The impact of genetic differences, voluntary exercise, and thermal injury on chronic pain and comorbidities in psychologically-stressed mice.

By

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Abstract

An estimated 80% of Americans suffer from at least one stress-related symptom during a month's time and the number is increasing year by year. Co-morbidity of pain and psychological disorders is very common, and both can be induced by stressful events. Psychological stress activates the Hypothalamic-Pituitary-Adrenal (HPA) axis and subsequently initiates and/or exacerbates symptoms related to both chronic pain and mood disorders. The impact of stress on allodynia and anhedonic behaviors in highly anxious individuals has not been well studied. The overall goal of this dissertation is to investigate stress-induced negative impacts on anxiety in individuals and search for potential non-pharmacological treatments for comorbid chronic pain and mood disorders in patients with anxiety.

By exposing anxiety-prone A/J mice to 10 consecutive days of foot shock stress, we established a feasible small rodent model for studying comorbid pain and depression in an individual with pre-existing anxiety. Foot shock-stressed A/J mice developed persistent bladder hypersensitivity, hind paw mechanical allodynia, and anhedonic behavior. Further, the HPA axis and inflammatory mediators were altered by foot shock stress. Considering other reports have identified that exercise has beneficial effects on both mood disorders and chronic pain, we tested the hypothesis that voluntary wheel running (VWR) can prevent persistent bladder, mechanical hypersensitivity, and anhedonic behavior in foot shock-exposed A/J mice. We discovered that four weeks of VWR decreased mechanical sensitivity, but failed to reach significance in stressed A/J mice. VWR also had no significant impact on either bladder hypersensitivity or anhedonic behavior. However, VWR normalized HPA axis output in stressed A/J mice. Interestingly, A/J mice displayed a unique running pattern after foot shock stress, suggesting that VWR behavior could be used as a potential biobehavioral marker of stress in mice. To explore how genetic

differences in baseline anxiety impact the response to stress, we compared hind paw mechanical and bladder sensitivity in anxiety-prone A/J and non-anxious C57Bl/6 mice following foot shock exposure. Both strains developed persistent hind paw allodynia, but bladder hypersensitivity was only present in A/J mice. By recording fecal output as a measure of anxiety during the foot shock exposure, we confirmed that C57Bl/6 mice adapted to the foot shock, whereas A/J mice did not. Finally, we adapted our foot shock model in A/J and C57Bl/6 mice to understand how underlying anxiety and stress exposure impacts the transition from acute to chronic pain following a burn injury. We revealed that psychosocial stress may be one of the key factors for developing persistent pain after burn injury. The whole of my dissertation has provided important and novel information on how stress and underlying anxiety may predispose an individual to develop chronic comorbid pain and mood disorders. Understanding how specific systems, such as the HPA axis and the inflammatory response are affected provides important therapeutic targets for the development of pharmacological and non-pharmacological treatments.

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Chapter 1: Introduction to dissertation

1.1 Psychological stress and HPA axis

Stress was first identified as a "*non-specific neuroendocrine response of the body to any demand*" by Hans Selye, who had a comprehensive outlined concept of stress in 1936 [1-3]. Stress is not always harmful and could be critically related to a species' survival, whereas long term intensive and uncontrollable stress would lead to a detrimental response [4]. Considerable evidence shows psychological stress exacerbates and triggers many diseases such as cancer [5], chronic pain [6, 7], mood disorder [8], and urogenital disorder [9, 10].

Stress triggers the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Initially, activation of the HPA axis is governed by neurons of the paraventricular nucleus (PVN) of the hypothalamus, which secretes corticotropin-releasing factor (CRF) and arginine–vasopressin (AVP). Release of CRF stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary [11]. Finally, ACTH increases the secretion of the glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal cortex. Glucocorticoids then react with multiple targets in peripheral tissues and the central nervous system (CNS). Glucocorticoids bind to their ubiquitous cytoplasmic receptors to elicit their effects. When bound to a ligand, glucocorticoid receptors translocate to the nucleus, interacting with specific glucocorticoid responsive elements (GREs) that activate appropriate genes [12]. Activation of glucocorticoid receptors inhibits several genes involved in the activation and growth of immune and other cells, whereas c-jun/c-fos and NF-kB will increase its transcription [13]. A negative feedback loop is formed to turn off activation of the HPA axis by decreasing secretion of CRF and ACTH from the hypothalamus and pituitary, respectively, and also at limbic structures, such as the amygdala and hippocampus, which regulate HPA axis activity [14].

Regulation of the stress response relies on transmitters that include CRF, AVP, opioid peptides, dopamine, and norepinephrine [15]. CRF is a 41 amino acid peptide [16] and is widely expressed in the CNS, as well as some peripheral tissues. In the CNS, CRF is highly expressed in the medial parvocellular subdivision of the PVN, bed nucleus of the stria terminalis (BNST), medial preoptic area, lateral hypothalamus, central nucleus of the amygdala, and dorsal motor complex. Studies show that in the periphery, CRF has also been identified in the adrenal gland, placenta, gastrointestinal tract (GI), thymus, skin, and bladder urothelial cells [17-19]. Recently, urocortin (Ucn)1 [20], Ucn2 [21], and Ucn3 [22] were identified and noted as stress coping-related peptides [18]. Two subtypes of the CRF receptor have been discovered and are known as CRF receptor type 1 (CRF1) and CRF receptor type 2 (CRF2). CRF1 is highly expressed in the anterior pituitary, cerebral cortex, hippocampus, amygdala, and cerebellum, but also found in peripheral tissues such as adrenal gland, testis, GI, ovary, mast cell, and bladder urothelial cells [19, 23]. In contrast, high levels of CRF2 are expressed in the heart, skeletal muscle, and skin and referred to as CRF type 2β receptor. Low levels of CRF2 are expressed in the CNS and also referred to as CRF type 2α receptor [24]. CRF and Ucns have different affinity for CRF1 and CRF2. CRF binds CRF1 with a 10-fold higher affinity than CRF2, Ucn1 binds CRF1 and CRF2 with equal affinity, and Ucn2 and Ucn3 both bind CRF2 [18]. CRF1 regulates the release of ACTH based on evidence of the phenotype of CRF1-deficient mice. Mice with CRF1 deficiency severely decrease their HPA-axis response to stress and display anti-anxiety-like behavior [25]. In contrast, CRF2-deficient mice display an amplified HPA response to stress and anxiety-like behaviors, but some studies report that CRF2 antagonists injected into the brain cause anxiolytic effects [18, 26, 27]. Two major mechanisms have been found for the glucocorticoid-mediated negative feedback pathway: delayed feedback system and fast feedback system, the latter of

which has been identified, but not well-characterized [28]. The delayed feedback system acts through glucocorticoid receptor (GR)-led transcriptional alterations in stress-responsive brain regions. Evidence supports that mineralocorticoid receptors (MRs) are up-regulated by forced swim stress in the amygdala and hippocampus [29]. Glucocorticoids have lower affinity for GR and GR is only fully-occupied during stress-induced elevated glucocorticoid secretion. In contrast, the affinity for MR is much higher. Therefore, studies suggest that MR regulates basal HPA activity while GR mediates glucocorticoid negative feedback following stress exposure [30, 31].

High instances of stress induce memory disorders through different mechanisms. Decades ago, GR and MR were found in the hippocampus and filled that gap between HPA axis and hippocampus function [32, 33]. Many studies suggest that chronic stress or a high level of corticosteroids can induce atrophy in the hippocampus in both humans and animals [34-36]. Further, chronic stress reduced the number of neurons [37], dendritic branches[38], dendritic synaptic terminals [37], and neurogenesis in the hippocampus [39]. Stress also disturbs long term potentiation in the hippocampal CA1 region [40, 41], as well as increases glutamate efflux to prefrontal cortex and contributes to chronic stress-induced memory loss [42, 43].

The functioning of the immune system is also closely related to stress. In the early 20th century, researchers found that people living under stress conditions had suppression of their immune system, which increased the risk of having tuberculosis [44]. More recently, stress-induced release of glucocorticoids has been reported as immune suppressant due to decreased lymphocytes, macrophages, as well as cytokines [45, 46]. Second, CRF acts to inhibit the release of growth hormone in the human hypothalamus [47] and evidence indicates that impaired immune system functioning has been found in growth hormone-deficient individuals, both in

human and pre-clinical studies [48]. Lastly, stress triggers norepinephrine release through the noradrenergic and sympathetic nervous systems [49]. It has been proven that catecholamine (such as norepinephrine and epinephrine) levels increase changes in lymphocyte, monocyte, and leukocyte functions and can ultimately lead to immunosuppressive effects [50, 51].

Stress also strongly impacts the cardiovascular system. A 1997 study showed that ischemia risk in people who have stress symptoms, such as tension, frustration and sadness, is at or above two folds higher compared to control patients [52]. Several other clinical studies have confirmed the relationship between stress and cardiovascular disease [53, 54]. Some studies suggest that endothelial dysfunction plays an important role in stress-induced cardiovascular disease [55, 56]. Atherosclerosis, a common cause of myocardial infarction, is a chronic inflammatory state of the arterial wall with accumulation of low-density lipoprotein, white blood cells, and macrophage particles [57]. Chronic stress or depression leads to chronic mild inflammation by increasing plasma C-reactive protein and pro-inflammatory cytokines like Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Interleukin (IL)-1β. [58-60]. Activation of the sympathetic nervous system by stressful events can result in elevated catecholamine secretion [61], which can induce hypertension and increase heart rate [62, 63]. Finally, an increase in the renin-angiotensin-aldosterone system was also found in open field-stressed rats and water avoidance stress-exposed mice [61, 64]. In humans, plasma renin activity was found to be significantly increased in people immediately after undergoing an exercise-induced mental stress exposure, as compared to measurements taken 15 mins after the challenge [65].

Stress can also alter nutritional intake and the gastrointestinal system. First, stress decreases appetite by inhibiting the glutamatergic N-methyl-D-aspartate (NMDA) receptor in the

ventral tegmental area and amygdala [66]. Second, stress or central injection of CRF inhibited gastric emptying through activation of the vagus nerve in both rat and dog models [67-69]. In contrast, CRF and stress exposure stimulate transit and motility in the descending colon by activating Barrington's nucleus and the sacral parasympathetic nucleus [70, 71]. Finally, studies also showed that stress induced inflammation in the gastrointestinal tract and increased the prevalence of irritable bowel syndrome [72, 73]. Increased substance P and CD4+ cells are also involved in stress-induced gastrointestinal tract inflammation and change of permeability [74-77]. Another study also indicated that decreased IL-10, an anti-inflammatory cytokine, was found in patients with irritable bowel syndrome [72].

Sex differences in stress have been discussed for decades, but the underlying mechanisms have not been fully delineated. Studies from the 1990's indicate that women tend to have higher reported anxiety levels and are more likely to express emotional feelings compared to men [78, 79]. Women may also be more susceptible to emotional distress and related disorders [80, 81]. However, other studies suggested that both sexes have equivalent stress and anxiety-related vulnerability, but tend to present with different psychiatric disorder [82, 83]. More women suffer from depression and anxiety disorders, but the prevalence of externalizing disorders (e.g. aggression and substance abuse) is higher in men [80, 84]. A recent study supports the previous argument by discovering that men and women have different areas of activation in the CNS during stressful events [85].

1.2 Stress-induced pain

Physical or psychological stressors can induce both hyperalgesia and analgesia, which is increased or decreased pain perception, respectively. In general, the analgesia effect occurs in acute, robust, intense stress, whereas chronic or repeated stress induces hyperalgesia [86-90].

Stress-induced allodynia, a painful response to non-noxious stimuli, or hyperalgesia was observed in both clinical and preclinical (mostly rodent model) studies [91, 92]. In clinical settings, the close relationship between stress-related disorders like anxiety and depression is demonstrated by the effectiveness of anti-depressants and anxiety medication such as amitriptyline [93], pregabalin [94, 95] and duloxetine [96, 97], to treat some forms of chronic pain. Although there are comparably fewer studies in human, compared to rodent, Crettaz et al. and Strigo et al., provided solid evidence that stress and increased emotional activity significantly increase sensitivity to thermal stimuli [92, 98]. Over the past three decades, various rodent models for studying stress-induced allodynia or hyperalgesia have been developed (see appendix 1). Stressors that have been used to generate allodynia or hyperalgesia in rat models include forced swim [99], repeated cold [100], restraint [89], immobilization [101], social defeat [102], water avoidance [103], chronic mild stress [104], maternal separation [105], noise [106] and vibration stress [107]; mouse models include forced swim [108, 109], repeated cold [110], immobilization [111], water avoidance [112] and maternal separation [113-115].

Multiple areas in the brain are affected by stress and modify the pain pathways that ultimately lead to the somatosensory cortex [116]. Evidence shows that stress-related psychiatric disease alters cortical function and structure [117, 118]. Increased grey matter thickness in cortex, S1 cortex, anterior cingulate cortex (ACC), and prefrontal cortex have been observed in chronic pain patients [119-121]. Interestingly, pain patients with greater negative emotions report higher pain scores, as well as greater activation of the ACC (measured by functional magnetic resonance imaging (fMRI) and magnetoencephalography (MEG)), compared to patients with lower negative emotion [122, 123]. An animal study revealed that abnormal activation of the ACC may trigger C-fiber activation and lead to increased pain experience [124-126]. The

amygdala not only plays a role in pain, but also modulates anxiety and fear [87, 127]. Injection of corticosterone into the central nucleus of the amygdala in rats resulted in increased anxiety–related behavior, and somatic and visceral sensitivity [128-130]. On the other hand, injection of mifepristone or spironolactone (GR or MR antagonist, respectively) into the central nucleus of the amygdala ameliorated visceral hypersensitivity induced by water avoidance stress [131]. The periaqueductal grey (PAG) was found to be activated in early life stress via colorectal distention, which was further amplified by later water avoidance stress exposure [132]. A separate study revealed that, in female rats, the first exposure to stress decreased c-fos expression, whereas re-exposure increased c-fos expression in the PAG [133]. A study using restraint stress-induced hyperalgesia in Sprague–Dawley rats showed a drop in glial fibrillary acidic protein (GFAP) and excitatory amino acid transporter 2 protein levels in PAG [134]. This indicated that the descending pain modulatory system is involved in restraint stress-induced hyperalgesia in rat [134]. A higher number of P-ERK-positive and tryptophan hydroxylase-positive neurons was observed in the rostral ventromedial medulla (RVM) in rats with chronic restraint stress-induced thermal hypersensitivity [135]. Lesion of the RVM can mitigate mechanical allodynia resulting from forced swim or air stress [136, 137]. A similar study injected cholecystokinin 2 receptor antagonist into the RVM and show attenuated social defeat-induced anxiety and allodynia [102]. In short, evidence indicates that the ACC, amygdala, PAG, and RVM may be potential areas involved in mediating stress-induced hyperalgesia or allodynia.

Differential neurotransmitter production and activity also contributes to stress-induced allodynia and hyperalgesia. Injection of ketamine, an NMDA receptor antagonist, prevented allodynia following forced swim stress in rat [138]. Two studies from Okano et al., showed that intrathecal administration of (2R)-amino-5-phosphonopentanoate (APV), a partial NMDA

receptor antagonist, decreased mechanical withdrawal thresholds and aversive behaviors in rats that underwent repeated cold stress, but had no effect on naïve rats [139, 140]. Glutamatergic receptor involvement has also been associated with stress-induced visceral hypersensitivity. Water avoidance stress, which has been shown to induce visceral hypersensitivity, results in a dysregulation of glutamate (Glu) homeostasis in spinal glia including a decrease in expression of Glu transporter 1 (GLT-1) and the Glu conversion enzyme glutamine synthetase (GS), but an upregulation in L-Glu-L-aspartate transporter (GLAST) [141]. The NMDAR antagonist, MK-801, also eliminated visceral hypersensitivity in the water avoidance stress group [141]. Most studies have reported a decreased level of gamma aminobutyric acid (GABA) in animal models of stress-induced hyperalgesia [99, 142, 143]. Reductions in spinal GABA levels have been found in forced swim-stressed rats. Administration of diazepam, a GABA-A receptor positive allosteric modulator, prior to the water avoidance stress procedure reduced pain scores during a formalin test [144], which could be reversed by flumazenil, a GABA-A receptor antagonist [90, 99]. Cholecystokinin and its receptor are highly expressed in the central nervous system and are related to pain and emotion control [145-147]. Deletion of the cholecystokinin receptor led to upregulation of μ- and δ-opioid receptors and decreased hyperalgesia in a mouse neuropathic pain model [146, 148]. Systemic treatment with a cholecystokinin receptor antagonist, CI-998, prevented social defeat stress-induced increases in formalin-evoked pain-like behavior [149]. Both 5-hydroxytryptamine (5-HT) and noradrenaline (NA) play a role in stress-induced allodynia and hyperalgesia. 5-HT levels were reduced in the hypothalamus, thalamus, medulla oblongata, and spinal cord of rats that were exposed to repeat cold stress [150]. Hind paw mechanical hypersensitivity induced by chronic restraint or chronic mild stress was reversed by injection of fluoxetine, a selective serotonin reuptake inhibitor [151]. Exposure to 15 minutes of

vibration-induced mechanical allodynia in rats was prevented by IP injection of clonidine, a central α2-adrenoceptor agonist, to inhibit NA release [152]. A similar study also showed that milnacipran, a SNRI (serotonin–norepinephrine reuptake inhibitor), alleviated muscle hyperalgesia induced by forced swim stress in rats [153]. The endocannabinoid system also plays a role in stress-induced allodynia and hyperalgesia. Cannabinoid receptor type 1 (CB1) expression in L6-S2 DRGs was significantly decreased in rats with visceral hypersensitivity resulting from water avoidance stress exposure [154, 155]. A subsequent study by the same group also discovered that 10 days of subcutaneous CORT injection or water avoidance stress in rats also resulted in a decline of CB1 receptor expression [155]. A separate study showed that treatment with CB1 receptor agonist, ACEA, could prevent partial restraint stress-induced visceral hypersensitivity, whereas treatment with a CB1 receptor antagonist, SR141716A, worsened visceral hypersensitivity [156]. Finally, as described in section 1.1, the HPA axis is involved in stress-induced allodynia and hyperalgesia. Increases in both visceral and mechanical hypersensitivity were observed following chronic delivery of corticosterone to the amygdala in rats [130, 157-159]. An increase in HPA axis output was observed following several different stress exposures, including maternal separation, foot shock, and water avoidance stress, all of which also induced hind paw allodynia [114, 160-162]. In summary, neurotransmitters and systems within the CNS are involved in the development of stress-induced hyperalgesia and allodynia, including glutamate, GABA, cholecystokinin, NA, serotonin, endocannabinoids, and the HPA axis.

1.3 Stress-induced mood disorder

Among the general public, the prevalence of anxiety disorders is around 3.8-25% with a huge increase in people with chronic disease (1.4-70%) [163, 164]. Stressful events are highly

related (OR, 3.09; 95% CI, 2.43-3.94) to escalation of generalized anxiety [165]. Although psychological stress plays an important role in anxiety disorder, it is not feasible to quantify the stressor level [164]. Therefore, few data were published. Increases in pro-inflammatory mediators can be the key link between stress and generalized anxiety disorder (GAD) [166]. Escalated plasma C-reactive protein was found in an anxiety disorder population [167]. Broader studies also discovered that pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), macrophage inflammatory protein (MIP)-1a, interleukin (IL)-6, IL-1α, IL-1β and IL-17, were significantly higher in GAD patients than control[168, 169]. Interestingly, increased pro-inflammatory cytokines, like interferon (IFN)- γ, IL-1β and IL-6, after psychological stress were also broadly reported [170-172]. Stress can activate an inflammation reaction by several different mechanisms. First, psychological stress evokes release of damage-associated molecular patterns that stimulate systemic inflammation through either NF-κB or MAPK pathways [173-175]. In a psychologically-stressed student, pro-inflammatory cytokines such as, TNF-α, IL-6, IL-1 receptor antagonist (IL-1Ra), and IFN-γ, show significantly higher expression levels [176]. Other meta-analyses report that IL-6 and IL-1β are robustly elevated after acute stress [177]. Stress promotes cytokine production by several mechanisms. Stress-induced NF-κB activation in peripheral blood mononuclear cells depends on norepinephrine and can be inhibited by α1-adrenoceptor blocker [178]. β-adrenoceptor stimulation and vagal withdrawal also contribute to NF-κB activation, which ultimately cause cytokines to be released and leads to subsequent inflammation [179, 180]. Finally, chronic stress modulates the HPA axis and leads to inflammation. Inflammatory responses have been observed in psychologically stressed individuals. During acute stress, HPA axis increases its output leading to increased CORT levels [181]. Once CORT binds to GR, it inhibits NF-κB activation and creates a powerful anti-

inflammatory effect [182]. However, during chronic stress, persistent high cortisol levels result in glucocorticoid resistance and failure to reduce NF-κB transcription for proinflammatory cytokines [183, 184]. This leads to a proinflammatory reaction.

The relationship between stress and the development of depression has been reported in many studies including humans [185, 186] and rodents [187]. In a clinical survey, an increase in stressful life events lead to a raised rate of depression symptoms, major depression, and suicide [188]. Although the pathophysiology of major depression is still poorly understood, the degree of overlap between antidepressant treatment and the molecular mechanisms of neuroplasticity is striking [189]. The most well-characterized studies perhaps those that examine learning-related synaptic plasticity in brain regions such as the hippocampus [190]. Chronic or severe stress and high dose treatment with glucocorticoids have deleterious effects on hippocampus-dependent memory not only in rodents [191], but in humans, as well [192, 193]. One of the important mechanisms of hippocampus-dependent memory formation is hippocampal synaptic plasticity, as modeled by long-term potentiation (LTP) [194]. Sufficiently severe stress will impair LTP in rodent hippocampus [195]. N-methyl-d-aspartate (NMDA) activation seems to play a role in behavioral stress modification in hippocampal plasticity studies [196]. Excess glucocorticoids [197] or chronic stress [198] will also cause atrophy and retraction of the apical dendrites of hippocampal pyramidal cells, which has also been reported in patients with major depression [199]. Chronic stress also impairs apical dendrites of pyramidal cells and reduces the number of dendritic spines in the prefrontal cortex (PFC) [200]. The similar morphological change is not only found in animals with chronic corticosterone administration [201], but also in patients with major depressive disorder [202]. Although the size and activity is decreased in the hippocampus and PFC in major depression patients, the amygdala's size and activity are increased [203].

Correspondingly, stress can increase dendritic spines and synaptic connectivity in the amygdala and the size of the amygdala [204, 205]. This is mechanistically important as the amygdala, hippocampus, and PFC provide important limbic control of the HPA axis. The amygdala drives activation of the HPA axis, while the hippocampus and PFC dampen its activation, all via interneurons synapsing onto the PVN of the hypothalamus [206].

Multiple mechanisms underlying psychological stress-induced inflammation have been reported and were discussed in the previous paragraph. Some evidence supports the belief that inflammation plays a key role in depression or anhedonic-like behavior. As mentioned, patients with major depressive disorders exhibit increased expression of pro-inflammatory cytokines in peripheral blood and cerebrospinal fluid (CSF). The expression of IL-1β, IL-6, TNF, Toll-like receptor 3 (TLR3), and TLR4 were elevated in suicide victims that had depression [207]. Further, administration of inflammatory cytokines (IFNα) or inducers (endotoxin) to healthy patients will induce depression-like symptoms [208, 209]. One potential molecular pathway for inflammatory effects to induce depression-like behavior might be reduced synaptic availability of monoamines via enhanced mitogen-activated protein kinase (MAPK) activity [210]. One study showed that the cytokine-inducer, lipopolysaccharide (LPS), will stimulate brain serotonin reuptake transporter (SERT) activity via induction of p38 MAPK activity, leading to a decrease in serotonin synaptic availability and depression-like behavior [211]. Another potential molecular pathway could be that inflammatory cytokines promote microglia activation [212], leading to astrocytic activation, and a reduction of glutamine (Glu) reuptake and stimulation of astrocytic Glu release [213]. Eventually, excessive glutamate binding to extrasynaptic NMDA receptors will lead to increased excitotoxicity, decreased production of BDNF, and cell death in the dorsal anterior cingulate cortex [214], which can lead to depression-like behavior [215].

Depression can lead to a loss of serotonergic fibers and dendritic spine density in the hippocampus; however, BDNF can enhance the growth and survival of serotonergic neurons via signaling cascades downstream of its receptor trkB [216].

It is important to note that both chronic stress and depression might be highly related to microglial activation. Under conditions like infection, injury, neurodegeneration, or markedly altered neuronal activity in the brain, microglial structure and function are rapidly altered to gain an activated status [217]. Evidence also suggests that microglial activation is associated with neuroinflammation, which evolved to help eliminate pathogenic challenges. However, activated microglia can damage neurons and glial cells, and result in neurodegenerative diseases under many circumstances [218]. In human studies, administration of endotoxin (LPS) or Salmonella typhi to activate microglia induced depression symptoms. A severe increase in the production of inflammatory cytokines was also observed in those patients [209, 219]. Animal studies provide more direct evidence about the involvement of microglia in LPS-induce depression by stating the following: 1) LPS-induced depressive-like symptoms can be treated by minocycline, a microglial inhibitor [220]; 2) activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in microglia is essential for LPS-induced depressive-like symptoms [221]; 3) LPS-induced depressive-like symptoms are more severe in mice with microglial hyper-reactivity [222].

1.4 Genetic variability in anxiety

Many studies have reported A/J mice as one of the most anxiety-prone inbred mouse strains [223-225]. One study compared male and female C57BL/6J and A/J mice in a battery of anxiety-provoking behavioral tests. This study showed that A/J mice of both sexes scored higher than C57BL/6J in all tests [223]. An earlier study also showed that, compared to 13 other inbred strains (e.g. BALB/cJ, NIH/Nude, C3H/HeN, DBA/2J, AKR/J, C57BL/6ByJ, C57BL/10J), A/J

mice were one of best models for anxiety-based studies on many behavior tests such as open field, light/dark transitions test, and elevated plus maze [225]. Finally, a study discussed the stability of behavior and brain size between different inbred mouse strains between laboratories. These studies were pursued for decades and indicated A/J mice as an anxiety-prone strain [224]. To investigate why A/J mice have anxiety behavior compared to other strain, Hovatta, I., et al. screen genes expression in central nervous system and found 17 genes' expression levels are correlated with anxiety pattern[226]. Compared to other 16 genes glyoxalase I (Glo1) involved most brain area such as, cingulate cortex, hippocampus, hypothalamus, periaqueductal grey and pituitary[226]. Glyoxalase 1 (GLO1) is one of two enzymes that detoxifies toxic byproducts of glycolysis, including methylglyoxal (MG). Elevated MG can induce protein modifications (advanced glycation end-products) and increase oxidative stress. A/J mice have more copies of Glo1 and has higher Glo1 mRNA expression (5 fold) compared to C57Bl/6 [226]. Other reports in mice observed that observed anxiety level was correlated with Glo1 expression in the amygdala and hippocampus and that knockdown of Glo1 by siRNA was able to increase the time spent in the center during an open field test [227].

High comorbidity between anxiety and chronic pain has been consistently reported, yet the mechanistic connection between these two disorders remains unknown [159, 228-232]. A/J mice have been recognized as an anxiety prone stain, yet few pain-related studies have been performed on this strain. In rat, the Wistar-Kyoto (WKY) inbred rat strain is a high anxiety strain of rat. Compared to Sprague-Dawley (SD) rats, WKY rats showed a significant increase in the time spent in the periphery during the open field test and, in the elevated plus maze test, WKY rats tend to spend more time in the close arms [233-236]. Further, increased immobility during the forced swim test was also found WKY rat [237-240]. WKY rats also have a higher

physiological response to stressful events compared to SD rat, exemplified as a significantly higher plasma ACTH level following immobilization stress [241]. Visceral hypersensitivity has been observed in WKY rat, which was associated with a higher expression level of CRF1 in the paraventricular nucleus, central nucleus of the amygdala, and hippocampus compared to SD rat [242]. Implanted micropellets of CRF1 receptor antagonist, CP 376395, in WYK's central nucleus of the amygdala (CeA) rat ameliorated anxiety-induced colonic hypersensitivity [243].

1.5 Effects of Physical activity on pain and mood disorders

An increasing number of diseases like type 2 diabetes, cardiovascular and pulmonary diseases, rheumatoid arthritis, and osteoporosis incorporate physical activity as therapy and recently, a guideline for prescribing exercise as therapy was published [244, 245]. Evidence shows that exercise provides a therapeutic benefit to neuropathy patients in addition to pharmaceutical treatments. Previous work from our lab indicates that exercise could reverse mechanical allodynia caused by a high fat diet [246, 247]. Further, more and more studies recommended that physical activity with or without pharmacological therapy can effectively improve various type of pain, including lower back pain, muscular pain, fibromyalgia and rheumatoid arthritis [248-251]. Many clinical studies suggest that exercise also benefits depression patients, but, due to a lack of animal studies, the mechanism of how exercise improves depression has not been fully understood [252].

Exercise improves symptoms of chronic pain diseases such as fibromyalgia [253], migraine [254], and low back pain [255]. In both humans and animals, aerobic exercise has been shown to be associated with an analgesic response [256]. Earlier studies from our lab indicate that voluntary exercise in mice reverses diet- and maternal separation stress-induced mechanical allodynia [247, 257]. Exercise provides strong anti-inflammatory effects, which are associated

with both depression and chronic pain [258-260]. The potential mechanism might be related to the activation of the skeletal muscle, which markedly increases both cellular and circulating levels of IL-6 over a short duration. This effect will then trigger anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1RA), increasing their levels as well as releasing cortisol [261]. The secretion of IL-6 inhibits endotoxin-induced TNF-alpha [262], which is known to cause neuropathic pain [263]. Other mechanisms have been proposed stating that exercise stimulates the HPA axis and the sympathetic nervous system (SNS). The end product, cortisol, has potent anti-inflammatory effects and releases catecholamines (adrenaline and noradrenaline), which downregulate the LPS-induced production of cytokines by immune cells [264-266]. In stress models, the peak of plasma corticosterone level shows no difference between voluntary wheel running and sedentary mice after exposure to restraint stress. However, the corticosterone level rapidly decreases to baseline in running mice compared to sedentary mice [267].

Many studies suggest that physical activity has beneficial effects on depression in patients. A meta-analysis that included 35 studies and 1356 participants reported that exercise had a greater benefit for depression patients compared to control interventions like placebo, meditation, or relaxation [252]. In animal studies, chronic stress can have a negative impact on brain plasticity by causing neurons to undergo morphological changes such as dendritic atrophy in hippocampal CA3 pyramidal neurons, which has been shown to be associated with depression-like behavior [268, 269]. Exercise consistently increases BDNF expression in several regions including the hippocampal formation and cerebral cortex, increasing brain plasticity and stimulating neurogenesis [270]. Aerobic exercise increases the size of the anterior hippocampus by 2%, which is associated with elevation of serum BDNF levels [271]. Others suggest the

mechanism of exercise-driven changes in the HPA axis is involved in exercise treatment for depression [272].

1.6 Study significance

The American population largely suffers from stress-induced symptoms like anxiety, depression, or worry. The percentage of people reporting having experienced symptoms of stress in the past month have escalated from 71% in August 2016 to 80% in January 2017 [273] and an increasing number are being diagnosed with stress-related health disease. The statistics on anxiety have risen from 9 percent in 2014 to 12% in 2015 and depression has risen from 12% in 2014 to 16% in 2015 [274]. Further analysis also indicates that younger generations are more prone to stress in comparison to older generations [274]. The prevalence of pain (126 million) is larger than people suffering from all kinds of cardiovascular diseases (102.7 million) [275] and cancer (14.5 million) [276] combined. In a clinical setting, it is well known that negative emotions will influence pain and the positive link between chronic pain and anxiety is well documented [277]. Currently, there is no well-developed treatment for chronic pain patients. Understanding the mechanisms underlying stress-induced chronic pain on anxiety patients is critically important. Nevertheless, few studies focus on this important issue and a convincing animal model has not yet been developed.

Physical activity has recently become a powerful and practical therapeutic tool for many diseases. In meta-analysis clinical trials, exercise has been reported to reduce fibromyalgia [253], rheumatoid arthritis [278], hip osteoarthritis [279], non-specific low back pain [255], depression, and anxiety [280], whereas research on how exercise alleviates painful disease is still incomplete. Current studies show that exercise increases BDNF and insulin-like growth factor (IGF-1) [281], which both have been reported to have exercise-induced benefits in depression

[282, 283] and chronic pain [284]. It has been proposed that exercise provides anti-inflammatory responses in an attempt to subdue inflammatory pain [285] and stress induced neuro-inflammation that can cause mood disorders like depression [8]. However, whether physical activity benefits stress-induced mechanical allodynia, bladder hypersensitivity, and anhedonic behavior associated with underlying anxiety, as well as the potential mechanism of action, still needs more investigation.

Over one million burn injury patients seek medical attention worldwide and 11 million people was reported to attend medical facility for burn injury [286, 287]. Guides to treat burn injury and acute pain have been established, yet a large percentage of burn patients will go on to develop chronic pain. Due to the underlying complexity of this condition, no effective treatment has been established [288-290]. One study reported that 11 years post-injury, nearly 52% of patients still experience chronic pain associated with their burn injury [291]. Psychological diseases have a close relationship and high comorbidity with burn injury. A high prevalence of anxiety has been broadly reported after thermal trauma [292, 293]. In a one-year-long clinical study, 82% of burn patients were identified as having anxiety [294]. Multiple reports indicate that stressful events will increase pain in both rodent and human models [7, 295, 296]. Therefore, it is urgent and critical to develop a preclinical model to study mood disorder and stress effects on chronic pain resulting from thermal injury.

The goal of the first study (Foot shock stress generates persistent widespread hypersensitivity and anhedonic behavior in an anxiety-prone strain of mice) was to investigate the effect of psychological stress on an anxiety-prone group and the molecular mediators associated with it. First, we developed a practical small rodent model for studying the impact of stress on a subject with underlying anxiety. We discovered that 10-day mild foot shock stress has

acute and chronic effects on hind paw allodynia and bladder hypersensitivity. By measuring sucrose preference and nesting building, we also found that depression-like behaviors start around day 3-4 of the foot shock stress exposure and can last for 28 days after the cessation of the foot shock procedure. Additionally, elevated CORT levels at 1 day post- but not 28 days post-foot shock indicate that that HPA axis increased its output acutely, but returned to normal, despite the continued presence of behavioral changes. Still, we investigated neurotrophic and HPA axis related molecular markers in the hypothalamus and hippocampus. We learned that inflammation occurred in the bladder after foot shock stress and may contribute to bladder hypersensitivity. Finally, we confirmed the previous study of anxiety-prone rat strains which showed elevated mast cell degranulation in the bladder compared to normo-anxious strains [297-299].

In the second study (The effect of voluntary exercise on foot shock-induced changes in A/J mice), we investigated the potential therapeutic effectiveness of voluntary exercise for repeated foot shock stress in an anxiety-prone strain of mice. We discovered that 38 days of access to a running wheel had a non-significant impact on stress-induced mechanical allodynia in A/J mice. 28 days of physical activity before foot shock stress failed to prevent bladder hypersensitivity in A/J mice. We also revealed a unique running pattern in foot shock-exposed A/J mice. Voluntary running distance was significantly increased during the first hour immediately following foot shock exposure in A/J mice. However, overall running distance was significantly lower in this same group, compared to sham-exposed (naïve) A/J mice. Regardless of the lack of significant behavioral changes, voluntary wheel running significantly lowered serum CORT levels in sham- and shock-exposed A/J mice, suggesting that physical activity can prevent elevated HPA axis output due to foot shock stress.

The goal of the third study (Genetic differences in susceptibility to foot shock-induced mechanical and visceral hypersensitivity) was to investigate the effect of different genetic backgrounds in response to foot shock stress, in an effort to identify potential therapeutic targets for treatment of stress-induced mechanical and visceral hypersensitivity in anxiety-prone individuals. First, we recorded body weight changes in both A/J and C57Bl/6 strains before and after foot shock stress exposure. We discovered that both strains developed acute and persistent hind paw allodynia after foot shock stress. However, visceral hypersensitivity was only found in foot shock stress-exposed A/J mice, but not in C57Bl/6 mice. Finally, we also assessed fecal output as a measure of adaptation to the repeated stress exposure and found that A/J mice are less-likely to acclimate to the repeated stressor than C57Bl/6 mice.

In the final study (The impact of foot shock-induced stress on pain-related behavior associated with burn injury), we investigated key factors that facilitate the transition from acute pain to chronic pain after burn injury. Interestingly, compared to C57Bl/6 mice, A/J mice had a shorter post-burn recovery time for resolving hind paw allodynia. We identified that foot shock stress exposure resulted in persistent hind paw allodynia in both C57Bl/6 and A/J mice, indicating that psychological stress may play a critical role in the development of chronic pain.

In summary, this project established a preclinical mouse model for chronic pain/co-morbid mood disorder and post burn-induced chronic pain. We investigated new information on mechanisms underlying the effects of stress and exercise on nociceptive sensitivity and mood disorders, especially in an anxiety-prone model. We ruled out and identified some potential key factors that facilitate the transition from acute to chronic pain. Finally, several potential novel treatments for comorbid chronic pain and mood disorder were also tested and established.

Chapter 2: Foot shock stress generates persistent widespread hypersensitivity and anhedonic behavior in an anxiety-prone strain of mice.

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2.1 Introduction

According to the American Psychological Association, nearly three-quarters of American adults in 2017-2018 stated they experienced at least one stress-related symptom over the past month [300]. This percentage jumps to 91% for Gen Z adults between 18 and 21 years of age. Stress is a well-known trigger for most chronic pain disorders, causing an onset or exacerbation of ongoing symptoms [7, 301]. Conversely, stress-related psychological disorders, such as depression, anxiety, and panic disorder, are more prevalent among chronic pain patients [231, 302-305]. Patients diagnosed with urologic chronic pelvic pain syndrome (UCPPS), including interstitial cystitis/painful bladder syndrome (IC/PBS) and/or chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), that also experience widespread pain have increased comorbidities, including fatigue, depression, anxiety, psychological stress, and higher negative affect scores compared to patients with only pelvic pain [306]. It is hypothesized that UCPPS patients with widespread sensitivity and high comorbidity have a centralized pain phenotype, due to adaptations within the central nervous system, and are more susceptible to acute and chronic stress exposure [306, 307].

Many patients with comorbid chronic pain and mood disorders display either hyper- or hypocortisolism, indicative of an improperly functioning hypothalamic-pituitary-adrenal (HPA) axis [307, 308]. The hypothalamus responds to stress by releasing corticotropin-releasing factor (CRF), which causes a downstream release of cortisol in humans or corticosterone (CORT) in rodents [206, 309]. Peripheral CRF and CORT impact both metabolic functions and neuroimmune interactions that can drive inflammation and increase pain sensitivity [307]. They also bind their respective receptors, CRF receptors 1 and 2 (CRF1 and CRF2), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), at each level of the HPA axis to diminish

signaling once the stressor is gone [206]. Higher limbic structures, including the amygdala and hippocampus, also express CRF and glucocorticoid receptors and have been shown to positively and negatively regulate the HPA axis, respectively [206]. Brain imaging studies of UCPPS patients showed increased gray matter volumes and functional connectivity between somatosensory and limbic cortices [310], which was related to higher choline levels in the anterior cingulate cortex (ACC) [311] and correlated with negative mood, supporting a role for modified central processing in chronic pelvic pain syndromes associated with stress.

We have previously shown that early life stress exposure negatively impacts the HPA axis, increases urogenital and hind paw sensitivity, and evokes mast cell degranulation in male and female mice, all of which is exacerbated following adult stress exposure [160, 312, 313]. Other groups have shown that both anxiety-prone [299, 314] and normative [315] rat strains display urogenital hypersensitivity following repeated stress exposure. The high-anxiety Wistar-Kyoto strain of rats also exhibited greater cortical activation in response to passive bladder distention following exposure to water avoidance stress (WAS) [316]. Here, we have adapted these previous studies to the anxiety-prone A/J mouse strain to provide evidence that exposure to repeated foot shock stress leads to persistent bladder and hind paw hypersensitivity, anhedonia, and increased downstream activation of the HPA axis.

2.2 Methods

2.2.1 Animals

All experiments in this study were performed on adult (>12-week-old) female A/J mice (Stock No: 000646, Jackson lab, Bar Harbor ME USA) housed in the Research Support Facility at the University of Kansas Medical Center. Mice were housed 4-5/cage in Techniplast Touch Slim Line Individually Ventilated Cages (Tecniplast North America, West Chester, PA) with corn cob

bedding in a climate-controlled room on a 12-hour dark-light cycle (lights on 6AM-6PM) and received water and food ad libitum. Animal use protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of IASP.

2.2.2 Foot shock stress exposure

Mice were exposed to foot shock stress for 10 consecutive days according to the following paradigm. Mice were transferred to a sound-proof room and placed 4 at a time into a Tru Scan Arena system cage equipped with a shock floor (26 × 26 × 39 cm, Coulbourn Instruments Holliston, MA, USA). Mice in the shock cohort were exposed to one of five random foot shock sequences consisting of 30 0.4 mA shocks over a 15-minute period. Mice in the sham cohort were held in the Tru Scan Arena system cage for 15 minutes, but received no-shocks. Mice were returned to their home cages after foot shock stress or sham exposure.

2.2.3 Hind paw mechanical withdrawal threshold

Mice were tested for hind paw mechanical withdrawal threshold prior to foot shock stress exposure and again at either 1d or 28d after the final foot shock stress exposure. Mice were acclimated to the testing room for two days prior to assessment of hind paw mechanical thresholds. On the day of testing, mice were placed in individual clear plastic chambers (11 x 5 x 3.5cm) on a 55cm-high wire mesh table and allowed to acclimate for 30 mins. The up-down method was performed using a series of monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.08, 4.31, 4.74 g; North Coast Medical, Inc Morgan Hill, CA, USA) applied to the right hind paw. The test started with application of the 3.22g monofilament. A negative response was followed by the next larger monofilament, whereas a positive response (brisk withdrawal of the paw) was followed by the next smaller monofilament. Four additional filaments were tested after the first

positive response. The 50% withdraw threshold was calculated for each mouse and group means were determined as previously described [317].

2.2.4 Hind paw thermal latency

Mice were tested for hind paw withdrawal latencies to radiant heat stimulus prior to foot shock exposure and again at 1d after the final foot shock exposure. Following mechanical withdrawal threshold testing, mice were placed in individual clear plastic chambers (11 x 5 x 3.5cm) on top of the heated glass surface of a thermal analgesiometer (UARDG; Department of Anesthesiology, University of California San Diego, La Jolla, CA) and allowed to acclimate for 30 minutes. A high intensity light (4.25A) was directed at the plantar aspect of the hind paw and the latency to withdraw from the stimulus was automatically recorded within 0.01s. Alternating hind paws were tested a total of three times with a minimum of 5 minutes between applications. The stimulus automatically shut off after 20 seconds to avoid tissue damage. Individual responses were averaged per mouse and group means were determined as previously described [160].

2.2.5 Urinary bladder distention

Mice were tested for urinary bladder sensitivity at either 1d or 28d after the final foot shock exposure. 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 implanted into the left and right abdominal muscle using a 26-gauge needle and the free ends were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA). A 24-gauge angiocatheter (EXELINT, Los Angeles, CA, USA) was inserted into the bladder via the urethra and secured to the tail with tape. Isoflurane was reduced to approximately 1% until hind limb reflexes, but not escape behaviors, were present. Body temperature was

maintained at approximately 37°C using a heating pad. The bladder was distended with compressed nitrogen gas controlled by a custom-made distention control device (The University of Iowa Medical Instruments, Iowa City, IA), manually adjusted by a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO), and verified by a separate pressure monitor (World Precision Instruments, Sarasota, FL). After stable responses to 60mmHg were confirmed, each pressure (15, 30, 45, 60mmHg) was applied in triplicate for 20 seconds with a 2-minute rest period in-between. Electromyographic activity was amplified, filtered, and recorded (Spike 2, Cambridge Electronic Design, Cambridge, UK) and the visceromotor response (VMR) was quantified, averaged between triplicate pressure applications, and expressed as a percentage of baseline activity immediately prior to the distention.

2.2.6 Sucrose preference testing

All mice were tested for sucrose preference for four days prior to sham- or foot shock- exposure to obtain a baseline preference measurement and then throughout the subsequent ten days. Mice were housed individually with free access to two identical polysulfone drinking bottles (BioDAQ Liquid Choice Unplugged Allentown cages, Biological Data Acquisition, New Brunswick, NJ). Mice were acclimated to caging conditions for 24 hours with both bottles containing standard drinking water. One bottle was then filled with 1% or 2% sucrose and bottle weights were measured and bottle positions were interchanged daily. Volume and percentage of 1% or 2% sucrose was calculated for each mouse.

2.2.7 Nest building test

Mice were individually transferred to a clean cage containing no environmental enrichment outside of a 3.0 g nestlet square one hour before the start of the dark phase (5pm). Seventeen hours later, the nest and intact nestlet pieces were photographed and weighed. Two blinded

experimenters scored the nests on a 1-5 scale according to previous publications [318, 319] and the average of their scores is presented here.

2.2.8 Serum corticosterone

Mice were deeply anesthetized with inhaled isoflurane (>5%) and trunk blood was collected. Blood was allowed to clot on ice for 1 hour and then centrifuged at 10,000 rpm for 10 minutes. Serum (clear supernatant) was collected and stored at −20°C until analysis. Serum corticosterone (CORT) was quantified using an ELISA kit according to the manufacturer's instructions (ALPCO, Salem, NH).

2.2.9 mRNA extraction and qRT-PCR

Mice were deeply anesthetized with inhaled isoflurane (>5%) and bladder and whole brains were removed and snap frozen in liquid nitrogen or on dry ice, respectively. Hypothalamus and hippocampus were dissected, immediately snap frozen in liquid nitrogen, and stored at -80°C. All tissue was homogenized in Trizol reagent (Ambion, Austin, TX, USA), followed by manufacturer's instructions of RNeasy Micro Kit (QIAGEN, Valencia, CA, USA). The concentration of RNA was determined by a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and cDNA was made by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Finally, 5 μL of cDNA was mixed with SsoAdvanced SYBR Green Supermix (Bio-Rad) and indicated primers (Table 1; Integrated DNA Technologies, Coralville, IA), and quantitative RT-PCR was performed using a Bio-Rad CFX manager 3.1 real time PCR system. GAPDH and β-actin were used as control genes for brain and bladder tissue, respectively. Samples were run in triplicate with negative controls. Efficiency values were derived for each individual sample (LinRegPOCR software v2012.3) and

threshold cycle values were subtracted from those of the control gene and percentage of fold change over sham was calculated using the Pfaffl method [320].

2.2.10 Statistical analysis

Statistical analyses were performed using Two-way analysis of variance (ANOVA), with or without repeated measures, followed by Bonferroni's posttest or Fisher's least squared difference, as denoted in the manuscript (IBM SPSS Statistics v. 24, IBM Corporation, Armonk, NY; GraphPad Prism 8, GraphPad Software, Inc., La Jolla, CA). All data are expressed as mean ± SEM and $p < 0.05$ was considered significant.

Table 1. Primers used for real-time PCR analysis

Gene	**Forward (5' – 3')**	**Reverse (3' – 5')**
CRF	CCTCAGCCGGTTCTGATCC	GCGGAGGAAGTATTCTTCACCC
CRF_1	CCCTGCCTTTTTCTACGGTGT	TTCCCGGTAGCCATTGTTTGT
BDNF	CAGGTTCGAGAGGTCTGACGA	CGCGTCCTTATGGTTTTCTTCG
GR	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
MR	GAAAGGCGCTGGAGTCAAGT	CCATGTAGCTGTTCTCATTGGT
IL10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
IL6	CTGCCAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
SCF	CCCTGAAGACTCGGGCCTA	CAATTACAAGCGAAATGAGAGCC
Art	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
MCP-1	AGGTCCCTATGGTGCCAATGT	CGGCAGGATTTTGAGGTCCA
NGF	ACACTCTGATCACTGCGTTTTTG	CCTTCTGGGACATTGCTATCTGT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT
β-actin	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT

2.3. Results

Foot shock stress induced acute and persistent bladder hypersensitivity and hind paw allodynia in female A/J mice

Female A/J mice were exposed to 10 days of foot shock stress treatment and then tested for bladder sensitivity 1 or 28 days later. Compared to sham-exposed mice, shock-treated mice had a significant increase in the visceromotor response (VMR) during urinary bladder distention (UBD) 1 day after the last shock treatment (Figure 1A). This was also observed for 30mmHg and 60mmHg pressures at 28 days after the final shock treatment (Figure 1B). Comparisons of the area under the curve for the VMR at both time points revealed a significant effect of foot shock stress exposure across both time points (Figure 1C).

Hind paw mechanical withdrawal thresholds were also measured at 1- and 28-days post-foot shock stress exposure. Stress-exposed mice had significantly lower withdrawal thresholds than their baseline measurements or those of sham-exposed mice, at 1-day post-foot shock stress exposure (Figure 2A). At 28 days post-foot shock stress, the foot shock stress-exposed mice maintained a significant reduction in withdrawal threshold, compared to their own baseline measurements, but were not significantly different from sham-exposed mice (Figure 2B). Hind paw thermal withdrawal latencies were also measured prior to and 1-day post-foot shock stress exposure and no differences were observed between sham- and shock-exposed mice (sham: 7.02 sec ± 0.913; shock: 7.06 sec ± 0.40; $p>0.05$, $n=6$).

Figure 2. 1 Foot-shock stress induced baldder hypersensitivity

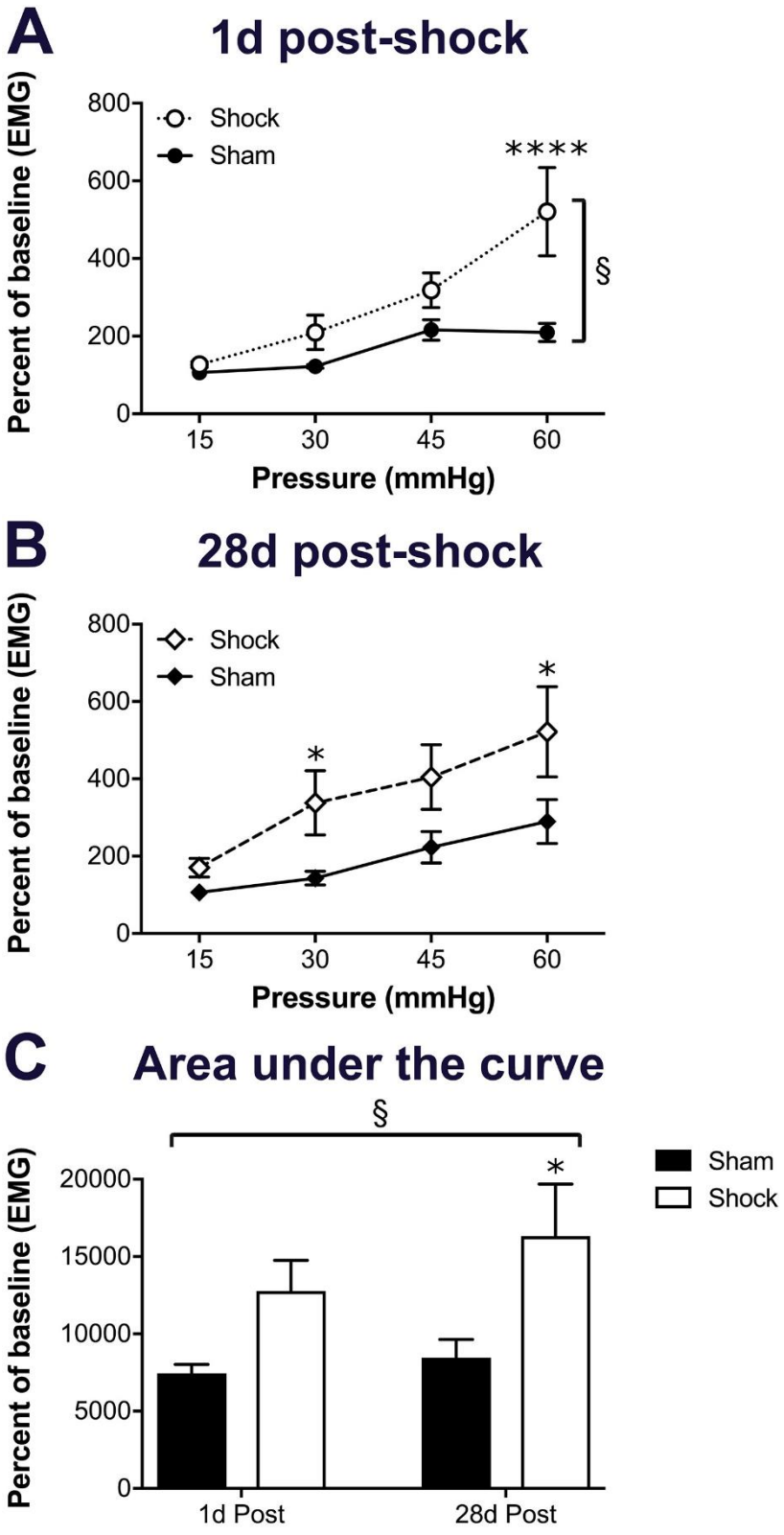


Figure 2. 2 Foot-shock stress induced Hind paw mechanical allodynia

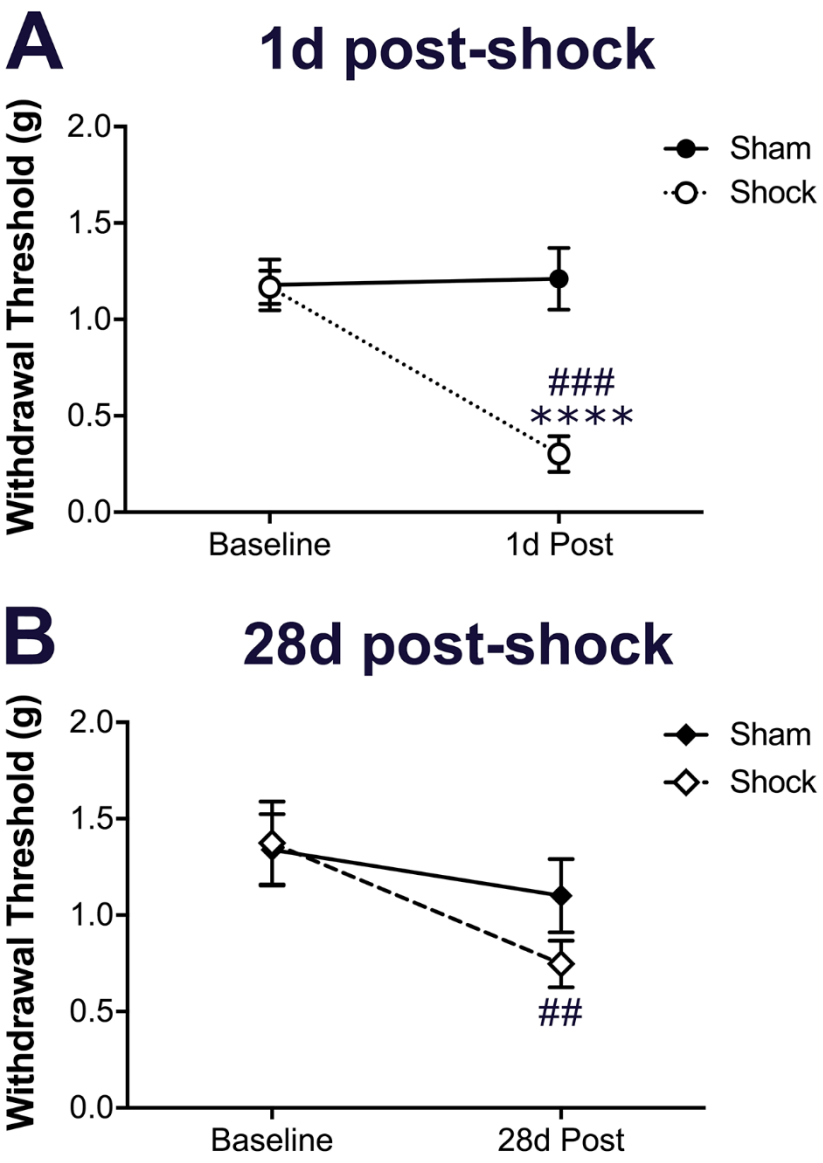


Figure 2.1: The visceromotor response (VMR) during urinary bladder distention (UBD) was measured 1 day and 28 days after the final foot shock exposure. (A) At 1 day after foot shock, there was a significant impact of foot shock stress, especially at the 60 mm Hg pressure. (B) At 28 days after foot shock stress, the shock group had a significantly higher VMR at 30 mm Hg and 60 mm Hg. (C) Area under the curve (AUC) measurements revealed a significant impact of foot shock stress, particularly in the 28-day group compared with sham. Brackets indicate a significant effect of shock (§$P < 0.05$), two-way RM ANOVA (A and B), or two-way ANOVA (C); *, ****$P < 0.05$, 0.0001 vs sham, Bonferroni posttest. n = 5 to 8 for all groups. RM ANOVA, repeated-measures analysis of variance.

Figure 2.2: Hind paw mechanical withdrawal thresholds were measured 1 day and 28 days after the final foot shock exposure. (A) The foot shock stress–exposed group had significantly lower mechanical withdrawal thresholds at 1 day compared with both their own baseline measurements and the sham-exposed group. (B) The foot shock stress–exposed group maintained significantly decreased mechanical withdrawal thresholds at 28 days after foot shock stress, compared with their baseline measurements, but were not significantly different from sham-exposed thresholds. Two-way RM ANOVA; ****$P < 0.05$, 0.0001 vs sham, ##, ###$P < 0.01$, 0.001 vs baseline, Bonferroni posttest. n = 8 for all groups. RM ANOVA, repeated-measures analysis of variance.

Depression-like behavior during and after foot shock stress exposure.

Anhedonia was assessed both during and after foot shock stress exposure to determine depression-like outcomes related to stress in female A/J mice. Preference for 1% or 2% sucrose was measured for four days prior to foot shock stress exposure to obtain a baseline preference and then continually during the 10 days of foot shock stress exposure. Compared to sham-exposed mice, the percentage of 1% sucrose consumed by foot shock stress-exposed mice decreased significantly during 10 days foot shock stress, particularly on days 3-4 and 9-10 (Figure 3A). In contrast, preference for 2% sucrose was not significantly impacted by foot shock stress; however, there was a significant variance due to time across both groups (Figure 3B).

Nest building was assessed to evaluate anhedonia at 1- and 28-days post-foot shock stress exposure. Nest scores and the weight of remaining intact nestlet were both significantly impacted by foot shock stress and time (Figure 3C-D). Although the 1- and 28-day measurements were significantly different from their sham-exposed counterparts, the 28-day measurements had slightly improved and were significantly different from the 1 day measures (Figure 3C-D).

Foot shock stress alters expression of selected mRNAs in the bladder but not mast cell infiltration or degranulation

Previous studies revealed that early life stress exposure increased mast cell degranulation and pro-inflammatory cytokine and growth factor expression in the bladder, which may contribute to urogenital hypersensitivity [313]. Therefore, we investigated the effect of foot shock stress exposure on mast cell infiltration and degranulation and levels of selected mRNAs in the bladder. There was no difference in the number of infiltrated mast cells or in the mast cell degranulation rate between bladders from 1-day post-foot shock stress and sham exposed mice (Figure 4A-C). A significant impact of foot shock stress or time was observed for every mRNA

evaluated. The mRNA levels of interleukin-10 (IL- 10), an anti-inflammatory cytokine, were significantly decreased by foot shock stress exposure, particularly at the 1-day time point (Figure 4D). The mRNA levels of both artemin and stem cell factor (SCF) were significantly elevated 28 days after foot shock stress exposure compared to 1 day, driving an overall time effect for both mRNAs (Figure 4D). Finally, levels of mRNA encoding monocyte chemoattractant protein 1 (MCP-1) were significantly decreased by foot shock stress exposure, particularly at the 28-day time point (Figure 4D).

Foot shock stress exposure transiently increases HPA axis output and regulation

To understand how foot shock stress impacts the output and regulation of the HPA axis in female A/J mice, we measured serum CORT and levels of selected mRNAs in hypothalamus and hippocampus. Serum CORT was significantly increased 1 day after completion of the foot shock paradigm compared to sham exposed mice (Figure 5A). Serum CORT levels in sham- and shock-exposed mice were not different 28 days later (Figure 5A). A significant overall effect of time was observed for mRNAs encoding both brain-derived neurotrophic factor (BDNF) and MR in the hypothalamus, largely driven by a trend toward increased levels at 1-day post-shock that were significantly lower in 28-day post-shock samples (Figure 5B). In the hippocampus, GR mRNA levels were significantly higher in the sham group at 28 days compared to 1-day post-sham and 28 days post-shock (Figure 5C).

Figure 2. 3 Foot-shock stress induced anhedonic behavior

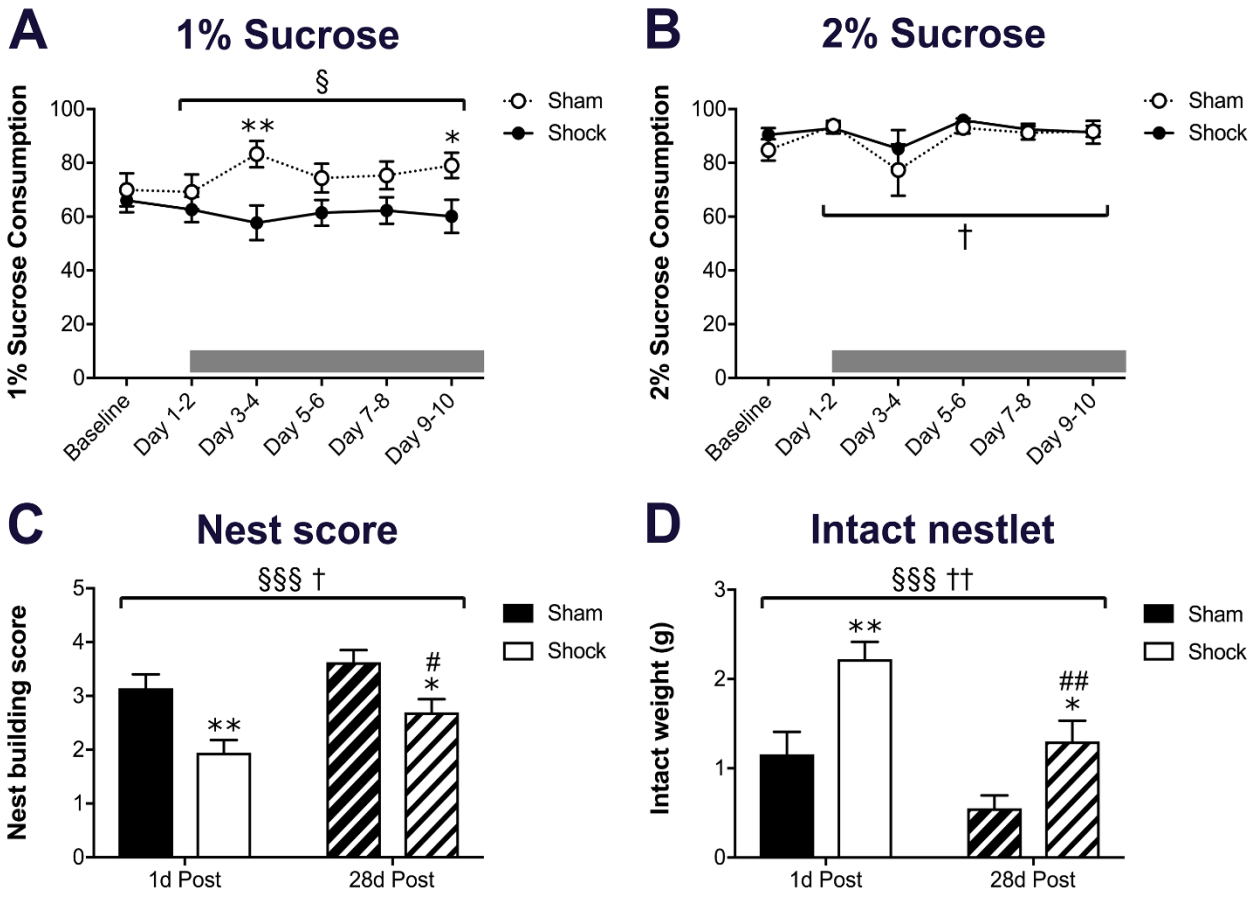


Figure 2.3: Sucrose preference testing and nest building were performed to assess anhedonic behavior resulting from foot shock exposure. Preference for 1% (A) and 2% (B) sucrose was measured for 4 consecutive days before and during (gray bar) the 10 days of foot shock exposure. (A) The percentage of 1% sucrose that was consumed, compared with standard drinking water, was significantly impacted by foot shock stress, particularly on days 3 to 4 and 9 to 10. (B) The percentage of 2% sucrose that was consumed was significantly impacted over time, but not by foot shock exposure. Nest quality (C) and the weight of intact nesting material (D) were both significantly impacted by foot shock exposure and time. At 1 day and 28 days, both measurements were significantly different between sham- and foot shock–exposed mice. The 28-day measurements in foot shock–exposed mice were significantly different from 1 day. Brackets indicate a significant effect of shock stress(§, §§§P < 0.05, 0.001) or time (†, ††P < 0.05, 0.01), two-way RM ANOVA (A–B), or two-way ANOVA (C and D); *, **P < 0.05, 0.01 vs sham, #, ##P < 0.05, 0.01 vs baseline, Bonferroni posttest. n = 7 to 8 for all groups. ANOVA, analysis of variance; RM ANOVA, repeated-measures analysis of variance.

Figure 2. 4 Foot shock stress effect on bladder mast cell and bladder mRNA

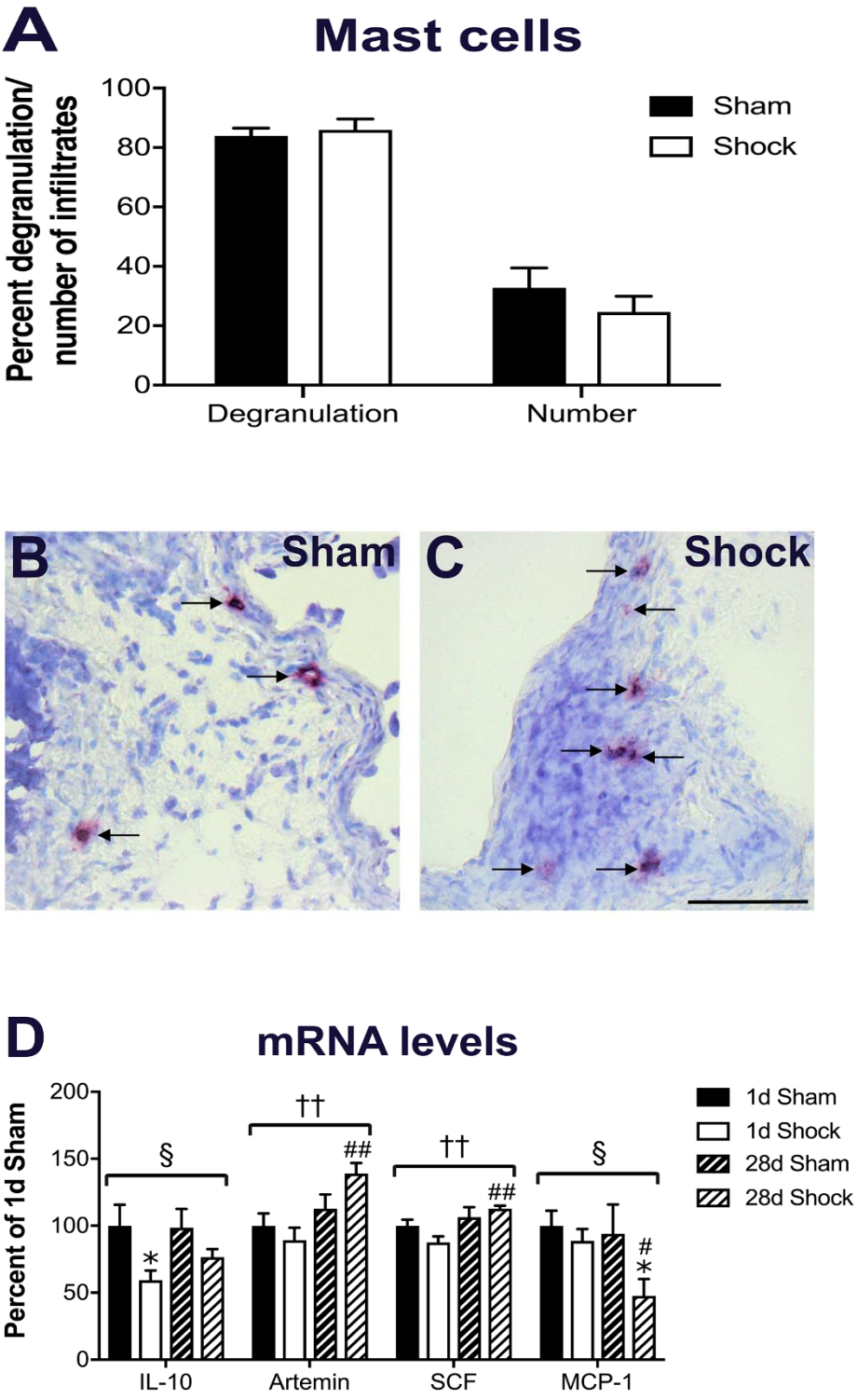


Figure 2.4: Bladder mast cell infiltration and degranulation, along with inflammatory-related mRNAs, were measured after foot shock stress exposure. Mast cells were visualized in the bladder using acidified toluidine blue in both sham- (B) and foot shock–exposed (C) mice. Nearly all mast cells displayed some degree of degranulation, evidenced as faint metachromasia and free granules within the cytoplasm and extruded outside of the cell borders (arrows). (A) At 1 day, there were no differences in the percentage of degranulation or the number of mast cells in the bladders between sham- or foot shock–exposed mice. (D) The level of IL-10 mRNA was significantly impacted by foot shock stress exposure, particularly at the 1-day time point. Artemin and SCF mRNA levels were similarly impacted over time with 28-day foot shock stressed bladders having significantly higher levels than 1-day bladders. MCP-1 mRNA levels were also significantly impacted by foot shock stress, with 28-day foot shock bladder having significantly lower levels than 28-day sham or 1-day foot shock bladder. Brackets indicate a significant effect of shock stress (§$P < 0.05$) or time (††$P < 0.01$), two-way ANOVA; *$P < 0.05$ vs sham, #, ##$P < 0.05$, 0.01 vs 1-day shock, Bonferroni posttest. n = 6 to 8 for all groups. ANOVA, analysis of variance; SCF, stem cell factor.

Figure 2. 5 Foot shock effect on HPA axis and CNS

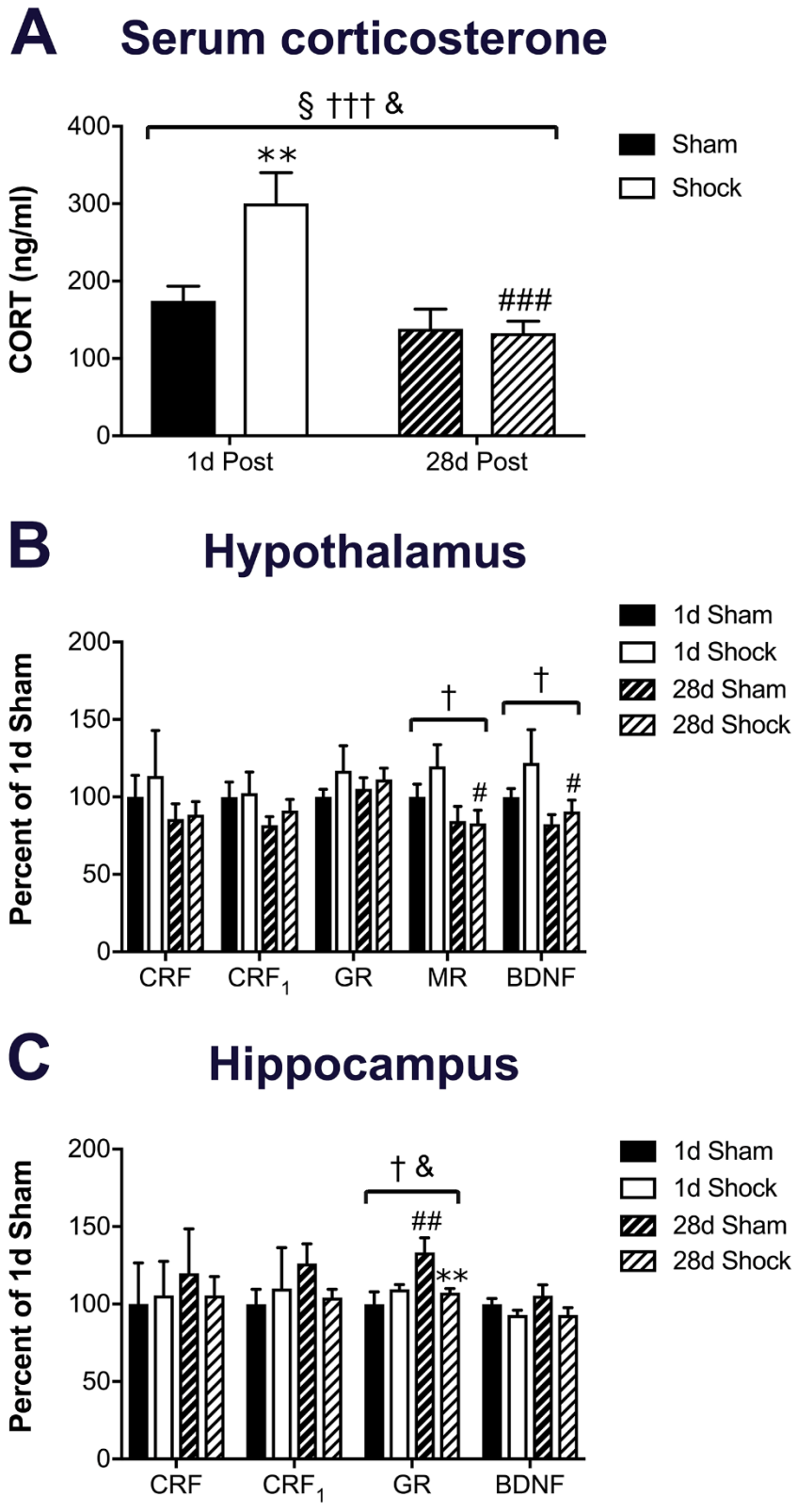


Figure 2.5: The output and regulation of the HPA axis was measured 1 day and 28 days after foot shock stress exposure. (A) Serum corticosterone was significantly impacted by shock stress and/or time, such that 1-day post–foot shock levels were significantly higher than 1-day sham and 28-day shock groups. (B) Only mineralocorticoid receptor (MR) and brain-derived neurotrophic factor (BDNF) mRNA levels were impacted by foot shock stress in the hypothalamus, with the 28-day shock groups having significantly lower levels of both mRNAs compared with 1-day shock. (C) A significant impact of time and a foot shock stress/time interaction effect was observed only for glucocorticoid receptor (GR) mRNA levels in the hippocampus. GR mRNA levels were significantly higher in 28-day sham compared with 1-day sham or 28-day shock bladder. Brackets indicate a significant effect of shock stress (§$P < 0.05$), time (†, †††$P < 0.05$, 0.001), or a shock stress/time interaction (&$P < 0.05$), two-way ANOVA; **$P < 0.01$ vs sham, #, ##, ###$P < 0.05$, 0.01, 0.001 vs 1 day, Bonferroni posttest. n = 4 to 8 for all groups. ANOVA, analysis of variance; HPA, hypothalamic–pituitary–adrenal.

2.4 Discussion

It is well-established that stress exacerbates symptoms of chronic pain and that anxiety is a common comorbidity with widespread pain [231, 302, 306]. The influence of stressors on bladder sensitivity has been explored in rat models with genetically enhanced [299, 314] or comparatively normal [315] levels of behavioral anxiety. Here we have investigated A/J mice, an inbred mouse strain that exhibits comparatively high levels of anxiety, for bladder-specific and more widespread hypersensitivity, as well as for measures of depression and regulation of the HPA axis following a 10 day-long exposure to foot shock stress.

Strain differences in behaviors related to anxiety, depression, and pain have been widely reported for both mice and rats. A comparison of C57BL/6J and A/J mice in a series of anxiety-provoking behavioral tests showed that A/J mice of both sexes scored higher than C57BL/6J in all tests performed [223]. A/J mice were also one of the more anxiety-prone strains in many applied behavioral tests, such as open field, light/dark transition test, and elevated plus maze when compared to 13 other inbred strains (e.g. BALB/cJ, NIH/Nude, C3H/HeN, DBA/2J, AKR/J, C57BL/6ByJ, C57BL/10J) [321]. Significant strain effects on visceral sensitivity and behavioral measures of anxiety and depression were reported and most pronounced in the CBA/J strain from Harlan, which is derived from the same progenitor line as the A/J mice used in this study, albeit with multiple decades of genetic drift. [322]. Finally, Wistar-Kyoto rats have been identified as an anxiety-prone strain and display significant defeat behaviors during forced swim [323] and sex-specific anxiety-like behaviors, depending on the applied test [324].

Our paradigm of repeated exposure to foot shock stress in an anxiety-prone strain of mice in the current study paralleled previous studies incorporating stress to exacerbate hypersensitivity in anxiety-prone rat strains [299, 314, 325]. Robbins, et al. [314] demonstrated that chronic (10

days), but not acute (1 day), exposure to water avoidance stress (WAS) increased VMR during UBD in Wistar-Kyoto but not Sprague Dawley rats. Additional studies have demonstrated that Wistar-Kyoto rats develop both acute and persistent bladder hyperalgesia, lasting up to 61 days [299], and altered micturition patterns [325] following 10 days of WAS exposure. Here, we observed a significant increase in bladder sensitivity both at 1 day and 28 days after 10 days of foot shock stress exposure. Chronic (7 days) exposure to foot shock stress has been shown to acutely increase bladder sensitivity in Sprague Dawley rats [315]; however, the duration of the increase was not reported, making it difficult to determine the influence of the strain on chronicity of hypersensitivity. Regardless, it is likely that genetic signatures in anxiety-prone strains make them more susceptible to outcomes related to chronic stress exposure, including long-lasting visceral hypersensitivity.

The A/J mice also displayed acute and persistent hind paw mechanical allodynia following 10 days of foot shock stress exposure. A sustained increase in hind paw mechanical withdrawal responses to a non-noxious stimulus was also reported by Lee et al., [299] following chronic WAS in Wistar-Kyoto rats. In contrast, a separate study in Wistar-Kyoto rats demonstrated decreased mechanical and thermal hind paw sensitivity after 1 WAS exposure, while a 10-day exposure had no effect compared to sham-exposed controls [314]. Differences in the outcomes of these studies are likely due to an apparent ceiling effect on mechanical thresholds in the first study and a perceptible habituation to WAS exposure in the latter study, as evidenced by a gradual decrease in fecal output during the WAS exposure.

Sucrose preference is a commonly used method with high validity for evaluating reward behavior, as preference is decreased or increased following stress exposure or antidepressant treatment, respectively [326]. In this study, we observed a significant decrease in preference for a

1% sucrose solution during the duration of the foot shock stress exposure. Previous studies have investigated genetic contributions to sucrose preference and have identified variants in relevant taste genes, particularly Tas1r3 [327]. A specific deleterious mutation in Tas1r3 was identified in the low sucrose-preferring 129X1/J strain, as well as in four other strains including A/J, that was not present in the high sucrose-preferring C57Bl/6 strain [328]. However, in a comparison study of 11 different strains using 9 concentrations of sucrose, the A/J strain was one of the highest sucrose-consuming strains tested, suggesting that sucrose preference is under polygenic control [329]. Our reported ~70% preference for a 1% sucrose solution is similar to that reported in A/J mice by Lewis et al. [329], and the foot shock stress-induced decrease in preference suggests a reduction in reward-seeking behavior. The study by Lewis et al., [329] also reported a lack of correlation between preference of different concentrations, as well as a compensatory effect of reduced caloric intake from chow as sucrose intake increased, particularly in the A/J strain. This suggests that there may be increasing metabolic influences regulating sucrose intake at higher concentrations, making these observations less dependent upon reward-based behaviors, which may underlie the lack of foot shock stress-induced reduced preference for 2% sucrose in the current study.

Nest building is a complex and innate behavior that is negatively impacted by hippocampal damage [330], and has been used to assess rodent models of pain disorders, schizophrenia, and neurodegenerative disease [331]. Nest building scores were lower in a model of social defeat stress and reversed by select anti-depressant treatments [332], suggesting that it is an appropriate measure for depressive-like behaviors. Here, we observed a persistent decrease in nest quality following exposure to foot shock stress. The decreased nest building is likely more indicative of general distress rather than persistent pain, as a previous study showed that carprofen analgesia

had no impact on nest building in a model of post-surgical pain [333]. The persistence of anhedonic behavior, coinciding with the increased bladder and hind paw sensitivity, supports the validity of this model for the study of stress-induced, widespread hypersensitivity.

The role of CRF in influencing pain signaling has been well-described in both the peripheral and central nervous system. Peripherally-released CRF activates mast cells [334] and binds receptors expressed by urothelium [19], which in turn induce neurogenic inflammation and urothelial leakiness, respectively. Here, we showed no increase in mast cell infiltration or degranulation following foot shock stress exposure; however, it should be noted that the level of degranulation for both sham- and shock-exposed mast cells in the bladder was markedly high (>80%). This is much higher than we reported in C57Bl/6 bladder (~30%) [313] and is similar to reports of the high anxiety strain of Wistar-Kyoto (WKY) rats (~75%), which also showed no increase in degranulation after a 10-day WAS protocol [297-299]. The comparatively high level of serum CORT in the sham cohorts (~170ng/ml in A/J vs. 20ng/ml in C57Bl/6 [115]) suggests that over-activity within, or downstream from, the HPA axis may be driving increased mast cell degranulation in A/J mice. Increased NGF and pro-inflammatory cytokine levels were observed in sera from IC/PBS patients [335] and from cold cup biopsies, which also had an increase in mast cell infiltration [336]. Here, we observed an overall pro-inflammatory shift in gene expression in the bladder with a decrease in IL-10 and an increase in both artemin and SCF mRNA levels. The persistent increase in artemin mRNA levels mimics other rodent studies of inflammation- or nerve injury-induced persistent pain [337] [338]. Pre-clinical studies have implicated SCF and the kit pathway in overactive bladder [339, 340]. MCP-1 has been reported to play an important role in inflammatory-related diseases such as atherosclerosis and rheumatoid arthritis [341, 342], and MCP-1 levels are higher in urine and bladder from rodent

models of IC/PBS [343, 344]. Our observation of significantly decreased MCP-1 mRNA levels at 28 days after foot shock stress was somewhat surprising and may represent a compensatory response. Although central changes were not as robust, the significant reduction in hypothalamic MR mRNA level indicates a potential loss of ambient glucocorticoid signaling and diurnal tone [30, 345] that could lead to greater HPA axis activation. Similarly, reduced GR mRNA levels in the hippocampus indicate a probable loss of negative regulation onto the hypothalamus [206].

2.5 Conclusion

In conclusion, exposure to foot shock stress elicited prolonged widespread hypersensitivity in the anxiety-prone A/J mouse strain, evidenced as increased bladder sensitivity and decreased hind paw mechanical withdrawal thresholds. Anhedonia was also observed as evidenced by decreased sucrose preference during the foot shock stress exposure and poor nest quality. A prolonged shift towards a pro-inflammatory gene expression profile in the bladder occurred following a transient increase in serum CORT levels. Together, these data suggest that A/J mice may provide an excellent model to understand the mechanisms contributing to widespreadness of pain and increased comorbidity in a subset of UCPPS patients.

Chapter 3: The effect of voluntary exercise on foot shock stress-induced changes in A/J mice.

3.1 Introduction

As the previous chapter mentioned, 80% of Americans experienced at least one stress-related symptom in the past three months and the number is increasing [273, 274]. Under stressful conditions, the HPA axis increases its output: CRF is released from the hypothalamic paraventricular nucleus and travels to the anterior pituitary to trigger ACTH release, which travels through the systemic circulation and stimulates the release of glucocorticoids, cortisol in humans and corticosterone in rodents, from the adrenal cortex [206, 346-348]. Evidence shows that many diseases, including osteoporosis, hyperglycemia, cardiovascular disease, and mood disorder, are related to persistent excess glucocorticoid production [349-352]. Excess glucocorticoids have been found in patients with major depressive disorder, however, the potential underlying mechanisms have yet to be discovered [198, 201, 202]. Severe stress and treatment with high-dose glucocorticoids have deleterious effects on hippocampus-dependent memory [191-193]. One of the important mechanisms of hippocampus-dependent memory formation is hippocampal synaptic plasticity, as displayed by long-term potentiation (LTP) [194]. Sufficiently severe stress will impair LTP in the rodent hippocampus [195]. Chronic corticosterone administration in animals induced atrophy and retraction of the apical dendrites of hippocampal pyramidal cells [197]. Other evidence found that chronic stress impairs apical dendrites of pyramidal cells and reduces the number of dendritic spines in the prefrontal cortex (PFC) [200]. Long-term stress will also induce widespread pain or abdominal pain. In a human study, pre-exposure to mental stress lowered pain tolerance in the cold pressor test [353]. Another study also suggested that people exposed to standardized psychosocial stress test tend to have higher sensitivity to thermal stimuli [301]. Abundant evidence of stress-induced hyperalgesia or allodynia was found in rodent, including visceral or mechanical hyperalgesia and

allodynia following repeat cold, immobilization, or water avoidance stress exposure [104, 112, 354-357]. Our previous study described in chapter 2 also showed that hind paw allodynia, bladder hypersensitivity, and mood disorder were observed in foot shock-stressed anxiety-prone A/J mice [161].

Evidence shows that physical activity ameliorates many symptoms related to chronic stress [358, 359]. Exercise increases BDNF expression in several regions of the brain, including the hippocampal formation and cerebral cortex, increasing neuroplasticity and stimulating neurogenesis [270]. Another study reported that elevation of serum BDNF levels by aerobic exercise triggered a 2% increase in the volume of the anterior hippocampus [271]. Exercise-driven changes in the HPA axis are also attributed to treatment for depression [272]. Recently, more and more evidence indicates that exercise benefits chronic pain. Systematic reviews show that exercise improves symptoms of fibromyalgia and knee osteoarthritis [360-362]. Studies also support that physical activity improves low back pain and chronic musculoskeletal pain in adults [363, 364]. Some potential mechanisms have been investigated. First, exercise stimulates the sympathetic nervous system (SNS) and the resultant increase in adrenaline and noradrenaline production can downregulate LPS-induced production of cytokines, such as IL-1 beta and TNF-alpha [264-266]. Second, activation of skeletal muscle increases both cellular and circulating levels of IL-6, over a short duration, which can trigger an increase in anti-inflammatory cytokines IL-10 and IL-1RA [261]. The secretion of IL-6 inhibits endotoxin-induced TNF-alpha [262], which is known to cause neuropathic pain [263]. Finally, both aerobic and resistance exercise increase cannabinoid receptors in the center nervous system [365]. Endocannabinoid receptors were highly expressed in spinal cord and brain and reports also showed that other activation of endocannabinoid receptors induced analgesia [366-368].

Our previous chapter investigated stress-induced hind paw allodynia, bladder hypersensitivity, and mood disorder in an anxiety-prone strain of mice. Comorbid chronic pain and mood disorder is often observed clinically (51.8–59.1% in chronic pain patient) [369, 370], and, as described above, physical activity has a beneficial effect on both depression and chronic pain. Here, we hypothesize that exercise will alleviate stress-induced mechanical allodynia, visceral hypersensitivity, and mood disorder in A/J mice through normalization of the HPA axis and stimulation of anti-inflammatory cytokine release. We also investigated the use of voluntary running distance as a biobehavioral marker of comorbid mood disorder.

3.2 Method

3.2.1 Animals

All experiments in this study were performed on adult (>12-week-old) female A/J mice (Stock No: 000646, Jackson lab, Bar Harbor ME USA) housed in the Research Support Facility at the University of Kansas Medical Center. Mice were housed in a climate-controlled room on a 12-hour dark-light cycle and received water and food ad libitum. Animal use protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of IASP.

3.2.2 Foot shock stress exposure

Mice were exposed to foot shock stress for 10 consecutive days according to the following paradigm. Mice were transferred to a sound-proof room and placed 4 at a time into a Tru Scan Arena system cage equipped with a shock floor (26 × 26 × 39 cm, Coulbourn Instruments Holliston, MA, USA). Mice in the shock cohort were exposed to one of five random foot shock sequences consisting of 30 0.4 mA shocks over a 15-minute period. Mice in the sham cohort were held in the Tru Scan Arena system cage for 15 minutes but received no-shocks. Mice were returned to their home cages after foot shock or sham exposure.

3.2.3 Voluntary wheel running

Mice in exercise groups were pair-caged with access to a stainless-steel running wheel (Mini Mitter; Bend, OR) beginning at 8-weeks of age (4 weeks before the first foot shock stress procedure). Mice maintained access to the running wheel during the 10 days of foot shock and until sacrifice or before the nest building test. Sedentary mice were pair-housed in standard cages with IACUC-compliant enrichment standards. Running distance was monitor by Vital View Acquisition and Analysis software (Harvard Apparatus, Holliston, MA) every half-hour.

3.2.4 Hind paw mechanical withdrawal threshold

Mice were acclimated to the testing room for two days prior to assessment of hind paw mechanical thresholds. On the day of testing, mice were placed in individual clear plastic chambers (11 x 5 x 3.5cm) on a 55cm-high wire mesh table and allowed to acclimate for 30 mins. Withdraw threshold was measured by an electronic monofilament 2390 series (IITC Life Science Inc. Woodland Hills, CA), equipped with a semi-flexible tip, applied five times to the plantar surface of the right hind paw with at least a one-minute interval between applications. Withdrawal threshold was calculated by averaging the middle three measurements, after removing the highest and lowest measurements.

3.2.5 Urinary bladder distention

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 implanted into the left and right abdominal muscle using a 26-gauge needle and the free ends were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA). A 24-gauge angiocatheter (EXELINT, Los Angeles, CA, USA) was inserted into the bladder via the urethra and secured to the tail with tape. Isoflurane was reduced to approximately 1% until hind limb reflexes, but not escape behaviors, were present. Body temperature was maintained at approximately 37 °C using a heating pad. The bladder was distended with compressed nitrogen gas controlled by a custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA), manually adjusted by a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO), and verified by a separate pressure monitor (World Precision Instruments, Sarasota, FL). After stable responses to 60mmHg were confirmed, each

pressure (15, 30, 45, 60mmHg) was applied in triplicate for 20 seconds with a 2-minute rest period in-between. Electromyographic activity was amplified, filtered, and recorded (Spike 2, Cambridge Electronic Design, Cambridge, UK) and the visceromotor response (VMR) was quantified and expressed as a percentage of baseline activity immediately prior to the distention.

3.2.6 Sucrose preference testing

Mice were housed individually with free access to two identical polysulfone drinking bottles (BioDAQ Liquid Choice Unplugged Allentown cages, Biological Data Acquisition, New Brunswick, NJ). Mice were acclimated to caging conditions for 24 hours with both bottles containing standard drinking water. One bottle was then filled with 1% or 2% sucrose and bottle weights were measured and bottle positions were interchanged daily. Volume and percentage of 1% or 2% sucrose was calculated for each mouse.

3.2.7 Nest building test

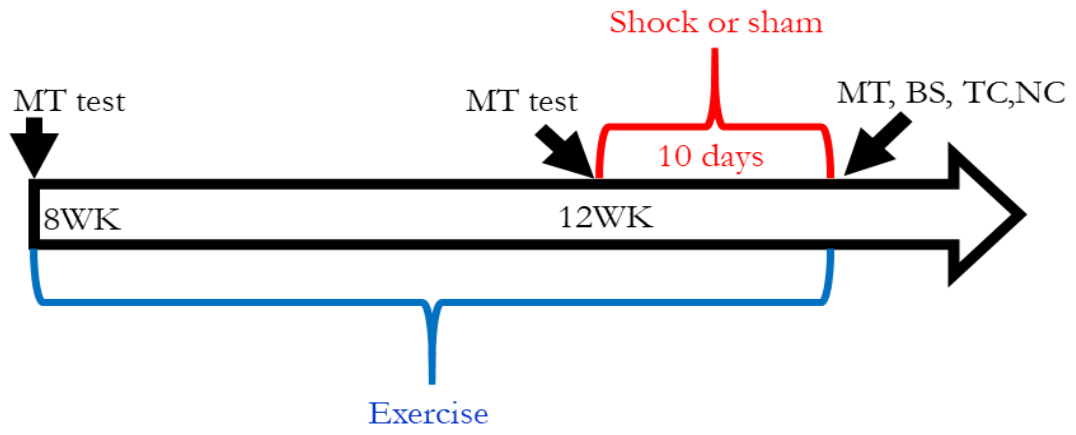
Mice were individually transferred to a clean cage containing no environmental enrichment outside of a 3.0 g nestlet square one hour before the start of the dark phase (5pm). Seventeen hours later, the nest and intact nestlet pieces were photographed and weighed. Two blinded experimenters scored the nests on a 1-5 scale according to previous publications [318, 319].

3.2.8 Serum corticosterone

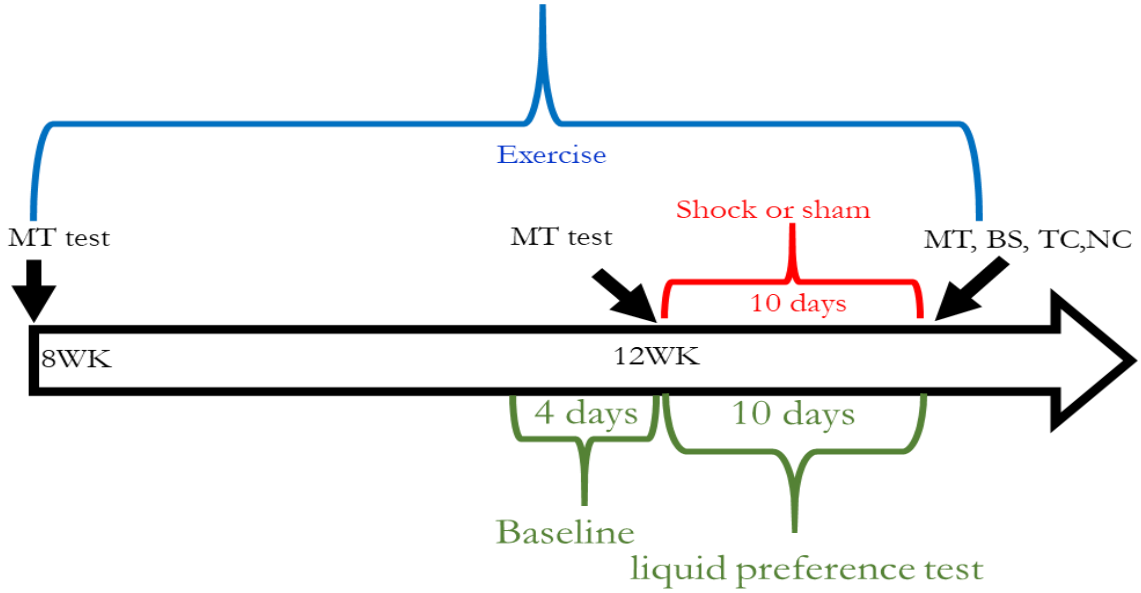
Mice were deeply anesthetized with inhaled isoflurane (>5%) and trunk blood was collected. Blood was allowed to clot on ice for 1 hour and then centrifuged at 10,000 rpm for 10 minutes. Serum (clear supernatant) was collected and stored at −20°C until analysis. Serum corticosterone (CORT) was quantified using an ELISA kit according to the manufacturer's instructions (ALPCO, Salem, NH).

Figure 3. 1 Experimental timeline

Group 1



Group 2



Group 1: pair housing, Group 2: single housing. MT: Mechanical withdrawal threshold test. BS: bladder sensitivity test. NC: nest construction test. TC: tissue collection.

3.3 Result

Voluntary exercise did not prevent foot shock stress-induced symptom.

Mechanical sensitivity was measured prior to (baseline) and 4-weeks after voluntary wheel running (VWR) or sedentary caging (sed). No significant difference was detected at either time point between sedentary and VWR mice (Figure 3.2 A). Withdrawal thresholds were measured again 24-hours after the 10^{th} and final foot shock treatment and there was no significant difference between sham-VWR and sham-sed mice (Figure 3.2 A). However, shock stress-VWR mice trended toward an increase in withdrawal threshold compared to shock-sed, but it did not reach significance (Figure 3.2 A).

Bladder sensitivity was also examined 1 day after the last foot shock. The shock groups were slightly higher than sham groups at 45 and 60 mmHg; however, there were no significant differences between sham-sed, shock-sed, sham-exe and shock-exe groups at any pressure tested (Figure 3.2 B). Overall, we also found no effect of VWR on bladder sensitivity in female A/J mice.

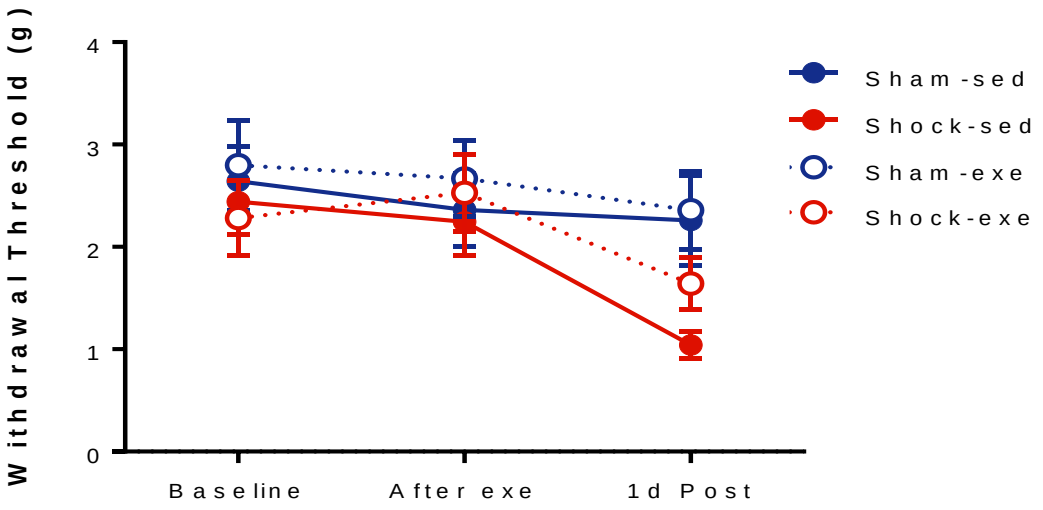
Foot shock decreases voluntary wheel running distance

We measured the wheel running distance of two separate groups of mice, before and during the 10 days of foot shock stress exposure, to determine if this could be a measure of depression-like behavior in mice. In both groups, foot shock stress significantly reduced running distances, beginning on day 5 in group 1 (Figure 3.3 A) and day 3 in group 2 (Figure 3.3 B), lasting through the final day of foot shock stress exposure. The average distance ran/day/pair was significantly lower in the stress-exposed mice, compared to both their pre-shock distances and sham-exposed mice (Figure 3.3 C). However, during the hour immediately following the foot

shock stress exposure, the shock group ran significantly longer than the sham group on foot shock days 7, 8, and 9.

Figure 3. 2 Mechanical sensitivity and bladder sensitivity tests on voluntary wheel running and foot shock-stress effect.

(A)



(B)

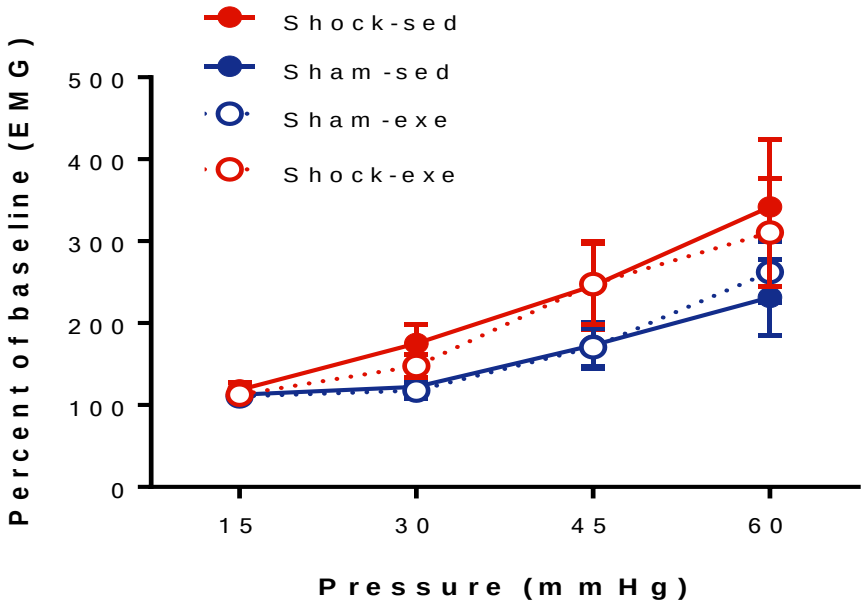
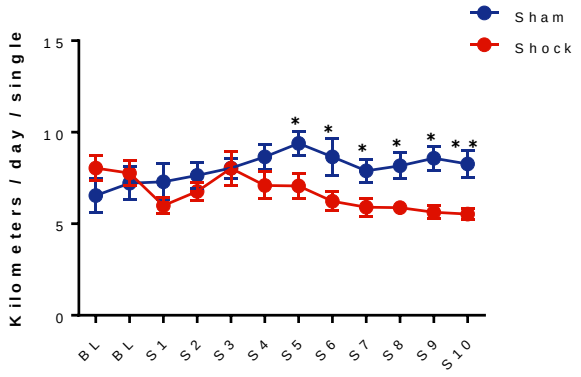


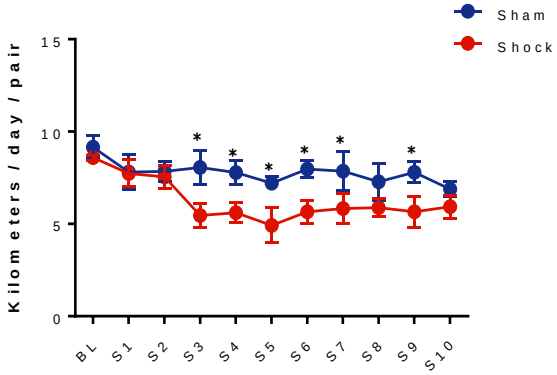
Figure 3.3 Mechanical sensitivity and bladder sensitivity tests on voluntary wheel running and foot shock-stress effect. (A) A/J mice withdrawal thresholds tested on the day before accessed to running wheel, or 28 days on running wheel and on day after the last foot shock paradigm. No significant effect was shown at all three time points. (B) abdominal EMG during bladder distension on 15, 30, 45 and 60 mmHg pressure one day after last foot shock. Exercise did not improve bladder sensitivity on every pressure tested.

Figure 3. 3 Voluntary wheel running distance

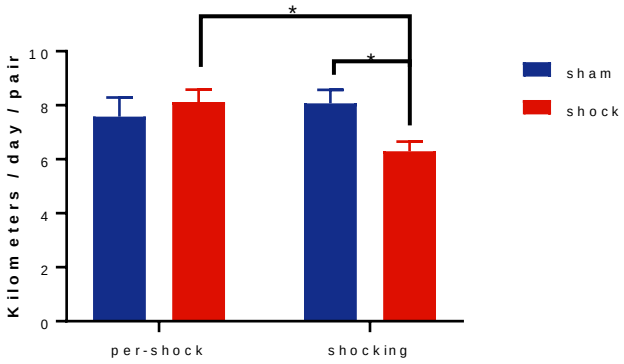
(A) Group 1



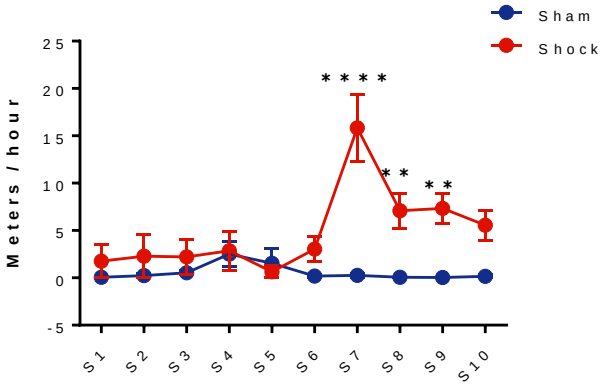
(B) Group 2



(C)



D)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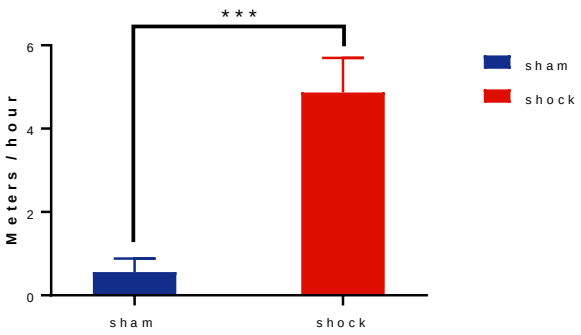
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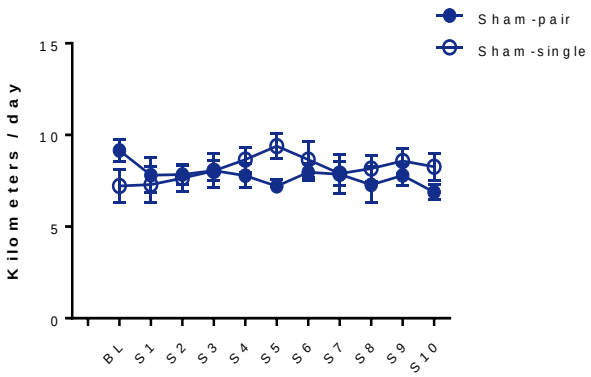
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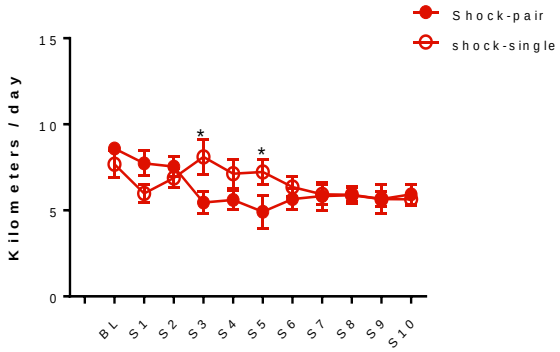
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Kilometers / day
15
10
5
0
*
*
BL
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10

Figure 3.1 Voluntary wheel running distance. Sham-Exercise and Shock-Exercise groups had access to running wheels four weeks prior to the foot shock procedure. Figure 3.1 only shows the day before foot shock and during the 10 days foot shock stress. (A) In the pair housing study, the shock group ran a significantly lower distance compared to sham group. * $p<0.05$, two-way RM ANOVA, Fisher posttest. (B) Similar running patterns were evident in single housing study. The shock group ran significantly shorter than sham group. * $p<0.05$, two-way RM ANOVA, Fisher posttest. (C) Running distance was analyzed and found during the 10-day shocking period A/J mice ran shorter distance compared to per-food shock and sham group during the foot shock. * $p<0.05$, two-way RM ANOVA, fisher posttest. (D) However, mice receiving foot shock stress ran significant longer during 1 hour after foot shock stress compared to sham mice starting from Day 7 until the last day of foot shock. **** $p<0.0001$, ** $p<0.01$two-way RM ANOVA, Fisher posttest. (E) Comparing 10 days average running distances, the shock group also ran a significantly smaller amount than the sham group. *** $p<0.001$ Student's t-test (F) In the sham group, no significant effects were apparent among mice in individual housing. (G) In the shock group, single housing has higher running count compared to pair housing on shock day 3 and 5 but no overall effect was found. * $p<0.05$, two-way RM ANOVA, Fisher posttest.

Exercise reduced serum corticosterone level but had no change on sucrose consumption.

To determine anhedonia-like behavior in A/J mice after foot shock stress and VWR, preference for 1% sucrose was measured before and during the 10-day foot shock procedure. There was no overall effect of foot shock stress or VWR on sucrose consumption (Figure 3.3 A and B). Despite having no effect on sucrose consumption, VWR significantly decreased HPA axis output. On 1 day after the final foot shock exposure, the sham-VWR group had a significantly lower serum corticosterone level compared to sham-sed group and the shock-VWR group was also significantly lower than the shock-sed group (Figure 3.3 D). An overall effect of exercise was also found on serum corticosterone level ($p<0.0001$, two-way ANOVA).

Figure 3. 4 Anhedonia-like behavior and alteration of HPA axis.

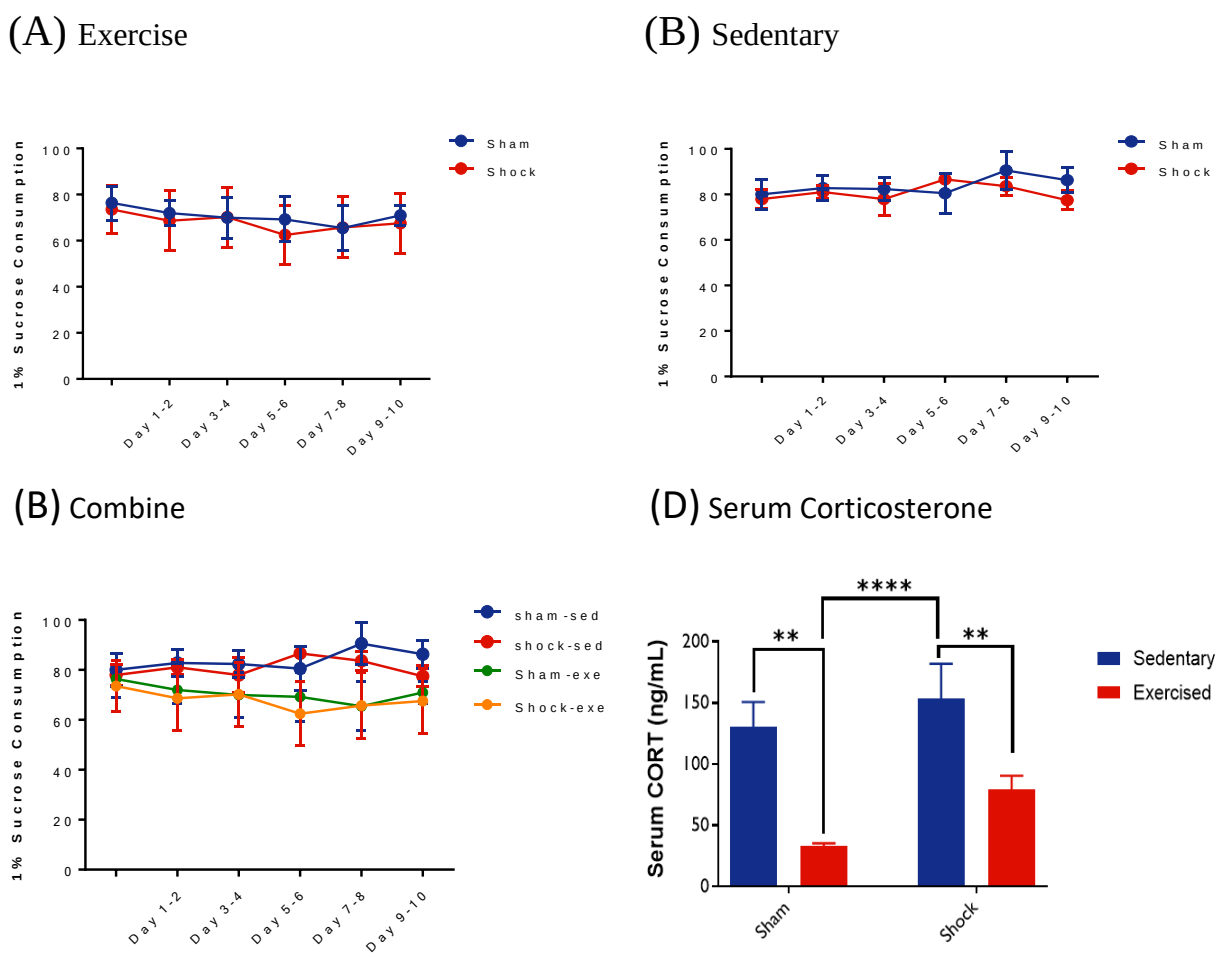


Figure 3.4 Anhedonia-like behavior and alteration of HPA axis. (A) Shock and sham group sucrose consumption in mice housed with running wheels. No significant effect was evident. (B) There was no significant differences between sham and foot shock-stressed mice in sedentary cages. (C) Combine graph for exercise and sedentary groups. (D) Serum corticosterone levels one day after the foot shock stress procedure. There was a significant decrease due to the effects of exercise in A/J mice compared to sedentary mice both in sham and foot shock stressed groups. A significant reduction in corticosterone levels was evident in sham-exercise compared to foot shock stressed-sedentary mice.

3.4 Discussion

Voluntary wheel running in rodents is a complex behavior influenced by mechanical sensitivity, psychological drive, physiological state, and genetic differences [115, 371-374]. Few studies have reported the effect of acute or chronic stress on rodent running distance and mixed results have been published. Our previous study showed that adult female mice that had undergone early life stress, in the form of neonatal maternal separation, ran shorter distances compared to naïve mice [257]. Male BALB/c mice, exposed to unpredictable chronic mild stress during the week, had significantly reduced running distances on the week days, compared to non-stressed mice, which was reversed on the weekends, when they received no stress exposure [375]. In a separate study, male C57BL/6J mice exposed to foot shock stress ran significantly longer distances compared to non-stressed mice during the first hour after foot shock [376]. Here, the effect of repeat stress on voluntary wheel running distance was uniquely studied on female A/J mice, which is an anxiety-prone stain. In our study, lower running distance per day was observed in foot shock-stressed A/J mice, compared to sham mice, especially after foot-shock day 5. However, we also noticed the running distance during one hour after foot shock stress was significantly higher in shock group compared to sham. These results interestingly paralleled with both of the previously-mentioned studies [375, 376].

Solitary-housing-induced psychological stress or mood disorder-like behavior has been widely discussed. One study showed that, compared to group housing, individual housing increased corticosterone level after a mild psychological stress, but not at baseline [377]. Another study showed that single housing induced anxiety- and depression-like behaviors and induced morphological changes in hippocampal CA1 neurons [378]. However, other studies reported no significant differences in stress-related behavior or to a stress response, including corticosterone

level and adrenal tyrosine hydroxylase, between singly-housed and group-housed mice [379, 380]. Recently, a study reported that single-housing had no significant impact on anhedonia or anxiety-like behavior, but increased corticosterone, ACTH, and BDNF level in Wistar rat's serum [381]. To prevent the possibility of additional psychological stressors other than foot shock, group 1 in our study was pair housed. However, in order to carry out the sucrose preference study, group 2 was housed in solitary conditions. Although our running wheels can accommodate two mice at the same time, the reliability of running distance data in pair-housed mice is obviously less than that of singly-housed mice, and few prior studies have addressed this discrepancy. In our study, we compared singly- and group-housed sham-exposed A/J mice and found no significant difference on running distance between the two groups. Nevertheless, there was a difference in running distance between singly- and group-housed foot shock-exposed A/J mice, specifically on days 3 and 5.

Multiple studies recently reported beneficial effects of voluntary wheel running on various forms of chronic pain and migraine [115, 371, 382-384]. We observed a trend toward increased mechanical withdrawal threshold in shock-VWR mice compared to shock-sedentary, but no significant effect. There are some potential reasons for the modest effect of VWR. First, the exercise period before foot shock stress exposure may have been too short. In our study, A/J mice had free access to a running wheel for 28 days before foot shock stress treatment. In contrast, Grace et. al, [371] allowed 6 weeks of access to a running wheel prior to chronic constriction injury. Second, we tested mechanical sensitivity 24 hours after the last foot shock. In Slivicki et. al. [384] found significant differences in paclitaxel-induced mechanical sensitivity was observed after 12 days of exercise. Benefits of VWR were observed on social defeat stress induced- hyperalgesia was observed at 16 days after the last stressor[384]. For chronic

constriction injury-induced allodynia, physical activity showed significant benefits around 2 (prevention) to 4 (treatment) weeks after injury [371]. Finally, according to data presented in Chapter 5, foot shock stress-induced allodynia has a more persistent effect than a second degree burn injury in mice, suggesting that a 10-day foot shock procedure in mice is a powerful insult that leads to persistent allodynia and mood disorder for up to 47 days.

Although we observed no significant benefit from exercise for hind paw mechanical and bladder sensitivity tests, VWR significantly reduced serum corticosterone levels compared to sedentary mice in the sham group. Similar findings have also been reported by other research group. Male Sprague-Dawley rats with access to VWR have lower plasma corticosterone after 30 mins of noise stress compared to sedentary mice [385]. Voluntary exercise also facilitates ACTH response 20 minutes after restraint stress in Sprague-Dawley rats [386]. A/J mice that had access to a running wheel for 4 weeks showed a significant decrease in baseline serum corticosterone levels compared to sedentary mice. To our knowledge, no study has showed that VWR reduces baseline corticosterone and some have even reported significantly elevated levels [385, 387-389]. One major difference is that none of these studies were performed on anxiety-prone strains of mice or rats. Two articles indicated that Sprague-Dawley rats had no change in serum corticosterone after VWR [385, 387]. Four weeks of VWR also did not have a significant effect on serum corticosterone in C57Bl/6J mice [389]. Other article even suggested that five weeks of VWR leads to an increase in serum corticosterone levels in C57B/6J mice [388]. As we mentioned in the previous chapter, A/J is a genetically anxious strain of mice with slightly elevated corticosterone [223, 224, 390, 391] and other evidence of anxiety-like behavior, which is highly corelated with corticosterone level [392-394]. The benefit of increased physical activity on anxiety has been widely reported [395-397]. Therefore, I believe in our experiments, the

beneficial effect of VWR on anxiety may have lowered HPS axis output, thereby reducing serum corticosterone compared to sedentary A/J mice.

3.5 Conclusion

This study revealed that 4-weeks of VWR prior to a 10-day foot shock procedure in A/J mice showed a trend toward decreased hind paw and bladder sensitivity. The VWR had no significant effect on foot shock stress-induced anhedonia-like behavior, but significantly decreased corticosterone level. For VWR as a biobehavioral marker, we discovered that foot shock-stressed A/J mice ran more during the 1-hour after foot shock stress exposure, but had overall significantly decreased running distance. We also validated that there is no significant difference on measured running distance between single- and pair-caged A/J mice.

Chapter 4: Genetic differences in susceptibility to foot shock stress-induced mechanical and visceral hypersensitivity.

4.1 Introduction

Anxiety disorders are highly prevalent and have a well-established link to chronic pain. Low back pain patients have a higher life-time prevalence of major anxiety disorder compared to healthy controls (30.9% vs. 14.3%) [398]. Sate-trait anxiety inventory test levels were elevated in non-cardiac chest pain patients compared to the reference group (OR = 3.27, 95% CI = 1.68–6.36; $p < 0.001$) and all anxiety-related symptoms were associated with non-cardiac chest pain[232]. Generalized chronic pain was positively associated with anxiety disorder in a study of more than 5000 participants [228]. Likewise, painful physical symptoms were twice as prevalent within generalized anxiety disorder (GAD) patients compared to people without GAD, and were even higher in GAD patients with comorbid major depressive disorder [399]. In other primary care studies, 61% to 93.5% of GAD patients reported to have pain symptoms [230, 400]. Interestingly, the main associated factors of painful physical symptoms in GAD patients were gastrointestinal disease (OR = 3.9), depression (OR = 2.2), or being women (OR = 1.5) [230].

In animal studies, exposure to various stressors (such as restraint, repeat cold, and early life stress) can cause both general mechanical and visceral hypersensitivity [299, 316, 355, 401-404]. Strong evidence suggests that the HPA-axis is involved in stress-induced pain. First, the HPA-axis was altered in chronically-stressed pain patients [405, 406]. IBS patients were found to have higher CRF level in plasma during the stress exposure [407]. Chronic corticosterone injections in rat resulted in visceral hypersensitivity, comparable with water avoidance stressed-mice [408]. Further proof is that RU-486, a glucocorticoid receptor antagonist, can effectively prevent chronic water avoidance stress-induced changes in transient receptor potential cation channel V1 (TRPV1) and cannabinoid receptor 1 (CB1) expression in L6–S2 dorsal root ganglions [155]. However, there are few studies that have compared stress-induced visceral pain

and general mechanical hypersensitivity in anxiety-prone and naïve groups. Further, although the pre-clinical model on comorbid chronic pain and anxiety has been well-established in rat [409, 410], there is no similar widely-used mouse model. Thus, we set out to compare the effect of foot shock stress on both hind paw mechanical sensitivity and visceromotor response during bladder distention in anxiety-prone A/J and non-anxious Bl6 mice.

Previously, chapter 2 reported that non-stressed A/J mice have surprisingly high bladder mast cell degranulation rate (~80%) and compared to Bl6 mice (~30%) [313]. Moreover, naïve Wistar-Kyoto (WKY) rats, a high-anxiety strain of rat, also has elevated mast cell degranulation rate (~75%) in the bladder, compared to non-anxious Sprague-Dawley rats (~30%) [298, 299, 325]. Therefore, we believe that these two separate studies suggest that high bladder mast cell degranulation rates are related to high anxiety levels. Increased bladder mast cell degranulation has been shown to underlie bladder hypersensitivity [313, 411-413]. Here, we hypothesize that A/J mice, an anxiety-prone stain of mice with comparably high bladder mast cell degranulation rates, will have higher visceromotor response during bladder distension following foot shock stress exposure, compared to non-anxious C57Bl/6 mice.

4.2 Method

4.2.1 Animals

All experiments in this study were performed on adult (>12-week-old) female A/J mice (Stock No: 000646, Jackson lab, Bar Harbor ME USA) and adult (>12-week-old) female C57Bl/6J (Stock No:000664, Jackson lab, Bar Harbor ME USA) mice housed in the Research Support Facility at the University of Kansas Medical Center. Mice were housed in a climate-controlled room on a 12-hour dark-light cycle and received water and food ad libitum. Animal use protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of IASP.

4.2.2 Foot shock stress exposure

Mice were exposed to foot shock stress for 10 consecutive days according to the following paradigm. Mice were transferred to a sound-proof room and placed 4 at a time into a Tru Scan Arena system cage equipped with a shock floor (26 × 26 × 39 cm, Coulbourn Instruments Holliston, MA, USA). Mice in the shock cohort were exposed to one of five random foot shock sequences consisting of 30 0.4 mA shocks over a 15-minute period. Mice in the sham cohort were held in the Tru Scan Arena system cage for 15 minutes but received no-shocks. Mice were returned to their home cages after foot shock or sham exposure.

4.2.3 Hind paw mechanical withdrawal thresholds

Mice were acclimated to the testing room for two days prior to assessment of hind paw mechanical thresholds. On the day of testing, mice were placed in individual clear plastic chambers (11 x 5 x 3.5cm) on a 55cm-high wire mesh table and allowed to acclimate for 30 mins. Withdraw threshold was measured by electronic monofilament 2390 series (IITC Life Science Inc. Woodland Hills, CA) for five times on right hind paws with at least a one-minute

interval between applications. A semi-flexible tip was used for every monofilament test. The withdrawal threshold was calculated by averaging the middle three measurements, following removal of the highest and lowest measurements.

4.2.4 Urinary bladder distention

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 implanted into the left and right abdominal muscle using a 26-gauge needle and the free ends were attached to a differential amplifier (Model 1700, A-M Systems, Sequim, WA). A 24-gauge angiocatheter (EXELINT, Los Angeles, CA, USA) was inserted into the bladder via the urethra and secured to the tail with tape. Isoflurane was reduced to approximately 1% until hind limb reflexes, but not escape behaviors, were present. Body temperature was maintained at approximately 37 °C using a heating pad. The bladder was distended with compressed nitrogen gas controlled by a custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA), manually adjusted by a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO), and verified by a separate pressure monitor (World Precision Instruments, Sarasota, FL). After stable responses to 60mmHg were confirmed, each pressure (15, 30, 45, 60mmHg) was applied in triplicate for 20 seconds with a 2-minute rest period in-between. Electromyographic activity was amplified, filtered, and recorded (Spike 2, Cambridge Electronic Design, Cambridge, UK) and the visceromotor response (VMR) was quantified and expressed as a percentage of baseline activity immediately prior to the distention.

4.2.5 Nest building test

Mice were individually transferred to a clean cage containing no environmental enrichment outside of a 3.0 g nestlet square one hour before the start of the dark phase (5pm). Seventeen

hours later, the nest and intact nestlet pieces were photographed and weighed. Two blinded experimenters scored the nests on a 1-5 scale according to previous publications [318, 319].

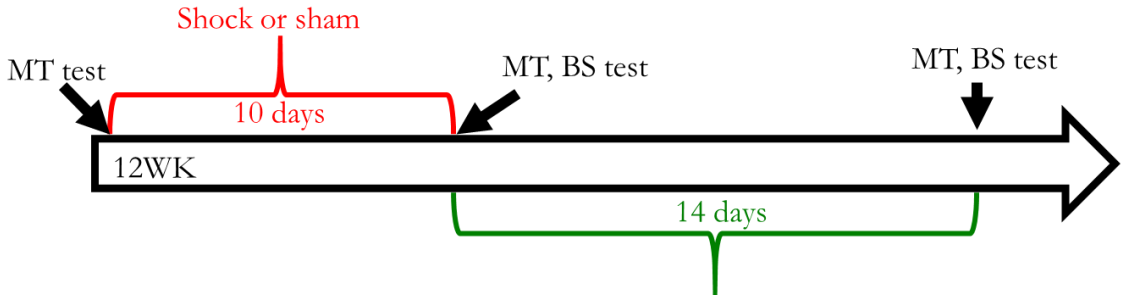
4.2.6 Serum corticosterone

Mice were deeply anesthetized with inhaled isoflurane (>5%) and trunk blood was collected. Blood was allowed to clot on ice for 1 hour and then centrifuged at 10,000 rpm for 10 minutes. Serum (clear supernatant) was collected and stored at −20°C until analysis. Serum corticosterone (CORT) was quantified using an ELISA kit according to the manufacturer's instructions (ALPCO, Salem, NH).

4.2.7 Fecal output

Fecal pellets from mice undergoing foot shock or sham exposure were counted daily, and averaged between 4 mice after each procedure.

Figure 4. 1 Experimental timeline



MT: mechanical withdraw threshold test. BS: bladder sensitivity test. NC: nest construction test. TC: tissue collection.

4.3 Result

Foot shock stress exposure had no effect on weight in either C57B/l6 or A/J mice.

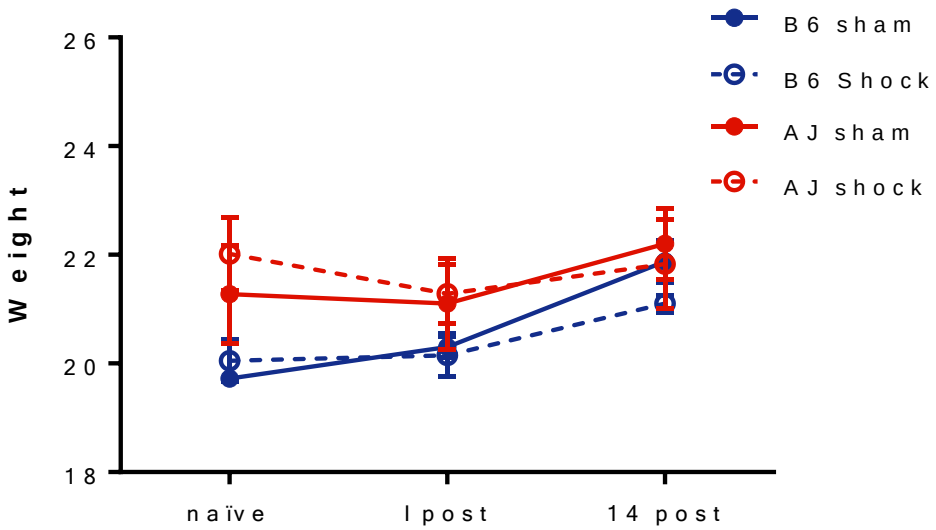
Whole body weights were measured before, 1 day and 14 days after the final foot shock stress procedure (Figure 4.2A). On average, A/J mice weighed more than C57Bl/6 mice, but it did not reach significance prior to foot shock stress exposure. At 1 and 14 days after foot shock stress exposure, there was no significant differences detected between the four groups. We observed that on 1 day post foot shock, C57Bl/6 mice had no change in body weight and the A/J mice had a slight, but not significant, decrease in body weight, compared to their baseline measurements. At 14 days after foot shock, we observed a similar pattern, such that all groups, outside of the shock-exposed A/J mice, had gained weight compared to their baseline measurements. There was a significant difference in weight change between sham-exposed C57Bl/6 and shock stress-exposed A/J mice at this time point (Figure 4.2B).

Both strains show significant hind paw allodynia after foot shock stress

At one day after 10 days of foot shock stress exposure, both A/J and C57Bl/6 mice had significantly lower hind paw mechanical withdraw thresholds compared to their respective sham-exposed groups (Figure 4.3A). Significant differences were also observed between both shock-exposed groups and their respective baseline measurements. 14 days after the final foot shock exposure, C57Bl/6 mice maintained significantly lower mechanical withdrawal thresholds compared to both their baseline measurements and sham-exposed counterparts (Figure 4.3B). Similar to what we reported in Chapter 2, the shock stress-exposed A/J mice had a significantly lower threshold compared to their baseline measurements, but not to the sham-exposed group. These results indicate that both strains are susceptible to foot shock stress-induced hind paw mechanical allodynia.

Figure 4. 2. Body weight change on shock stress or sham exposure in C57Bl/6 and A/J mice

(A)



(B)

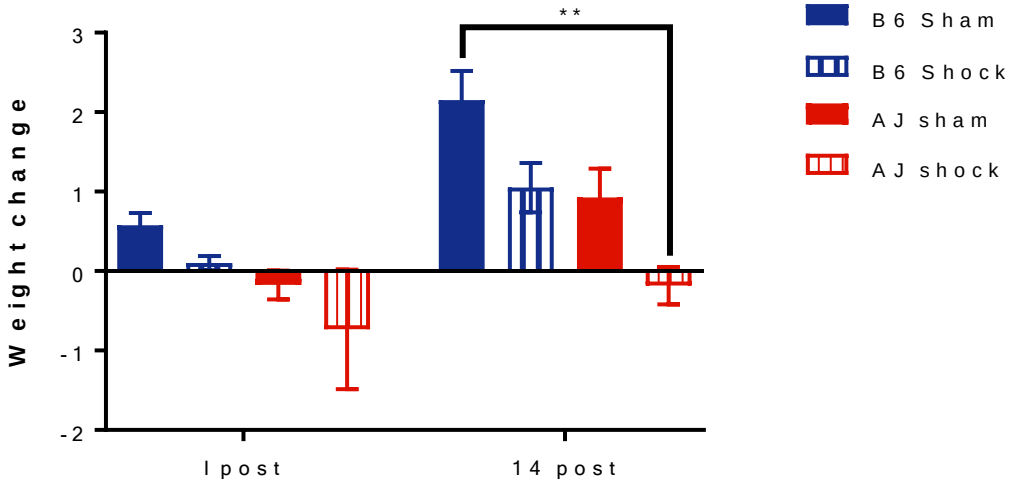
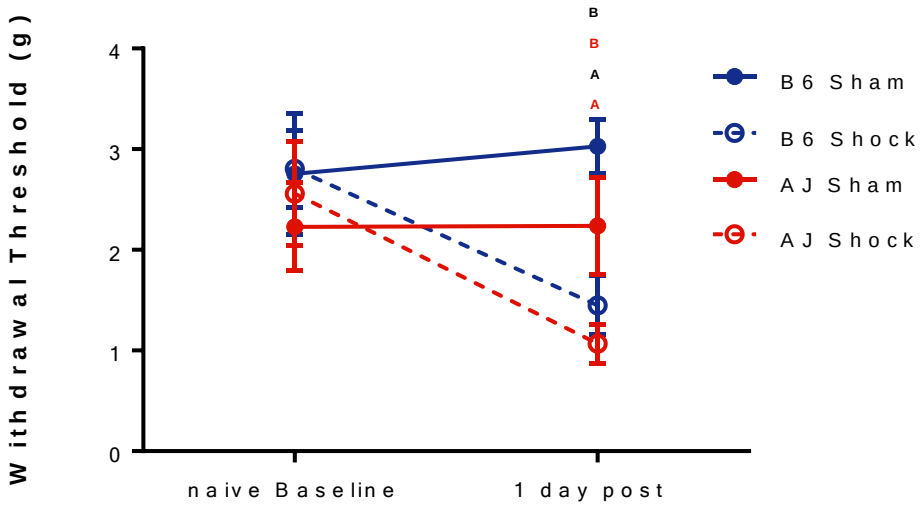


Figure 4. 3 Foot shock stress effect on hind paw mechanical withdrawal thresholds in A/J and C57Bl/6

(A)



(B)

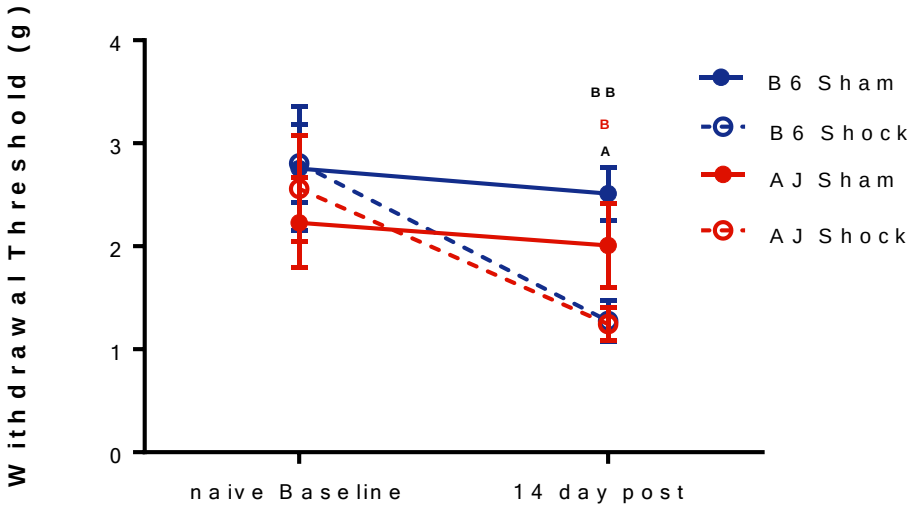


Figure 4.2 (A) Body weights were measured at baseline, 1 day, and 14 days after the final foot shock stress or sham exposure in C57Bl/6 and A/J mice. No significant differences were found between the four groups at any time point. (B) Weight change compared to baseline is shown for 1 day and 14 days after the final foot shock. No significant differences were found among the four groups at 1 day after foot shock. However, at 14 days post-foot shock, the A/J foot shock stress group had significantly lower weight changes compared to C57Bl/6 non-stressed mice. Two-way RM ANOVA; ** $p<0.01$, Bonferroni posttest. N=4

Figure 4.3 A) Hind paw mechanical withdrawal thresholds were measured in A/J and C57Bl/6 mice prior to (baseline) and 1 day after the final foot shock exposure. Both foot shock stressed A/J and C57Bl/6 mice showed a significant decrease in hind paw withdrawal thresholds compared to their respective sham groups and baseline measurements. (B) Hind paw mechanical withdrawal thresholds were again measured on 14 days after the final foot shock exposure. Foot shock stress had a significant effect on C57Bl/6 thresholds, compared to both its baseline and the C57Bl/6 non-stressed group. Foot shock stressed A/J mice remained significantly lower than their baseline, but not compared to sham-exposed mice. Two-way RM ANOVA; B, $p<0.05$ BB, $p<0.01$, B6 shock vs its baseline, B, $p<0.01$.B6 shock vs B6 shock, A, $p<0.05$.AJ shock vs its baseline, A, $p<0.05$ AJ shock vs AJ sham. fisher posttest.

Foot shock stress increases bladder sensitivity in A/J mice, but not C57Bl/6 mice.

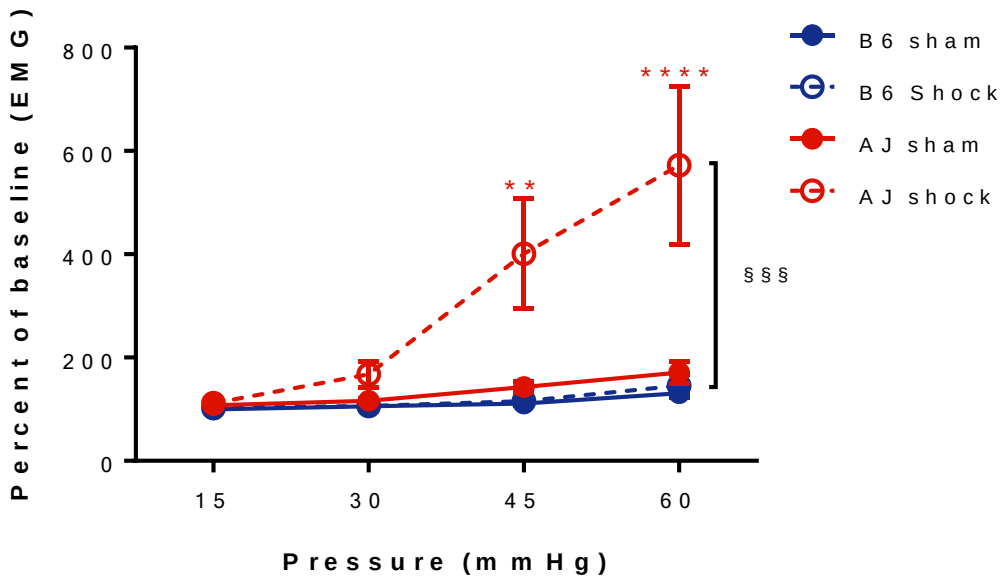
To determine changes in visceral sensitivity after foot shock stress, we measured bladder hypersensitivity by recording the visceromotor response (VMR) during bladder distension at 1d and 14d after the final foot shock (Figure 4.4). Overall, foot shock stress had a significant effect on A/J mice compared to non-stressed mice, and especially at the highest pressures of 45mmHg and 60mmHg. Surprisingly, foot shock stress did not significantly increase VMR in C57Bl/6 mice at either time point.

Fecal output during foot shock stress.

Fecal output was assessed following each foot shock stress exposure to determine habituation to the stimulus, as well as general anxiety. Overall, A/J mice had a higher fecal output compared to C57Bl/6, regardless of shock of sham exposure. Both strains had an increase in fecal output in response to foot shock stress which was maintained in the A/J mice and returned to sham levels in the C57Bl/6-stressed group. Due to our foot shock stress protocol exposing 4 mice at once, we were unable to perform comparative statistics on these data.

Figure 4. 4 Foot shock effect on A/J and C57Bl/6 mice's bladder sensitivity

(A) 1-day post foot shock



(B) 14-day post foot shock

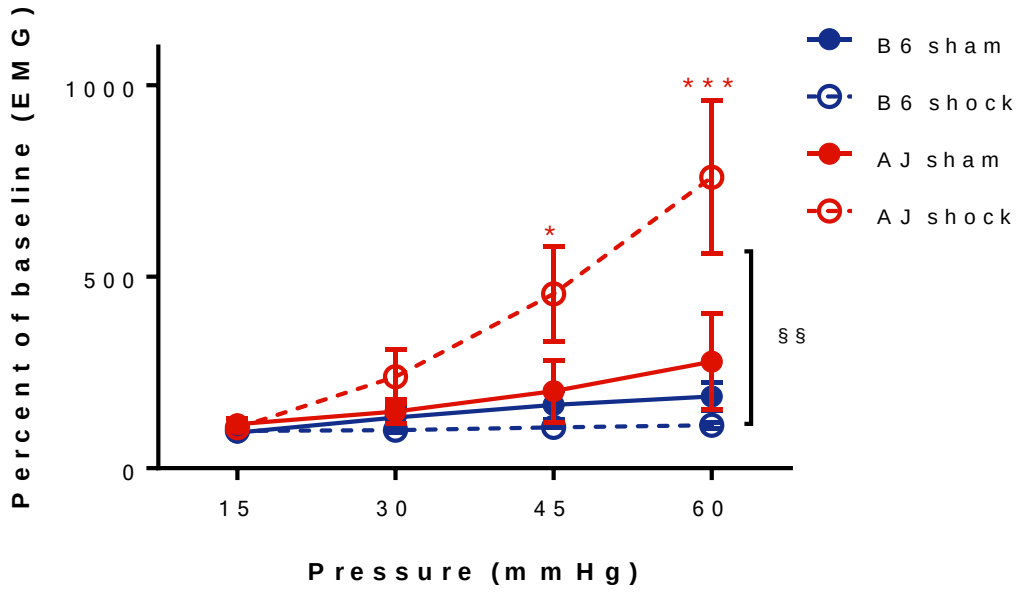


Figure 4. 5 Fecal output

(A)

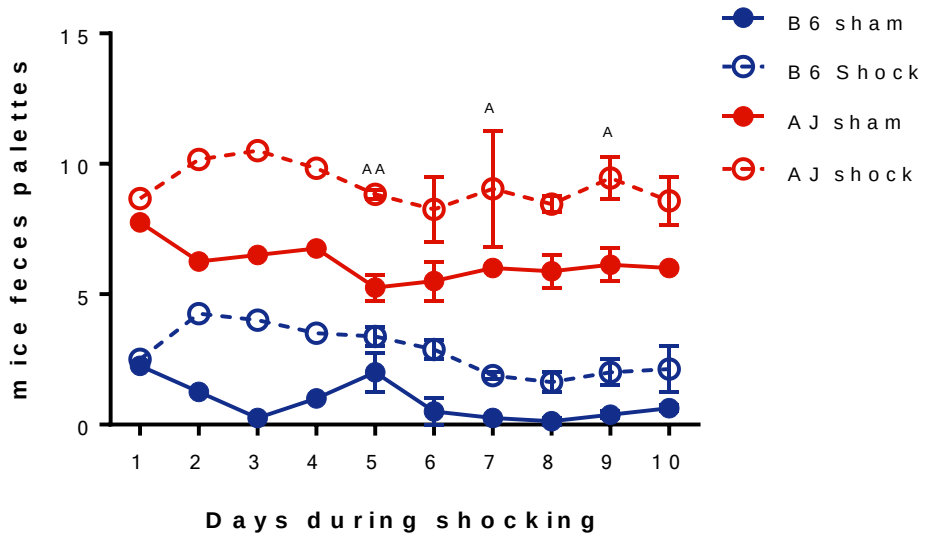


Figure 4.4 The visceromotor response (VMR) during urinary bladder distension (UBD) was measured 1 day and 14 days after the final foot shock. (A) 1 day after the final foot shock, the A/J shock group had significantly higher VMR compared to the A/J sham group with a significant difference overall and at 45 and 60 mmHg. No significant difference was found between C57Bl/6 shock stress and C57Bl/6 sham mice. (B) At 14 days after the final foot shock, there remained a significant difference between shock stress and sham exposed A/J mice, but not C57Bl/6 mice. Two-way RM ANOVA; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, A/J sham vs A/J shock, Bonferroni posttest.

Figure 4.5 Fecal output was assessed during the foot shock stress paradigm to determine habituation to the shock stimulus. The reported number of pellets are for four mice/group and repeated data are only available for days 5-10. Stressed- and non-stressed A/J mice had higher fecal output than C57Bl/6 mice across the entire experiment. Stressed A/J mice maintained higher fecal output, compared to non-stressed A/J mice, which was not observed in C57Bl/6 mice. Two-way RM ANOVA; A, $p<0.05$, AA, $p<0.01$ A/J sham vs A/J shock, Bonferroni posttest.

4.4 Discussion

Pelvic pain is highly associated with psychological disorders such as anxiety [414], depression [415, 416] and catastrophizing [417, 418]. Earlier study at chapter 2 suggested that sham A/J anxiety-prone mice has higher bladder mast cell degranulated rate compared to unstressed C57Bl/6 mice, which could involve bladder hypersensitivity. Further, women are higher risk group for both anxiety and pelvic pain compared to men [419, 420]. Miller-Matero LR et. la. also stated that to decrease functional impairment in chronic pelvic pain patient, an multidisciplinary approach including psychological treatment and pain management is critical [421]. Here, we compared the foot shock stress effect on A/J anxiety-prone mice and C57Bl/6 mice and invested potential intervention target for anxiety comorbid chronic pelvic pain.

Stress-induced mechanical hypersensitivity has been reported in many studies involving mice and rats [7, 296, 301, 354, 355]. Evidence also indicates that anxiety-prone strains exposed to psychological stress have increased allodynia or behavioral evidence of pain [161, 403]. Most studies have focused on pelvic or abdominal sensitivity and few studies have compared normo-anxiety and high-anxiety strains for stress-induced mechanical hind paw sensitivity. Here, we presented a comparison of normo-(C57Bl/6) and high-anxiety (A/J) mouse strains for hind paw mechanical withdrawal threshold after foot shock stress exposure. Our study confirmed that foot shock stress exposure significantly reduced the withdrawal thresholds in both C57Bl/6 and A/J mice, compared to their respective baseline and sham group measurements. This was observed at both 1 and 14 days after the final foot shock stress exposure. One slight difference was that 14 days after the final foot shock stress, only C57Bl/6 mice showed a significant decrease compared to the sham group, whereas shock stress-exposed A/J mice were only significantly different from their baseline measurements and not from sham-exposed mice. This suggests that C57Bl/6 and

A/J mice both have a long-lasting hind paw allodynia after foot shock stress, which confirms the results shared in Chapters 3 and 5.

Hypersensitivity in pelvic or abdominal organs has been related to stress in both clinical and preclinical settings. In women, early life stressful events had a significant impact on the development of chronic pelvic pain or general pain [422, 423]. It has been widely recognized that psychological stress plays a major role in irritable bowel syndrome and may involve corticotrophin releasing factor signaling pathways [401, 424, 425]. Stress-induced bladder hyperalgesia in a highly-anxious strain of rats, Wistar-Kyoto, has been reported in many studies [299, 426, 427]. Visceral hypersensitivity can be triggered by anxiety itself even without exposure to psychological stress. Colonic hypersensitivity was also discovered in naïve Wistar-Kyoto rats compared to normo-anxious Sprague Dawley rats [428, 429]. However, few studies have compared visceral hypersensitivity after psychological stress in high- and normo-anxious strains of mice. Here, as in chapter 3, foot shock stress-exposed A/J mice displayed long-lasting bladder hypersensitivity. This was observed at both 1d and 14d after the final foot shock. In contrast, C57Bl/6 mice showed no evidence of increased bladder sensitivity at either time point following foot shock stress exposure. Thus, we believe that anxiety-prone strains are highly susceptible to the development of visceral pain and that 10 consecutive days of foot shock stress exposure in A/J mice is a feasible model for stress-induced visceral discomfort such as that experienced by patients with Urologic Chronic Pelvic Pain Syndrome.

The influence of stress and genetically-high anxiety on defecation behavior has been reported on for decades. Clinically, patients with irritable bowel syndrome, which includes abdominal pain and diarrhea, commonly present with comorbid anxiety [430, 431]. In rodents, most evidence supports that strains with higher anxiety have a higher defecation number. Through a two-way

avoidance test, the RLA/Verh rat line (rapid acquisition) was considered to be more anxious and emotionally reactive than the RHA/Verh line (extremely poor acquisition) [432]. The RLA/Verh rats also showed higher defecation rates and self-grooming behavior than RHA/Verh rats [433, 434]. In a more recent study, anxiety-like behavior and the number of fecal boli during open field test were highly corelated [435]. Finally, a study comparing C57Bl/6 and A/J mice on alcohol preference, open-field activity, defecation, and rope climbing tasks showed that A/J mice have more fecal boli during the open-field test compared to C57Bl/6 mice [436]. Our study showed that, in general, the anxiety-prone A/J strain of mice had more fecal pellets, compared to C57Bl/J mice, during the 15 minutes spent in foot shock cage, which fits these previous studies. Stress-induced hyper bowel movement or increased defecation has been reported in rat and human, but there are few studies in mice [437]. In rat, water avoidance and restraint stress are the most common stressors used to induced defecation [438, 439]. Repeat swimming stress significantly increased fecal pellet production and induced colonic damage in female Sprague-Dawley rats [440]. Forelimb restraint stress and water avoidance stress significantly enhanced fecal pellet number and total weight during a 1 hour stress treatment in male Wistar rats [441]. Our result also supports the view that foot shock stress increased defecation in mouse strains with either high or low anxiety level. Further, we provided novel evidence that psychological stress in an anxiety-prone mouse strain further increased the number of fecal pellets.

4.5 Conclusion

This study compared the behavioral outcomes of two different strains of mice, the normo-anxious C57Bl/6 mouse and the highly-anxious A/J mouse, in response to 10 consecutive days of foot shock stress exposure. The foot shock stress prevented weight gain in the A/J mice, but not in C57Bl/J. Both C57Bl/6 and A/J mice developed long-lasting hind paw mechanical allodynia

after foot shock stress procedure, but C57Bl/6 tended to have more profound effect. On the other hand, bladder hypersensitivity was observed only in A/J mice and not C57Bl/6 mice. Finally, stress induced a significantly higher and sustained defecation behavior in anxiety-prone strain of mice that was not observed in C57Bl/6 mice.

Chapter 5: The impact of foot shock-induced stress on pain-related behavior associated with burn injury.

5.1 Introduction

According to the World Health Organization, over 1 million burn injuries require medical attention each year in United states and approximately 11 million people seek medical care worldwide annually [286, 287]. Acute pain is a debilitating complication following burn injury and has the potential in certain individuals to transition to chronic pain. As an example, one study reported that 11 years post-injury, nearly 52% of patients still experience chronic pain associated with their burn injury [291]. Overall, however, there remains little preclinical or clinical research to address this significant problem for humans suffering from a burn injury. To address this issue, we have developed an experimental model of burn injury in mice that will allow for mechanistic investigations into factors that are associated with the transition from acute to chronic pain associated with burn injury.

A relationship between psychological disease and burn injury has been demonstrated, as approximately 10-23% of burn patients go on to develop depression at one year after burn injury[442, 443]. The incidence of depression, however, increases to 42% with moderate or severe depression two years after burn injury[444]. In addition, a high prevalence of anxiety had been broadly reported after thermal trauma [292, 293]. In these studies, 82% of burn patients were identified with anxiety, including 26% patients with mild, 22% with moderate and 34% with symptoms of severe anxiety [294]. Additional studies have reported similar positive correlations between psychological disorders and chronic pain. Of note, 22% of chronic pain patients displayed symptoms of depression and 35.1% displayed symptoms of anxiety compared to general population (9.3% and 18.1%, respectively) [228]. Moreover, the prevalence of depression and anxiety in patients with chronic pain was very common (80% and 60% respectively) [445, 446]. This relationship between chronic pain and anxiety/depression has been

described as a vicious cycle, with pain and psychological disorders cyclically worsening each other [248, 447]. Despite this emerging clinical data, few animal models have been developed that incorporate both pain and psychological disorders. In this chapter, we present data that addresses these two intertwined components to begin to identify mechanisms that aid in this problem.

Stress imposed on an individual can also exacerbate pain, and different forms of stressful events will increase pain in both rodent and human models [295, 296]. In rodents, stress-induced allodynia has been widely studied, and various stressors used to experimentally modify pain include repeated cold exposure, restraint stress, chronic mild and early life stress [7]. In our own studies, we have identified that that stress derived from mild electrical shock of rodent's feet foot shock stress or early separation of mouse pups from their mother leads to decreased thresholds in hind paw mechanical sensitivity and increased sensitivity to bladder distension. Theses stressors were also associated with an increase in mast cell degranulation, suggesting a relationship with the immune system [113, 161, 313].

Here, we developed a murine model of mild burn injury and have tested the hypothesis that stress can modify the severity and duration of paw sensitivity in relation to the burn injury. These experiments were carried out in two different genetic strains of mice (A/J and C57BL/6 mice), as these strains are known to display differences in their response to stress and their level of anxiety [223, 224]. Our results suggest that the response to burn injury to the hind paw can be modified by introducing stress prior to the burn injury, and these two mouse strains display differences in their recovery, suggesting a genetic involvement in these responses.

5.2 Material and Methods

5.2.1 Animals

All experiments were performed on 12-week old male A/J (Stock No: 000646, Jackson lab, Bar Harbor ME USA) or C57BL/6 (Charles River, Wilmington, MA, USA) mice. All mice were housed within the research support facility at the University of Kansas Medical Center. All mice had access to water and food ad libitum and housed in climate-controlled cage with 12-hour dark-light cycle room. Animal was all group housed, whereas isolation cage applied when animal flight and recommend by animal facility. Animal use protocols approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

5.2.2 Foot shock stress

Mice were transferred from their home cage to a sound-proof room and placed into a Coulbourn Instrument's Tru Scan Arena system cage equipped with a mice shock floor (26 × 26 × 39 cm, Coulbourn Instruments Holliston, MA, USA). All foot shock procedure started and finished before noon. Shock group mice were placed in shock floor cage in group (4 at a time) and received one of five random sequences of 30 times 0.4 mA shock for 1 second over 15 minutes 10 consecutive days. In sham group, 4 mice were placed in the cage for 15 mins daily for 10 consecutive days without any foot shock applied. All the mice immediately returned to home cage after foot shock [161].

5.2.3 Second degree burn injury

Working with our veterinary staff and institutional animal care committee, we performed pilot studies on 2-3 mice to test different temperatures and heating durations to best model a second degree burn on the paw. Based on these pilot studies, 65°C for 15s produced a reliable burn that was well tolerated by the mouse and had reliable healing and recovery. One mouse was sacrificed after 4 days to examine the histology of the burn site using paraffin-sectioned,

hematoxylin-stained section through the mouse dermis and epidermis. All of the subsequent studies were conducted using 65°C for 15s as a protocol to induce burn injury.

Following 24 hours after the last session of foot shock, mice were organized by right hind paw mechanical threshold into four groups: no-burn/no-shock, no-burn/shock, burn/no-shock and burn/shock. Under Avertin anesthesia, (200 µL/10g body weight), the right hind paw of the mouse was placed on a metal block with a controlled surface temperature for 15s. The metal block was heated to either 65°C (burn group) or 25°C (sham group). During the heating period, a 5-g weighted pouch filled with small plastic beads was placed on the dorsum of the hind paw to maintain consistent pressure between the heel and the metal block. Mice were kept on heating pad to maintain their body temperature at 37°C and vital signs were monitored until the mouse recovered. To prevent infection associated the burn injury, silver sulfadiazine ointment was applied to the injured site twice daily until scar tissue formed (5-7 days).

5.2.4 Hind paw mechanical and thermal withdrawal thresholds

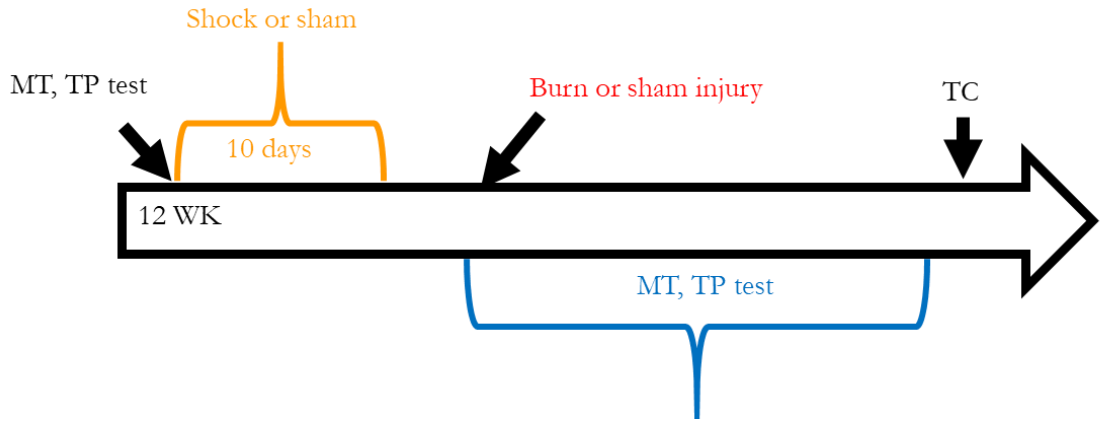
Assessment of pain behaviors included both mechanical and thermal pain threshold assessments to the hind paw before (baseline) and subsequently over a 55-day period. For mechanical thresholds, mice were placed in the testing room two days before testing day for one hour in order to acclimate them. On the test day, mice were placed on a metal wire mesh platform that is 55 cm high, allowing for access to the paws from below. Mice were acclimated on the table for 30 mins before testing began. Mechanical sensitivity testing started with 3.22g monofilament then was performed using the up down method by a series of monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.08, 4.31, 4.74 g; North Coast Medical, Inc Morgan Hill, CA, USA). If a positive response was noted, subsequent testing was followed using a lower monofilament, whereas a negative response was followed by using a higher monofilament[317]. For thermal thresholds,

mice were placed in individual clear plastic cages on a Hargreaves's apparatus and a 4.0 V radiant heat source was applied twice to each hind paw for a total of three tests. The time elapsed for each animal to withdraw the hind paw was counted as withdrawal latency (sec). Latencies from four applications were used to calculate the mean latency per animal and mean latencies were combined to calculate group means.

5.2.5 Statistical analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA) and if appropriate, followed by Fisher's least squared difference, as denoted in the manuscript (GraphPad Prism 8, GraphPad Software, Inc., La Jolla, CA). All data are expressed as mean ± SEM and $p < 0.05$ was considered significant.

Figure 5. 1 Experimental timeline



MT: mechanical withdraw threshold test. TP: Thermal sensitivity test. TC: tissue collection.

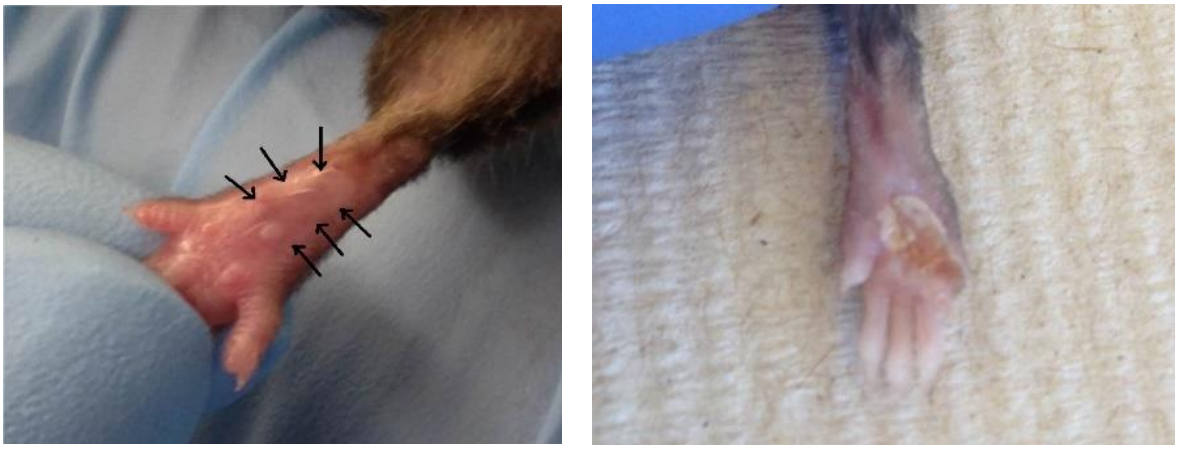
Results

Generation of A Second Degree Burn Injury to the Hind Paw

Mice received a mild burn injury by contacting the right hind paw to a heated metal block for 15 seconds. Two days after the burn injury, mice receiving the burn injury displayed signs of a second degree burn that included redness and swelling at the burn site. As shown in Fig. 5.2A, the burn injury consistently created a small blister with erythema and edema during initial acute phase of burn injury (Fig. 5.2A, left panel). Over time, a visible burn scar tissue was form commonly around 8 days after the burn injury (Fig. 5.2A, right panel). One mouse was sacrificed 4 days after the burn injury to assess the burn site. The cutaneous tissue from the burn injury was examined using bright field microscopy and H&E staining (Fig. 5.2B). Examination of the burn region revealed extensive damage and disruption of the epidermis and underlying dermis. The burn injury site was heavily infiltrated by immune cells.

Figure 5. 2 Thermal injury and histology

(A)



(B)

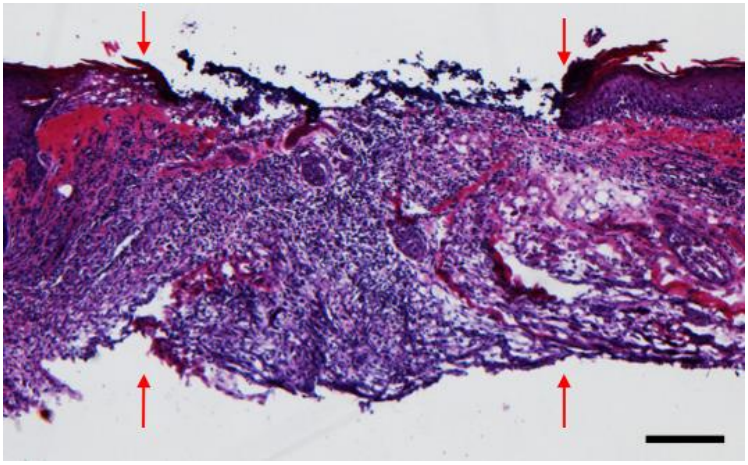
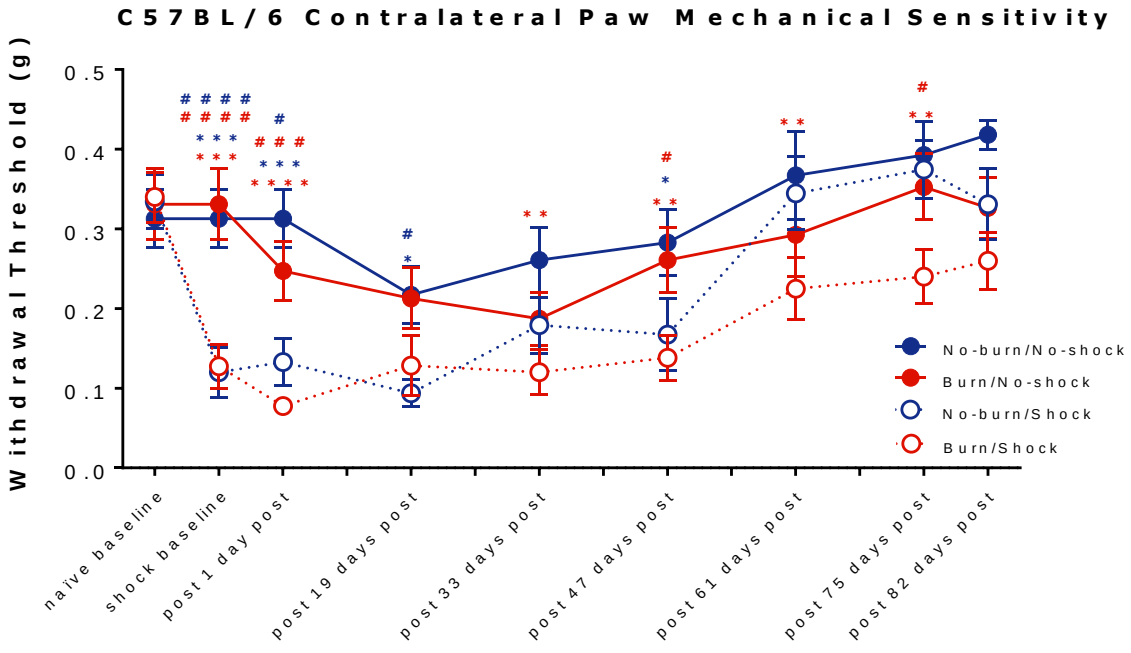


Figure 5. 3 Hind paw mechanical sensitivity in C57BL/6 mice

(A)



(B)

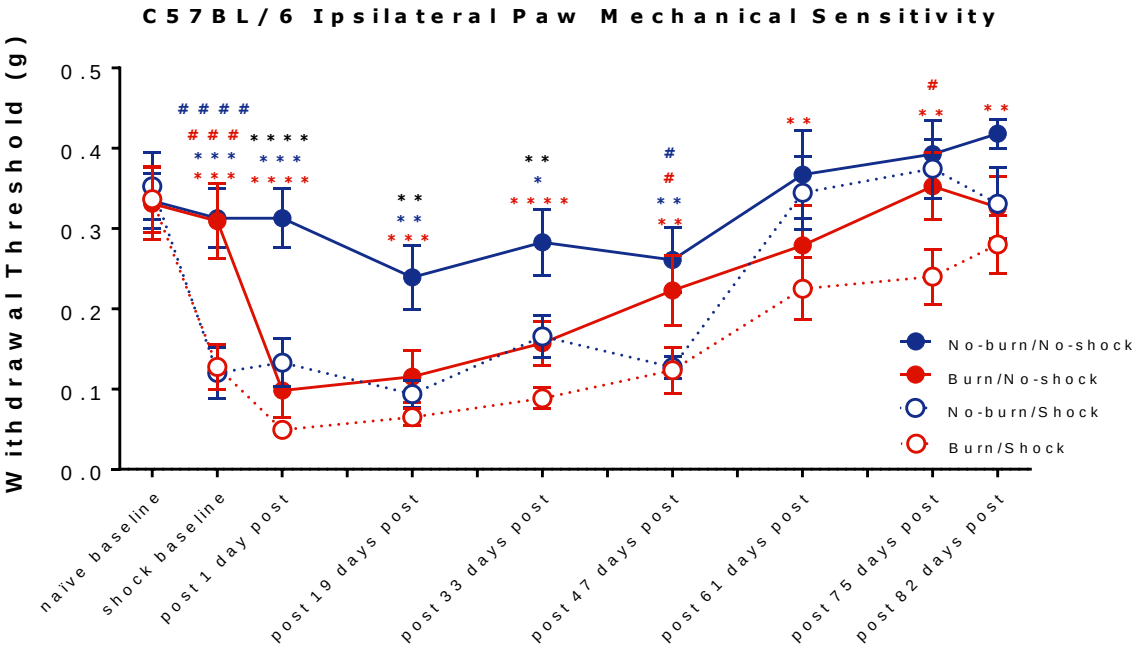


Figure 5.2 (A) Left inset shows a burn injury produced at 65°C for 15s in a male C57BL/6 mouse two days after the burn injury. Black arrows highlight the burn blister with erythema and edema produced during the initial phase following burn injury. Right inset shows the burn injury 8 days after the burn injury in the same mouse. Visible scar tissue is evident on the hind paw. (B) H&E stained section of the plantar surface of the paw 4 days after a burn injury. The burn results in disruption of the epidermis and dermis below the blister. The dermis is filled extensively with inflammatory cells. Scale bar equals 50 μms.

Figure 5.3 Hind paw mechanical sensitivity in C57BL/6 mice on naïve baseline, after foot shock stress, 1, 12, 19, 26, 34, 40, 47, and 54 post-burn injury. (A) After 10 days of the foot shock stress procedure, no-burn/shock and burn/shock group have a significant decrease in mechanical contralateral hind paw withdrawal threshold (LHP-WT) compared to the no-burn/no-shock group, and the foot shock stress effect lasts for more than 54 days. Mechanical LHP-WT on burn/non-shock group starts to decline on day after burn injury and was significantly lower on 12, 26, 34, 40 days post-burn injury compared to no-burn/no-shock group. (B) no-burn/shock and burn/shock groups have significant reductions in mechanical ipsilateral hind paw withdrawal thresholds (RHP-WT) for more than 54 days compared to no-burn/no-shock group. Burn/non-shock group's mechanical thresholds RHP-WT dramatic decrease on one day after burn injury and was significant difference at 1, 12, 19, 26, 34, 40 days after burn injury but not 47 and 54 days after burn injury. Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ vs. no-burn/no-shock, # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ vs. burn/non-shock, Fisher's LSD posttest. Black: burn/no-shock, Blue: no-burn/shock, Red: burn/shock.

C57BL/6 mice developed persistent mechanical allodynia due to foot shock stress and/or burn injury.

Hind paw mechanical sensitivity was tested in C57BL/6 mice in naïve (baseline), after foot shock, 1, 12, 19, 26, 34, 40, 47, and 54 post burn injury (Figure 5.3 A and B). There were no significant differences in baseline mechanical thresholds among the four groups. In the ipsilateral paw, 3 groups (no-burn/no-shock; no-burn/shock; and burn/shock) displayed significant decreases in mechanical thresholds after ten days ($P<0.05$). It should be noted that the decrease in mechanical threshold in the no-burn/shock group took longer to develop compared to the two groups that received shock stress and changes in mechanical thresholds were related to the burn injury. The two shock groups (no-burn/shock; burn/shock), regardless of receiving a burn or not both decreased their mechanical thresholds immediately after receiving the shock protocol (($P<0.05$; Figure 5.3B). This suggests that by itself, foot shock stress had a significant impact on mechanical thresholds. All three groups maintained significant decreased mechanical thresholds for 33 days ($p=0.0142$ vs burn/no-shock, $p=0.0219$ vs no-burn/shock, $p=0.0001$ vs burn/shock).

In the contralateral paw (Figure 5.3A) similar but less severe decreases in mechanical thresholds were observed. Foot shock stress led to rapid decreases in mechanical thresholds regardless of receiving a burn injury, and this persisted throughout the 54-day testing period. Mice in the no-burn/shock group also developed decreased mechanical thresholds that appeared after the burn injury but appeared to approach control levels by 34 days after the burn injury (Figure 5.3A)

C57BL/6 mice show thermal sensitivity on first three weeks on contralateral side.

Compared to mechanical sensitivity, thermal sensitivity was also affected but not to the same duration (Figure 5.4A, B). In the ipsilateral paw burn injury (no-burn/shock and burn/shock) did not cause a cause a significant change in paw withdrawal latency. For reasons not clear, the shock/no-burn mice had slightly longer paw withdrawal latencies for approximately two weeks following the sham burn procedure (Figure 5.4B). In the contralateral paw, burn injury decreased thermal thresholds only transiently during the first 13 days following burn injury (Figure 5.4 A). In contrast to mechanical sensitivity, foot shock stress had no impact on thermal sensitivity.

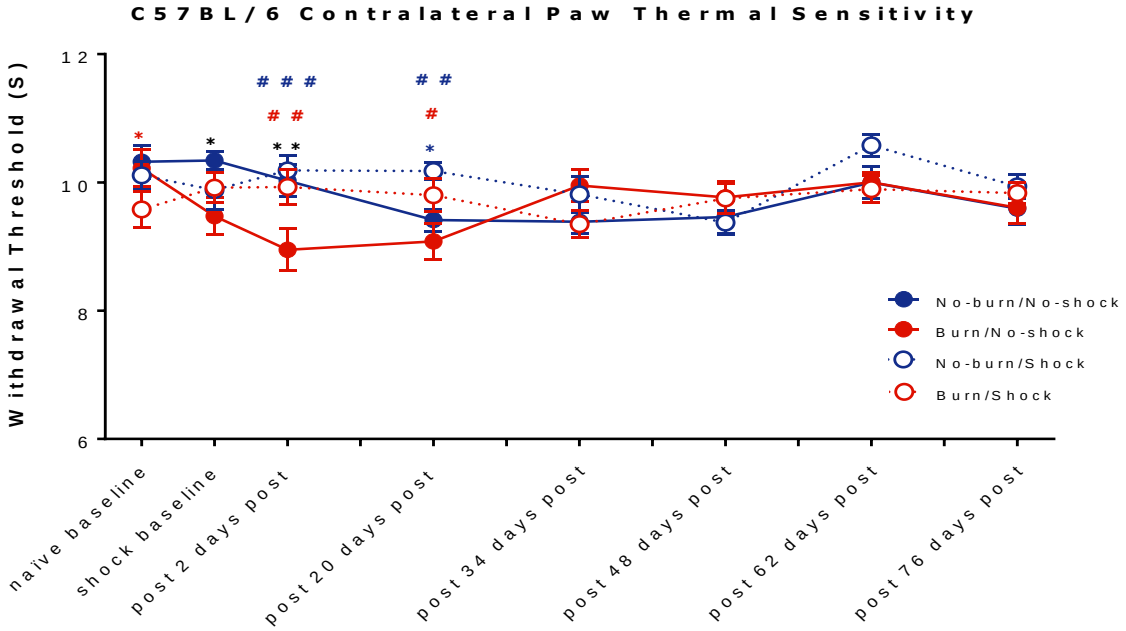
Persistence mechanical allodynia developed in A/J mice after either foot shock stress, burn injury or both.

Identical studies were performed in A/J mice in which mice were organized into four groups that received foot shock stress and/or a burn injury. A/J mice were then assessed for mechanical and thermal thresholds. For mechanical thresholds, no significant differences were evident in baseline mechanical thresholds in either hind paw (Figure 5.4 A, B). In the ipsilateral paw, both groups of A/J mice that underwent foot shock stress (shock/no-burn and burn/shock) displayed a rapid decrease in mechanical thresholds as a result of the foot shock stress (Figure 5.4 B). A/J mice that received a burn injury (no-burn/shock and burn/shock) developed significant mechanical allodynia. In comparison to the control group (no-burn/no-shock), the mechanical allodynia in all other three groups (no-burn/shock; shock/no-burn; burn/shock) persisted for the duration of the experiments up to 54 days. In the contralateral paw, the foot shock stress also reduced mechanical thresholds immediately after the foot shock stress. However, the burn injury in the absence of foot shock stress (no-burn/shock) on the opposite foot pad did not significantly change mechanical thresholds (Figure 5.4 B). The mechanical thresholds in A/J mice receiving

the foot shock stress remained significantly lower throughout the duration of the 54-day testing period.

Figure 5. 4 Hind paw thermal sensitivity in C57BL/6 mice

(A)



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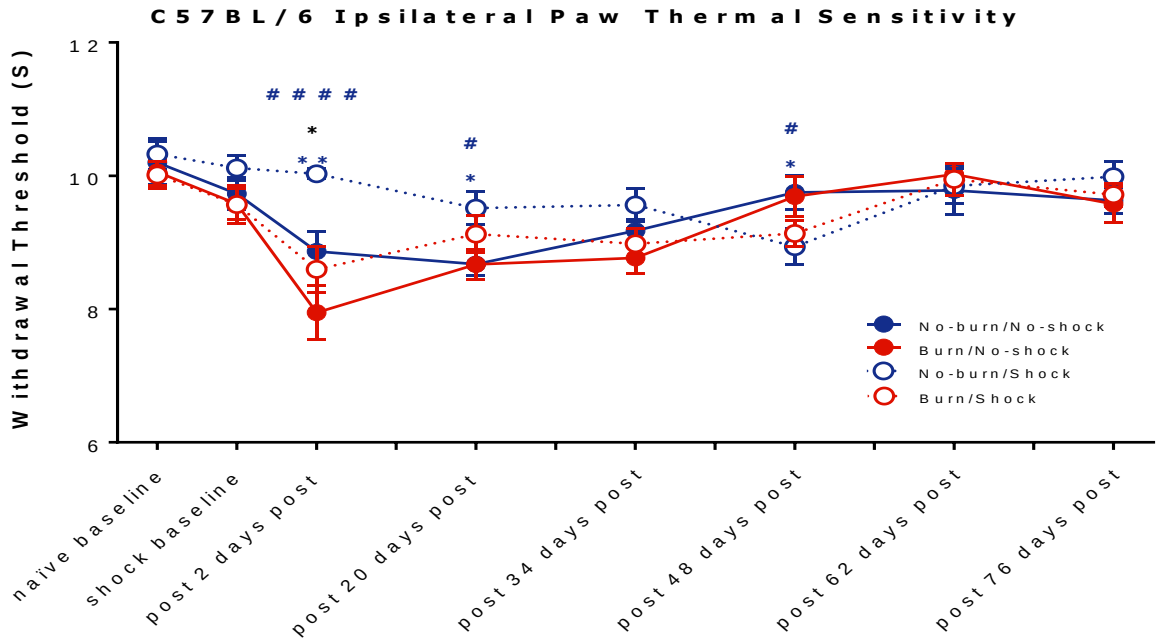
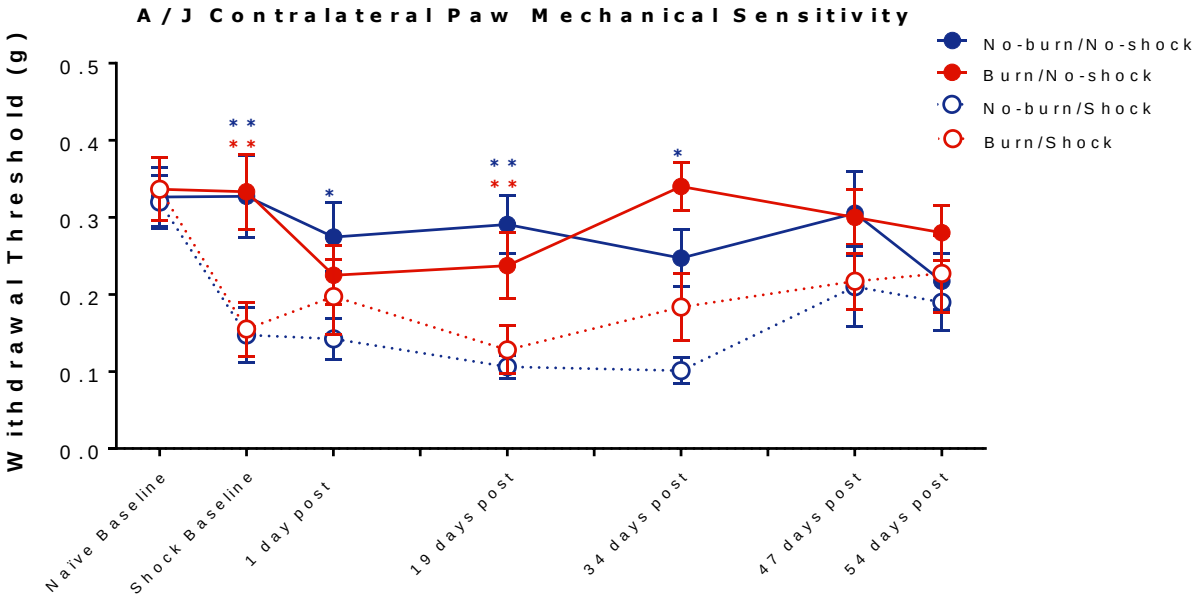


Figure 5. 5 Hind paw mechanical sensitivity in A/J mice

(A)



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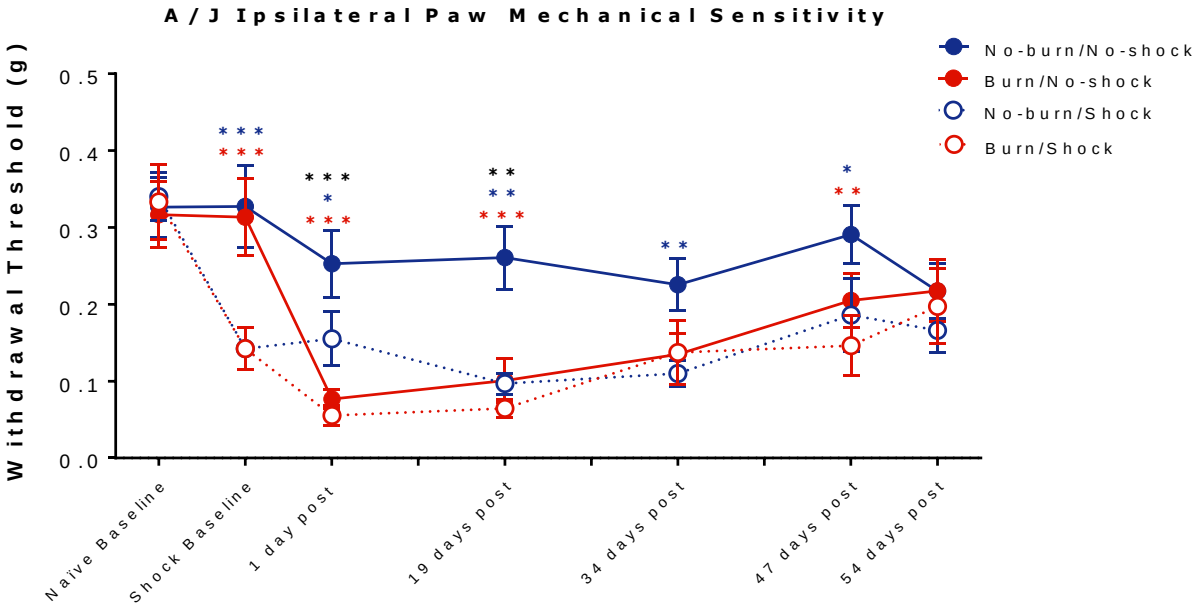


Figure 5.4 Hind paw thermal sensitivity in C57BL/6 mice on naïve baseline, after foot shock, 2, 13, 20, 27, 35, 41, 48 and 54 post burn injury. (A) Thermal sensitivity thresholds on contralateral hind paw (LHP-WT). Significant differences among groups were found in 2, 20 and 27 days after burn injury, but not in 35 days or later. (B) thermal sensitivity test on ipsilateral hind paws (RHP-WT). No-burn/no-shock, Burn/no-shock, and Burn/shock for RHP-WT were decreased after burn injury. No significant difference on naïve baseline, after foot shock, 35, 41 and 55 days after burn injury.

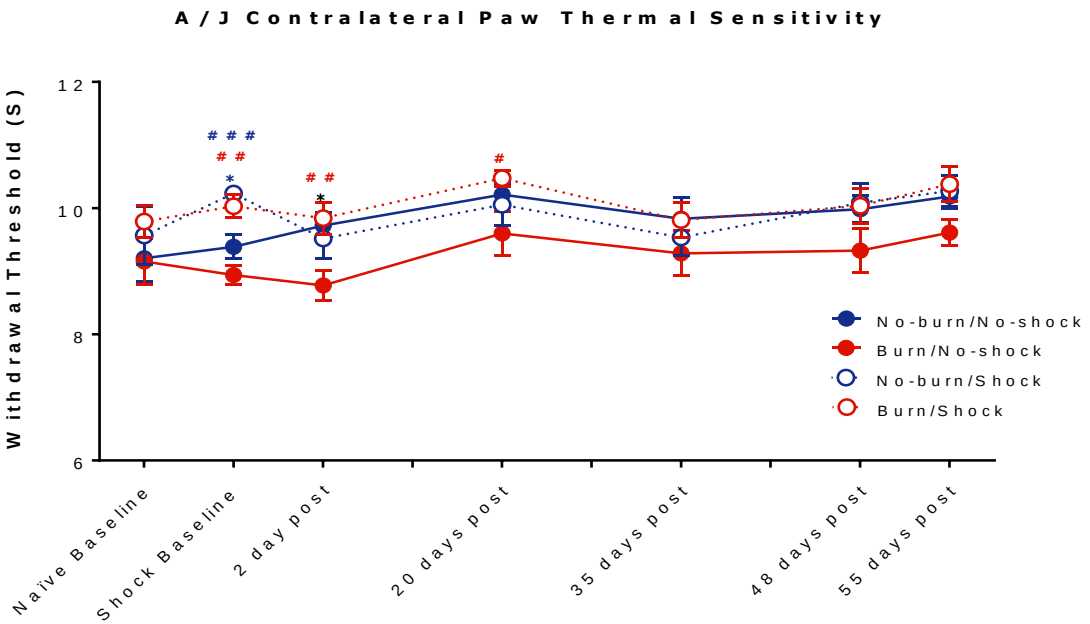
Figure 5.5 Hind paw mechanical sensitivity in A/J mice on naïve baseline, after foot shock, 1, 12, 19, 26, 34, 40, 47, and 54 days post-burn injury. (A) After 10 days foot shock stress procedure, no-burn/shock and/or burn/shock group have a significant decrease in contralateral hind paw mechanical withdrawal thresholds (LHP-WT) compared to no-burn/no-shock group as well as on 1, 12, 19, 26 and 34 days after burn injury. No significant differences were found between No-burn/no-shock and Burn/non-shock group's mechanical LHP-WT. (B) In response to a 10-day foot shock procedure, No-burn/shock and burn/shock groups had significantly reduced mechanical withdrawal threshold (RHP-WT) on shock baseline, 1, 12, 19, 26 and 47 days compared to the No-burn/no-shock group. Burn/no-shock group's mechanical RHP-WT dramatic decrease on 1 day after burn injury and was significant difference at 1, 12 and 19 days after burn injury compared to No-burn/no-shock group, but recuperated after 28 days post-burn injury. Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. No-burn/no-shock, Fisher's LSD posttest. Black: Burn/no-shock, Blue: No-burn/shock , Red: Burn/shock.

Burn injury but not foot shock stress alters thermal thresholds in A/J mice.

Compared to mechanical sensitivity, thermal sensitivity in A/J mice was not significantly affected by the foot shock stress. However, in the ipsilateral paw, both groups of A/J mice receiving burn injury (burn/no-shock and burn/shock) displayed a small decrease in thermal thresholds during the first week after the burn injury (Figure 5.5B). In the contralateral paw, neither foot shock stress or burn injury appeared to have any significant impact on thermal thresholds in A/J mice (Fig. 5.5 A).

Figure 5. 6 Hind paw thermal sensitivity in A/J mice

(A)



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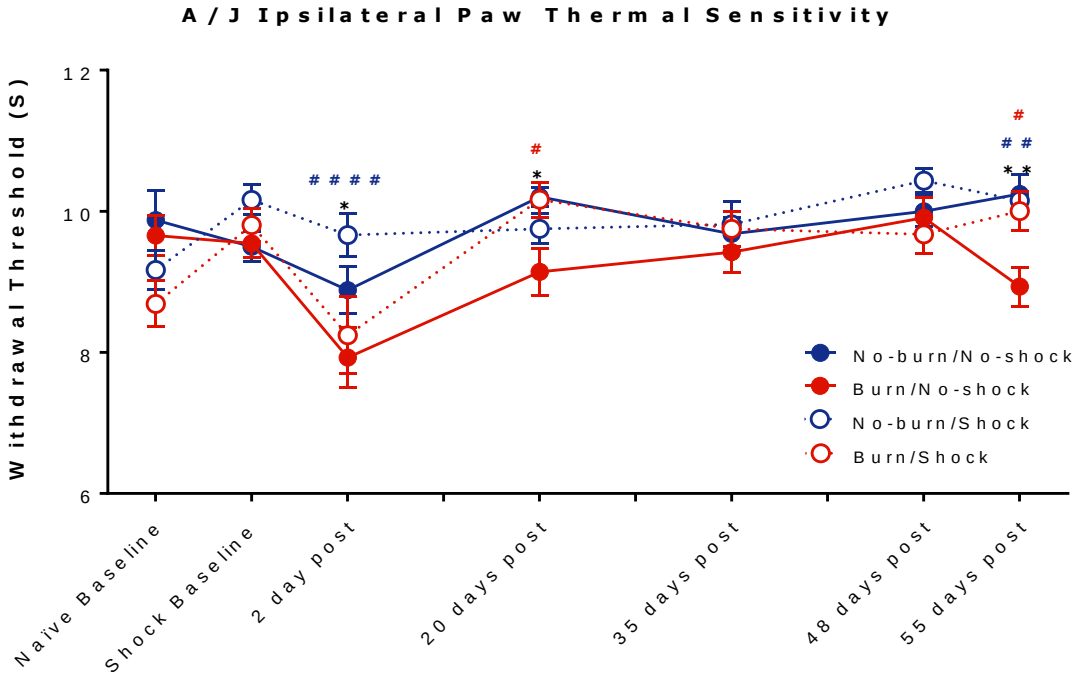

Figure 5.5 Hind paw thermal sensitivity in A/J mice on naïve baseline, after foot shock stress, 2, 13, 20, 27, 35, 41, 48 and 55 post burn injury. (A) Thermal sensitivity test on contralateral hind paw (LHP-WT). Significant differences were observed on shock baseline, 2, 13, 20 and 41-day post-burn injury and no differences were observed at 20, 35, 48 and 55 days after burn injury. (B) shows thermal sensitivity test on ipsilateral hind paw withdrawal threshold (RHP-WT). Significant differences were detected in 2, ,13, 20, 27,41 and 55 days after burn injury, but not in 35 days and 48 days. Two-way ANOVA, * $p<0.05$, ** $p<0.01$, vs. no-burn/no-shock, # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ vs. burn/no-shock, Fisher's LSD posttest. Black: burn/no-shock, Blue: no-burn/shock, Red: burn/shock.

Discussion

The current study has developed an experimental model in mice to safely induce a second degree burn on the plantar surface of the mouse paw and to follow the behavioral recovery of the mice related to mechanical and thermal withdrawal thresholds. The study also introduced a known modifier of pain (stress) to test whether these detrimental interventions modify the duration or severity of altered nociceptive thresholds. Our results demonstrate that a reliable second-degree burn can be induced in the mouse hind paw and this burn leads to behavioral changes associated with mechanical and thermal sensitivity. The burn also modestly impacts the sensitivity of the contralateral paw. Importantly the foot shock stress prior to the burn injury strongly impacts mechanical sensitivity. In C57Bl/6 mice, foot shock stress and a burn injury leads to long-lasting mechanical allodynia in both paws that is greater and longer in duration to only the burn injury, suggesting that prior stress can exacerbate and extend the recovery of mechanical thresholds. This finding adds to the view that stress can modulate pain thresholds and may be an important modifier of burn injury-related chronic pain.

Experimental Models of Burn Injury and Pain

Although relatively few, several previous studies have developed models in pigs, rats and mouse to explore the consequences of burn injury and pain. Pigs have a relatively thick epidermis similar to humans(30-140 μm) compared to rodents (< 25 μm) [448-450], however as a model, swine are expensive and complex to maintain also less amenable to genetic and molecular studies. [451]. In rodents, the epidermal layer in the paw is much thicker (41-47 μm) compared to its dorsal and ventral skin [452, 453]. One difference related to our study is that the previous rodent studies of burn injury applied the burn on the dorsal or ventral side of body instead of the foot or paw that maintains a thicker epidermis [454-456]. In rats, a thermal hind

paw burn injury model was developed in which investigators created third-degree hind paw burn injury by contacting rat paw with 75°C for 10 s. These studies reported a significant increase in mechanical sensitive but not thermal sensitivity at both 1 and 2 weeks(s) post burn injury [457, 458]. Another study created a burn injury on the dorsal surface of the rat's hind paw by immersing the paw into an 85 °C water bath for 4, 7 or 12 seconds, leading to mechanical hypersensitivity for 3-7 days [459]. One burn-induced pain study in mice utilized 52°C Peltier plate for 25 s but only measured mechanical allodynia for 72 hours after the burn injury [460]. In another study in mice by Shields et al., [461] wild-type C57BL/6 mice underwent a burn injury on their hind paw using 65°C metal plate for 15s. The authors reported that this temperature and duration of burn led to a focal second-degree burn that reliably produced reduced withdrawal thresholds to heat and mechanical stimuli present 2-4 hours after the injury and lasted 2-3 weeks. The authors also reported that multiple pilot studies were performed using higher temperatures (75°C or 85°C). They reported sensory changes at these higher temperatures that did not differ significantly in magnitude or duration from the 65°C model, but that more damage to the skin and digits were present. We also found in the current study that these parameters produced a consistent second-degree burn (65°C metal plate for 15s). Results from our study extended the timeline to reveal a very long-lasting during of mechanical hypersensitivity. For example, in the Shields et al study, the burn injury group at 21-days post burn showed no significant differences in mechanical sensitivity compared to non-burn sham group. However, our burn-no-shock group took 47 days to recovery. One potential reason is our mice in the no-shock group were transported daily and placed into a cage (26 × 26 × 39 cm) without lids. Although no foot shock stress was provided, the housing cage is nearly identical with the caging used for an open-field test. Open-field is not only a widely used behavior test for measuring anxiety [462] but also can

be used as a stressor [435, 463, 464] and it is plausible that the paradigm to transport and handle mice created stress for the mice.

Mechanisms of Burn-Injury Related Pain

Peripheral inflammatory response to acute burn injury is initiated within minutes of the injury. This response includes an excessive release of inflammatory mediators, including calcitonin gene-related peptide (CGRP) and substance P and results in activation of NMDA receptors. These irritant mediators sensitize and stimulate peripheral pain fibers throughout the inflammatory process[465]. As a result of this process, patients experience pain at the burn injury area in the form of allodynia and primary hyperalgesia [466, 467] and secondary hyperalgesia can also occur in surrounding uninjured tissues [468]. This tissue trauma, in conjunction with the inflammatory processes, can result in neuroplastic adaptations throughout the nervous system whereby the pain signals develop into hyperalgesia [467, 469] With time, these changes often become irreversible and lead to the development of chronic pain. This phenomenon involves sensitization of peripheral receptors, increased excitability in the spinal dorsal horn centered on NMDA receptor systems, and activation of descending supraspinal facilitative pathways [470]. Recently, a rodent model for partial-thickness burn injury was shown to induce rapid and sustained mechanical allodynia [471]. Electrophysiological studies then demonstrated that dorsal horn neurons became hyperexcitable and displayed significantly increased evoked activity after burn injury. These studies in experimental models demonstrate that a unilateral peripheral burn injury can produce long-lasting peripheral and central sensitization.

Foot Shock Stress as a Modifier of Pain

Foot shock to induce stress in rodents is widely used studies of context memory and less commonly but recently used to apply psychological stress [472, 473]. A summary of these studies revealed that shock-induced stress impacts the hypothalamic-pituitary-adrenal (HPA) axis pathway, which is then proposed to contribute to comorbid chronic pain and mood disorders [308, 474]. In this study, we observed that in both genetic mouse strains, shock with or without burn injury induced significant and long lasting bilateral mechanical allodynia soon after the foot shock procedure was completed. Further decrease of withdrawal thresholds appeared after burn injury in both C57Bl/6 and A/J strains in the ipsilateral paw. In general, the no-burn/shock group had prolonged mechanical allodynia both in the ipsilateral and contralateral hind paw compared to the burn/no-shock group. These results suggest our burn paradigm did create a huge impact on small rodents, but foot shock stress itself generates a lasting effect on both A/J and C57BL/6 mice. Consistent with our findings, one study reported that a 10-day foot shock procedure induced allodynia up to 28 days on A/J mice [161]. Here, we demonstrate that this stress-induced mechanical allodynia can last up to 54 days after the foot shock stress paradigm.

Strain Differences in Mice

Strain differences in anxiety, pain-behavior and depression between C57BL/6J and A/J have been widely reported. One study analyzed four different anxiety related behaviors (including open filed, light-dark transition test, elevated plus and elevated zero maze) from 3 various articles that compared A/J and C57BL/6. These studies concluded that A/J mice may serve as a good model to study genetic contributions for studying anxiety-related behaviors compared to C57BL/6 mice [223]. Other investigations of the longevity of anxiety-related behavior tests during over the past seven decades showed that A/J mice remain highly anxious now relative to other mouse strains and this increase in anxiety was also evident decades ago

[224]. To understand the effect of psychological stress and mental disorders on post-burn injury patient, we observed A/J and C57BL/6 mouse's mechanical and thermal sensitivity after our burn injury process. Surprisingly, despite a reputation for increased anxiety behaviors, A/J mice (40 days post) displayed slightly shorter recovery times for burn-induced mechanical allodynia compared to C57BL/6 mice (47days post) on the ipsilateral hind paw. Relative to the contralateral paw, only C57BL/6 mice displayed mechanical allodynia in response to burn injury, whereas A/J mice were much less affected. One limitation of our study is that we only used male mice, and subsequent studies should explore both sexes to identify potential sex difference that may exist in the response to burn injury and chronic pain.

Conclusions

In the current experiments examining the response of mice to a paw burn injury and the addition of stress, similar patterns of mechanical allodynia following burn injury were observed when comparing no-burn/shock C57Bl/6 and A/J mice. Second, we observed that elicitation of foot-shock stress prior to a burn injury lead to a significant and extended duration mechanical allodynia that was independent of a burn injury. Third, behaviors consistent with chronic pain after burn injury are apparent in both C57Bl/6 and A/J mice that received foot shock stress, suggesting that stress prior to burn injury may play a role in the development of chronic pain. Finally, A/J mice exposed to foot shock stress surprisingly had shorter post-bun recovery time compared to C57BL/6 mice. This indicated that an anxiety-prone genetic background in A/J mice appears not exacerbate the burn injury recovery process.

Chapter 6: Discussion

6.1 Development of a model to study the effect of psychological stress on individuals with pre-existing anxiety.

As far as we know, anxiety studies using animals can be tracked back to the 1978 work by File & Hyde studying individual differences among same strain of rats [475]. Follow-up research within the same year showed that treating male hooded rats (*Rattus norvegicus*) with chronic ACTH administration resulted in anxiety-like behavior [476]. Two years later, researchers successfully separated high and low anxiety pointer dogs, which is the first reported study on inherent high anxiety as we know [477]. Later studies successfully created rodent models of depression by socio-environmental deprivation (isolation); however, this intervention was not successful in producing an anxiety model in rodents [478-481]. In recent years, multiple rodent models for anxiety have been developed and can be roughly divided into genetic knockout/transgenics and inbred strain /selected lines. More than 20 genetically-modified rodent strains have been developed that show an anxiety phenotype. Most anxiety rodent models created by genetic modification relate to four major systems: Norepinephrine, serotonin (5-HT), gamma-Aminobutyric acid (GABA), and the hypothalamic-pituitary-adrenal (HPA) axis. For example, monoamine oxidase A knockout induced anti-anxiety behavior in an open field test [482]. By measuring open field, elevated plus maze, and elevated zero maze, many studies showed THAT 5–HT1A knockout increased anxiety behavior [483-487]. Transgenic mice with overexpression of corticotropin-releasing factor (CRF) had a higher anxiety phonotype compared to control mice in open field activity, elevated plus maze, and light/dark transition [488-491]. In my opinion, genetic modifications to create anxious rodent models are mostly based on already-identified anxiety targets and widely used drugs. Inbred strains or selective lines seem more valuable for research to identify new targets for anxiety treatment. In rats, a widely used anxiety model is the Wistar-Kyoto (WKY) strain [324, 492]. WKY rats show less activity in open fields, are more

immobile in the forced swimming test, and have faster adaption in the learned helplessness test [491]. More defecation was also observed in WKY rat during a forced swimming test and this indicated that anxious strains may have higher bowel movements while under stress [491]. In chapter 4, A/J mice also had higher defection behavior during the 15 minutes of foot shock stress compared to C57Bl/6 mice. Other anxiety rat models include Roman High and Low Avoidance rats [493-495], Maudsley Reactive and Non-reactive rats [496, 497], Fawn Hooded rat [498], Floripa High and Low rats [499] and HAB/LAB rats [500]. More rat strain comparisons on anxiety behavior tests was also investigated [501, 502]. In inbred strains of mice, BALB/c mice [503, 504], 129 mice [505] and BTBR T + tf/J mice [506] are more anxious. The mouse model in my studies, A/J mice, as mentioned in chapters 1 and 2, are highly anxious in open field and forced swimming tests compared to other strains. Many anxiety-related or induced disorders, including depression [507], cardiovascular disease [508], and schizophrenia [509] have been studied [510]. However, stress-induced mechanical and bladder hypersensitivity in high-anxiety individuals has rarely been studied. In particular, anxiety-prone mice are poorly study compared to the WYK rat model. Here, we identify the physical and psychological effect of stress-induced mood disorder and mechanical and visceral hypersensitivity.

Since the 1970's until today, more than 14 rodent stress model categories have been developed [89, 99-115]. In a study by Lee et. al., WYK rats exposed to a 10-day water avoidance stress for 1 hour every day had increased visceromotor response (VMR) during bladder distension and referred mechanical hyperalgesia in the suprapubic region [299]. A previous study by Robbins and Ness, showed that 10 days of uncontrollable foot shock stress induced significant bladder hypersensitivity in normo-anxious Sprague Dawley (SD) rats [315]. In this foot shock paradigm, an SD rat was placed in a foot shock cage and 30 1.0mA shocks were

applied over 30 minutes for 10 consecutive days. We chose to model our stress exposure to mimic this study and, therefore, A/J mice were treated with a similar foot shock paradigm. Because a mouse is much smaller than a rat, instead of using 1mA we tried to search for the optimal current for foot shock in mice. Studies have shown that the strength of the foot shock current does not significantly impact the extent of elevated plasma corticosterone (CORT) in rat [511]. Four A/J mice were tested with a foot shock of 0.8, 0.7, 0.6, 0.5, 0.4, 0.3 and 0.2 mA and we found that 0.4mA was the highest current that caused a behavioral jumping response, but did not elicit freezing, in A/J mice. This 10-day foot shock procedure successfully induced both acute and persistent mechanical allodynia, bladder hypersensitivity, and anhedonic behavior. Most importantly, according to hind paw histology at one day after foot shock, there was no viable skin damage.

6.2 HPA axis and downstream mediators change following foot shock exposure.

Although some stress stimuli (self-reported stress in neuropathic pain) is not related to increased CORT secretion [512-514], several reports indicate that various stressors trigger HPA axis activation and downstream increased CORT release [29, 206, 267, 394, 439, 515, 516]. However, not all stimuli that lead to an increase in CORT are categorized as a stressor. Stimuli like exercise, anxiolytic drugs, and sexual experience can also induce CORT release [511, 517-519]. In our study, we discovered that foot shock in anxiety prone A/J mice enhanced HPA axis output and serum CORT was significantly higher in the stressed group compared to the non-stressed group. CORT level is highly related to circadian rhythm [520]. In rodents, the plasma CORT levels between 9 a.m. to 12 p.m. are relatively low, followed by a dramatic increase between 2 p.m. and the beginning of the dark phase [242, 378]. All the A/J mice in our study

were sampled between 10am to 11:59am to avoid huge variations in the afternoon-associated increases in CORT.

High correlation between anxiety and CORT has been discovered in many models. One found that, after restraint stress, SD rats decreased the time spent on the open arm during the elevated maze test, indicating anxiety-like behavior [521]. Increased plasma CORT levels in restrained rats were also found in the same study [521]. Maternal exposure to lipopolysaccharide increased both anxiety and CORT levels in SD rats [522]. Others also showed evidence that increased CRF gene expression in amygdala and hypothalamus was related to anxiety behavior and CORT secretion increase [523]. However, the baseline CORT level in the A/J mice not exposed to foot shock stress in our study did not significantly differ from that of C57Bl/6 mice in previous research in our lab. The CORT level in the A/J sham group was around 175 ng/ml and was around 300ng/ml in shock group; CORT level in naïve C57Bl/6 mice was around 165 ng/ml [114]. Other studies reported that WYK rats have no significant difference in baseline CORT level compared to normo-anxious Wistar rats. However, after a stressful event, CORT levels in WKY rats were significantly higher compared to Wistar rats [524]. Further studies are needed to compare the ability to facilitate acute stress between high and low anxiety strains of mice.

Apart from the CORT change, we also found changes in the mRNA levels of MR and GR in the hypothalamus and hippocampus, respectively, in stressed mice. Stress can alter the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus and initiate mental disorder through decreased neurogenesis [525, 526]. Several stress models, including early life stress [527], social defeat [528], social isolation stress [529], and restraint stress [530] affect BDNF expression in the central nervous system. Specifically in foot shock stress, male rats had decreased BDNF expression in the dentate gyrus [531] and another study indicated that foot

shock in female, but not male, rats increased BDNF expression in hippocampus [532]. This could be one of the potential reasons that repeated foot shock stress exposure induces learned helpless in male, but not female, rats [533, 534]. Following repeated foot shock stress exposure, some studies showed a significant drop in BDNF expression prefrontal cortex of female, but not male, rats [532]. However, another study indicated that hippocampal BDNF expression decreased in both sexes after chronic unpredictable stress, which included foot shock stress [535]. In our study, a change in BDNF expression in the hippocampus after 10 days of foot shock was also found.

6.3 Voluntary wheel running effect on foot shock stressed A/J mice.

Physical activity can be a practical therapeutic tool for many diseases. Physical activity to alleviate chronic pain and mood disorder are two hot topics that people have investigated in recent years. In a meta-analysis of clinical trials, exercise has been reported to reduce fibromyalgia [253], rheumatoid arthritis [278], hip osteoarthritis [279], non-specific low back pain [255], depression, and anxiety [280]. Current studies show that exercise increases BDNF and insulin-like growth factor (IGF-1) [281], which both have been reported to have exercise-induced benefits in depression [282, 283] and chronic pain [284]. It has been proposed that exercise provides anti-inflammatory responses in an attempt to subdue inflammatory pain [285] and stress induced neuro-inflammation that can cause mood disorders like depression [8].

In most studies, voluntary wheel running has a significant effect on stress-induced mechanical and visceral sensitivity. Preventative physical activity can successfully reduce mechanical hypersensitivity associated with muscle pain [536-538] and chronic constriction injury [371] when carried out for 6 and 8 weeks, respectively. Voluntary physical activity showed a hypoalgesic effect in rodent models using complete Freund's adjuvant (CFA) [539],

Mono-iodoacetate-induced osteoarthritis [540], diabetes-induced pain model [541] and stress [384]. A previous study from our lab showed that 4 weeks of voluntary wheel running normalized bladder hypersensitivity in female C57Bl/6 mice that had undergone early life stress [115]. To our knowledge, only one very recent study has investigated the therapeutic potential of voluntary wheel running on improving stress-induced hypersensitivity in an anxiety-prone model. Sanford et al.,[542] exposed female WKY rats to 10 days of water avoidance stress and then allowed them to run at night for 5 days/week for 3 weeks. Voluntary wheel running significantly improved many measures of bladder function and had a moderate, but significant, impact on bladder sensitivity, most prominently at lower intravesicular pressures. In our study, A/J mice were allowed to run for 28 days prior to and during a 10-day foot shock stress exposure. Voluntary wheel running reduced hind paw withdraw thresholds in stressed A/J mice, but it did not reach significance. Voluntary wheel running also failed to prevent the development of bladder hypersensitivity following foot shock stress. Considering that Sanford et. al.,[542] saw a significant improvement with voluntary exercise occurring after the stress exposure, it would be interesting to repeat our studies using a similar post-foot shock wheel running intervention.

Acute exercise increases CORT level [543-545], but mixed data have been found in studies of chronic voluntary wheel running. Reports showed that 1-3 weeks of voluntary wheel running increased morning CORT by 2.5-3-fold [386, 546]. However, no significant difference was found after four weeks of running [386]. Another study indicated that afternoon plasma CORT increased around 1.8-fold after two weeks of wheel running in mice, compared to sedentary[547]. In contrast, another study reported no effect of 2 weeks of voluntary wheel running on afternoon CORT levels [548]. Following 12 days or 4 weeks of voluntary wheel running, the CORT levels during the dark phase were elevated by around 1.6 and 2-fold,

respectively [549, 550]. Finally, examination of fecal content over a 24 hour period showed that C57Bl/6 mice allowed to wheel run had 1.5-fold higher CORT levels compared to sedentary mice [551]. Differences in the length of wheel running, sampling time, and lack of described exercise time have resulted in un-unified opinions on the effect of chronic voluntary wheel running on CORT level. However, it has been widely recognized that long-term exercise can be a buffer for stress impacts on the HPA axis. Treadmill running can attenuate elevated CORT induced by acute stressors, such as restraint [552], single prolonged stress [553], and foot shock stress [554]. Similarly, long-term voluntary exercise also had a significant effect on normalizing CORT level after acute stress exposure, such as restraint [386, 555], tail shock[556], and noise stress [557]. Another study also reported that mice housed with running wheels had an earlier CORT peak, but this decayed much faster, after restraint stress [558]. Finally, after FWS, exercised and sedentary groups had a similar peak in CORT, but the exercised group returned to baseline faster than the sedentary group[559]. Not surprisingly, voluntary wheel running also benefitted the response to chronic stress exposure by buffering the HPA axis. Voluntary physical activity reduced CORT levels in 11-day noise stress [560], 28-day chronic unpredictable stress [561], and even 30 days of electric shock [562]. In our study, a dramatic decrease in CORT levels were observed in both the shock and sham exercised groups. This not only confirms all of the previous studies, but also provides a better idea of how exercise might mitigate the response to stress in individuals with underlying anxiety.

Finally, we also discovered a unique running pattern in A/J mice following a stressful event. Stressed A/J mice ran a longer distance during the immediate one hour after daily foot shock stress treatment. However, the overall running distance during the 10 days of foot shock was significantly lower in stressed A/J mice compared to sham. Very few studies have reported on

this phenomenon. As mentioned in Chapter 3, a study with male BALB/c mice exposed to unpredictable chronic mild stress showed a significant decrease in running distance in stressed mice compared to sham [375]. However, another study in male C57BL/6J mice exposed to foot shock stress indicated that stressed mice ran significantly more during the first hour after foot shock [376]. This indicates that running distance in A/J mice can potentially become a biobehavioral marker for stress and even can identify when an event is stressful.

6.4 Disparate phenotypes after foot shock stress caused by genetic differences.

Mast cell activity and HPA axis output closely influence each other. For example, CRF, the product of the HPA axis, can stimulate mast cells and trigger their degranulation in skin and brain [515, 563]. Interestingly, mast cells can also influence the HPA axis. Mast cells have been found in the pituitary and are especially enriched in the hypothalamus. Histamine, one of the major products of the mast cell, is an important regulator in the hypothalamus [564]. In fact, some studies discovered that hypothalamic mast cell activation led to stimulation of the HPA axis in an experimental animal [565, 566]. In chronic pain disorders, including irritable bowel syndrome and fibromyalgia, mast cell numbers increase [567, 568]. Mast cells are located next to unmyelinated nerves throughout the body, including skin, intestine, and trachea [569, 570]. Furthermore, a study associated with chronic pain-related inflammatory responses reported that mast cells release pro-inflammatory molecules that interact with peripheral nerve endings of sensory neurons, creating an inflammatory response [571]. Increase of HPA axis output and bladder hypersensitivity has been identified in foot shock stressed mice (chapter 2). Therefore, we hypothesized that increased HPA activity would trigger bladder mast cell degranulation, thereby contributing to bladder hypersensitivity. However, mast cell number and degranulation rates between the shock and sham group were not different and both were comparably high

(~80%). Cerebral histamine levels are highly associated with anxiety behavior [572-574]. In 2002, Ikarashi and Yuzurihara identified that mast cells, but not neuronal-derived histamine, play an important role in anxiety phonotype. We believed the high degranulation rate in A/J mice created a ceiling effect, such that no significant differences could be observed following foot shock stress exposure. Similarly, in WYK rats, approximately 70-75% of mast cells in the bladder from both sham and water avoidance stress groups were degranulated [298, 325]. In contrast, non-anxiety strains, like C57Bl/6 mice and SD rat, have a degranulation rate of 20-30% in bladder mast cells [313, 575].

Around 5-40 % of the world's population suffers from visceral pain [576-579]. Not only can stress affect sensitivity, but patients with psychiatric disorders such as depression and anxiety, have been shown to present with chronic visceral hypersensitivity [580-582]. Evidence indicates that using pharmacological or psychological interventions designed to treat stress symptoms can also improve symptoms associated with irritable bowel syndrome [583, 584]. In our study, stressed A/J mice showed a significant increase in VMR during bladder distension at both 1 and 14 day(s) post-stress, whereas foot shock stress had no significant impact on C57Bl/6 bladder sensitivity at either time point. A previous study from our lab showed that exposure to water avoidance stress also did not significantly alter VMR during bladder distension in C57Bl/6 mice [313]. Another study tested bladder function (micturition pressure) after 2-weeks of social defeat by an aggressive C57Bl/6 breeder on C57Bl/6 mice and anxious FVB mice. Although the power of social defeat from the same strain versus an alternate strain may be different, social defeat had a significant impact on the micturition pressure (threshold and baseline) in FVB mice, but not in C57Bl/6 [585]. All of the aforementioned studies have implied that C57Bl/6 mice have a higher resistance to stress-induced bladder hypersensitivity compared to other strains, such as

A/J and FVB. As mentioned in chapter 4, the WKY rat, a high anxiety-prone strain, is more vulnerable to visceral hypersensitivity compared to normo-anxious SD rats [428, 429]. In clinical studies, high comorbidity between anxiety and gastrointestinal disorders, like inflammatory bowel disease and irritable bowel syndrome, has been observed [586, 587]. The brain-gut axis is largely involved in mediating this link between visceral hypersensitivity and anxiety. Multiple reports have showed that germ-free mice show less anxiety behavior than specific-pathogen-free mice [588-590]. Other studies have also shown that the microbial environment in the gut alters the HPA axis response to stressful events [588, 591]. Correction of gut microbiota, including administration of probiotics or antibiotics, has been shown to ameliorate visceral pain or disorder [592-594]. Lastly, in human studies, probiotics with Lactobacilli, Bifidobacteria, Enterococci, Streptococci or/and Bacilli species had positive effects on irritable bowel syndrome symptoms in patients [595]. Finally, in chapter 4, both A/J and C57Bl/6 mice developed mechanical allodynia at 1 and 14 day(s) after the foot shock stress procedure. However, according to data from chapter 5, C57Bl/6 mice show a more persistent shock stress-induced mechanical allodynia phenotype compared to A/J mice.

6.5 Transition from acute to chronic pain in burn injury.

How to best prevent acute pain from transitioning into chronic pain has been discussed and investigated for a long time. A lot of studies have focused on persistent postsurgical pain in recent years and well-constructed reports in epidemiology, risk factors, and candidate mechanisms have been published [596-598]. Psychological factors, such as depression, anxiety, poor social support, and stress, have all been reported as rick factors [598]. However, apart from postsurgical chronic pain, few studies have investigated the transition from acute to chronic pain. Here, we tested whether genetic differences that lead to greater (A/J) or less (C57Bl/6)

behavioral evidence of anxiety and stress exposure may be risk factors for transitioning to chronic pain in a mouse model of burn, using a 15s exposure to 65°C to induce a second degree burn on the plantar surface of the right hind paw.

The results from studies described in Chapter 5 confirm that foot shock stress induced persistent allodynia that lasted up to 68 days post shock. Foot shock stress prolonged thermal injury recovery time in both stains of mice. More importantly, we recognized that foot shock stress was a robust modulator for chronic pain. As far as we understand, this is one of the longest studies measuring thermal and mechanical sensitivity after foot shock stress [599] [315, 474, 600]. As mentioned earlier, anxiety is closely related with chronic pain [228, 230, 400, 601, 602]. However, in our study, anxiety-prone A/J mice had a shorter recovery period for mechanical allodynia compared to C57Bl/6 mice. According to our results, C57Bl/6 mice were not only more susceptible to foot shock stress, but also to thermal injury itself. We are one of the first groups to compare the post-burn recovery period between A/J and C57Bl/6 mice. Further investigation will be needed to confirm our results and reveal the underlying mechanism. Finally, we found much shorter recovery times in thermal sensitivity, compared to mechanical sensitivity, with both strains sharing a similar recovery pattern.

6.7 Conclusion

Prevalence of anxiety and depression is very high and is escalating year by year [273, 274]. The population of people suffering from chronic pain is larger than that with cardiovascular disease and cancer combined [276] [275]. Further, co-morbid anxiety and chronic pain is common and the link has not yet been fully investigated [231, 602, 603]. Here, we first established a small rodent model for stress-induced chronic pain co-morbid with mood disorder in an anxiety-prone strain of mice. This rodent stress model has an acute and persistent impact on both hind paw

mechanical and bladder hypersensitivity. Our work helps us to determine the impact of stress on the HPA axis and other modulators, especially in an anxious individual. We also discovered that the HPA axis may have been triggered, with several possible targets, such as BDNF, MR and GR, showing potential for clinical intervention. We revealed that persistent and acute anhedonic behavior was triggered by our stress model. Voluntary physical activity showed benefit to normalize the stress-triggered HPA axis elevation in serum CORT levels. However, it did not significantly decrease the foot shock stress-induced hind paw allodynia or bladder hypersensitivity. Interestingly, a unique pattern of running distance in stressed A/J mice was discovered. This finding has potential to become a biobehavioral marker of stress, possibly indicating when and whether or not an event is stressful. This study also suggested that the degranulation rate of mast cells in the bladder may be reflective of the anxiety phenotype of a rodent. Both bladder sensitivity and fecal output indicated that A/J mice are more vulnerable to foot shock stress than C57Bl/6. The anxiety phonotype may also play a role in this. Finally, 11 million patients receive burn treatment annually and 52% of patient still have “ongoing burn-related pain” at 11 years after their initial burn injury [286, 287, 291].Therefore, we developed a small rodent burn model to search for clues into the transition from acute to chronic pain and revealed that stress may play an important role. Surprisingly, after thermal injury, mechanical allodynia in anxiety-prone A/J mice recovered faster compared to C57Bl/6. Studying the genetic differences between A/J and C57Bl/6 mice, and how they influence the various molecular and behavioral responses to different pain models, could provide crucial information that will help us understand how to prevent the development of chronic pain in both non-anxious and anxious individuals.

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Appendix: Stress-induce allodynia or hyperalgesia in rodents.

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Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Forced swim-stress				
Male Sprague-Dawley rats	Day 1: 10 min	Formalin test and hot plate test	Inflammatory and thermal hyperalgesia	Quintero et al., 2011, Quintero et al., 2003, Quintero et al., 2000, Suarez-Roca et al. (2008), and Suarez-Roca et al., 2006a, Suarez-Roca et al., 2006b
	Day 2–3: 20 min	Carrageenan intra-muscular injection followed by grip strength	Mechanical hyperalgesia as determined by reduced grip strength	Suarez-Roca et al. (2006a)
Male Swiss albino mice	Two swim stress sessions 6 min duration, 8 h apart	Hot plate	Thermal hyperalgesia	Suaudeau and Costentin (2000)
Male Wistar rats	5 min sessions daily for 5 days	Tail flick test	Thermal hyperalgesia	Fereidoni et al. (2007)
Adult male and female Swiss Webster mice	5, 15 or 30 min or 15 min for 2 days	Tail flick test and grip strength	Thermal and mechanical hyperalgesia	Abdelhamid et al., 2013a, Abdelhamid et al., 2013b
Male albino mice	6 min sessions daily for 15 days	Tail immersion test	Thermal hyperalgesia	Dhir and Kulkarni (2008)
Repeated cold stress/SART				
Male 4-week old ddY mice	Over 7 days: Alternating 24 °C/4 °C every hour for 7 h; 4 °C for final 17 h	Randall–Selitto apparatus	Mechanical hyperalgesia (days 5–7)	Ohara et al. (1991)

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Male Sprague-Dawley rats	Over 5 days: Alternating 24 °C/4 °C or −3 °C every 30 min for 7½ h; 4 °C/−3 °C for final 16½ h	Randall–Selitto test and the von Frey hair test	Mechanical hyperalgesia (greater in −3 °C group than 4 °C group)	Nasu et al. (2010)
Male Wistar Rats	Over 5 days: Alternating 24 °C/−3 °C every hour for 4 h; −3 °C for remaining 20 h	Randall–Selitto test	Mechanical hyperalgesia	Fujisawa et al. (2008)
Male Wistar rats	Over 5 days: Alternating 24 °C/−3 °C every 2 h for 6 h; −3 °C for remaining 18 h	Footshock on one of two floors	Decreased escape latency	Kawanishi et al. (1997)
Restraint stress				
Male and female (mixed estrous phases) Wistar rats	Daily 1 h restraint for 40 days	Tail flick test	Thermal hyperalgesia in males, no effect in females	Gamaro et al. (1998)
Male Wistar rats	*Acute*: 15 min, 30 min or 1 h restraint *Subchronic*: 1 h restraint for 3 days *Chronic*: 1 h daily, 5 days per week for 40 days	Formalin injection into the temporomandibular joint (TMJ)	Increased inflammatory pain in chronic restraint stress rats	Gameiro et al. (2006)
Male Sprague-Dawley rats	Daily 1 h restraint for 4 days over for 5 weeks	von Frey, Randall–Selitto, tail immersion test, acetone-induced cold allodynia, formalin test	Inflammatory, thermal and mechanical hyperalgesia	Bardin et al. (2009)

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Adult male Wistar rats	1 h daily restraints for 5 days per week over 8 weeks	Tail flick test[a] Formalin injection into TMJ[b]	Thermal and inflammatory hyperalgesia	da Silva Torres et al. (2003)[a] and Gameiro et al. (2005)[b]
Male Sprague-Dawley rats	6 h restraint once or over 1, 2 or 3 weeks	Tail flick test	Thermal hyperalgesia following 2 and 3 week restraint	Imbe et al. (2004)
Male Sprague[a] Dawley rats Male and female Wistar rats[b]	Acute restraint for 2 h in restraint cage	Colorectal distension	Visceral hyperalgesia	Ohashi-Doi et al. (2010)[a] and Eutamene et al. (2010)[b]
Male Sprague Dawley rats	2 h restraint stress 4 days	Colorectal distension	Visceral hyperalgesia	Shen et al. (2010)
Male Wistar rats	1 h restraint 5 days a week for 11 weeks	von Frey Test and hot plate	Mechanical allodynia and thermal hyperalgesia	Spezia Adachi et al. (2012)
Immobilisation stress				
Adult male Sprague Dawley rats	90 min daily for 7 days	Tail-Flick test	Thermal hyperalgesia	Costa et al. (2005)
Male ICR mice	1 h daily for 5 days	Formalin test	Inflammatory hyperalgesia	Seo et al. (2006)
Social defeat				
Male Sprague-Dawley rats (Long Evans rats as intruder)	Four daily intruder sessions divided into two periods (see above)	von Frey, Randall–Selitto test and formalin test	Mechanical and inflammatory hyperalgesia	Rivat et al. (2010)
Male Long Evans rats	Resident rats were vasectomised prior to testing. Five daily	Formalin test[a], thermal preference and	Inflammatory and thermal hyperalgesia	Andre et al. (2005)[a] and Marc

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
	intruder sessions divided into two periods (see above)	thermal escape tests[b]		inkiewcz et al. (2009)[b]
Water avoidance				
Male Wistar rats	1 h per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	Bradesi et al., 2006, Bradesi et al., 2007, Bradesi et al., 2009, Bradesi et al., 2005, Larauche et al. (2008) and Wang et al. (2013)
Male Sprague-Dawley rats	1 h per day for 10 consecutive days	von Frey test[a], colorectal distension[b]	Mechanical and visceral hyperalgesia	Chen et al. (2011)[a] and Green et al., 2011a, Green et al., 2011b[b]
Adult male C57Bl/6 mice	1 h per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	Hong et al. (2009) and Larauche et al. (2010)
Male Sprague-Dawley rats	Over three days: tones played over four frequencies over 30 min time period	Randall Selitto test[a], colorectal distension[b]	Mechanical and visceral hyperalgesia	Khasar et al., 2009, Khasar et al., 2005[a] and Green et al., 2011a, Green et al., 2011b[b]
Chronic mild stress				
Male Wistar rats	Unpredictable Chronic stress for 6 weeks;	von Frey and hot plate in normal and complete Freund's adjuvant chronic pain rat model and formalin test	Increased mechanical and thermal thresholds and inflammatory hyperalgesia	Shi et al. (2010a)

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Male Wistar rats	Unpredictable chronic stress for 6 weeks	Hot plate and von Frey tests in naive and SNL rats	Increased thermal and inflammatory pain thresholds for both normal and SNL rats	Shi et al. (2010b)
Rotation stress				
Male CBA/J mice	Rotational movement in spinning cages at 45 rpm for 10 min every hour daily for 2 weeks	Formalin test	Inflammatory hyperalgesia	Boccalon et al. (2006)
Maternal separation/Deprivation/Early life stress				
Wistar male rats[a] Sprague Dawley[b] male rats	Pups separated from mother for 180 min from days 2 to 14	Colorectal distension	Visceral hyperalgesia	Chung et al., 2007a, Chung et al., 2007b[a] and Zhang et al., 2009a, Zhang et al., 2009b, Zhang et al., 2008[b]
Long-Evans rats	Pups separated from mother for 180 min from days 2 to 14	Colorectal distension	Visceral hyperalgesia	van den Wijngaard et al. (2012), Wouters et al. (2012) and Welting et al. (2005)
Wistar male and female rats	24 h MD on PND 9	Hot Plate, von Frey, acetone test and prior to and after spinal nerve ligation	Thermal hypoalgesia, mechanical allodynia in females	Burke et al. (2013)
Sprague-Dawley rats	Mother and pups are placed in cages fitted with a stainless steel	Digital force transducer	Mechanical hyperalgesia	Alvarez et al. (2013) and Green et al.,

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
	mesh bottom on post-natal day 2–9			2011a, Green et al., 2011b
Noise stress				
Male Sprague-Dawley rats	105 dB tone of mixed frequencies, ranging from 11 to 19 kHz over 30 min over 3–4 days	Paw-withdrawal threshold	Enhanced inflammatory pain	Khasar et al., 2009, Khasar et al., 2005
Vibration stress				
Male and female Wistar rats	4 Hz applied to restraint tube for 5 min	Tail flick test	Hyperalgesia developed at 2–10 min after stress in male rats. Thermal hyperalgesia and female responding was oestrus dependent	Devall et al. (2009) and Devall and Lovick (2010)
Whisker pad stimulation				
Male Sprague-Dawley rats	Light tactile whisker pad stimulation: 10 applications/session, 4 sessions/h in 1 day, sessions on days 1–5 and 8–12	von Frey test	Mechanical hyperalgesia	Reynolds et al. (2011)
Air stress				
Male Sprague-Dawley rats	Continuous stream of air at room temperature was directed at the face for 30 min	von Frey test	Mechanical hyperalgesia	Wagner et al. (2013)
PTSD model				

Species, sex and strain	Frequency/duration	Pain test	Pain behaviour observations	Reference
Male Sprague-Dawley rats	2 h restraint, 20 min swim followed by 15 min rest, inhalation of an ether until unconscious	von Frey test and paw withdrawal to heat stimulus	Mechanical allodyina and thermal hyperalgesia	Zhang et al. (2012)
Male Sprague-Dawley rats	2 h restraint, 20 min swim followed by 15 min rest, inhalation of an ether until unconscious then footshock when conscious	Colorectal distension	Visceral hyperalgesia	He et al. (2013)