the percentage of fold change over naïve controls was calculated using the Pfaffl method (362).

Protein analysis

Upon confirmation of estrus, mice were overdosed with inhaled isoflurane (>5%) and, following decapitation, dura mater from the supratentorial calvarium was dissected and flash frozen in liquid nitrogen while the urinary bladder was longitudinally bisected (as mentioned above) and flash frozen in liquid nitrogen. Total protein was isolated using Cell Extraction Buffer containing Halt protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham MA) and Na_3VO_4 . Protein concentrations were determined using a D_c protein assay (ThermoFisher Scientific, Waltham, MA). Samples were reduced by heating to 95°C for 5 minutes in the presence of 2-mercaptoethanol, subjected to SDS-PAGE (Criterion 4% to 12% Bis-Tris gels; Bio-Rad Laboratories), and transferred to Nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) by Criterion Blotter wet transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature in 5% milk in Tris-buffered saline with Tween-20 then incubated overnight at 4°C with antisera to PAR-2 (1:500; Abcam, Cambridge, MA) and GAPDH

(1:1000; Cell Signaling Technology, Beverly, MA). Membranes were washed with Tris-buffered saline with Tween-20 and incubated for 1 hour with anti-rabbit secondary antibody (1:10,000; Cell Signaling Technology, Beverly, MA). Densitometry was performed using Quantity One 4.6.9 software (Bio-Rad Laboratories).

Age of mice at experimental time points

Within the early and late exercise paradigms, separate cohorts of mice were used for behavioral tests: UBD, micturition, SPP, and forepaw sensitivity at the indicated ages (Table 4.2). Approximately 1 week post-UBD, animals were euthanized for tissue collection to be used in mast cell degranulation assay, western blotting, or qRT-PCR as described above. Multiple cohorts were chosen because behavioral testing might be considered an acutely stressful experience, which has been shown to alter expression of inflammatory mediators in the pelvic organs and the genes involved in the regulation of the HPA axis in the hippocampus (150).

Statistical analysis

Calculations were performed on Microsoft Excel and statistical analysis was performed using 2-way (with or without repeated measures) analysis of variance (ANOVA) followed by Bonferroni's or Tukey's posttest (GraphPad Prism 6, GraphPad Software, Inc, La Jolla, CA) as denoted in the results section. All data are expressed as mean \pm SEM. A *p* value of less than 0.05 was considered significant.

Table 4.2 Age of mice at experimental time points.

	Running wheels	UBD	Micturition	Sucrose preference	Mechanical forepaw
Early exercise					
Sedentary		8	8	8	8
Exercised	4-8	8	8	8	8
Late exercise					
Baseline		8			
Post-Exercise	8-12	12			

Table 4.2 NMS and corresponding naïve mice underwent behavioral testing at the above noted ages (in weeks). Mice that underwent early exercise were housed in cages with free access to running wheels from 4-8 weeks of age. At 8 weeks of age, individual cohorts underwent urinary bladder distension (UBD), micturition analysis, two-choice sucrose preference test, or mechanical forepaw sensitivity testing. Mice in the late exercise group underwent baseline UBD testing at 8 weeks of age, and afterward, were immediately housed in cages with free access to running wheels until 12 weeks of age at which point UBD was retested. Tissue for mRNA, protein, and mast cell analysis was utilized from naïve and NMS mice 1 week following UBD: 9 weeks of age in early exercise mice (mast cells only) and 13 weeks of age in late exercise mice.

4.4 Results

Exercise ameliorates bladder hypersensitivity and dysfunction in NMS mice

To determine whether voluntary exercise could modify bladder hypersensitivity and over-activity resulting from NMS, mice were housed with free access to running wheels beginning at either 4 (early exercise) or 8 (late exercise) weeks of age. Mice given access to running wheels (-Ex group) were compared to sedentary (-Sed) counterparts in the early exercise experiment and to their baseline measurements (-BL) in the late exercise experiment to determine if exercise could prevent or reverse NMS-induced changes, respectively. The VMR was measured during UBD to determine bladder sensitivity and all groups exhibited a significant effect of intravesicular pressure, indicating a physiological response to UBD (Figure 4.1). In both experiments, sedentary NMS mice at 8 weeks of age (NMS-Sed and NMS-BL) exhibited a significantly greater VMR over the entire distension series, compared to corresponding sedentary naïve mice (naïve-Sed and naïve-BL, respectively) (Figure 4.1). NMS mice that were given exercise wheels at 4 weeks (Figure 4.1A) or 8 weeks (Figure 4.1B) of age displayed significantly reduced VMR compared to either NMS-Sed or NMS-BL mice, respectively, and were not significantly different than their exercised naïve counterparts. Exercise had no significant impact on bladder sensitivity in naïve mice.

Changes in micturition frequency or habit are commonly observed symptoms in IC/PBS (409). Therefore, we evaluated the impact of NMS and exercise on void frequency during a 1 h testing period. The number of void events was significantly increased in NMS-Sed mice compared to naïve-Sed mice (Figure 4.2). However, micturition frequency was not significantly different between NMS and naïve mice given

Figure 4.1

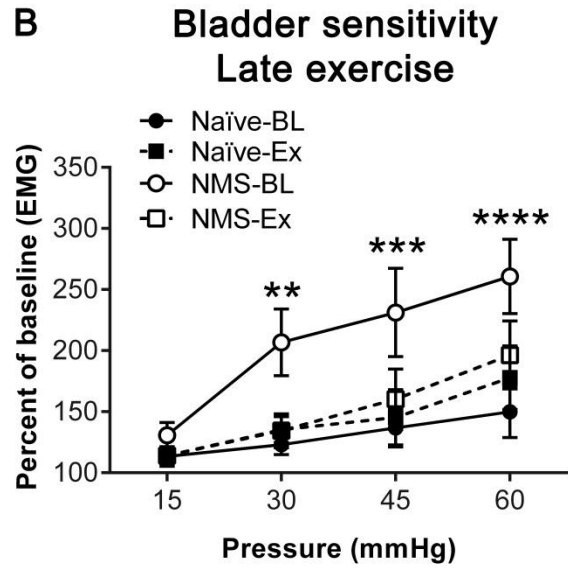
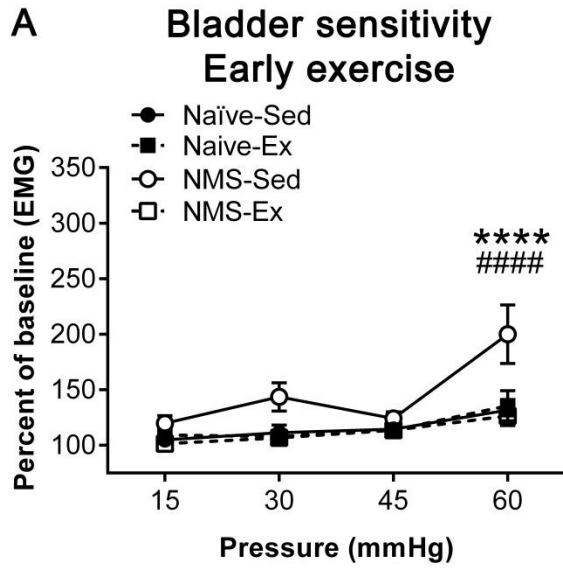


Figure 4.1 The visceromotor response (VMR) during urinary bladder distension (UBD) was measured to determine the effect of early (A) or late (B) exercise intervention on neonatal maternal separation (NMS)-induced bladder hypersensitivity. **A)** Sedentary (-Sed) NMS mice displayed significant urinary bladder hypersensitivity over the entire distension series, compared to naïve-sed mice ($p=0.0053$). Exercised (-Ex) NMS mice that were given running wheels at 4 weeks of age displayed a significantly reduced VMR compared to NMS-Sed ($p=0.0064$). **B)** Similar to the early exercise group, at baseline, NMS mice displayed significantly greater VMR than naïve mice ($p=0.0062$). Following 4 weeks of exercise, the same group of NMS mice displayed a significantly reduced VMR compared to their baseline measurements ($p=0.0048$). Two-way RM ANOVA; *, **, *** $p<0.05$, 0.01, 0.001 vs. naïve-sed (**A**) or naïve-baseline (BL; **B**); ##, ### $p<0.01$, 0.001 vs. NMS-Ex; Bonferroni posttest.

Figure 4.2

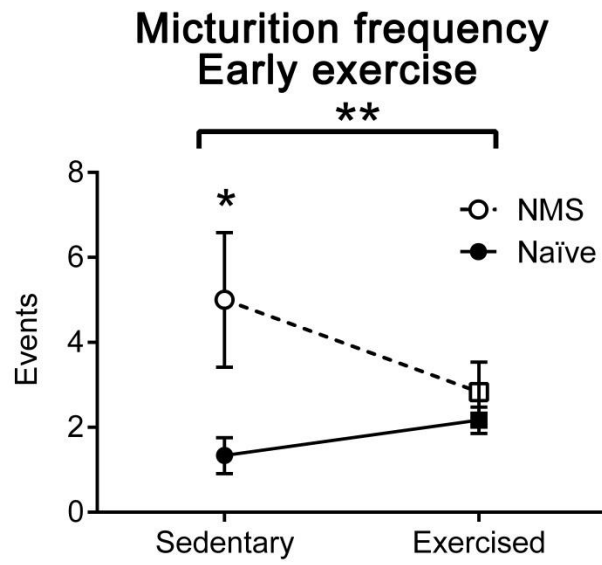


Figure 4.2 Micturition frequency was measured over a 1 h test period to determine changes in bladder function due to neonatal maternal separation (NMS) or exercise. Void frequency, measured as the number of voiding events during a 1 h test period, was significantly increased in female sedentary NMS mice compared to sedentary naïve mice. This difference was not observed between NMS and naïve mice given running wheels at 4 weeks of age. $p=0.0092$, two-way ANOVA; ** $p<0.01$ vs. naïve, Bonferroni posttest.

running wheels at 4 weeks of age (Figure 4.2). Exercise had no impact on micturition frequency in naïve mice.

Exercise stabilizes mast cell infiltrates in NMS bladder

Many studies report evidence of increased mast cell activity in the bladder of IC/PBS patients (410-413). Mast cells are also acutely responsive to stress and both secrete- and respond to- stress ligands, such as corticotropin-releasing factor (CRF) (120). Therefore, we quantified the percentage of degranulated to total mast cells in the bladder of NMS and naïve mice with or without access to running wheels. In both groups of mice, sedentary NMS mice at 8 weeks of age exhibited a significant increase in mast cell degranulation, compared to sedentary naïve counterparts (Figure 4.3). Exercise beginning at 4 weeks (Figure 4.3A) or 8 weeks of age (Figure 4.3B) significantly decreased the percentage of degranulated mast cells in NMS bladders, compared to sedentary or baseline counterparts. Exercise moderately decreased the percentage of degranulated mast cells in naïve bladder; however, the impact was not significant.

Exuded mast cell granules contain the secretory protein tryptase, which can bind and activate PAR2 located on adjacent free nerve endings (363, 414). Protein levels of PAR2 were moderately increased in sedentary NMS bladder; however, exercise beginning at 4 weeks of age significantly reduced PAR2 protein levels in NMS bladder compared to NMS-Sed (Figure 4.4A). The mRNA levels of PAR2 were also significantly reduced in NMS-Ex bladder compared to NMS-Sed levels (Figure 4.4B). Exercise had no impact on PAR2 protein or mRNA levels in naïve bladder.

Figure 4.3

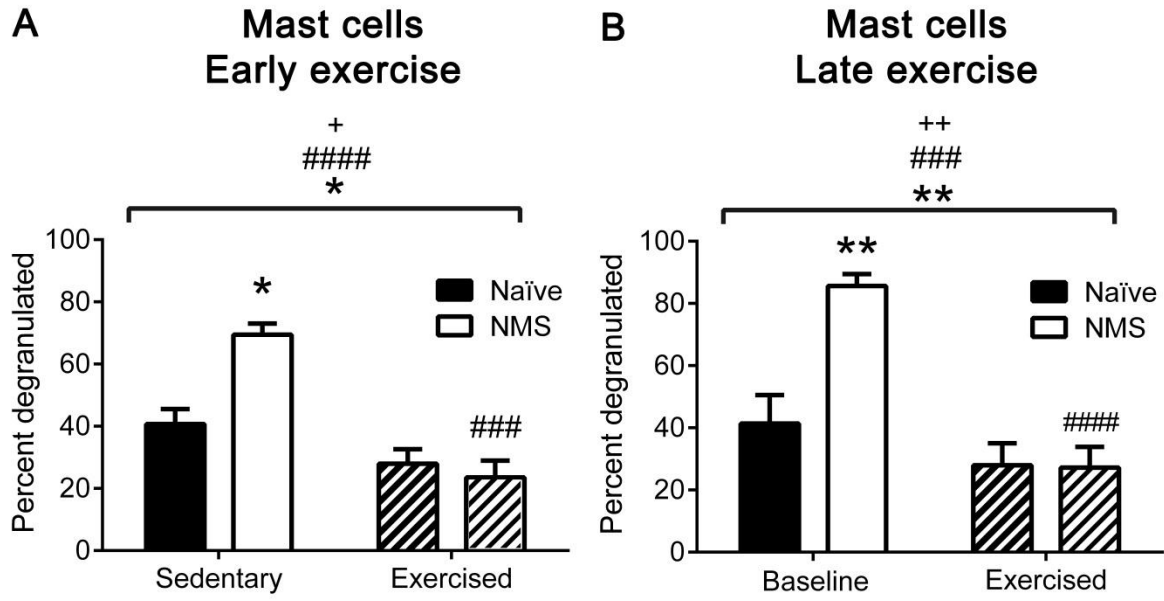


Figure 4.3 The extent of mast cell degranulation was determined in bladders from neonatal maternal separation (NMS) and naïve mice that underwent early (A) and late (B) exercise interventions. Sedentary NMS mice in both experiments exhibited a significant increase in the percentage of degranulated mast cells in the bladder, compared to sedentary naïve counterparts. Exercise beginning at 4 weeks of age (A) or 8 weeks of age (B) significantly decreased the percentage of degranulated mast cells in female NMS bladders. Brackets indicate a significant impact of NMS (*, ** $p < 0.05, 0.01$), exercise (###, #### $p < 0.001, 0.0001$) or an NMS/exercise interaction (+, ++ $p < 0.05, 0.01$), two-way ANOVA; *, ** $p < 0.05, 0.01$ vs. naïve, ###, #### $p < 0.001, 0.0001$ vs. sedentary, Bonferroni posttest.

Figure 4.4

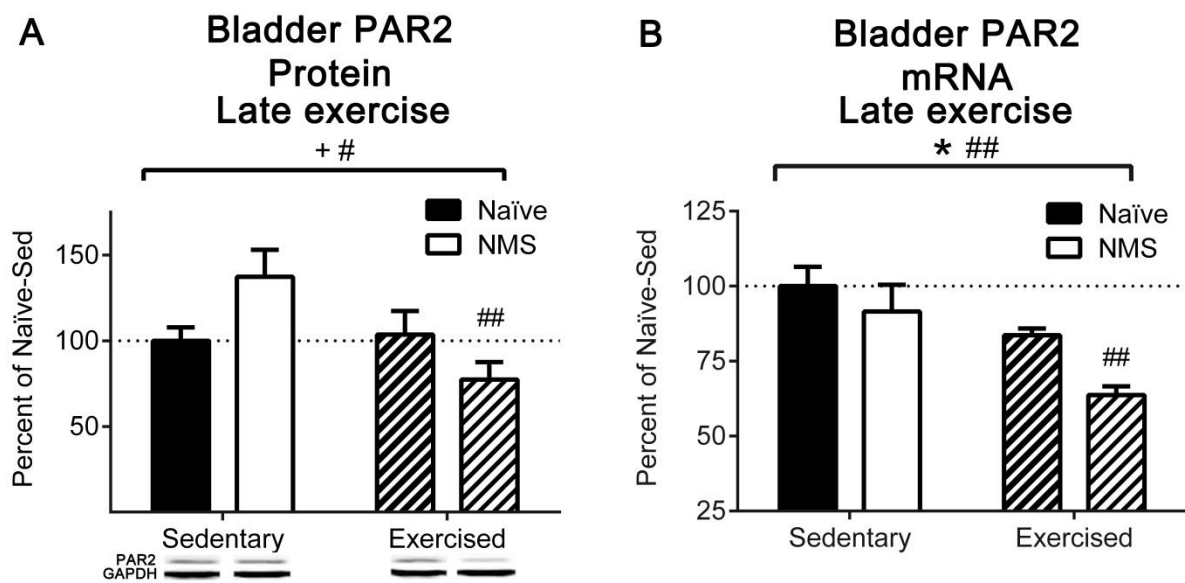


Figure 4.4 The protein and mRNA levels of the tryptase receptor, protease-activated receptor 2 (PAR2), were evaluated in the bladders from neonatal maternal separation (NMS) and naïve mice that underwent late exercise intervention. **A)** PAR2 protein levels were moderately increased in sedentary NMS bladder. Exercise beginning at 8 weeks of age significantly decreased both protein **(A)** and mRNA levels of PAR2 in NMS bladder **(B)**. Bracket indicates a significant impact of NMS (* $p < 0.05$), exercise (#, ## $p < 0.05, 0.01$) or an NMS/exercise interaction (+ $p < 0.05$) two-way ANOVA; * $p < 0.05$ vs. naïve, ## $p < 0.01$ vs. sedentary, Tukey's posttest.

Exercise influences hippocampal gene expression

To evaluate the impact of exercise on early life stress-imposed influences on limbic regulators of the HPA axis, we evaluated the hippocampal mRNA levels of BDNF and GR in sedentary and exercised NMS and naïve mice from the late exercise group. Exercise beginning at 8 weeks of age significantly increased BDNF expression in the hippocampus of both naïve and NMS mice (Figure 4.5A) compared to their sedentary counterparts. Meanwhile, NMS significantly increased GR expression in the hippocampus; however, no significant effects of exercise were observed in either naïve or NMS mice (Figure 4.5B).

Exercise influences altered reward behavior in NMS mice

Previous studies have shown that the psychopathology associated with behavioral indicators of depression (415, 416) including anhedonia (417), and sensitivity of reward pathways (416, 418), have defined neurodevelopmental origins, which are associated with dysfunction of the HPA axis (65, 350, 419). In a two-choice preference test, sedentary NMS mice consumed a significantly greater volume of 1% sucrose than their naïve counterparts (Figure 4.6A). Exercise beginning at 4 weeks of age increased sucrose consumption only in naïve mice, with a modest increase in NMS mice (Figure 4.6A). No significant difference in total volume of liquids consumed (drinking water and sucrose combined) was observed between groups (data not shown). A significant correlation was observed between sucrose preference and micturition frequency only in NMS mice, such that lower sucrose preference correlated with greater micturition frequency (Figure 4.6B). Running distance was calculated as a measure of reward behavior and NMS mice given running wheels at 4 weeks of age ran significantly

Figure 4.5

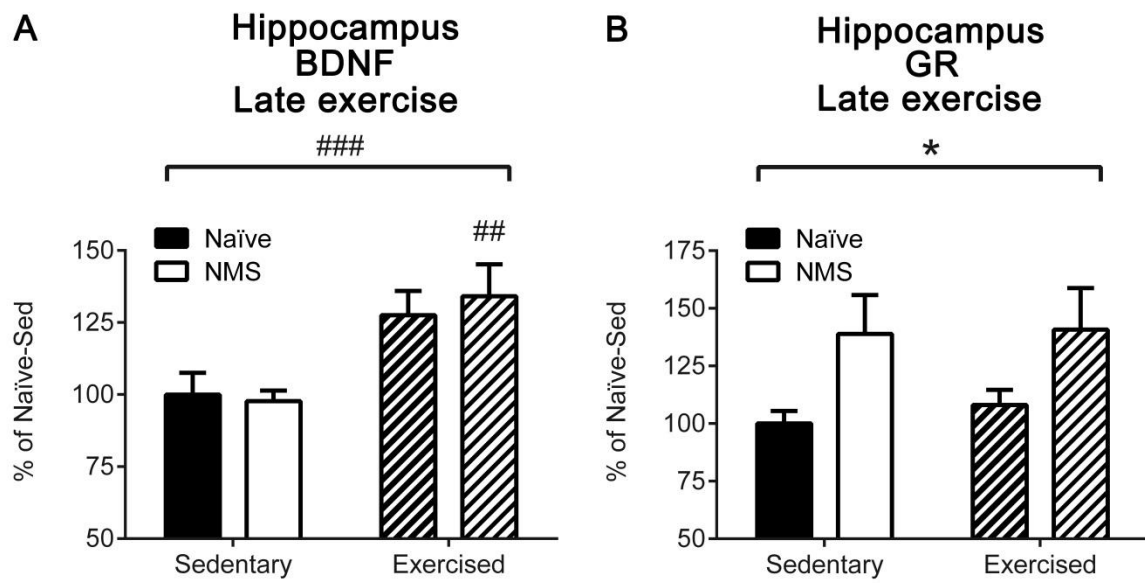


Figure 4.5 The mRNA levels of brain-derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) were measured in the hippocampus from neonatal maternal separation (NMS) and naïve mice that underwent late exercise intervention. **A)** No difference in hippocampal BDNF mRNA levels were observed between sedentary NMS and naïve mice; however, exercise beginning at 8 weeks of age significantly increased BDNF mRNA levels in the hippocampus of both naïve and NMS mice. **B)** NMS had a significant overall impact on increasing GR mRNA levels in the hippocampus. Late exercise did not have an impact on GR mRNA levels in either naïve or NMS hippocampus. Bracket indicates a significant impact of NMS (* $p < 0.05$) or exercise (### $p < 0.001$) two-way ANOVA; ## $p < 0.01$ vs. sedentary, Tukey's posttest.

Figure 4.6

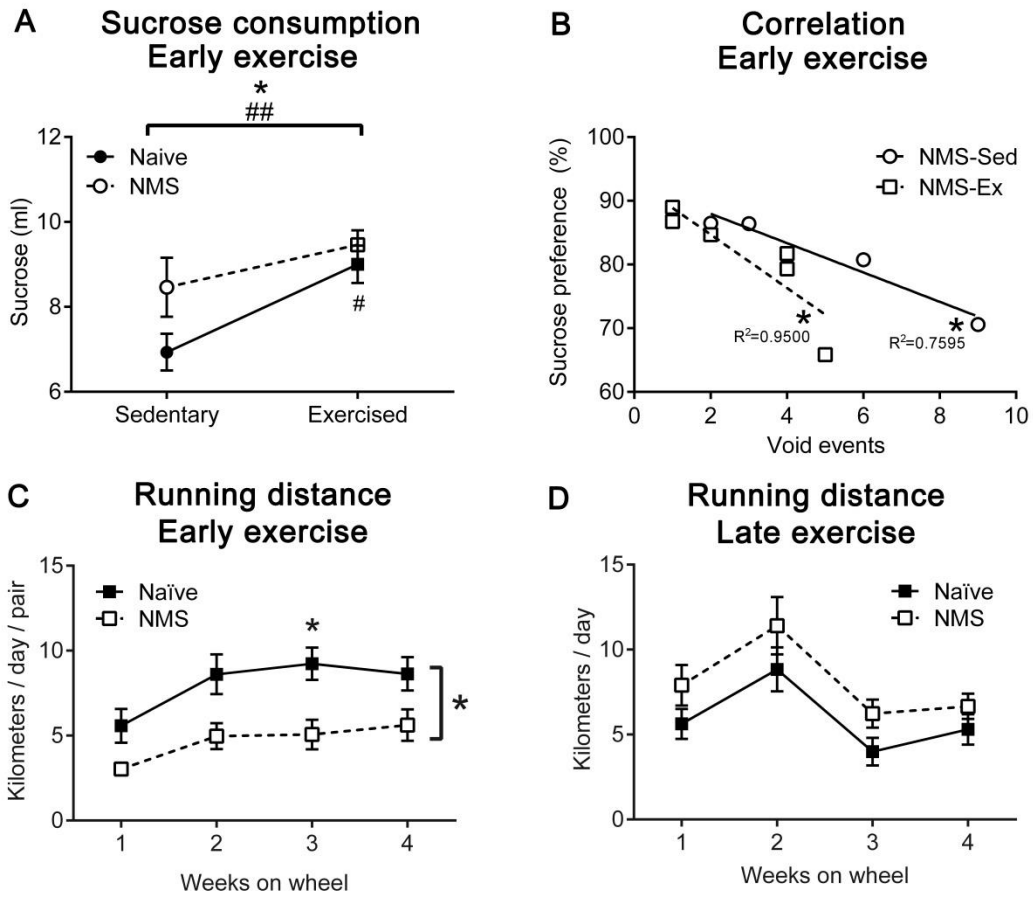


Figure 4.6 Reward behaviors were measured in naïve mice and those that underwent neonatal maternal separation (NMS) to determine functional output of the hippocampus and the influence of an early or late exercise intervention. **A)** In a two-choice preference test, sedentary NMS mice consumed significantly more sucrose than their naïve counterparts. Exercise beginning at 4 weeks of age significantly increased sucrose consumption in only naïve mice. **B)** A significant correlation between sucrose preference and void frequency was observed in female NMS mice, such that increased micturition frequency correlated with decreased sucrose preference. **C)** NMS mice given running wheels at 4 weeks of age ran significantly shorter distances per day (km/day) compared to naïve mice ($p=0.0441$) **D)** NMS mice given running wheels at 8 weeks of age trended toward an increase in distance ran (km/day) compared to naïve mice ($p=0.0809$). **A:** Bracket indicates a significant impact of NMS ($*p<0.05$) or exercise ($##p<0.01$), two-way ANOVA; $#p<0.05$ vs. sedentary, Tukey's posttest. **B:** $*p<0.05$, Pearson correlation. **C, D:** Two-way RM ANOVA.

shorter distances per day (km/day) compared to their naïve counterparts across the study (Figure 4.6C); whereas late exercise NMS mice trended toward an increase in average distance ran per day (km/day) compared to naïve mice (Figure 4.6D).

Late exercise reverses evidence of somatic comorbidities associated with HPA axis dysfunction

Somatic co-morbidities are commonly experienced by IC/PBS patients and are associated with dysfunction within the HPA axis or limbic circuits (420, 421). Here, we evaluated forepaw mechanical sensitivity and mast cell degranulation within the dura mater as correlates to the musculoskeletal dysfunction exhibited by fibromyalgia and migraine patients, respectively. Forepaw mechanical sensitivity was significantly increased in NMS-Sed mice compared to naïve-Sed at 8 weeks of age, but after 4 weeks of exercise, forepaw sensitivity was no longer significantly different between NMS-ex and naïve-Ex. Naïve-Sed was significantly more sensitive compared to both NMS-Ex and naïve-Ex after 4 weeks of exercise, but the withdrawal thresholds in NMS-Sed remained significantly decreased compared to naïve-Sed (Figure 4.7A). A greater percentage of mast cells were degranulated in NMS-Sed dura mater compared to naïve-Sed (Figure 4.7B). This difference was not observed in NMS-Ex animals exercised at 8 weeks of age. No significant impact of NMS or exercise was observed on PAR2 protein levels in the dura mater (Figure 4.7C).

Figure 4.7

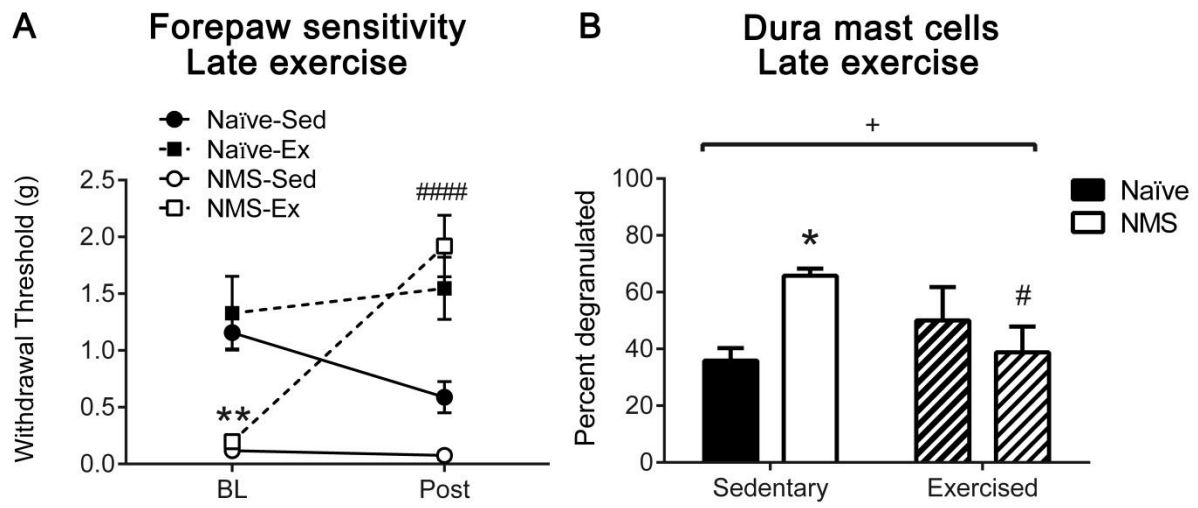


Figure 4.7 Forepaw mechanical sensitivity and dural mast cell degranulation were evaluated as phenotypic markers of somatic comorbidities in mice that underwent neonatal maternal separation (NMS). **A)** Sedentary NMS mice exhibited significant forepaw mechanical allodynia, which was reversed following 4 weeks of exercise beginning at 8 weeks of age. A significant increase in forepaw mechanical sensitivity was observed in sedentary naïve mice. Two-way RM ANOVA with Bonferroni posttest; ** $p < 0.01$ vs. naïve, #### $p < 0.0001$ vs. baseline. **B)** The percentage of mast cells with evidence of degranulation was significantly elevated in the dura mater of sedentary NMS mice, compared to sedentary naïve mice. Exercise beginning at 8 weeks of age significantly decreased the percentage of degranulated mast cells present in NMS dura mater. Bracket indicates a significant NMS/exercise interaction ($p < 0.05$) two-way ANOVA with Bonferroni posttest. * $p < 0.05$ vs. naïve-Sed, #### $p < 0.0001$ vs. NMS-Sed.

4.5 Discussion

Voluntary physical activity and exercise training exerts a wide range of metabolic, neuroendocrine, and neurobiological effects. While exercise physiology has been studied for many decades, the full potential of health benefits derived from exercise has not been explored. Specifically, no published studies have evaluated the impact of exercise on animal models of chronic urogenital pain. For several decades NMS has been used to investigate long-term changes in behavior and physiology resulting from early life stress, including alterations in limbic, reward, and stress circuitry-central brain regions (106, 185, 186), areas that are also heavily impacted by exercise (187). Clinically, exercise reduces the effects of depression and anxiety and associated perceptions of pain (33-35). In this chapter, we investigated the efficacy of an early or late exercise intervention on reversing NMS-induced behavioral and molecular changes.

Clinically, IC/PBS is characterized by recurrent pain in the bladder or surrounding region and at least one additional urological symptom such as frequency, urgency, or nocturia (422). In the present study, we quantified bladder sensitivity by measuring VMR during UBD in NMS and naïve mice that were either housed in cages with or without free access to running wheels beginning at two different experimental time points. In both paradigms, NMS-Sed mice at 8 weeks of age displayed significantly increased VMR during UBD as compared to their naïve-Sed counterparts, confirming our results in chapter 3 that NMS induces long-lasting urinary bladder hypersensitivity in female mice. Early exercise animals were given access to running wheels at 4 weeks of age to evaluate the potential for exercise to prevent an adult-onset phenotype

whereas late exercise animals were given access to running wheels at 8 weeks of age after the adult-onset phenotype was established. Introducing running wheels at 4 weeks of age prevented the onset of bladder hypersensitivity, as the VMR during UBD was not significantly different between naïve-Ex and NMS-Ex mice. Likewise, NMS-Ex mice given access to running wheels after the establishment of bladder hypersensitivity at 8 weeks of age exhibited a significantly lower VMR during UBD, as compared to their baseline measurements, and were not significantly different from naïve-Ex mice. Furthermore, NMS-Sed, but not NMS-Ex mice exhibited polyuria, measured as increased voiding events over a 1 h testing period, compared to their naïve counterparts. Together, these data suggest that voluntary exercise is capable of both preventing and reversing NMS-induced bladder hypersensitivity and micturition frequency.

Mast cells have been shown to contribute to inflammatory states in a variety of diseases associated with stress onset, exacerbation, or predisposition (363). Circulating or local glucocorticoids and other stress ligands can stimulate the release of numerous pro-inflammatory mediators such as histamine, tryptase, or proteases from mast cells (120, 423). Mast cells are part of the innate immune response and primarily reside in tissues interfacing with the environment, including the integument, meninges, and respiratory-, gastrointestinal-, and genitourinary tracts (94-96). Many studies have found increases in mast cell infiltration in biopsies of affected tissue from IC/PBS patients (291, 424-428). The observed increase in mast cell degranulation in NMS bladders both in this chapter and chapter 3 suggests downstream dysregulation of neuroimmune regulators of visceral inflammation. Mast cell-released tryptase binds to

and activates PAR2, which is located on neuropeptide-containing nerve endings in the viscera, and has been shown to drive visceral hypersensitivity (124, 125, 429). As in chapter 3, we observed an increase in PAR2 protein levels in NMS-Sed bladder, suggesting an increase in the “nerve-mast cell axis”, which is thought to contribute to chronic pain.

Clinically, exercise has been shown to improve markers of chronic inflammation (154, 430) and here we report that voluntary exercise, beginning at 4 or 8 weeks of age, decreased mast cell degranulation in NMS bladder. Furthermore, we observed that exercise beginning at 8 weeks of age decreased both protein and mRNA levels of PAR2 in NMS-Ex bladder, compared to NMS-Sed mice. Previous work has shown that intravesicular installation of zymosan during the second postnatal week resulted in adult bladder hypersensitivity without a concordant change in mast cell degranulation (325). Our observation of increased mast cell degranulation in NMS bladders suggests that stress and injury experienced during neonatal development induce bladder hypersensitivity via different mechanisms, with the former likely recruiting the involvement of higher brain centers. This is further supported by our findings showing that bladder hypersensitivity, over-activity, and associated mast cell degranulation can be prevented or reversed by exercise, which has been shown to impact activity of the hippocampus and downstream activation of the HPA axis in exercised individuals (431).

Clinically, early life stress reduces hippocampal volume (432) and functional connectivity within limbic structures of individuals with co-morbid depression (433), and improper hippocampal development systemically disinhibits the HPA axis (434) in a

BDNF-dependent manner. In concordance with these findings, NMS, adult stress, and increased glucocorticoids have been shown to decrease BDNF expression specifically in the hippocampus (415, 435-437), and similar findings were reported in human patients with histories of childhood trauma (438, 439). Diminished neurogenesis, specifically in the dentate gyrus of the hippocampus, blunts negative feedback on the HPA axis and induces depression-like behaviors in rodents (440). The present study demonstrates increased GR mRNA levels in the hippocampus of mice subjected to NMS with no corresponding change in BDNF mRNA levels. This observation is striking in that dexamethasone treatment or genetic overexpression of GR has been shown to increase hippocampal BDNF mRNA or protein levels and, conversely, knockdown of GR decreased BDNF protein content in the hippocampus (369, 370). Previous studies have shown evidence of epigenetic changes indicative of glucocorticoid resistance (441, 442), which may be at play in our NMS mice. GR resistance may be described in terms of decreased affinity for the ligand or ability to translocate to the nucleus for binding glucocorticoid response elements (443); for instance, phosphorylation states of the GR in response to stress may mediate GR responsiveness (444). Deficits in negative feedback can also be related to elevations in GR expression such as those observed in animal models of post-traumatic stress syndrome (PTSD). For instance, female patients with PTSD specifically associated with sexual or physical abuse display lower circulating cortisol compared to control (445) while increased GR (446) and decreased BDNF (447) expression in the hippocampus have been observed in animal models. Understanding altered GR function, such as evidence of glucocorticoid resistance, is of critical importance to understanding disorders characterized by impaired HPA axis

activity and negative feedback. While hippocampal GR mRNA levels were unaffected by exercise, they were increased in NMS mice compared to naïve, but BDNF mRNA levels were increased in both naïve-Ex and NMS-Ex hippocampus, suggesting increased neurogenesis or plasticity within the hippocampus and provides evidence of a mechanism by which exercise mediates positive effects. The interplay between BDNF and glucocorticoids within the hippocampus is complex (366), but given that BDNF promotes neurogenesis and synaptic plasticity within circuitry that also regulates the HPA axis, reinstatement of proper HPA axis negative feedback may be one mechanism by which NMS-induced behavioral phenotypes are ameliorated by exercise.

Changes in limbic circuitry and the emotional response to reward have long been studied by exercise physiologists and may provide insight into specific mechanisms by which exercise could improve the limbic regulation of the HPA axis following NMS. Behavioral and hippocampal changes in rats exposed to prenatal glucocorticoids (448) or NMS (272) were shown to be normalized with exercise. Clinically, exercise has also been shown to improve symptom scores of depression and anxiety (195-197), including depression among fibromyalgia patients (193, 194). In the present study, NMS-Sed mice consumed significantly more sucrose likely reflecting diminished processing of rewarding stimuli, a finding also observed in depressed individuals (449). A significant correlation was found between anhedonia and voiding events only in NMS mice, such that decreased sucrose preference corresponded with increased voidance events, suggesting that behaviors indicative of depression and polyuria are comorbid in NMS mice. This observation persisted in NMS-Ex mice despite a decrease in the number of voidances in this population, indicating that voluntary exercise evoked a systemic

improvement in NMS-related behaviors rather than specifically improving bladder sensitivity and function alone.

Interestingly, average distances ran per week differed between naïve and NMS mice in an age-dependent manner. In the early exercise paradigm, NMS mice ran significantly shorter distances per week compared to naïve across the entire testing period. In contrast, during the late exercise paradigm, we observed that NMS mice trended toward increased running distance per week compared to naïve. Interpretation of this data is somewhat complicated by the housing situation between the two paradigms, as early exercised mice were pair-housed to avoid potential complications arising from social isolation and late exercised mice were singly housed. The elevated running distance in the naïve animals observed in early exercise might be due to dual runners in the cage while NMS animals ran in tandem or one did not run at all. However, we do not believe the latter to be the case as early exercise improved behavioral measures of bladder sensitivity, micturition frequency, and forepaw sensitivity across all animals in the pair-housed NMS group. Naïve animals that were singly housed in the late-exercise paradigm ran an average distance of 5-10 km/day while pair-housed animals in the early exercise paradigm also ran an average distance of 5-10 km/day, suggesting that the effects observed were due to differences in the running patterns of NMS, rather than naïve, mice. Together these data suggest age-dependent alterations in the sensitivity of reward pathways in NMS mice. Additional studies should be undertaken to elucidate specific regulatory mechanisms that might be driving these changes.

Early life adversity increases the likelihood of comorbid symptomology or diagnoses of multiple functional pain disorders and is a significant risk-factor for the development of psychiatric disease in adults (265, 266, 450, 451). Furthermore, estimates suggest up to 80% of individuals with severe cases of depression and 90% of individuals with severe cases of generalized anxiety have additional psychiatric comorbidities, including affective, anxiety, and substance abuse disorders (452). Patients with IC/PBS are not only at increased risk of developing mood disorders, but also chronic pain in non-pelvic regions, such as that experienced by patients diagnosed with fibromyalgia or migraine (12, 453). The data from this chapter suggest that NMS may be an initiator of behaviors reflecting these comorbid, centralized pain conditions. To the best of our knowledge, this is the first report of forepaw hypersensitivity following NMS. While hindpaw sensitivity has previously been investigated in NMS animals (150) and may in part be driven by spinal convergence of hind limb and pelvic organ innervation, hypersensitivity of the forepaw is unlikely to be attributed to dichotomous innervation or secondary convergence with the pelvic organs. Cutaneous hypersensitivities in patients with CPP vary in regard to somatotopic organization (454-456), but demonstrations of lower (457) and upper (458) extremity somatic sensitivity are observed, delineating forepaw sensitivity as a novel finding in NMS mice. Exercise reversed the forepaw sensitivity observed in NMS-Sed animals and, strikingly, mechanical sensitivity of the forepaw was increased in naïve animals that remained sedentary over time, although not to the extent of NMS-Sed animals. These data highlight an incredible point about behavioral studies in rodents that are otherwise natural runners. Mice are mobile in the wild (459) and while the limitations of laboratory

manipulation cannot take into account all aspects of the natural environment, it is likely that the sedentary caging condition utilized in animal studies is a covariate that should be taken into account.

Broadening the impact of these findings, we also evaluated mast cell degranulation in the dura mater as a potential biomarker of migraine. Animal models of migraine are often chemically induced by dural application of mast cell mediators known to sensitize meningeal nociceptors (460). Here we report that, indeed, mast cell degranulation was significantly elevated in dura from NMS-Sed mice, compared to naïve-Sed mice, and that exercise beginning at 8 weeks of age significantly reduced the percentage of degranulated mast cells in the dura of NMS-Ex mice, compared to NMS-Sed. As mast cells in particular are thought to contribute to the pathophysiology of migraine (461), reducing their state of degranulation with exercise as demonstrated here has potential implications for migraineurs, particularly those whom also suffer from CPP. These data, again, support the theory that voluntary exercise resulted in systemic improvements in comorbid sensitivity and molecular markers of neuroendocrine dysfunction that are likely being driven by improper regulation of the HPA axis.

4.6 Conclusion

This study showed that maternally-separated adult female mice displayed increased urinary bladder sensitivity and micturition rate. Consumption of sucrose was greater in NMS mice, the preference of which correlated with urinary dysfunction, and changes in sucrose consumption and running distance suggest NMS impacted reward pathways. Peripherally, increased mast cell degranulation and PAR2 protein levels

were elevated in NMS bladder. Four weeks of voluntary exercise beginning at either 4 or 8 weeks of age abolished many of the abnormal behavioral and molecular changes attributed to NMS, including forepaw sensitivity and dura mater mast cell degranulation, findings reflective of comorbid hypersensitivity. Centrally, voluntary exercise significantly increased BDNF mRNA levels in the hippocampus of both naïve and NMS mice. Combined with our data from the previous two chapters, our results here further suggest that hippocampal input to the HPA axis may be disrupted in NMS mice and voluntary exercise can prevent or reverse negative outcomes following early life stress.

Chapter 5

Dissertation discussion and conclusion

Significant subsets of female patients with functional pelvic pain disorders, including the urogenital disorders IC/PBS and vulvodynia, report a history of adverse childhood events and exacerbation of their symptoms during stressful periods (227, 228). This disrupted stress response has been attributed to dysfunction within the HPA axis, which regulates the stress response and influences the perception of pain. Stressors experienced early in life, such as childhood neglect or abuse, low socioeconomic status, and witnessing domestic violence, parental discord, or crime in the home, profoundly impact behavior and also serve as risk factors for developing functional pain disorders (257, 266, 275-278, 284, 462).

Understanding the links between early life stress and adult-onset pelvic pain has been the purpose of the studies described in this dissertation. Despite an increased prevalence for CPP syndromes and comorbid mood disorders in women, animal models of female CPP are lacking. In pursuit of this question, the presented studies used an animal model of early life stress, NMS, as a means to study behaviors reflecting the clinical conditions observed in CPP. Experiments in this dissertation evaluated the impact of NMS on visceral and somatic sensitivities, anxiety-like and depression-like behaviors, and the response to acute stressors in adulthood. These studies expanded upon our understanding of these altered behaviors by evaluating the expression of factors that regulate the HPA axis within the central nervous system, including the limbic structures. These studies went on to investigate the downstream effects of a dysregulated HPA axis on the immune system, specifically mast cell degranulation, cytokine expression, and peripherally-expressed stress ligands. The final study in this

dissertation evaluated the potential for exercise to prevent or reverse the aberrant behaviors attributed to NMS.

5.1 NMS alters adult behavior

Psycho-emotional components are often observed in conjunction with CPP syndromes. A national study found prevalence rates and severity of chronic pain conditions with comorbid depression were twice as high in women, as compared to men (463). Among women, depression was related to severity of pain, whereas, among men, depression was related more to functional impairment (464). Two generations ago, these correlations led medical professionals to attribute a patient's pain to peripheral manifestations of a psychogenic origin (465-468).

Epidemiological evidence suggests a common rate of comorbidity between urogenital pain syndromes and chronic pain syndromes affecting other body locations, such as fibromyalgia, temporomandibular joint disease, and migraine, as well as with mood disorders such as depression (22). While a number of studies have clearly indicated strong correlations between adverse experiences during childhood and psychiatric impairment in adulthood, behavioral changes have largely gone understudied in the female mouse model of NMS (58, 395, 469-473). Therefore, investigating the impact of NMS on adult behaviors indicative of anxiety or depression was an important aim in this dissertation. As reported in chapter 2, following exposure to an adult stressor (VBD), female NMS mice spent a significantly higher percentage of their total distance traveled in the perimeter of an open field, compared to naïve mice, indicating an increase in anxiety-like behavior. Studies in chapter 4 of this dissertation

evaluated anhedonic behaviors in NMS mice, defined as the blunted capacity to experience pleasure, a hallmark characteristic of major depressive disorder (474). A diminished lack of interest in palatable food was determined by a two-choice sucrose preference test, which has been shown to be an indicator of an anhedonic state in rodents (475). This lack of enthusiasm for normally rewarding behaviors such as consumption of sucrose may be reversed by administration of antidepressant drugs (476, 477) and correlates to successful pharmacological anti-depressant responsiveness in humans (478). Interestingly, sedentary NMS mice consumed greater volumes of sucrose compared to their naïve counterparts. While the impact of stress on regulation of appetite was previously discussed, this finding likely reflects metabolic derangement rather than absence of anhedonia. However, further analysis of the data found significant correlations between decreased sucrose preference and severity of polyuria in NMS, but not naïve mice. Together, these findings suggest early life stress negatively impacts mood in adulthood, comorbidities common in CPP.

In addition to mood disorders, women diagnosed with urogenital pain syndromes are more likely to have chronic pain in other body locations. Somatic hypersensitivities may be measured by a range of quantitative tests which include measuring thresholds to temperature, mechanical, or ischemic pain, findings of which have all been found in association with CPP syndromes (457) and IC (479).

Cutaneous hypersensitivities in patients with CPP vary in regard to somatotopic organization (454-456). Zhou et al., (457) found that IBS patients demonstrated decreased threshold and tolerance to cold and thermal stimuli on the lower but not

upper extremity. There were no differences in mechanical cutaneous sensitivity among IBS patients compared to healthy control, but tolerance and pain threshold was significantly decreased in IBS patients during tourniquet exsanguination of the upper extremity. They also reported that immediately following the ischemia and cold sensitivity tests, serum cortisol of IBS patients was significantly increased over baseline levels, as well as compared to that of healthy controls that underwent the same procedure. Another study reported thermal hyperalgesia in IBS patients compared to healthy controls on both upper and lower extremities (458). Viscero-visceral and viscerosomatic sensitivities are also found among patients with CPP syndromes. Female IBS patients hypersensitive to colorectal distension also exhibited increases in rectal pain scores during somatic stimulation of a lower extremity (480). Similar dysfunction in pain facilitation has been found in patients with fibromyalgia and IC/PBS (479, 481), especially between organ interactions with common spinal projections. Women diagnosed with both dysmenorrhea and urinary calculi had more genitourinary and referred abdominal hyperalgesia than women with endometriosis or urinary calculi alone. Interestingly, alleviation of symptoms associated with one diagnosis not only improved symptoms associated with the other but also improved the referred somatic hyperalgesia, a finding which implicates the importance of spinal convergence (482). However, unlike organically derived chronic pain, functional pain disorders are associated with no apparent organ pathology, have elusive onsets, and respond poorly to traditional therapies. In the work presented in this dissertation, female NMS mice developed somatic hypersensitivities as demonstrated by decreased withdrawal latencies to thermal stimuli and decreased withdrawal thresholds to

mechanical stimuli applied to the hindpaw. The sensitivity to thermal and mechanical stimuli was observed both at baseline and following VBD, a stressful event in adulthood. Uncertainty existed whether the somatic sensitivity observed in this model was due to convergence in lumbosacral spinal processing between visceral and lower extremity afferents or due to widespread sensitization. Therefore, mechanical sensitivity of the forepaw was tested in chapter 4 of this dissertation. Indeed, sedentary NMS mice exhibited forepaw hypersensitivity, a novel finding in stress neurobiology that suggests a widespread sensitization in the NMS model, extending beyond the lumbosacral spinal region.

Neonatal stress or injury in rodents produces a phenotype in adulthood similar to what is seen in IBS patients, such as visceral hyperalgesia, increased firing rates of dorsal horn neurons responding to colonic distension, and increased expression of TRP channel subtypes in sensory neurons that innervate the colon (146-149). These studies have largely concentrated on abdominal withdrawal or VMR during CRD in male rats. Equivalent mouse models have been less consistent (311, 318, 319). One study of male mice that deviated from the standard NMS model by combining unpredictable NMS with unpredictable maternal stress showed increased VMR during CRD in adulthood (319). The traditional NMS model has otherwise been shown to increase colorectal hypersensitivity, aberrant responses to future stress, and susceptibility to infection or experimental colitis in adulthood (146, 229, 230). While NMS is well established in rats, until the work presented in this dissertation, it has not been used to study the effect on urogenital hypersensitivity in either rats or mice. By quantifying the VMR during organ distension, the data presented here demonstrated both vaginal and

urinary bladder hypersensitivity in NMS mice compared to naïve. Furthermore, acute stress in the form of WAS significantly enhanced the bladder hypersensitivity observed in NMS mice. On the other hand, NMS mice displayed a diminished VMR during CRD compared to naïve, a finding that persisted following acute stress. Women commonly exhibit comorbidities between IC/PBS and gynecological pain, but the mechanisms underlying this comorbidity are unclear (313, 483, 484). Anatomically, the distal vagina, vulva, and bladder arise from a more common embryological origin compared with the hindgut (485-487). While dichotomizing afferents have been reported between the bladder and colon in dorsal root ganglia (488), the vagino-vesicosphincteric reflex, which inhibits urinary bladder activity and increases contraction of urethral sphincters during vaginal distension, is evidence of neurological circuitry within the genitourinary system, a finding which is less apparent between the urogenital organs and colon (489, 490). In support of NMS-induced urogenital specific effects, the studies presented in this dissertation revealed increased expression of cytokines and growth factors in vaginal and bladder tissue that largely spared the colon in comparison (chapter 2, 3). In the context of pelvic sensitivity, this work provides an anatomical and molecular basis for mechanisms of shared stress-induced pathophysiology between the urogenital organs, as well as evidence of comorbid somatic sensitivities and mood disorder.

5.2 NMS influences central regulation of the HPA axis

NMS has generally been shown to increase the output of the HPA axis, as evidenced by stimulation of anxiety-like behaviors and increased duration of ACTH and CORT release following a stressful event (64, 241-243). Activity of the HPA axis is

regulated by positive and negative feedback from glucocorticoids both within the hypothalamus and from higher limbic structures. Under homeostatic conditions, the amygdala and hippocampus stimulate and inhibit CRF production and secretion from the hypothalamus, respectively. Generally, these limbic regions work in opposition to one another to control the activity of the HPA axis (65, 90).

Hippocampal inhibition of the HPA axis can be compromised by chronic stress or long-term administration of corticosteroids (65). Decreased glucocorticoid feedback to the hippocampus due to either 1) decreased receptor expression or 2) GR resistance (93), reduces descending inhibition onto the hypothalamus and results in increased CRF release and subsequent glucocorticoid production (65, 90). Decreased hippocampal MR expression has been previously reported to occur following a single sustained acute stress in rats and has been theorized to contribute to post-traumatic stress disorder (338). Even within a given anatomical area, the concept of balanced activity from distinct brain regions extends to a molecular level when regarding the GR:MR ratio and adaptability to stress (491). The fast- and slow-acting activities of MR and GR in the amygdala, hippocampus, or prefrontal cortex typically serve to promote energy redistribution, hone focus, increase vigilance, and enhance learning or memory and are essential in the mammalian adaptability to stress. Maladaptive responses, on the other hand, are related to improper MR:GR expression, particularly in higher regions that regulate the HPA axis. For instance, genetic disruption of GR expression in the PVN of the murine hypothalamus increased CORT, impaired negative feedback via dexamethasone suppression testing, and increased adult adiposity, but did not influence anxiety-like or despair-like behaviors (492). On the other hand, conditional

GR knockout model in the forebrain, hippocampus, and basolateral/medial amygdala, but not the PVN, resulted in a depression- and anxiety-like phenotype, impaired negative feedback onto the HPA axis, and interestingly, decreased MR expression in the hippocampus after restraint stress (493). Functionally, GR knockdown within the hippocampal neurogenic niche accelerated neuronal differentiation, migration, spinogenesis, and synaptic contacts (494). Similarly, diminished neurogenesis and accelerated atrophy in the *Cornu Ammonis* 3 and 1 and the subiculum subfields of the hippocampus, the primary circuit that provides inhibitory influence over the PVN (495), was associated with CORT exposure associated with chronic stress conditions, including early life adversity (496, 497). Thus, characterization of GR and MR expression in limbic structures and the hypothalamus was an important finding in the NMS model presented in this dissertation. As shown in chapter 2, expression of GR was increased in the hypothalamus, unchanged in the amygdala, and decreased in the hippocampus of NMS mice. Likewise, expression of MR was decreased in NMS hippocampus yet unchanged in hypothalamus and amygdala. Similarly, following WAS, expression of GR and MR were increased in NMS and naïve hypothalamus, but decreased in the NMS hippocampus (chapter 3). Together, these studies provide evidence for corticosteroid receptor resistance in the hypothalamus and diminished negative feedback from the hippocampus, which ultimately decreases descending inhibition onto the HPA axis. Targeting the dysfunctional regulation of the HPA axis from the hippocampus could provide a mechanism by which to normalize HPA axis activity and restore downstream homeostasis within the pelvic viscera, and ultimately, improve chronic pelvic pain.

5.3 Impact of NMS on the pelvic viscera

Immunoendocrine signaling in the periphery is modulated by downstream signaling from the HPA axis and previous studies have shown that regulators of this process differ across the pelvic organs. On one hand, CRF₁ has been implicated in colorectal hypersensitivity and gut-barrier dysfunction, which results in the presentation of gut luminal antigens to resident immune cells by toll-like receptors (318, 386). In fact, the CRF₁ antagonist GW876008 completed phase II clinical trial as a potential treatment for IBS, and is currently under investigation for unexpected, off-target side effects (498, 499). However, CRF₂ antagonism blocked the release of the proinflammatory cytokine IL6 from cultured cardiomyocytes (387) and expression of TNF α and IL10 were increased in cytotrophoblast cells by a CRF₂-mediated mechanism (388). Similarly, stress-induced bladder hypersensitivity and vascular permeability have also been shown to be driven by a CRF₂-, rather than a CRF₁-, mediated mechanism and peripheral expression of the cytokines TNF α and IL10 are also upregulated by a CRF₂-mediated mechanism (144, 387, 388). Furthermore, activation of peripherally versus centrally expressed CRF₁ and CRF₂ is not as well delineated in function. Most CRF receptor activation is coupled to G α_s and cAMP signaling, but there are examples of CRF receptor expression in testis and placenta that instead activates MAPK, intracellular Ca²⁺/PKC, or IP₃ pathways and stimulation of such divergent signaling cascades as to include NF- κ B, ERK1/2, GSK-3 β , and Wnt/ β -catenin pathways (145, 500, 501). The G-protein subtype involved and subsequent downstream signaling cascade is also tissue dependent (501). For instance, in the hippocampus and other neuronal cells such as pituitary corticotrophs, CRF receptor signaling leads to activation

of ERK1/2, but in cell cultures of female reproductive tissue such as breast, uterus, and placenta, CRF receptor signaling leads to inhibition of ERK1/2 cascades (502-505). It is still unclear which endogenous CRF receptor ligands are involved in these processes. Opposing tissue specific effects of CRF receptor agonists have also been shown regarding activation or inhibition of immunomodulatory activity, intracellular signaling cascades, and gene expression (371, 387, 506, 507). Acknowledgement of the peripheral neuroendocrine response to stress is becoming clearer, but localization across cell types in various tissues remains uninvestigated. The differing intracellular pathways stimulated by CRF receptor subtypes might account for the differential impact of stress across the pelvic organs.

Despite their potential involvement in stress-related pathologies and immunomodulatory effects, expression characteristics of peripheral stress ligands and their receptors and the mast cell-nerve axis had previously not been investigated in early life stress-induced urogenital hypersensitivity. While work presented in chapter 2 of this dissertation does not necessarily suggest that the increased expression of the tested cytokines and growth factors was due to a CRF₂ or CRF₁ mediated mechanism, differential patterns of such expression between NMS vagina, bladder, and colon emerged. This differential impact of NMS and adult stress on the expression of factors representing the local inflammatory state of the viscera was also reflected in data presented in chapter 3.

Mast cells have been shown to contribute towards the visceral inflammation and pain associated with nearly all CPP-related disorders and have specifically been

investigated in IC/PBS for several decades (291, 411, 424-428, 508). Previous studies have shown that mast cells are acutely responsive to CRF and other stress ligands, and that release of their contents helps drive neurogenic inflammation and increase local production of TNF α (105, 106, 119, 129). Interestingly, we observed an initial decrease in the mRNA level of TNF α in the vagina of NMS mice that was significantly increased following VBD procedure (chapter 2). Mast cell activation also been shown to increase *de novo* synthesis and release of NGF (509), which has been shown to mediate stress-induced gut-barrier dysfunction, influence plasticity in central pain circuitry, and has been suggested as a biomarker for IC/PBS (296, 510-514). As shown in chapters 2 and 3, NGF mRNA levels are increased in urinary bladder tissue of adult NMS mice. Increased infiltration and degranulation of mast cells in the NMS urinary bladder and vagina (chapter 3), but not colon (data not shown) were observed. Furthermore, selective overexpression of NGF in the bladder has been shown to result in hyperreflexia, hyperinnervation, and increased mast cell infiltrates (170). Hyperexcitability of bladder afferents was also reported following intrathecal administration of NGF (171). Behavioral indicators associated with experimental cystitis could be attenuated with systemically-administered NGF neutralizing antiserum or intravesicular infusion of a nonspecific Trk receptor antagonist (177) that together highlights a dominant role for this neurotrophin with direct relevance to IC/PBS patients (178). Due to perpetuation by the nerve-mast cell axis, increased sensory innervation in the NMS urinary bladder and vagina should be analyzed in future studies.

Secreted mediators from mast cells can lead to, among other signal transduction pathways, activation of nociceptor-expressed TRP channels, the chronic activation of

which can drive sensitization (515). Mast cell paracrine functions involving degranulated tryptase and histamine binding to PAR and histamine receptors, respectively, have been shown to sensitize TRPA1 and TRPV1 *in vivo* (139, 142) and likely contributes to chronic functional pain (124, 125). Furthermore, data presented in chapters 2 and 3 showed that NMS and WAS increased TRPA1 protein expression in bladder, while elsewhere, experimental cystitis has been shown to induce a TRPA1-, but not TRPV1-, dependent hyperalgesia (392). The mRNA expression of metabotropic glutamate receptors was also upregulated primarily in bladder rather than lumbosacral spinal cord, of mice with experimental cystitis (516). Clinically, an increase in TRPV1-immunopositive innervation was reported in biopsies of affected tissue from vulvodynia patients with dyspareunia (517). Interestingly, this study also found increased TRPV1-immunopositive innervation in skin samples from vulvodynia patients. A separate study evaluated widespread sensitivity in vulvodynia patients via injection of subdermal capsaicin in the upper and lower limbs and reported increased hyperalgesia and allodynia compared to controls (518).

While the extent of systemically increased TRPV1, and/or TRPA1, expression in vulvodynia patients has yet to be determined, the contribution of TRP channels, and nociceptor sensitization in general, towards comorbid symptomology is likely attributed to downstream alterations in centrally-regulated stress responses (519, 520). Indeed, vulvodynia patients given subdermal capsaicin exhibited evidence of dysautonomia in the form of increased resting heart rate and lower blood pressure, consistent with a hyperactive stress reaction (518). Risk of vulvodynia is elevated among women with antecedent mood disorders (54), suggesting that higher levels of chronic stress among

these patients serves to increase symptom severity (55). Indeed, vulvodynia patients demonstrate blunted serum cortisol cycles that manifests in a variety of emotional and physiological indicators (56). Hyperalgesic responses to capsaicin and increased expression of TRPV1 in vulvodynia patients are well documented, but clinical studies have yet to evaluate the role of TRPA1 in CPP syndromes despite its established role in the development and maintenance of visceral hypersensitivity (149, 392, 521). Given that TRPA1 is almost exclusively co-expressed with TRPV1 by afferents innervating viscera and other deep tissues (522-525) it is likely that the changes within this afferent population among patients with CPP also reflect potential increases in TRPA1 expression and/or function.

5.4. Exercise as an intervention for NMS-associated phenotypes

Recently, exercise been shown to confer protective benefits for neurological disorders such as Parkinson's disease, Alzheimer's disease, stroke, and traumatic brain injury (267-269). Clinically, exercise reduces the effects of depression and anxiety and associated perceptions of pain (195-197, 199, 270, 271) and improves symptom severity in patients with IBS (198-203) and fibromyalgia (192-194). Despite the general acceptance that exercise improves a wide variety of disease outcomes, little investigation has been done to evaluate the impact of exercise on animal models of many human diseases, including chronic pain disorders. Voluntary wheel running has been shown to attenuate colorectal hypersensitivity exhibited by prediabetic mice (526), indicating that exercise has the potential to reverse visceral hypersensitivity; however,

the therapeutic potential of interventional exercise has not been evaluated in animal models of stress-associated pelvic pain.

Exercise in rodents has been shown to attenuate neuromolecular and behavioral changes associated with a dysfunctional HPA axis induced either by hyperactive function or diminished negative feedback, the latter implicated in NMS-induced visceral hypersensitivity. Prenatal exposure to glucocorticoids via dexamethasone injection in pregnant rats resulted in elevated levels of CORT and anhedonic-like behaviors in the adult offspring that were attenuated by a 4-week swimming exercise (448). Running exercise also normalized altered behavior and hippocampal changes in Sprague-Dawley rats that underwent NMS in the post-natal period (272). Following NMS, rats in this study spent decreased amounts of time exploring either the center of an open field or the open arms of an elevated plus maze and also spent more time immobile in a forced swimming test, a behavior that has largely been shown to be responsive to antidepressant therapy (527). Both the NMS-induced behavioral findings and hippocampal gene expression changes were ameliorated by providing the rats access to running wheels beginning in the post-weaning period (272), likely due to exercise-induced restoration of negative feedback on the HPA axis or potentiation of dopaminergic reward in the mesolimbic system (70-72, 528-531). Interestingly, this study also revealed that providing the animals a palatable, high-fat diet beginning in the post-weaning period resulted in the same outcomes as providing access to running wheels. A wealth of studies have demonstrated that ghrelin, a gut-derived peptide known to stimulate food intake, is intimately involved in regulating behaviors related to food reward and promoting activity in the mesolimbic-dopaminergic reward centers

particularly in response to high-fat diet (532-541). In keeping with these findings, we observed that sedentary NMS mice consumed greater volumes of sucrose compared to sedentary naïve animals (chapter 4). Sucrose consumption was further increased in exercised animals; however, the underlying drive for increased sucrose intake could have been due to an increased caloric need. The effects of NMS on the hypothalamic regulation of appetite have not been explored, but mouse models of systemic disease that involve gene knockouts of appetite-regulators (542) highlight how this interaction might confound behavioral studies involving exercise, a physiology closely tied to metabolic state.

Changes in limbic circuitry and the emotional response to reward have long been studied by exercise physiologists and may provide insight into mechanisms by which exercise could improve the limbic regulation of the HPA axis following NMS. Improper hippocampal development resulting in HPA axis disinhibition has been shown to be induced by lentiviral knockdown of BDNF (434). This implicates developmental BDNF expression as imperative in hippocampal management of HPA axis circuitry because the same chronic elevations in corticosterone were not observed when the lentiviral knock-down was administered later in life. In concordance with these findings, NMS, adult stress, and increased glucocorticoids have been shown to decrease BDNF expression specifically in the hippocampus (415, 435-437) with similar findings in human patients with histories of childhood trauma (438, 439). No statistically significant changes in BDNF expression were observed in NMS hippocampus compared to naïve at baseline or following WAS in the current study (chapter 3), but exercise did increase expression in both groups compared to their sedentary counterparts (chapter 4).

Exercise was also effective at either preventing or reversing the urinary bladder hypersensitivity, related polyuria, and mast cell degranulation resultant from NMS. A similar pattern was observed in the forepaw sensitivity study (chapter 4) that demonstrated decreased sensitivity in the exercised groups compared to sedentary naïve mice, otherwise considered the control group. Taken together, this provides evidence for one mechanism by which NMS-induced behavioral phenotypes might be ameliorated by an exercise-induced increase in BDNF and subsequent reinstatement of proper HPA axis negative feedback. Ultimately, these data highlight an incredible point about behavioral studies in rodents that are otherwise natural runners. Mice are mobile in the wild and while the limitations of laboratory manipulation cannot take into account all aspects of the natural environment, it is likely that the sedentary caging condition utilized in animal studies is a covariate that should be taken into account in the future.

5.5 Conclusion

Functional pelvic pain disorders, such as IC/PBS and vulvodynia, are diagnosed symptomatically in the exclusion of other, clinically identifiable pathologies. A disparate array of etiologies has been shown to underlie these disorders, including infection, peripheral sensory and/or motor neuron dysfunction, improper immune function, and psychological disturbances (278, 279, 543-545). As a result, patient populations display similar symptomology that manifests via different underlying pathology, partially explaining the difficulty in identifying effective treatment strategies. Subsets of CPP patients are more likely to report histories of early life adverse events that, in turn, increase the risk of developing a CPP syndrome later on in life. Understanding the

relationship between functional pain syndromes and early life stress is of vital importance considering current trends of early childhood adversity and the profound impact it imparts on adult health, disease, and mortality. The work presented in this dissertation generated novel insight on how early life stress drives female urogenital pain, contributes to mood disorders, and alters neurodevelopment. Finally, this work provides the first report of exercise as an intervention method for visceral pain, a finding that will be of particular benefit for those most at risk for developing stress-induced chronic pelvic pain, that is, patients with histories of early life stress.

Chapter 6

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