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A Single Neonatal Injury Induces Life-Long Adaptations In Stress And Pain Responsiveness

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A SINGLE NEONATAL INJURY INDUCES LIFE-LONG ADAPTATIONS IN STRESS AND PAIN
RESPONSIVENESS

by

NICOLE C. VICTORIA

Under the Direction of Anne Z. Murphy, Ph.D.

ABSTRACT

Approximately 1 in 6 infants are born prematurely each year. Typically, these infants spend 25 days in the Neonatal Intensive Care Unit (NICU) where they experience 10-18 painful and inflammatory procedures each day. Remarkably, pre-emptive analgesics and/or anesthesia are administered less than 30% of the time. Unalleviated pain during the perinatal period is associated with permanent decreases in pain sensitivity, blunted cortisol responses and high rates of neuropsychiatric disorders. To date, the mechanism(s) by which these long-term changes in stress and pain behavior occur, and whether such alterations can be prevented by appropriate analgesia at the time of injury, remains unclear. We have previously reported in rats that inflammation experienced on the day of birth permanently upregulates central opioid tone, resulting in a significant reduction in adult pain sensitivity. However, the impact on early life pain on anxiety- and stress-related behavior and HPA axis regulation is not known. Therefore the goal of this dissertation was to determine the long-term impact of a single neonatal inflammatory pain experience on adult anxiety- and stress-related responses. Neuroanatomical changes in stress-

associated neurocircuits were also examined. As the endogenous pain control system and HPA axis are in a state of exaggerated developmental plasticity early in postnatal life, and these systems work in concert to respond to noxious or aversive stimuli, this dissertation research aimed to answer the following questions: (1) Does neonatal injury produce deficits in adult stress-related behavior and alter stress-related neuroanatomy through an opioid-dependent mechanism? (2) Does neonatal injury alter receptor systems regulating the activation and termination of the stress response in adulthood? (3) Are stress- and pain-related neurotransmitters altered within the first week following early life pain? (4) Is early activation of the pain system necessary for the long-term changes in anxiety- and stress-related behavior? Together these studies demonstrate the degree, severity and preventability of the long-term deficits in stress responding associated with a *single* painful experience early in life. The goal of this research is to promote change in the treatment of infant pain in the NICU to reduce long-term sensory and mental health complications associated with prematurity.

INDEX WORDS: Hypothalamic pituitary adrenal axis, Amygdala, Lateral septum, Periaqueductal gray, Corticosterone, Glucocorticoid receptor, Corticotrophin releasing factor receptors, Endogenous opioids, Enkephalin, Endorphin, Morphine

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Georgia State University

2013

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DEDICATION

To my parents, *Peter and Kathleen Victoria*, for instilling in me the importance of education, dedication, persistence and leadership, and for your patience, love and support.

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Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning. - *Albert Einstein*

Life is either a daring adventure or nothing at all. Character cannot be developed in ease and quiet. Only through experience of trial and suffering can the soul be strengthened, vision cleared, ambition inspired, and success achieved. - *Helen Keller*

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1 CHAPTER ONE: INTRODUCTORY OVERVIEW

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1.1 Premature Birth And Pain Treatment In The Neonatal Intensive Care Unit

Premature Birth

Each year, 16.5% of infants worldwide and 12% of infants in the United States are born prior to 37 gestational weeks and are considered preterm (Martin et al., 2006). The etiologies underlying preterm birth are complex and not completely understood, but risk factors include maternal diabetes, hypertension, smoking, prenatal substance use, lack of prenatal care and assisted reproductive therapies (PeriStats, 2011).

Following birth, the majority of premature infants are admitted into the Neonatal Intensive Care Unit (NICU), where they spend an average of 25 days (PeriStats, 2011). During their stay in the NICU, preterm infants undergo 10-18 invasive procedures each day, including repeated heel lance, endotracheal intubation, surgery, and respiratory and gastric suctioning, (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008; PeriStats, 2011). Despite the fact that the majority of these procedures produce pain and inflammation, less than 35% of NICU patients receive pre- and/or post-emptive analgesia or anesthesia (Simons et al., 2003). As this treatment occurs during a period of sensitive developmental plasticity, serious concerns from parents, practitioners and researchers have surrounded the immediate and long-term consequences of these NICU practices (Anand et al., 1987b; Carbajal et al., 2008; McGrath, 2011; Rodkey and Pillai Riddell, 2013).

Modern Absence Of Pain Treatment Early In Life And Historical Origins

The rationale for withholding pain treatment is complex and multifaceted. Historically, infants and children were considered as lower 'castes' of people, unable to engage in sensory processing beyond the brainstem and thalamus, and lacking the cognitive capacity to remember early life events (Rodkey and Pillai Riddell, 2013). With this assumption, investigations of the 19th and 20th centuries interpreted infant responses to surgery, pin-pricks or electric shock as reflexive or non-specific to the procedures (Rodkey and Pillai Riddell, 2013). These perspectives guided medical training and practices, and by the 21st century, respiratory support and paralytics were deemed sufficient for preterm infants undergoing surgery (Wesson, 1982). The validity and ethics of such practices were strongly called into question in the 1980s (Anand et al., 1987a; Purcell-Jones et al., 1988). However, issues of reliable pain assessment, age-appropriate dosing, opioid tolerance and long-term consequences associated with pharmacological intervention became points of concern that continue to hinder neonatal therapies for pain (Anand and Hickey, 1987; Anand et al., 1987a; Anand, 2000; Anand et al., 2005b; Anand et al., 2005a; Qiu, 2006b; Cignacco et al., 2009; Bellieni, 2012).

Evidence over the last 30 years has demonstrated that, indeed, premature and term infants can discriminate noxious stimuli. NICU procedures induce robust secretion of stress hormones (Anand et al., 1987b), elevated heart rate and facial reactivity (Grunau et al., 2005; Grunau et al., 2010). Further, preterm infants as young as 25 gestational weeks display evoked cortical activity in response to noxious stimulation (Bartocci et al., 2006; Slater et al., 2006). While insufficient and sporadic administration of pain therapy persists in the modern NICU (Carbajal et al., 2008), the vast majority of modern pediatric physicians acknowledge that preterm infants feel pain (Purcell-Jones et al., 1988).

Efficacy Of Neonatal Analgesia

A number of clinical studies have demonstrated the benefit of acute opioid analgesia for infants undergoing invasive procedures in the NICU. For example, administration of opioid analgesics significantly decreases cortisol, norepinephrine, epinephrine and β -endorphin release, decreases sepsis and prevents death, both operatively and post-operatively in comparison to controls (Anand et al., 1987b; Anand and Hickey, 1992). Morphine administration before endotracheal suctioning, central venous catheterization or heel lance reduces blood flow to the skin and decreases facial responses to procedural pain (McCulloch et al., 1995; Moustogiannis et al., 1996; Scott et al., 1999). These studies together suggest that specific and appropriate analgesia has immediate antinociceptive benefits for preterm infants.

Despite the effectiveness of opioid analgesics for infant pain, concerns of tolerance, dependence and side effects such as bradycardia, hypotension, apnea, urinary retention and reduced gastrointestinal motility (Anand et al., 2011) make their use controversial. A number of studies have aimed to test the long-term impact of opioid analgesia in the NICU. However, the majority of these efforts have been challenged by small sample sizes, inclusion of infants with illnesses such as hypotension or pre-existing neurological impairment, or dosing that is age-inappropriate for the infant (MacGregor et al., 1998; Bouwmeester et al., 2001; Anand et al., 2004; Roze et al., 2008; de Graaf et al., 2011). Therefore, it is unclear what, if any, long-term consequences are associated with early life opioid analgesia. As a precaution, the International Association for the Study of Pain (IASP) currently recommends judicious use of morphine and its derivatives, and slow-speed infusions of fentanyl and other potent synthetic opioids for premature and term infants for moderate to severe procedural pain, pre-operative sedation, surgical pain and post-operative care (Anand et al., 2011). While caution is prudent, it is important to note a recent follow-up study reported that former preterm infants at 8-9 years old who received morphine in the NICU for pain management had improved executive functioning and reduced problems with externalization relative to infants that received placebo (de Graaf et al., 2013).

Non-opioid analgesics have also been shown to be effective in infants and are recommended for procedural pain. For example, topical analgesics, such as tetracaine gel (4%) or lidocaine cream (5%), decrease composite pain profiles in response to venipuncture (Gradin et al., 2002) or intravenous cannulations (Moore, 2001). Lidocaine before circumcision decreases facial reactivity, duration of crying, heart rate and O₂ saturation (Benini et al., 1993; Lander et al., 1997; Woodman, 1999; Taddio et al., 2000). By contrast, acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) have limited use in infants due to hepatic and/or renal toxicity, hypothermia, platelet dysfunction and gastrointestinal bleeding (Anand et al., 2011).

A number of studies have examined oral sucrose for procedural pain relief in infants with varying outcomes. Slater et al. (2010) reported that 24% oral sucrose does not attenuate the nociceptive-specific brain, spinal cord and limb flexion activity elicited by heel stick (Slater et al., 2010). However, a Cochrane Review, which systematically reviewed primary research in human health care and health policy between 1950 and 2011, found 24% oral sucrose or 30% glucose safe and effective for reducing pain associated with skin breaking procedures in preterm and term neonates (Anand et al., 2011; Stevens et al., 2013). In particular, oral sucrose administration before heel lance or assessment of retinopathy decreases total duration of crying, grimacing and O₂ saturation, suggesting that sucrose confers analgesia and reduces physical discomfort to the infant (Stevens et al., 2013).

Non-pharmacological options are also effective for managing pain in infants. Physical analgesia, such as skin-to-skin contact, gentle massage or non-nutritive sucking (Corbo et al., 2000; Jain et al., 2006; Golianu et al., 2007), reduces crying, grimacing and heart rate during heel-stick for preterm infants as young as 28 gestational weeks (Gray et al., 2000; Johnston et al., 2003; Johnston et al., 2008). Breastfeeding during heel lance or venipuncture confers analgesia in term infants (Carbajal et al., 2003; Shah et al., 2012), with a recent study indicating it is also effective for reducing pain during acute procedures in preterm infants between 32-36 gestational weeks (Simonse et al., 2012). Combination therapies that involve the use of physical

analgesia and sensory saturation with gentle tactile, auditory, visual, olfactory, gustatory and vestibular stimuli are also recommended (Bellieni et al., 2001; Bellieni et al., 2007). Whether such therapies alleviate nociceptive signals and stress hormone release associated with procedural pain, or simply distract the infant from the painful intervention, remains to be systematically investigated (Cignacco et al., 2009).

1.2 Early-Life Pain Impairs Stress And Pain Responding

Clinical Studies

Clinical studies show early life pain has an immediate impact on response to stress- and pain-evoking stimuli. For example, preterm infants undergoing surgical procedures without analgesic treatment have significantly higher concentrations of catecholamines and cortisol during and after surgery as compared with infants receiving analgesia (Anand et al., 1987b). Heart rate, facial reactivity and cortisol levels of preterm infants are initially high in response to procedural pain. However, these behavioral, neuroendocrine and autonomic responses become significantly blunted as the number of invasive procedures experienced in the NICU increases (Grunau et al., 2005; Grunau et al., 2010). This suggests that repeated, unalleviated pain results in immediate changes or 'adaptations' in systems mediating pain and stress that may become permanent.

At 4 and 8 months, former preterm infants display decreased facial responsiveness to immunization pain in comparison to full-term peers (Oberlander et al., 2000). Toddlers born prematurely into the NICU exhibit blunted nociceptive responses and are rated by parents as less sensitive to pain in comparison to term-born controls (Grunau et al., 1994a). At 9-12 years of age, children that experienced infant cardiac surgery with limited pain therapy display global alterations in both mechanical and thermal somatosensory processing (Schmelzle-Lubiecki et al., 2007). Further, adolescence and teenagers that spent time in the NICU as infants are less

sensitive to thermal pain (Hermann et al., 2006; Walker et al., 2009b) and display attenuated stress-induced analgesia (Wollgarten-Hadamek et al., 2011) in comparison to controls.

A number of studies have now associated NICU treatment with long-term changes in autonomic and cortisol reactivity. For example, basal and immunization pain-induced cortisol release is blunted at 3 and 4 months, respectively, for former preterm infants as compared with term controls (Grunau et al., 2007; Grunau et al., 2010). At 6 months, tighter coordination of stress-related cortisol, heart rate and vagal tone is observed, suggesting an altered autonomic response pattern relative to term peers (Haley et al., 2010). As physiological changes in stress responding are associated with disorders of anxiety, depression, obsessive compulsion, panic and post-traumatic stress (Heim et al., 2000; Heim et al., 2001; Chrousos, 2009), such findings indicate that preterm infants are at higher risk for developing later-life changes in affective functioning.

Indeed, altered cortisol reactivity for former preterm infants at age five is significantly associated with issues of internalization, emotional reactivity, anxiety, depression, inattention, and higher rates of negative verbalization during mother-child interactions (Bagner et al., 2010). By middle school, these children are at least 28% more likely than term peers to have clinical symptoms of anxiety, depression and inattention (Botting et al., 1997; Hayes and Sharif, 2009). Psychological social-stress testing evokes blunted cortisol responses in 8-14 yr old former preterm infants relative to age- and gender-matched term controls (Buske-Kirschbaum et al., 2007). Lastly, parents and teachers of former NICU patients report significantly higher rates of neuro-behavioral and neuropsychiatric impairments at 20 years of age relative to term peers, including issues with internalizing and externalizing, reduced cognitive and behavioral flexibility, and higher rates of anxiety and depression (Hack et al., 2004; Aarnoudse-Moens et al., 2009; Hayes and Sharif, 2009; Sullivan et al., 2012).

Early life pain is also associated with changes in brain development and later-life functioning. For example, magnetic resonance imaging spectroscopy and diffusion tensor imaging of

preterm infants at 32 and 40 gestational weeks shows significant decreases in white and gray matter maturation that is positively correlated with the number of skin breaking procedures experienced (Brummelte et al., 2012). Pain-related stress in the NICU is associated with reduced spontaneous cortical gamma-to-alpha ratio oscillations during perceptual reasoning in childhood at 7-8 yrs of age that is independent of illness severity, days on mechanical ventilation, cumulative morphine exposure and general intelligence, (Doesburg et al., 2013). Notably, gamma and alpha band activity are thought to occur during active recruitment of brain regions for perception (Jensen et al., 2007; Doesburg et al., 2008) and resting state (Pfurtscheller et al., 1996; Klimesch et al., 2007; Doesburg et al., 2013), respectively. As alterations in gamma band oscillations are significantly correlated with neurological and psychiatric disorders (Uhlhaas and Singer, 2006), these findings further suggest that early life pain represents a significant risk factor for later-life pathology.

Animal Studies

Animal models of early life pain support clinical findings demonstrating a long-term impact on subsequent responses to pain, physiological markers of stress and brain development. Acute or repeated exposure to early life pain induced by foot shock, surgery, or inflammatory agents results in general thermal or mechanical hypoalgesia for adult rodents (Shimada et al., 1990; Bhutta et al., 2001; Sternberg et al., 2005; LaPrairie and Murphy, 2007, 2009). Consistent with clinical findings showing blunted stress hormones in former preterm infants, early life inflammatory pain in male rats reduces adult release of stress hormones, corticotrophin releasing factor (CRF), arginine vasopressin (VAS) and adrenocorticotrophin releasing hormone (ACTH) following acute swim stress (Anseloni et al., 2005). Anatomically, early life pain results in cortical thinning and increases the number of apoptotic cells throughout the brain, specifically in the cortex, septum, hypothalamus and hippocampus. Reduced expression of cortical and thalamic proteins that control neuronal differentiation, migration and synaptic connections have also been

reported (Duhrsen et al., 2013). Notably, administration of morphine before early life injury prevents long-term reductions in pain sensitivity and alterations in brain development (LaPrairie et al., 2008; Duhrsen et al., 2013). While the mechanisms underlying these long-term changes in pain and stress responding and brain development are not well understood, recent evidence suggests that decreased sensitivity to pain later in life results from upregulated endogenous opioid tone in key brain regions mediating pain (LaPrairie and Murphy, 2009). As endogenous opioids have organizational effects on the developing brain (Zagon and McLaughlin, 1983, 1991) and contribute to the perception of stress (Akil et al., 1984), it is possible that the injury-induced increase in opioid tone underlies the long-term reduction in stress sensitivity as well.

1.3 The Nociceptive System Early In Life

Over the last several decades, animal studies have allowed for examination of the nociceptive system and its development (Fitzgerald, 2005). In response to noxious/tissue damaging stimulation, nociceptive information ascends via the spinal cord for processing in the brain. In turn, nociceptive sensations are dampened through descending pain modulatory circuits that include the midbrain periaqueductal gray (PAG), rostral ventromedial medulla (RVM) and spinal cord dorsal horn (DH) (Basbaum and Fields, 1978, 1984).

Neonates are significantly more sensitive to thermal and mechanical noxious stimulation in comparison to adults (Grunau et al., 2005; Hathway et al., 2012). Early in life, neurons of the DH are highly excitable, have large receptive fields, and noxious stimulation evokes prolonged action potentials of high amplitude (Fitzgerald, 2005). In addition, the neonatal DH is heavily innervated with myelinated A δ fibers that transmit phasic nociceptive signals and a lower frequency of mIPSCs in comparison to adults (Fitzgerald, 2005).

Over the first 2-3 postnatal weeks, the balance between excitation and inhibition develops to result in adult-like descending inhibition of pain (Hathway et al., 2009). Inhibitory responses to electrical stimulation of the dorsal lateral funiculus, which connects supraspinal and

spinal sites of pain modulation, emerge by postnatal day 6 (P6) (Fitzgerald and Koltzenburg, 1986). Between P3-P21, electrical stimulation of the PAG (van Praag and Frenk, 1991) or RVM (Hathway et al., 2009) of rat pups facilitates nociceptive reflexes in the hindlimbs and promotes neuronal firing in the DH. The opposite effects are observed by P40 (Hathway et al., 2009), indicating that early in life nociceptive sensitivity is high and becomes attenuated with age.

Paradoxically, morphine administration provides analgesia for rat pups given hindpaw inflammation between P1-P21 (Abbott and Guy, 1995; Gupta et al., 2001), suggesting that electrophysiological and EMG stimulation studies reveal only a partial profile for development of descending modulation. Indeed, the use of anesthesia for surgical implantation of electrodes (Fitzgerald and Koltzenburg, 1986), may tip the balance of descending modulation in favor of the excitatory state in the immature antinociceptive circuit.

Both, enkephalin and β -endorphin, key neuropeptides in the endogenous descending pain modulatory circuit, are present in the brain at birth, as are their receptors (Tsang and Ng, 1980; Tsang et al., 1982; Rius et al., 1991). Injection of the μ - or δ -opioid receptor agonists, DAMGO or DPDPE respectively, into the ventral PAG or RVM results in thermal analgesia for rat pups on P3 (Barr and Wang, 2013). Further, morphine injected into the ventrolateral PAG (vIPAG) of rat pups produces analgesia that is naloxone-reversible on P3, P10 and P14 (Tive and Barr, 1992).

Recent studies show activation of the endogenous opioid system is essential for development of the descending pain modulatory circuit. For example, subcutaneous morphine administration from P7-P14 accelerates normal development of antinociception, as electrical stimulation of the RVM decreases reflex excitability in rat pups to mimic adult-like responses (Hathway et al., 2012). In contrast, blockade of endogenous opioid signaling early in life delays nociceptive circuit development (Hathway et al., 2012). Indeed, such findings suggest that specific activation of the endogenous opioid system early in life is required to produce analgesia before the balance of excitation and inhibition are established at adult levels.

1.4 The Stress System Early In Life

The hypothalamic-pituitary-adrenal (HPA) axis is used by the nervous system to mount physiological responses to stressors, promote survival in the presence of physical threats, and mediate psychological perturbations successfully, with the ultimate goal of reinstating homeostasis. In response to stressors, a variety of forebrain and brainstem regions are recruited to stimulate the release of CRF from the paraventricular nucleus of the hypothalamus (PVN) (Vale et al., 1981). Through the hypophyseal portal system, CRF stimulates the anterior pituitary gland to release ACTH (Dallman et al., 1987). In turn, ACTH acts on the cortex of the adrenal gland to promote release of glucocorticoids (cortisol in humans; corticosterone in rats: CORT)(Dallman and Jones, 1973; Guillemin et al., 1977). CORT then feeds up to the pituitary and PVN to terminate further release of neurohormones, and to the hippocampus where binding to the glucocorticoid receptor (GR) reinstates inhibition of the PVN (Sapolsky et al., 1984a; Dallman et al., 1987; Ulrich-Lai and Herman, 2009).

All components of the HPA axis are functional and interfaced by mid-gestation for humans and rodents (Kandel et al., 2000). In rodents, stressors such as ether or laparotomy stimulate the release of fetal CRF (Hiroshige and Sato, 1971) and CORT (Negellen-Perchellet and Cohen, 1975), indicating stress has specific effects on HPA activity during gestation. At birth, adrenal gland weight and circulating CORT concentrations are elevated (Corbier and Roffi, 1978b). Over the first postnatal week, CORT decreases to nearly detectable levels to initiate the stress hypo-responsive period (SHRP), which spans approximately P2-P14 in rat pups (Sapolsky and Meaney, 1986; Walker et al., 1986); for humans a similar process occurs over the first months of life (Mantagos et al., 1998; Grunau et al., 2007). Although stressors such as ether, electric shock and hypoxia can activate the HPA axis, this period of adrenal quiescence allows glucocorticoid levels to remain low and promote neurogenesis, axonal outgrowth, synaptogenesis, myelination, and rise of endogenous CRF and ACTH levels (Sapolsky and Meaney,

1986; Walker et al., 1986; Baud et al., 2005) (Antonow-Schlorke et al., 2009; Du et al., 2009; Liston and Gan, 2011).

HPA axis dysfunction and the manifestation of neuropsychiatric disorders have been significantly associated with exposure to trauma early in life (Heim et al., 2001; Heim et al., 2008). As such, the influence of early life perturbation on later-life outcomes has been studied extensively in rodents. These studies have revealed that the type of stress (e.g. acute, chronic, mild, severe, homotypic, heterotypic), developmental stage of presentation (e.g. prenatal, postnatal, peripubertal), and in some cases the sex of the offspring, programs the HPA axis to be hyper- or hypo-responsive. For example, male but not female, offspring exposed to mild chronic variable stress during the first week of gestation show adult increases in CRF expression, stress-induced CORT and an increase in depression-related behaviors in both the forced swim (FST) and tail suspension tests (Mueller and Bale, 2008). Hyperactivity of the HPA axis is also observed in adult rats given the corticosteroid dexamethasone chronically during the last week of gestation (Shoener et al., 2006). In contrast, chronic restraint stress from gestational day 14-21 increases CRF expression and CORT output for adult females, whereas CRF expression decreases, ACTH increases, and no change in CORT is observed in males (Garcia-Caceres et al., 2010). Postnatal stress on P3 in the form of acute peripheral inflammation significantly decreases adult anxiety- and depression-related behaviors in the elevated plus maze (EPM) and FST, respectively, and blunts stress-induced CRF and ACTH release (Anseloni et al., 2005), suggesting HPA hyposensitivity. In contrast, chronic reduction of maternal resources for nest building on P2-P9 significantly increases basal CORT for offspring as adults (Rice et al., 2008). In response to mild peripubertal stress from P27-29, adult male and female rats show increases in CORT, and decreases in the number of central entries in the Open Field (OF) and time in the open arms of the EPM (Jacobson-Pick and Richter-Levin, 2010). In a model of chronic juvenile social subjugation, similar effects occur primarily in adult females (Weathington et al., 2012). Although the mechanisms underlying the maintenance of these changes in gene expression

and behavior are not completely understood, modification to the methylation and histone profiles of CRF and GR system genes are commonly observed in adult offspring exposed to early life stress (Weaver et al., 2004; Mueller and Bale; Elliott et al., 2010; Rodgers et al., 2013). In addition, epigenetic influence of miRNAs on the embryonic germ line have sex-specific, transgenerational effects on adult stress-related profiles (Morgan and Bale, 2011, 2012; Rodgers et al., 2013), suggesting that production of sexually dimorphic phenotypes is more complex than organizational influence of sex steroids (Carruth et al., 2002; Konkle and McCarthy, 2011; McCarthy et al., 2012).

1.5 Interaction Between Systems Mediating Pain And Stress Responding

Notably, the endogenous opioid system works in concert with classic systems regulating HPA axis activity. For example, CRF and CRF receptors (CRFR) co-localize and co-express with endogenous opioids in the neurosecretory hypothalamus, thalamus, septum, hippocampus, amygdala, locus coeruleus, cortex, PAG and DH (Rivalland et al., 2005; Mousa et al., 2007) (Sakanaka and Magari, 1989; Larsen and Mau, 1994; Chalmers et al., 1995; Dumont et al., 2000; Marchant et al., 2007). Acute or chronic stressors such as restraint, inflammatory pain, or osmotic distress simultaneously increase hypothalamic expression and release of CRF and enkephalin, and circulating β -endorphin, ACTH and CORT (Guillemin et al., 1977; Lightman and Young, 1989; Shippenberg et al., 1991; Taylor et al., 1998). Dexamethasone or CORT completely blocks stress-induced increases in CRF, preproenkephalin and proopiomelanocortin (POMC) mRNA (Beaulieu et al., 1988; Harbuz and Lightman, 1989), and GR transcriptionally regulates expression of both the endogenous opioid and CRFR systems (Schoneveld et al., 2004). Together these data suggest that the stress system recruits and regulates endogenous opioids to aid in the response to homeostatic perturbations. As enkephalin or morphine administration significantly reduces cortisol concentrations (McDonald et al., 1959; Stubbs et al., 1978), and opioid receptor antagonists naloxone or naltrexone, increases plasma β -endorphin,

ACTH and cortisol (Wand and Schumann, 1998; al'Absi et al., 2004), such findings support the role of opioids in reducing stress reactivity.

While the CRFR system is well known for regulating responses to anxiety and stress (Smith et al., 1998; Bale et al., 2000; Coste et al., 2000; Weaver et al., 2004), it participates in antinociception as well. For example, administration of CRF in either humans or rodents stimulates the release of β -endorphin from the anterior pituitary to produce analgesia in response to noxious thermal heat (Hargreaves et al., 1987; Hargreaves et al., 1990). Adrenalectomy does not abolish CRF antinociception, indicating that analgesia results from central rather than peripheral release of CRF (Vit et al 2006). In addition, dexamethasone prevents analgesia produced by DAMGO or β -endorphin (icv) in response to hot plate or tail flick testing (Pieretti et al., 1994), suggesting that endogenous glucocorticoids can regulate analgesia.

Enkephalin and endorphin dampen responses to anxiety and stress-related behavior (Akil et al., 1984). For example, pharmacological activation of μ - or δ -opioid receptors, to which enkephalin and endorphin bind, significantly reduces fear-potentiated startle (Glover and Davis, 2008) and decreases stress-induced anxiety in the EPM (Randall-Thompson et al., 2010). In contrast, blockade of endogenous opioid signaling through these receptors with naltrexone decreases activity in the center of the OF (de Cabo de la Vega et al., 1995). In addition, prepro-enkephalin knockout mice display reduced time in the open area of the light-dark test, decreased entries and time spent in the inner area of the OF and increased startle amplitudes relative to wild-types. (Konig et al., 1996; Bilkei-Gorzo et al., 2008; Kung et al., 2010). Significant reductions in basal CORT and prolonged recovery time from stress are also observed in these mice (Bilkei-Gorzo et al., 2008), suggesting enkephalin is an important regulator for neuroendocrine response and recovery from stress. Similar to mice lacking enkephalin, β -endorphin knockout mice show decreases in total time and percent entries into the open arm of the EPM (Grisel et al 2008). However, loss of β -endorphin increases stress-induced ACTH, blunts basal and peak stress-induced CORT while maintaining CORT recovery time similarly to wild-types

(Bilkei-Gorzo et al., 2008). Together these data suggest that although enkephalin and endorphin reduce anxiety, they have independent and unique rolls in regulating stress hormone responses.

In contrast to enkephalin and β -endorphin, dynorphin promotes anxiety and stress through binding to the κ -opioid receptor. For example, activation of κ -opioid receptor with U-50488H significantly decreases time in the center of the OF and percent time in the open arms of the EPM for mice (Wittmann et al., 2009). In contrast, anxiogenic effects are reversed by kappa antagonist nor-Binaltorphimine (Wittmann et al., 2009). Similarly, loss of prodynorphin increases time in the center of the OF, time in the open arms of the EPM and immobility in the tail suspension test (Wittmann et al., 2009; Kastenberger et al., 2012). Prodynorphin knockout mice show accelerated CORT peak following stress, yet negative feedback is prolonged in comparison to wild-type animals (Bilkei-Gorzo et al., 2008). These data suggest that dynorphin promotes anxiety, stress and has a specific role in stress hormone regulation. Collectively, the above findings suggest interplay between pain and stress systems, such that neuropeptides of the stress system participate in analgesia, whereas endogenous opioids dampen or increase sensitivity to anxiety and stress.

1.6 Dissertation Goals

Clinical findings indicate that early life exposure to repetitive pain, and therefore stress, in the NICU results in polysystemic adaptations. Former preterm infants have decreased sensitivity to pain, blunted cortisol reactivity, altered autonomic coordination, differential gray and white matter development, differential perceptual processing and experience neurobehavioral and psychiatric disorders at significantly higher rates than full-term peers. These issues emerge early in the NICU and persist in children, teens and young adults, indicating that adaptations associated with neonatal pain-related stress are permanent. The mechanism(s) by which these long-term changes in stress and pain behavior and physiology occur in humans are not known.

Further, whether such alterations can be prevented by appropriate analgesia at the time of injury remains to be investigated.

Animal studies indicate that permanent upregulation of endogenous opioids result from early life injury and are necessary for hypoalgesia in adult rats (LaPrairie and Murphy, 2007, 2009). In contrast, mechanisms whereby long-term changes in anxiety- and stress-related behavior and HPA axis regulation occur as a result of early life pain remain unclear. Therefore the goal of this dissertation was to determine the long-term impact of a single neonatal inflammatory pain-experience on adult stress-related responses and neuroanatomy. As the endogenous pain control system and HPA axis are in a state of exaggerated developmental plasticity early in postnatal life, and these systems work in concert to respond to noxious or aversive stimuli, we hypothesized that (1) a *single* injury on the day of birth produces permanent adaptations in stress-, anxiety- and pain-related responses; (2) early life injury alters neurotransmitter circuits underlying responses to noxious and aversive stimuli; and (3) as males and females often respond differently to stress, anxiety and pain, at least some of the long-term adaptations resulting from early pain are sexually dimorphic. This dissertation research tested these hypotheses via the following questions: (1) Does neonatal injury produce deficits in adult stress-related behavior and alter stress-related neuroanatomy through an opioid-dependent mechanism? (2) Does neonatal injury alter receptor systems regulating the activation and termination of the stress response in adulthood? (3) Are stress- and pain-related neurohormones altered within the first week following early life pain? (4) Is early activation of the pain system necessary for the long-term changes in anxiety and stress-related behavior?

Together these studies demonstrate the degree, severity and preventability of the long-term deficits in stress responding associated with a single painful experience early in life. The clinical goal of this research is to promote change in the treatment of pain in the NICU to reduce long-term sensory and mental health complications associated with prematurity.

2 CHAPTER TWO: A SINGLE NEONATAL INJURY INDUCES LIFE-LONG DEFICITS IN RESPONSE TO STRESS

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2.1 Abstract

Approximately 500,000 infants are born prematurely each year in the United States. These infants typically require an extensive stay in the Neonatal Intensive Care Unit (NICU), where they experience on average 14 painful and invasive procedures each day. These procedures, including repeated heel lance, insertion of intravenous lines, and respiratory and gastric suctioning, typically result in an inflammatory response, inducing pain and stress in the newborn. Remarkably, the majority of these procedures are performed in the complete absence of pre- or

post-emptive analgesics. Recent clinical studies report that former NICU patients have increased thresholds for pain and stress later in life as compared with term born infants. However to date, the mechanisms whereby early life inflammation alters later life response to stress and pain are not known. The present studies were conducted to determine if neonatal injury impairs adult responses to anxiety- and stress-provoking stimuli. As we have previously reported that early life pain results in a significant increase in opioid peptide expression within the midbrain periaqueductal gray, the role of endogenous opioids in our behavioral studies was also examined. Male and female rats received an intraplantar injection of the inflammatory agent carrageenan (1%) on the day of birth (P0). In adulthood, animals were assessed for changes in response to anxiety- and stress-provoking stimuli using the Open Field and Forced Swim Tests, respectively. Injury-induced changes in sucrose preference and stress-induced analgesia were also assessed. As adults, neonatally injured animals displayed a blunted response to both anxiety- and stress-provoking stimuli, as indicated by significantly more time spent in the inner area of the Open Field and a 2-fold increase in latency to immobility in the Forced Swim Test as compared to controls. No change in sucrose preference was observed. Using *in situ* hybridization and immunohistochemistry, we observed a 2-fold increase in enkephalin mRNA and protein expression, respectively, in stress-related brain regions including the central amygdala and lateral septum. Administration of the opioid receptor antagonist naloxone reversed the attenuated responses to forced swim stress and stress-induced analgesia, suggesting the changes in stress-related behavior were opioid-dependent. Together, these data contribute to mounting evidence that neonatal injury in the absence of analgesics has adverse effects that are both long-term and polysystemic.

2.2 Introduction

Premature birth, defined as birth prior to 37 weeks of gestation, occurs at alarmingly high rates worldwide. According to the World Health Organization, 16.5% of all infants are born prema-

ture, with over 500,000 preterm babies born each year in the United States alone (Martin et al., 2006). Preterm infants spend, on average, 25 days in the Neonatal Intensive Care Unit (NICU), where they undergo 10-18 invasive procedures each day, including repeated heel lance, endotracheal intubation, surgery, and respiratory and gastric suctioning (www.marchofdimmes.com/peristats/) (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008). Despite strong evidence that pain and stress circuitry are established and functional in preterm infants, 65% of these procedures are performed in the complete absence of analgesics (Anand et al., 1987b; Simons et al., 2003; Grunau et al., 2005; Slater et al., 2006; Carbajal et al., 2008). Unfortunately, it is becoming increasingly clear that pain experienced during the critical perinatal developmental period has long-lasting effects on adult responses to pain-, anxiety- and stress-provoking stimuli (Hack et al., 2004; Hermann et al., 2006; Wollgarten-Hadamek et al., 2011).

Clinical studies report that children with prior NICU experience display decreases in pain and stress sensitivity that persist long after discharge from the NICU (Hermann et al., 2006; Grunau et al., 2007). For example, former preterm infants have significantly decreased nociceptive sensitivity and blunted cortisol responses before and after immunization pain as compared with full term controls (Grunau et al., 2005; Hermann et al., 2006; Walker et al., 2009b; Grunau et al., 2010). In middle school, former preterm infants are at least 28% more likely to suffer from disorders of externalization and internalization (Botting et al., 1997; Hayes and Sharif, 2009). At 20 years of age, parents and teachers of former NICU patients report significantly higher rates of neurobehavioral impairments relative to control counterparts (Levy-Shiff et al., 1994; Hack et al., 2004; Hayes and Sharif, 2009; Sullivan et al., 2012). The mechanism(s) by which these long-term changes in pain and stress behavior occur in humans are not known.

While previous studies in rodents have reported decreased pain sensitivity following early life injury, its impact on adult stress responsiveness is not known (Shimada et al., 1990; Bhutta et al., 2001; Ren et al., 2004; Sternberg et al., 2005; LaPrairie and Murphy, 2007).

Therefore, the present studies were conducted to determine whether a single inflammatory insult on the day of birth impairs adult male and female responses to anxiety- and stress-provoking stimuli. We have previously reported that a *single* inflammatory insult (1% carrageenan; hind paw) administered on the day of birth (P0) significantly increases leu- and met-enkephalin protein levels in the ventrolateral periaqueductal gray (vlPAG) of adult rats (LaPrairie and Murphy, 2009). Indeed, in both male and female rats, a 180% increase in met-enkephalin protein levels was observed. As enkephalin has been implicated previously in responses to anxiety and stress (Lightman and Young, 1987; Konig et al., 1996; Bilkei-Gorzo et al., 2008), the role of opioid peptides in mediating the effects of early life pain was examined.

Using behavioral pharmacology, *in situ* hybridization and immunohistochemistry, we present evidence for the first time that one inflammatory insult experienced on the day of birth attenuates adult responses to stress through an opioid-dependent mechanism. Parallel changes in adult proenkephalin mRNA and met-enkephalin protein expression were observed in the central amygdala, lateral septum and vlPAG, regions previously implicated in pain, anxiety- and stress-related behaviors (Rizvi et al., 1991; Franco and Prado, 1996; Hunt and Mantyh, 2001; Ulrich-Lai and Herman, 2009; Herman, 2010).

2.3 Materials And Methods

Animals

Pregnant Sprague-Dawley rat dams were obtained on gestational day 14 (G14) (Charles River). Dams were housed individually under 12:12 hr light:dark cycle with *ad libitum* access to food and water. On the day of birth (P0), pups were sexed by examination of anogenital distance and subjected to neonatal treatment. All litters were reared identically, weaned on P21 and housed with same sex littermates in groups of 2-3. Male and female rats were used in all experiments and tested on separate days (LaPrairie and Murphy, 2007). All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP, and were

approved by the Georgia State University Animal Care and Use Committee. Behavior experiments were conducted during the light phase (10:00-17:00), animal order was randomized and the experimenter was blinded to neonatal treatment. All experiments were conducted with different cohorts of neonatally injured and handled adults. To ensure participation in previous behavioral experiments did not influence enkephalin expression, separate cohorts of animals were used for the anatomical studies.

Neonatal Treatment

Acute neonatal inflammatory injury was induced as in our previous studies (LaPrairie and Murphy, 2007, 2009). Briefly, male and female rat pups were injected with 5 μ L Carrageenan (1% dissolved in saline; Sigma, USA) into the intraplantar surface of the right hind paw or handled identically within 24 hours of birth on P0. This well-established model causes acute, local inflammatory pain that persists for 24-72 hours and does not alter maternal behavior (Ren et al., 2004; LaPrairie and Murphy, 2007). Intraplantar saline control was not used, as we noted in our previous studies that this results in 24-48 hours of inflammation (LaPrairie and Murphy, 2007). Pups were separated from their dam for <20 minutes and returned to the home cage as a group. Each litter received a single treatment. Animals are largely undisturbed until adulthood (P60).

Test Of Anxiety-Like Behavior

Adult anxiety-like behavior was assessed using the Open Field (OF), a well-established test sensitive to detecting the effects of early life manipulations on anxiety (Joffe et al., 1973). Animals (P65-70; n = 6-8/treatment/sex) were habituated to the testing room daily for 60 minutes, 3 days before and on the day of testing. Adults were gently placed in the OF (gridded Plexiglas box 120 cm x 120 cm x 30 cm) facing the same direction. Testing occurred under red light. Each animal experienced the OF one time for 5 minutes. Behaviors were recorded digitally

with Noldus Observer 5.0 (Noldus, USA) and observed remotely with a video monitor. The testing apparatus was cleaned thoroughly with 70% ETOH between each animal; vapors were allowed to evaporate completely before the next session commenced. Scoring of anxiogenic (OF: duration in outer perimeter), anxiolytic behaviors (duration in inner area) and locomotor behavior (number of lines crossed) occurred post-hoc by an experimenter blinded to neonatal treatment. Data were expressed as duration or frequency.

Tests Of Stress-Related Behavior

Adult stress-related behavior was assessed with the modified Forced Swim Test (FST), Sucrose Preference Test (SPT) and Stress-Induced Analgesia (SIA) test. Animals (P70-90) were habituated to the testing room daily for 60 minutes, 3 days before and on the day of testing. For SIA testing, animals were habituated to the Paw Thermal Stimulator daily for 60 minutes 3 days before and on the day of testing. To avoid any carryover effects, different cohorts of neonatally injured and handled adults were used for each test (FST, SPT, SIA).

Forced Swim Test

Water was maintained at 25°C and filled to height such that animals could neither escape nor could the tail touch the bottom (63.5 cm) (Porsolt et al., 1977; Porsolt et al., 1978). On day one of the FST, adults (n = 8-9/treatment/sex) were placed in a circular swim tank (71.2 cm x 62.5 cm x 56 cm) for 5 minute pre-swim to elicit “behavioral despair”(Armario et al., 1988). On day two, animal were placed in the swim tank for a 5-minute FST; all behaviors were digitally recorded. Following the FST, animals were dried with a clean towel and placed in clean cages. Fecal boli were counted and removed from the tank between each test. The tank was cleaned with detergent and ETOH between tests. The following behaviors were scored post-hoc: (1) latency to immobility (LTI), defined as the first cessation of swimming with arched-back floating (Porsolt et al., 1978); (2) duration of immobility, characterized by arched-backed floating and

movement only necessary to keep the head above water or prevent drowning (Porsolt et al., 1978). A separate cohort of animals ($n = 8-9/\text{treatment}/\text{sex}$) received naloxone HCl (1 mg/kg or 5 mg/kg; i.p; Sigma, USA) dissolved in saline (0.9%) or equivolume saline 15 minutes before testing. Data are expressed as frequency and duration.

Sucrose Preference Test

Animals ($n = 9-16/\text{treatment}/\text{sex}$) were singly housed for 7 days prior to testing. Separate water bottles were filled with 500 mL of tap water or sucrose solution (1%), weighed and placed in the cage in randomized order to control against place preference. During the 48-hour test, rats had *ad libitum* access to both bottles. After the first 24 hours, bottles were removed and immediately replaced with new bottles oriented in the opposite order. After the second 24-hour period elapsed, bottles were removed; final bottle weights (WT_F) were subtracted from initial weights (WT_I) yielding a difference score (D) for each animal. Percent sucrose preference was calculated using the following formula: $[(WT_D \text{ sucrose bottle}) / (WT_D \text{ sucrose bottle} + WT_D \text{ H}_2\text{O bottle})] \times 100$, where $WT_D = (\text{day 1 } WT_I - WT_F) + (\text{day 2 } WT_I - WT_F)$.

Stress-Induced Analgesia

Immediately before (pre-stress) and after (post-stress) 30 minutes of restraint in acrylic restraint cylinders, pain threshold was tested using a Paw Thermal Stimulator (UCSD, San Diego, CA) (Aloisi et al., 1994; Costa et al., 2005). Animals ($n = 6/\text{treatment}/\text{sex}$) were placed in clear Plexiglas chambers mounted on the glass surface. A radiant beam of light was focused on the plantar surface of each hind paw and the latency for the animal to withdraw its paw in response to the noxious thermal stimulus (50-51°C) was recorded in seconds as the paw withdrawal latency (PWL) (Hargreaves et al., 1988). Average PWL for three trials was calculated for each animal. The Paw Thermal Stimulator was set to produce latencies between 8-10 seconds and terminated after 20 seconds if no withdrawal occurred. Testing chambers and apparatus

were cleaned thoroughly with 70% ETOH between session; vapors were allowed to evaporate completely before the next session commenced.

Twenty-four hours later, animals received naloxone (5 mg/kg; i.p.) or saline. Fifteen minutes after naloxone, PWL was measured immediately before (pre-stress) and after (post-stress) 30 min of restraint stress as stated. Data are represented as raw latencies (in sec).

Sample Collection

Behaviorally naïve neonatally injured and control animals (P75-80) underwent perfusion fixation for immunohistochemistry (n=7/treatment/sex) or rapid decapitation for *in situ* hybridization (n=5/treatment/sex). Tissue fixation occurred as reported previously (LaPrairie and Murphy, 2009). Briefly, animals were given a euthanizing dose of sodium pentobarbital (160 mg/kg) intraperitoneally (i.p.) and perfused transcardially. Heparin-sodium (0.1 mL) was injected into the heart to prevent blood coagulation. Blood was removed from the brain with 0.9% sodium chloride and 2% sodium nitrite solution (250 mL). Fixation was achieved using 4% paraformaldehyde in 1 M phosphate buffer containing 2.5% acrolein (350 mL) (Polysciences, USA). A final wash of sodium chloride-sodium nitrite solution (250 mL) removed residual acrolein. Brains were stored in 30% sucrose at 4°C until sectioned. Alternatively, animals were placed in a decapacone (VWR, USA) and decapitated with a razor sharp guillotine. Immediately thereafter, brains were extracted, flash frozen in 2-methylbutane (VWR, USA) chilled on dry ice and stored at -80°C until sectioned.

Immunohistochemistry

Perfusion-fixed brains were sectioned in 1:6 series at 25 µM through the rostrocaudal axis. Sections were removed from the cryoprotectant-antifreeze solution, rinsed extensively in potassium phosphate buffer (KPBS), and reacted for 20 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were incubated in primary antibody solution directed

against methionine enkephalin in KPBS containing 1.0% Triton X for 1 hour at room temperature (RT), followed by 48 hours at 4°C. Met-enkephalin (Lot # 1004002) immunoreactivity was identified using polyclonal rabbit anti-met-enkephalin antibody at a concentration of 1:50,000 (Immunostar; Hudson, WI, USA). Staining was completely eliminated by pretreatment with 5 µg met-enkephalin per mL diluted antiserum, but not 5 µg leucine enkephalin per mL diluted antiserum (manufacturer technical information). For chromagen staining, tissue was rinsed in KPBS, incubated for 1 hour in biotinylated goat-anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, USA; 1:600) solution containing KPBS and 0.4% Triton X, rinsed again, and incubated for 1 hour in 0.009% avidin-biotin peroxidase complex (ABC Elite Kit; Vector Labs, USA). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), antigens were visualized using nickel sulfate-intensified 3,3-diaminobenzidine solution containing 0.083% hydrogen peroxide in sodium acetate buffer. The reaction was terminated after 20-25 min by rinsing in sodium acetate buffer. Sections were mounted out of KPBS onto gelatin-subbed slides, air dried overnight, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with Permount. For fluorescent staining, sections were washed in KPBS, incubated for 2.5 hours at RT in goat-anti-rabbit IgG DyLight488 secondary antibody (Jackson ImmunoResearch, USA; 1:50). Tissue was rinsed in KPBS, mounted as above and immediately coverslipped with VectaShield Hardset (Vector Labs, USA).

Proenkephalin In Situ Hybridization

Fresh frozen brains were sectioned in 1:6 series at 20 µM and mounted on SuperFrost Plus slides (Fisher Scientific, USA). Sections were stored at -80°C until time of assay. Sense probe was hybridized to control for specific binding. Preproenkephalin *in situ* hybridization was performed using oligonucleotide probe as reported previously (Lim et al., 2004). Briefly, 50-base oligonucleotide

(5'TCATCTGCATCCTTCTTCATGAAACCGCCATACCTCTTGGCAAGGATCTC-3'), comple-

mentary to bases 715-764 of the rat proenkephalin mRNA (Genebank accession number NM_017139) was labeled with ^{35}S -dATP at the 3' end using terminal deoxynucleotidyl transferase. Sections were fixed with 4% paraformaldehyde/PBS, acetylated, and hybridized with the antisense probe. After the hybridization, slides were washed, dried, and exposed to Kodak Bio-Max MR films (Kodak, USA).

Densitometry

Chromagen immunohistochemistry was quantified in pain- and stress-related regions with high density of met-enkephalin protein expression according to Bregma and region size defined in Paxinos and Watson, 5th edition: ventrolateral periaqueductal gray (vlPAG; Bregma: rostral -6.72 to caudal -8.76), central amygdala (CeA; Bregma: rostral -1.44 to caudal -3.24), lateral septum (LS; Bregma: rostral 2.28 to caudal -0.48), paraventricular nucleus (PVN; Bregma: rostral -0.84 to caudal -2.04), nucleus accumbens (NAcc; Bregma: rostral 2.52 to caudal 0.84). For each region of interest (ROI), 12-bit grayscale images of each section were captured with a 20X objective on a Nikon Eclipse E800 microscope using a QImaging Retiga EXi CCD camera and quantified with iVision Software (BD Biosciences, USA; Apple, USA). For each ROI, three sections per animal were sampled randomly. The mean grayscale pixel value was measured from a box of fixed size (vlPAG: 1.5 mm²; CeA: 1.5 mm²; LS 2.0 mm²; PVN 1.0 mm²; NAcc 2.0 mm²) and recorded. Measures were corrected for nonspecific binding by subtracting background adjacent to the ROI that lacked immunoreactivity. Mean specific immunoreactivity was reported as the relative optical density (ROD). For *in situ* hybridization data, C-14 microscopes (GE Healthcare Life Sciences, USA) were used to create standard curves ($R^2 > 0.99$) for each assay. For each ROI, sections were selected and captured using the above criteria with Scion Image (NIH and Scion Corp., USA), MTI CCD 72 camera and Northern Light box (Imaging Research, Inc., CN). The mean pixel value was recorded and measures were corrected for non-

specific binding by subtracting background adjacent to the ROI that lacked hybridization. Mean specific hybridization was reported as the disintegrations per minute per mg of tissue (dpm/mg).

Statistical Analysis

Significant main effects of neonatal treatment and sex or neonatal treatment and drug were assessed using two-way ANOVA or Repeated Measures ANOVA. Where effects of sex were not observed, data are collapsed by treatment for simplicity. Student's unpaired or paired t-tests were used for post-hoc analyses to determine differences between groups. Where applicable, values ≥ 2 standard deviations from the mean were eliminated as outliers. All comparisons were *a priori* specified. Confidence was set to $p < 0.05$ and considered statistically significant.

2.4 Results

Neonatal Injury And Adult Affective Behaviors

To test the impact of neonatal injury on adult responses to anxiety-provoking stimuli, male and female rats were exposed to the Open Field (OF). Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatally injured adults spent more time in the inner area than controls ($F_{(1,23)} = 50.24$; $P < 0.0001$) independent of sex ($F_{(1,23)} = 1.21$; $P = 0.28$) (fig. 1a). Neonatal treatment had no effect on the number of lines crossed ($F_{(1,23)} = 1.13$; $P = 0.35$) (fig. 1b) indicating no effect of injury on locomotion. Together, these data suggest that early life pain dampened behavioral responses to anxiety-provoking stimuli.

To test the effect of neonatal injury on adult stress-related behavior, rats were exposed to the forced swim test (FST). A 2-way ANOVA was used to test for significant main effects of treatment and sex. Latency to immobility (LTI) was significantly increased in neonatally injured adults as compared with controls ($F_{(1,30)} = 23.03$; $P < 0.0001$) (fig. 2a), suggesting reduced sensitivity to stress. No significant main effect of sex was observed ($F_{(1,30)} < 1$; $P = 0.38$). Neonatally

injured adults also excreted significantly less fecal boli ($F_{(1,30)} = 8.84$; $P = 0.0058$) (fig. 2b) consistent with the injury-induced decrease in sensitivity to stress. No significant change in the duration of immobility was observed ($F_{(1,30)} < 1.0$; $P = 0.97$) (fig. 2c). Together, these data suggest that early life pain dampened sensitivity to stress-provoking stimuli.

Neonatal Injury And Anhedonia

The sucrose preference test was used to assess for neonatal injury-induced changes in hedonic state. Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatal injury did not change adult preference for sucrose over water as compared with controls ($F_{(1,49)} < 1$; $P = 0.98$). Similarly, no effect of sex was observed ($F_{(1,49)} = 2.10$; $P = 0.15$) (fig. 2d). These results suggest that early life pain had no impact on an animal's hedonic state.

Enkephalin Mrna And Protein Increase In Pain And Stress-Related Brain Regions

To test whether neonatal injury increased adult enkephalin mRNA in pain and stress-related brain regions, density of *in situ* hybridized proenkephalin was measured in the vIPAG, CeA, LS, NAcc and PVN (fig. 3). These regions were selected as they contain high levels of enkephalin and have been previously implicated in an organisms' response to stress (Lightman and Young, 1987; Sanchez et al., 1992; Ulrich-Lai and Herman, 2009). Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatal injury significantly increased proenkephalin mRNA expression in the vIPAG (56%; $F_{(1,16)} = 18.12$; $P < 0.0006$), CeA (66%; $F_{(1,16)} = 96.80$; $P < 0.0001$) and LS (32%; $F_{(1,16)} = 4.51$; $P = 0.049$) as compared with controls. No change in proenkephalin mRNA was observed in the NAcc ($F_{(1,16)} = 0.54$; $P = 0.47$) or PVN ($F_{(1,16)} = 3.33$; $P = 0.087$) (data not shown). Sex differences were not observed in any of the brain regions examined.

To test whether the injury-induced increase in proenkephalin mRNA expression increased met-enkephalin protein, density of protein immunoreactivity was measured in the above regions of interest (fig. 4). Two-way ANOVA was used to test for significant main effects of treatment and sex. Neonatal injury significantly increased the expression of met-enkephalin protein in the vIPAG (138%; $F_{(1,24)} = 180.81$; $P < 0.0001$), CeA (101%; $F_{(1,24)} = 136.83$; $P < 0.0001$) and LS (55%; $F_{(1,24)} = 34.78$; $P < 0.0001$) as compared to controls. No change in met-enkephalin protein was observed in the NAcc ($F_{(1,24)} = 1.10$; $P = 0.30$) or PVN ($F_{(1,24)} = 0.17$; $P = 0.68$) (data not shown). Consistent with proenkephalin mRNA expression, sex differences in met-enkephalin protein expression were not observed in any of the brain regions examined.

Opioids Are Necessary For Impaired Stress Response

Our anatomical data demonstrated a significant increase in enkephalin expression in neonatally-injured animals. Therefore, we next determined whether the increase in stress threshold observed in the FST was opioid-mediated. Rats ($n=8-9$ /treatment/sex) were given the opioid receptor antagonist, naloxone HCl intraperitoneally (i.p.) 15 minutes before FST (fig. 5a). As no significant effect of sex was observed in our previous studies (see fig. 2), data are collapsed across sex and analyzed for significant main effects of treatment and drug using a 2-way ANOVA. Systemic naloxone significantly reduced LTI of neonatally injured adult rats in comparison to vehicle control injured rats (drug: $F_{(2,43)} = 9.58$; $P = 0.0004$) (fig. 5a). Importantly, latency to immobility of neonatally injured adults was similar to control levels in the presence of 1 mg/kg or 5 mg/kg naloxone (fig. 5a). Again, we observed no effect of treatment ($F_{(1,43)} < 1$; $P = 0.51$) or drug ($F_{(2,43)} < 1$; $P = 0.97$) in the duration of immobility (fig. 5b) and no significant change in fecal boli were observed (data not shown).

Neonatal Injury Impairs Adult Stress-Induced Analgesia

It is well established that acute stress activates neural systems that inhibit pain. As our data show that neonatal injury decreases adult sensitivity to stress (fig. 2, fig. 5), we next assessed whether adult stress-induced analgesia was altered by early life pain. Paw withdrawal latency (PWL) was measured using a Paw Thermal Stimulator before and after 30 minutes of restraint stress. No effect of sex was observed so data are collapsed. Restraint stress significantly increased PWL from baseline in the left paw of controls, as compared with injured adults (Repeated Measures ANOVA: $F_{(1,22)} = 5.58$; $P = 0.028$)(fig. 6a). Specifically, PWL for controls increases by 86% from baseline indicating the induction of stress induced analgesia (post-hoc paired t-test: $t_{(11)} = 8.25$; $P \leq 0.0001$)(fig. 6a). By contrast, neonatally injured adult rats did not display stress-induced analgesia (post-hoc paired t-test: $t_{(11)} = 1.53$; $P = 0.16$) (fig. 6a) (PWL before stress 11.4 seconds versus 12.9 seconds after stress; 14% change from baseline). A similar trend was observed in the right paw, such that PWL increased by 88% for controls, but only 44% for injured adults following restraint (data not shown). Data are collapsed by neonatal treatment, as no effect of sex was observed for the change in PWL from baseline in either the left (Repeated Measures ANOVA: $F_{(1,20)} < 1$; $P = 0.84$) or right paws (Repeated Measures ANOVA: $F_{(1,20)} < 1$; $P = 0.53$).

To determine whether neonatal injury impaired stress-induced analgesia through an opioid-dependent mechanism, naloxone HCl (5 mg/kg; i.p.) was administered 15 prior to restraint stress. Naloxone completely blocked stress-induced analgesia in the left (Repeated Measures ANOVA: $F_{(3,20)} = 5.078$; $P = 0.0089$) and right (Repeated Measures ANOVA: $F_{(3,20)} = 3.41$; $P = 0.038$; data not shown) paw of controls and neonatally injured adults (fig. 6b). Consistent with fig. 6a, vehicle treated controls, but not neonatally injured adults, displayed stress-induced analgesia.

2.5 Discussion

The present studies were conducted to determine whether a single inflammatory insult administered on the day of birth impacted adult responses to stress- and anxiety-provoking stimuli through an opioidergic mechanism. Our results show that neonatally injured adults had significantly decreased anxiety-like behaviors and decreased sensitivity to stress as compared with controls. Administration of the opioid antagonist naloxone HCl attenuated stress thresholds in both the forced swim and stress-induced analgesia tests, suggesting the opioid system is necessary for the observed deficits in stress responsiveness. Neonatally injured adults showed significantly increased proenkephalin mRNA and met-enkephalin protein expression in the vPAG, CeA, LS but not in the NAcc or PVN relative to controls. These data are the first mechanistic demonstration that early life inflammatory pain changes adult responses to stress through alterations in the endogenous opioid system. Moreover, these data contribute to the growing number of animal studies addressing issues surrounding early life pain, analgesia and anesthesia and their long-term consequences (Anand et al., 1999; Alvares et al., 2000; Bhutta et al., 2001; LaPrairie et al., 2008; Medeiros et al., 2011; Medeiros et al., 2012).

A Single Neonatal Injury Decreases Behavioral Sensitivity To Aversive Stimuli

Neonatal injury impacted adult anxiety-like behavior in the OF such that adults spent significantly more time in exposed areas than controls, independent of locomotor behavior. These data suggest that injury on P0 significantly decreases adult anxiety-like behaviors or sensitivity to anxiogenic stimuli. In the FST neonatally injured adults had a significantly higher threshold for stress, taking approximately 10-15 seconds longer (97%) than controls to become immobile. Administration of the opioid antagonist naloxone attenuated the stress threshold of neonatally injured adults such that it was similar to controls, suggesting that our observed decrease in sensitivity to stress-provoking stimuli occurs through an opioid-dependent mechanism. In parallel, neonatally injured adults excreted less fecal boli in the FST. As opioids are known to regu-

late behaviors in the FST and decrease colonic motility, these data are consistent with increased expression of endogenous opioids (Gillan and Pollock, 1980; Amir, 1982).

We hypothesize that the observed changes in anxiety- and stress-related behaviors result from injury-induced augmentation of the enkephalineric system. In support, viral overexpression of preproenkephalin or direct administration of enkephalin potentiates the anxiolytic effects of benzodiazepines, increases time in the open arms and blocks swim-stress induced anxiety in the EPM (Kang et al., 2000; Randall-Thompson et al., 2010). Conversely, knockout of the enkephalin gene in mice increases anxiety-like behavior in the OF, suggesting that enkephalin has anxiolytic properties (Konig et al., 1996; Kang et al., 2000; Randall-Thompson et al., 2010).

Importantly our data are consistent with clinical studies showing that former NICU patients display significantly reduced sensitivity to stress, suggesting early life trauma reduces sensitivity to aversive stimuli (Hack et al., 2004; Hermann et al., 2006; Grunau et al., 2007; Hayes and Sharif, 2009). Reports also show former preterm infants experience impairments of self-concept relative to their environment, altered ability to externalize, reduced ability to adapt and cognitive inflexibility (Levy-Shiff et al., 1994; Hayes and Sharif, 2009). Although we cannot specifically delineate many of these behaviors in rodents, increased exploration into an open area exposed to predation or reduced behavioral sensitivity to acute or severe stressors can be likened to insensitivity to salient cues in the environment, inattention and impaired ability to adapt.

Neonatal Injury Mitigates Stress-Induced Analgesia

Consistent with our previous studies, we found that neonatal injury resulted in a significant increase in basal pain sensitivity (LaPrairie and Murphy, 2007, 2009). Further, we now report that injury on the day of birth attenuates restraint stress-induced analgesia by greater than 100% in adults in comparison to controls. This impairment in stress-induced analgesia was ob-

served in both the neonatally injured and uninjured paws of adults relative to controls. Systemic naloxone HCl prevented stress-induced analgesia (Mogil et al., 1996), suggesting that changes in the endogenous opioid systems are responsible for dysregulating normal functioning of the stress system. These findings are consistent with our previous studies showing opioid-dependent increases in basal pain threshold (LaPrairie and Murphy, 2007, 2009), and threshold for forced swim stress.

Previous studies have reported that early life stress in mice decreases adult stress-induced analgesia (Sternberg and Ridgway, 2003). Furthermore, our observed impairment is consistent with clinical reports of attenuated stress-induced analgesia in adolescents and teens that experienced burns early in infancy (Wollgarten-Hadamek et al., 2011). Consistent with our observed increase in endogenous opioid-tone, high levels of enkephalin are known to dampen the perception of noxious or aversive stimuli, including pain associated with formalin inflammation, anxiety in the EPM and fecal boli excreted in response to immobilization stress (Tanaka et al., 1989; Kang et al., 1998; Randall-Thompson et al., 2010). In the context of our observed increases in anxiolytic behaviors and opioid-dependent increases in stress and pain thresholds (LaPrairie and Murphy, 2007, 2009), our stress-induced analgesia data are consistent with a general hyposensitivity to noxious or aversive stimuli.

Neonatal Injury Site-Specifically Augments Expression Of Enkephalin

The most profound increases in proenkephalin mRNA and met-enkephalin protein occurred in the VIPAG, CeA and LS. Increases in enkephalin expression in these regions provide potential neuroanatomical substrates for impaired responses to stress observed in our neonatally injured adults. Increases in mRNA and protein expression in each region occurred in parallel, and with similar magnitude, suggesting changes in expression are maintained through transcriptional or post-transcriptional epigenetic mechanisms. These findings are in line with other models of early life perturbation showing concomitant, unidirectional changes in mRNA and protein in

adult animals (Weaver et al., 2005; Schwarz et al., 2011). For example, preproenkephalin mRNA and met-enkephalin protein increase significantly in stress-related brain regions, including the CeA, in response to perinatal stress induced by dam restraint or pup exposure to an infanticidal adult male rat (Sanchez et al., 1992; Wiedenmayer et al., 2002). Overexpression of enkephalin or direct administration of an enkephalin analogue into the CeA dampens anxiety in the EPM and produces naloxone-reversible analgesia (Kang et al., 1998; Kang et al., 2000; Randall-Thompson et al., 2010). Conversely, loss of proenkephalin increases anxiety-like behavior, as reflected by a decrease in the number of inner area entries into the OF, decrease in latency to attack an intruder and increase in startle amplitude in response to acoustic perturbations (Konig et al., 1996; Bilkei-Gorzo et al., 2008). Collectively, these data suggest enkephalin is critical for reducing responses to anxiety- and stress-provoking stimuli.

The contribution of other endogenous opioids, such as β -endorphin and leu-enkephalin, to the long-term changes in affective behavior we observed cannot be ruled out. Previously, we reported that neonatal injury increases β -endorphin in the vIPAG, but fiber number was low and distribution sparse (LaPrairie and Murphy, 2009). Injury also increases leu-enkephalin (LaPrairie and Murphy, 2009), however, preproenkephalin is known to yield four times more met-enkephalin protein as compared with leu-enkephalin (Yoshikawa et al., 1984).

Our working hypothesis is that early life pain experienced during this critical period of development (P0-P8 (LaPrairie and Murphy, 2007)) increases afferent drive to brain regions responsive to noxious input (e.g. vIPAG, CeA and LS). This increase in afferent nociceptive drive results in the activation of supraspinal pain and stress circuits (Walker et al., 1986; Fitzgerald, 2005; LaPrairie and Murphy, 2009). Subsequent release of endogenous opioid peptides dampens perception, produces analgesia and promotes recovery from the inflammatory insult. As the inflammation associated with intraplantar carrageenan persists for 24-72 hours, the release of endogenous opioids is sustained. It is highly probable the continuous demand for enkephalin

programs the methylation or chromatin profile of the enkephalin promoter, such that high production of enkephalin becomes the basal state and persists throughout life.

Our data fit within a broad framework of studies documenting the long-term impact of early life experience on stress responsiveness (Weaver et al., 2004; Anseloni et al., 2005; Benetti et al., 2007; Korosi et al., 2010; Morgan and Bale, 2011; Schwarz et al., 2011). To our knowledge this is the first study to mechanistically establish that early life pain impairs adult stress through an endogenous opioid-dependent mechanism. Collectively, these data argue that insensitivity to stress and pain (LaPrairie and Murphy, 2007, 2009) we observed are adaptations to the early life environment. However, decreased ability to evaluate or respond to aversive or noxious stimuli, such as venturing into an area open to predation, excess energy expenditure when faced with the threat of drowning or inability to produce appropriate analgesia, can have severe, even mortal consequences. Thus, these behavioral changes are potentially maladaptive. Admittedly, medical sequelae surrounding prematurity are diverse and complex. However, clinical studies report former preterm infants experience significantly higher behavioral and hormonal thresholds for stress and pain as compared with term-born controls, suggesting reduced sensitivity to noxious or potentially harmful stimuli (Hermann et al., 2006; Grunau et al., 2007). Here, our studies delineate specific long-term effects associated with a *single* neonatal injury. These findings should be considered by the clinical community to promote changes in analgesic regimens for NICU patients. Our observations advocate for consistent and appropriate analgesia regimens for NICU patients to reduce the potential for mental health complications associated with premature birth.

2.6 Chapter 2 Figures

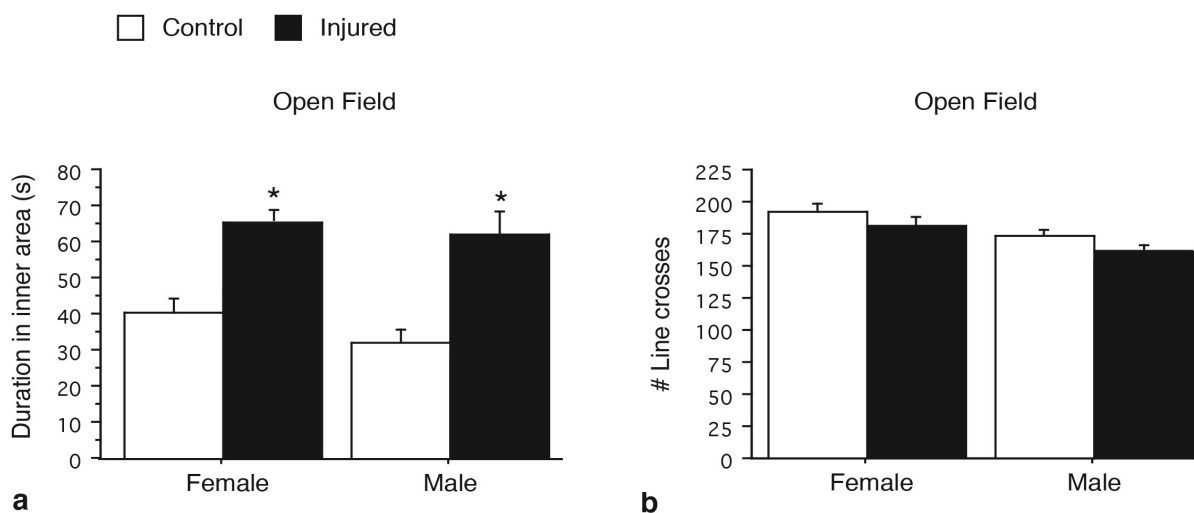


Figure 2.1 Neonatal injury increases adult anxiolytic behaviors

(a) Duration spent in the inner area of the Open Field was significantly increased by neonatal injury for both females and males similarly. (b) Locomotor behavior, as measured by the number of lines crossed in the Open Field, was not affected by neonatal injury. Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 6-8$ subjects per group. Significant main effect of treatment was observed in (a). (*) Denotes significant group differences as measured post-hoc by Student's t-test. $P < 0.05$.

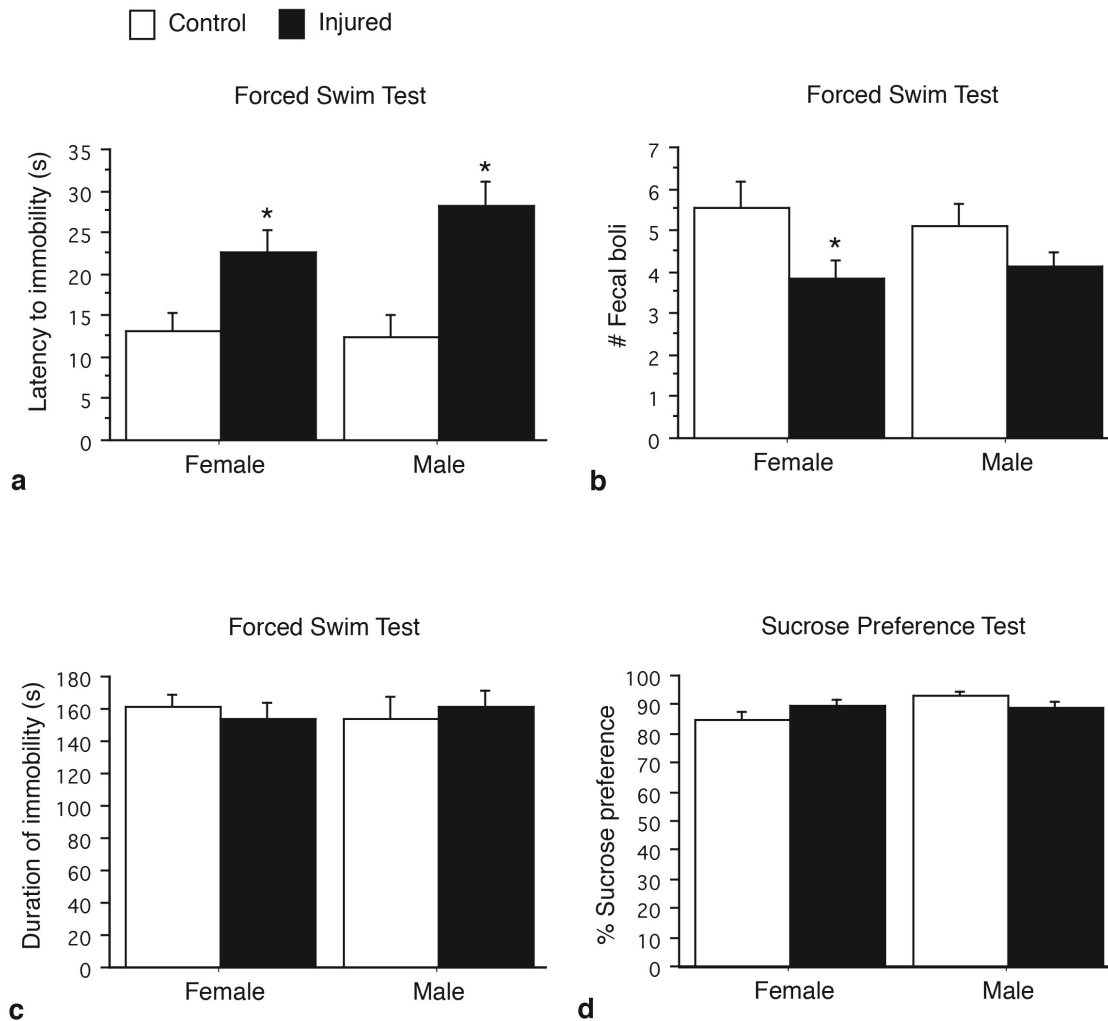


Figure 2.2 Neonatal injury increases adult threshold for stress but does not produce anhedonia

(a) Latency to immobility, the first display of immobile behavior in the Forced Swim Test (FST), was significantly increased by neonatal injury in females and males similarly, relative to handled controls. (b) Number of fecal boli excreted during the 5 minute FST were significantly reduced by neonatal injury. (c) Duration of immobility in the FST was not affected by neonatal injury. (d) Percent preference for 1% sucrose solution over a 48 hour period was not changed by neonatal injury. Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 8-9$ subjects per group in FST; $n = 9-16$ subjects per group in SPT. Significant main effect of treatment was observed in (a-b). (*) Denotes significant group differences as measured post-hoc by Student's t-test. $P < 0.05$.

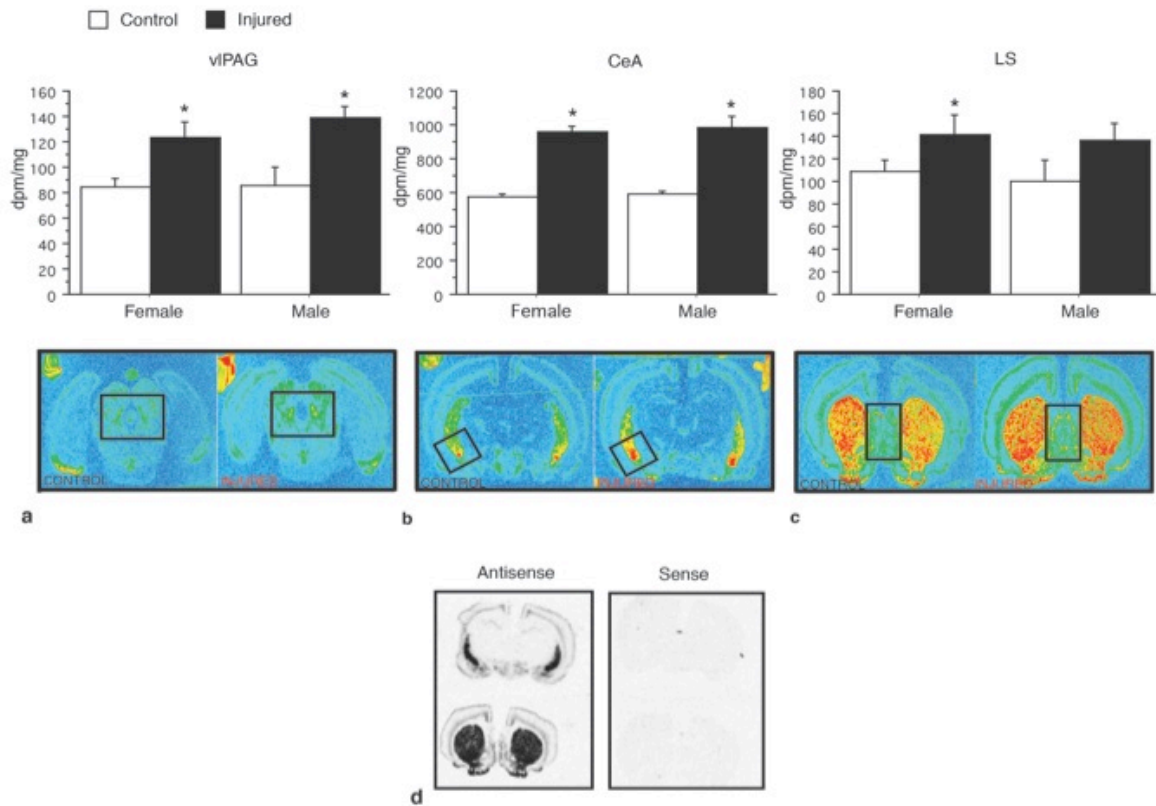


Figure 2.3 Neonatal injury increases adult expression of proenkephalin mRNA in pain and stress-related brain regions

Neonatal injury increases adult expression of proenkephalin mRNA in pain and stress-related brain regions. Proenkephalin mRNA was visualized on film via *in situ* hybridization using an oligo-proenkephalin probe. Relative optical density of proenkephalin mRNA was significantly increased by neonatal injury in the (a) mid through caudal ventral lateral PAG (VIPAG), (b) central amygdala (CeA), (c) lateral septum (LS). (d) Specificity of oligo-antisense probe for proenkephalin sequence as demonstrated by lack of hybridization with sense control. S-35 disintegrations per minute per milligram tissue (dpm/mg). Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 5$ subjects per group. Significant main effect of treatment was observed in (a-c). (*) Denotes significant group differences as measured post-hoc by Student's *t*-test. $P < 0.05$.

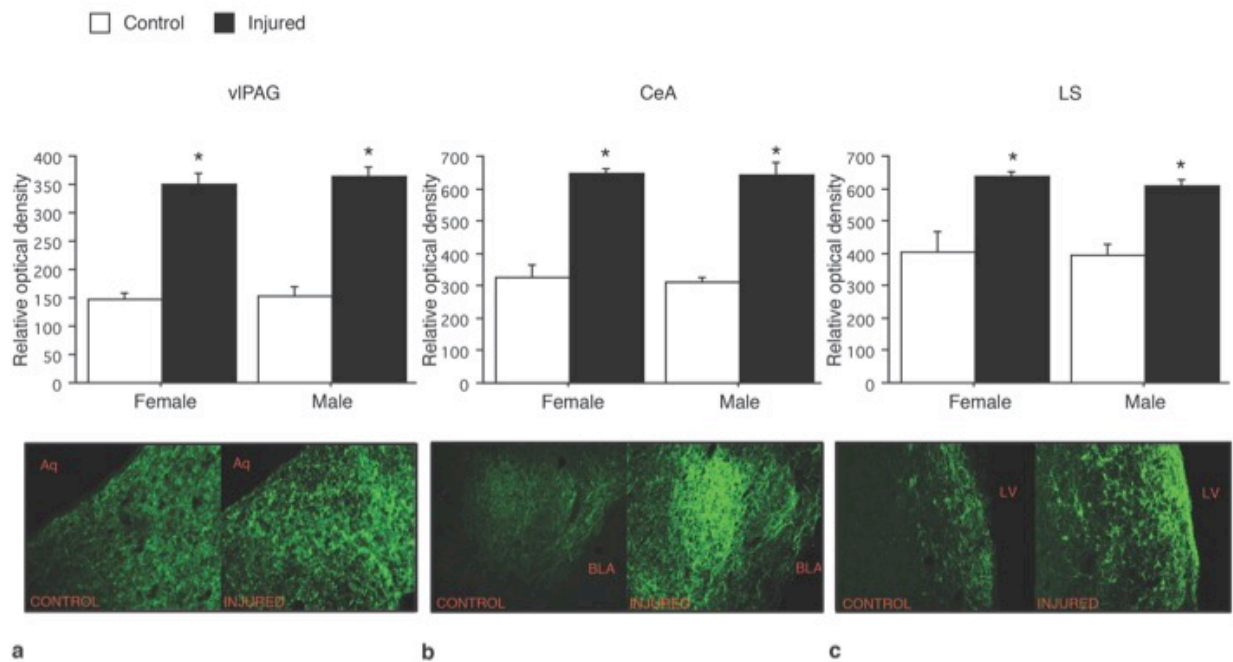


Figure 2.4 Neonatal injury increases adult met-enkephalin protein immunoreactivity in pain and stress-related brain regions

Consistent with proenkephalin mRNA expression, relative optical density of met-enkephalin immunoreactivity was significantly increased by neonatal injury in the (a) mid through caudal ventral lateral PAG (vIPAG) consistent with our previous reports, (b) central amygdala (CeA), (c) lateral septum (LS). Aquaduct (Aq), basolateral amygdala (BLA), lateral ventricle (LV). Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 7$ subjects per group. Significant main effect of treatment was observed in (a-c). (*) Denotes significant group differences as measured post-hoc by Student's t-test. $P < 0.05$.

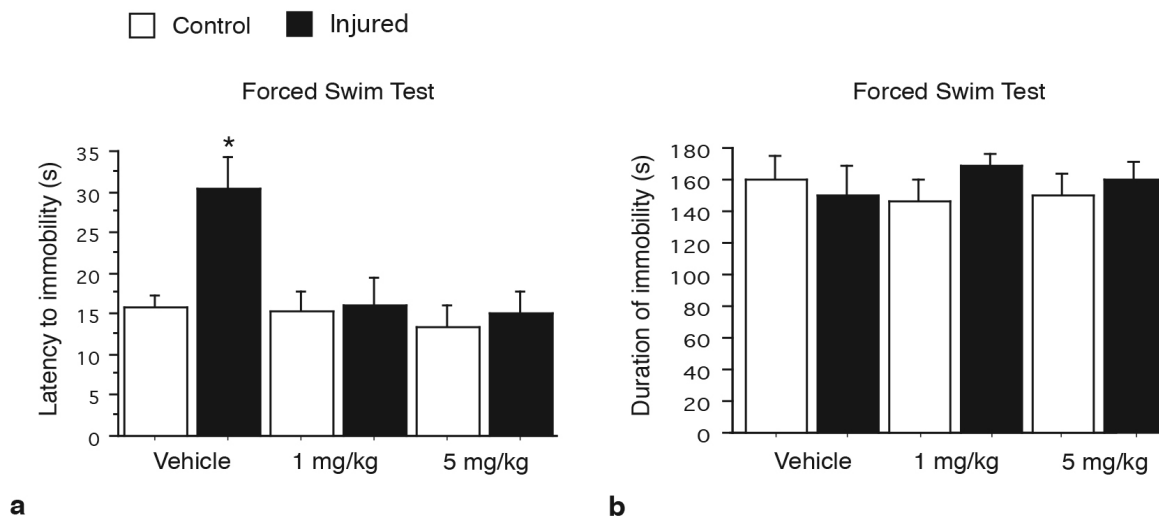


Figure 2.5 Naloxone attenuates the injury-induced increase in adult stress threshold

(a) Neonatally injured adults given vehicle had significantly increased latency to immobility (LTI) as compared with controls. Injured adults given naloxone HCl (1 mg/kg or 5 mg/kg; i.p.) 15 minutes before FST show significantly reduced LTI such that LTI became similar to controls. (b) Duration of immobility in the FST was not affected by naloxone treatment. Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 8-9$ subjects per group. Significant main effects of treatment and drug were observed in (a). (*) Denotes significant group differences as measured post-hoc by Student's t-test. $P < 0.05$.

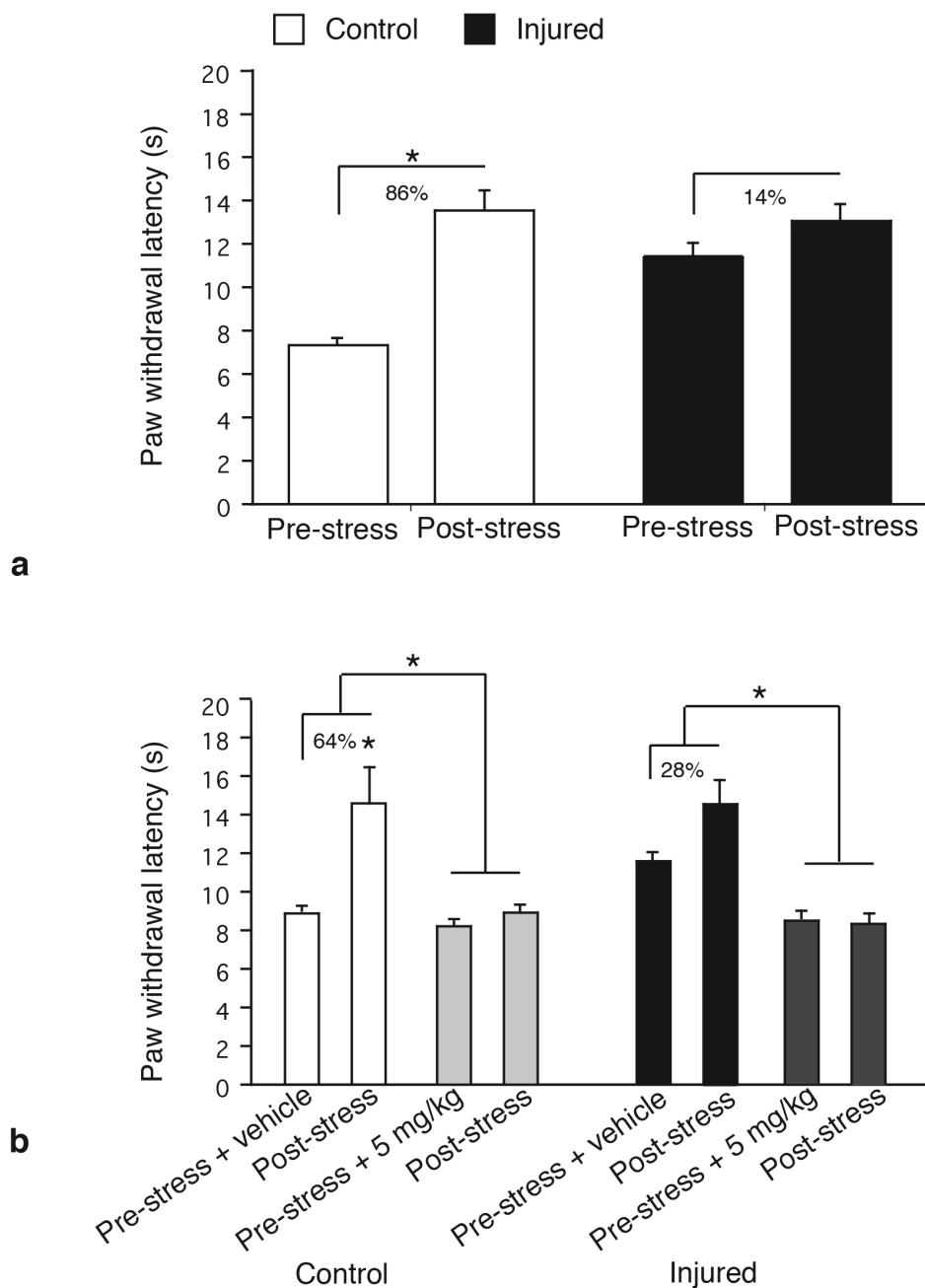


Figure 2.6 Neonatal injury impairs adult stress-induced analgesia (SIA) through an opioidergic mechanism.

(a) Paw withdrawal latency (PWL) before and after 30 minutes of restraint stress. Restraint significantly increased PWL in controls but not neonatally injured adults. (b) Naloxone HCl (5 mg/kg) significantly reduced PWL in neonatally injured adults similarly to controls demonstrating that SIA is opioid-based. As in (a), neonatally injured adults given vehicle did not exhibit SIA as compared with controls. Data are shown as Repeated Measures ANOVA (Mean \pm SEM) for the left paw as the same trend was observed in the right paw; $n = 6$ subjects per group. Significant main effect of treatment was observed in (a-b). (*) Denotes significant group differences as measured post-hoc by a paired Student's t-test. $P < 0.05$.

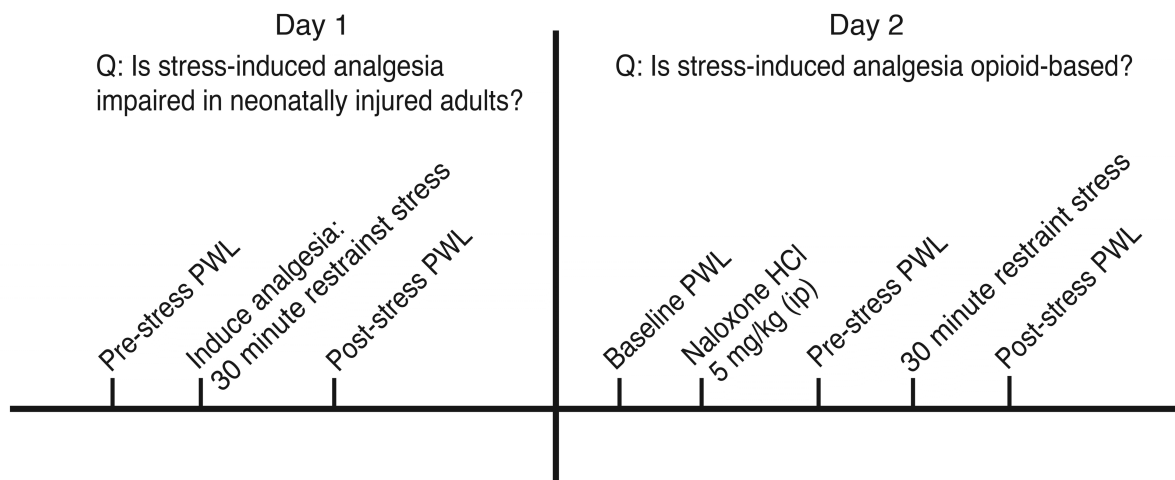


Figure 2.7 Protocol for testing the effect of neonatal injury on adult stress-induced analgesia

**3 CHAPTER THREE: LONG-TERM DYSREGULATION OF BRAIN CORTICOTROPHIN
AND GLUCOCORTICOID RECEPTORS AND STRESS REACTIVITY BY SINGLE
EARLY-LIFE PAIN EXPERIENCE IN MALE AND FEMALE RATS**

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3.1 Abstract

Inflammatory pain experienced on the day birth (postnatal day 0: PD0) significantly dampens behavioral responses to stress- and anxiety-provoking stimuli in adult rats. However to date, the mechanisms by which early life pain permanently alters adult stress responses remain unknown. The present studies examined the impact of inflammatory pain, experienced on the day of birth, on adult expression of receptors or proteins implicated in the activation and termi-

nation of the stress response, including corticotrophin releasing factor receptors (CRFR1 and CRFR2) and glucocorticoid receptor (GR). Using competitive receptor autoradiography, we show that Sprague Dawley male and female rat pups administered 1% carrageenan into the intraplantar surface of the hindpaw on the day of birth have significantly decreased CRFR1 binding in the basolateral amygdala and midbrain periaqueductal gray in adulthood. In contrast, CRFR2 binding, which is associated with stress termination, was significantly increased in the lateral septum and cortical amygdala. GR expression, measured with *in situ* hybridization and immunohistochemistry, was significantly increased in the paraventricular nucleus of the hypothalamus and significantly decreased in the hippocampus of neonatally injured adults. In parallel, acute stress-induced corticosterone release was significantly attenuated and returned to baseline more rapidly in adults injured on PD0 in comparison to controls. Collectively, these data show that early life pain alters neural circuits that regulate responses to and neuroendocrine recovery from stress, and suggest that pain experienced by infants in the Neonatal Intensive Care Unit may permanently alter future responses to anxiety- and stress-provoking stimuli.

3.2 Introduction

Approximately 12% of live births in the United States occur before 37 gestational weeks and are considered premature (<http://www.marchofdimes.com/peristats/>). These infants spend on average 25 days in the Neonatal Intensive Care Unit (NICU) where they endure 10-18 painful and inflammatory procedures per day, including heel lance, endotracheal intubation, respiratory and gastric suctioning and surgery (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008)<http://www.marchofdimes.com/peristats/>). Despite strong evidence that pain and stress circuitry are established and functional in preterm infants (Anand et al., 1987b; Grunau et al., 2005; Bartocci et al., 2006; Slater et al., 2006), 65% of these procedures are performed in the absence of analgesia (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008).

Clinical studies suggest early life pain has an immediate and long-term impact on re-

sponses to stress- and anxiety-provoking stimuli (Sullivan et al., 2012). Intrinsically, painful NICU procedures activate the stress response (Anand et al., 1987b; Grunau et al., 2005; Grunau et al., 2010). For example, preterm infants undergoing surgical procedures without analgesia have significantly higher concentrations of catecholamines and glucocorticoids (corticosterone: CORT) during and after surgery as compared with infants receiving analgesic treatment (Anand et al., 1987b). While initially heart rate, facial reactivity and cortisol levels of preterm infants are high in response to procedural pain, they become significantly blunted as the number of skin breaking procedures increases (Grunau et al., 2005; Grunau et al., 2010). Even in early childhood, cortisol release in response to painful stimuli remains blunted in former preterm infants (Grunau et al., 2007; Grunau et al., 2010), and represents a known risk factor for adult psychopathologies such as depression and post-traumatic stress disorder (PTSD)(Chrousos, 2009).

We have previously reported that a *single* inflammatory insult (1% carrageenan; hind paw), administered on the day of birth (postnatal day 0: PD0), significantly dampens behavioral responses to stress-, anxiety-, and pain-provoking stimuli in adult rats (LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b). Our studies further show that these behavioral changes are due to alterations in the endogenous opioid system (LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b). Most recently, we reported that early life pain permanently upregulates enkephalin mRNA and protein in the central amygdala, lateral septum and midbrain periaqueductal gray, brain regions that are highly responsive to stress (Victoria et al., 2013b). Notably, the endogenous opioid system works in parallel with classic systems regulating hypothalamic pituitary adrenal (HPA) axis activity. For example, enkephalin and corticotrophin releasing factor (CRF), which is essential for activation of the HPA axis (Vale et al., 1981), are simultaneously released from the hypothalamus in response to stress (Lightman and Young, 1989). Further, CRF and CRF receptors (CRFR) co-localize with and co-express endogenous opioids throughout the brain (Rivalland et al., 2005; Mousa et al., 2007). The glucocorticoid receptor (GR) system,

which terminates HPA activity (Dallman et al., 1987), regulates expression of both the endogenous opioid and CRFR systems (Lightman and Young, 1989). Given that early life pain alters the endogenous opioid system, which interacts with factors regulating the stress axis, the present study was conducted to test the hypothesis that a single inflammatory insult on the day of birth alters CRFR and GR systems in adulthood.

3.3 Materials And Methods

Animals

Pregnant Sprague-Dawley rat dams were obtained on gestational day 14 (GD14; Charles River, USA). Dams were housed individually under 12:12 hr light:dark cycle with *ad libitum* access to food and water. On the day of birth (PD0), pups were sexed by examination of anogenital distance and subjected to neonatal treatment. All litters were reared identically, weaned on PD21 and housed with same sex littermates in groups of 2-3. Male and female rats were used in all experiments and tested on separate days. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain, and were approved by the Georgia State University Animal Care and Use Committee. Behavior experiments were conducted during the light phase (9:00-12:30), animal order was randomized and the experimenter was blinded to neonatal treatment.

Neonatal Treatment

Acute neonatal inflammatory injury was induced as in our previous studies (LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b). Within 24 hours of birth on PD0, male and female rat pups received an injection of 5 μ L carrageenan (CGN, 1% dissolved in saline; Sigma, USA) into the intraplantar surface of the right hind paw. This time point is developmentally comparable to 24 weeks of gestation in humans (Workman et al., 2013). Intraplantar CGN is a well-established model of early life inflammatory pain that results in local edema lasting approximate-

ly 24-72 hours in pups (Lidow et al., 2001; Ren et al., 2004; LaPrairie and Murphy, 2007). Separate litters were handled as a control. Intraplantar saline was not administered as it results in an inflammatory response (<24 hrs). Pups were separated from their dam for no more than 20 minutes, maintained on a warm surface and returned to the home cage as a group. We have previously reported that this protocol does not alter maternal behavior (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009). In total, 26 litters (10-12 pups each) were generated, from which 117 animals were used in the present study. Approximately half of the animals were used for measures of anatomy (n = 60), while the remaining half were tested for HPA reactivity (n = 57). Therefore, while all measures do not contain pups from each litter, treatment consisted of pups from multiple litters in all measures (n = 5-12/treatment/sex per dependent variable). Each litter received a single treatment. Animals were undisturbed except for routine cage changes until adulthood (PD60). A summary of the experimental protocol is provided in Figure 1.

Estrus Cycling

Two weeks before euthanization or stress testing, vaginal lavage (starting \geq PD60) was performed once daily to track the estrus cycle of females (Fig. 1). Estrus stage was defined by presence of stage-specific epithelial cells in \geq 90% of the cell population. Specifically, proestrus, estrus, diestrus I and diestrus II were defined by presence of nucleated epithelial cells, cornified epithelial cells, leukocytes and all cell types, respectively (Becker et al., 2005). Males were handled one time per day to control against an effect of handling on stress responsiveness in females. Similar to females, males were removed from their cage by the base of the tail, placed on a clean cart for 10 s (time for vaginal sample collection) and then returned to their cage. Cycling and handling occurred in the morning between 08:00-10:00. Non-cycling females were eliminated from the study. Blood and tissue samples were collected across all cycle stages.

Euthanization

Behaviorally naïve neonatally injured and control animals (PD75-80) underwent perfusion fixation for immunohistochemistry or decapitation for receptor autoradiography and *in situ* hybridization (Fig. 1). For tissue fixation, animals were given a euthanizing dose of sodium pentobarbital (160 mg/kg; i.p.) and perfused transcardially with 0.9% sodium chloride and 2% sodium nitrite solution (250 mL; before and after fixation). Fixation was achieved using 4% paraformaldehyde in 1 M phosphate buffer (pH 6.8) containing 2.5% acrolein (350 mL) (Polysciences, USA). Brains were stored in 30% sucrose at 4°C until sectioned. Alternatively, animals were placed in a decapacone (VWR, USA) and decapitated with a razor sharp guillotine. Immediately thereafter, brains were extracted, flash frozen in 2-methylbutane (VWR, USA) chilled on dry ice and stored at -80°C until sectioned.

CRF Receptor 1 And 2 Autoradiography

Fresh frozen brains were sectioned in 1:6 series at 20 µM and mounted on SuperFrost Plus slides (Fisher Scientific, USA). Sections were stored at -80°C until time of assay. Competitive autoradiography for CRFR1 and CRFR2 was conducted with CRFR agonist ¹²⁵I Sauvagine (Perkin Elmer, USA) as the radioligand, and the CRFR1-selective antagonist CP-154,526 (Tocris, USA) or CRFR2-selective antagonist Astressin-2B (Sigma, USA) as competitors to reveal CRFR2 and CRFR1, respectively. Procedures were performed as previously published (Lim et al., 2004; Ahern and Young, 2009). Briefly, slides were thawed at room temperature (RT) until dry and fixed in 0.1% paraformaldehyde (pH 7.4; 2 min). Slides were then washed in 50 mM Tris buffer (pH 7.4; 10 min; 2 washes) and incubated for 2 hrs in tracer buffer containing 50 mM Tris buffer, 10 mM MgCl₂, 0.1% bovine serum albumin, 0.2 nM ¹²⁵I Sauvagine and either 500 nM Astressin-2B for visualization of CRFR1 or 500 nM CP-154,526 for visualization of CRFR2. Slides were incubated in 0.2 nM ¹²⁵I Sauvagine without antagonists as a positive control; 0.2 nM ¹²⁵I Sauvagine, 500 nM CP-154,526 and 500 nM Astressin-2B were applied to

slides to reveal nonspecific binding. Next, slides were washed in 50 mM Tris containing 0.2% MgCl₂ for 5 min at 4°C (4 washes) then for 30 min at RT (1 wash). Slides were briefly dipped in ddH₂O, allowed to dry at RT and then exposed to BioMax MR film (Sigma, USA) for 72 hrs.

Glucocorticoid Receptor In Situ Hybridization

In situ hybridization was used to quantify GR mRNA. Fresh frozen brains were sectioned in 1:6 series at 20 µM, mounted on SuperFrost Plus slides (Fisher Scientific, USA), and stored at -80°C until time of assay. To measure rat GR mRNA, a GR fragment was amplified from cDNA of adult prairie vole brain with rodent GR primers (forward: 5' GGACTTTCATAAAACCCTAAGGG 3'; reverse: 5' ACCCAGCAGAAAACCTCCAAATCC 3') (Integrated DNA Technologies, USA) using polymerase chain reaction. The 524 base pair nucleotide sequence of prairie vole is 90.3% identical to base pairs 97-234 and 292-680 of rat GR sequence (Genbank accession number: NM_012576). ³⁵S (Perkin Elmer, USA) UTP-labeled sense and antisense probes for GR mRNA were generated with GTP, CTP and ATP, spermidine, DTT, RNasin and RNA polymerase, using a linearized GR template by incubating for 2 hrs at 37°C (Inoue et al., 2004; Burkett et al., 2011). Sense and antisense probes were purified, dehydrated and applied to slides in hybridization buffer for 16 hrs at 55°C in a humidified chamber. Sections were stringently washed and excess probe was removed using RNase digestion buffer containing RNaseA. Following a final high stringency wash and dehydration, sections were dried at RT and exposed to FujiFilm imaging plates (GE Healthcare Life Sciences, USA) for 4 days. Plates were processed with BAS5000 and Multigauge (FujiFilm, JP) for photomicrographic presentation. Sections were then laid on BioMax MR film (Sigma, USA) for 26 days for analysis and quantification.

Immunohistochemistry

To confirm neonatal injury-induced changes in GR mRNA, perfusion fixed brains were sectioned in 1:6 series at 25 μ M and processed immunohistochemically for visualization of GR protein as previously described (LaPrairie and Murphy, 2009). GR was visualized using a polyclonal IgG rabbit anti-GR antibody at a concentration of 1:40,000 (Santa Cruz Biotech Inc., USA; sc-1004 (M20)). This rabbit anti-serum was prepared against a peptide mapping at the N-terminus of GR α of mouse origin. In Western Blotting, this antibody recognizes the 95/90 kDa GR α / β protein. For chromagen staining, tissue was incubated for 1 hour in biotinylated goat-anti-rabbit IgG secondary antibody (Jackson Immunoresearch, USA; 1:600) solution, rinsed and incubated for 1 hour in 0.009% avidin-biotin peroxidase complex (ABC Elite Kit; Vector, USA). After rinsing, antigen was visualized using nickel sulfate-intensified 3,3'-diaminobenzidine solution containing 0.083% hydrogen peroxide in sodium acetate buffer. The reaction was terminated after 20-40 min. Sections were mounted onto gelatin-subbed slides, air dried overnight, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with Permount.

Densitometry

Binding, hybridization and immunoreactivity (ir) were quantified in regions previously implicated in the activation, termination and processing of stressful stimuli (Ulrich-Lai and Herman, 2009). As CRFR1 and CRFR2 are expressed differentially (Chalmers et al., 1995), measures were taken only in regions where specific binding was detectable from background. Selective binding of CRFR1 was quantified in the medial prefrontal cortex (mPFC; Bregma 3.72 to 2.52), lateral septum (LS; Bregma 2.28 to -0.48), BNST (BNST; Bregma 0.12 to -0.84), paraventricular nucleus (PVN; Bregma -1.32 to -2.04), medial amygdala (MeA; Bregma -1.44 to -3.60), basolateral amygdala (BLA; Bregma -1.72 to -2.16) and ventrolateral periaqueductal gray (vIPAG; Bregma -6.72 to -8.76). Selective binding of CRFR2 was quantified in mPFC, LS, BNST, PVN, MeA, cortical amygdala (CoA; Bregma -3.96 to -5.64) and ventral hippocampus (vHPC; Bregma

-4.56 to -6.12). Positive control slides exhibited binding in regions known to express mRNA for both receptors (Chalmers et al., 1995). The presence of both antagonists prevented all binding in the negative control slides. GR mRNA hybridization was measured in the PVN, dorsal CA1 of the hippocampus (dCA1; Bregma -2.52 to -4.20), ventral CA1 of the hippocampus (vCA1; Bregma -4.56 to -6.72) and central amygdala (CeA; Bregma -2.40 to -3.24). ^{125}I and ^{14}C microscalers (ARC, USA; GE Healthcare Life Sciences, USA, respectively) with known tissue equivalent activities (disintegrations per minute per mg of tissue, dpm/mg) were used to create standard curves ($R^2 > 0.99$) for each receptor autoradiography and *in situ* hybridization assay, respectively. ROI's for receptor autoradiography and *in situ* hybridization were selected and captured using the above criteria with Scion Image Software (NIH), MTI CCD 72 camera and Northern Light box (Imaging Research, Inc., CN). Bregma and region size from Paxinos and Watson (2005), along with a series of adjacent sections stained with Neutral Red were used for anatomical reference. The mean pixel value was recorded from a box of fixed size (LS: 4.0 mm²; mPFC: 2.5 mm²; vHPC: 2.0 mm²; BNST, PVN, MeA, BLA, CoA, CeA, vCA1, vIPAG: 1.5 mm²; dCA1: 1.0 mm²). Measures were corrected for nonspecific binding (NSB) by subtracting background adjacent to the ROI that lacked binding or hybridization (mean NSB \pm SEM was 2.38 ± 0.15 , 2.70 ± 0.46 , 15.53 ± 0.92 dpm/mg for CRFR1, CRFR2 and GR, respectively). Mean specific binding or hybridization was reported as (dpm/mg).

GR-ir was quantified in the PVN, dCA1, vCA1 and CeA. For each ROI, 12-bit grayscale images of each section were captured with a 10X objective for quantification (4X objective for photomicrograph presentation) on a Nikon Eclipse E800 microscope using a QImaging Retiga EXi CCD camera and quantified with iVision Software (BD Biosciences, USA; Apple, USA). For each ROI, three sections per animal were sampled randomly. The mean grayscale pixel value was measured from a box of fixed size as above and recorded. Measures were corrected for nonspecific binding by subtracting background adjacent to the ROI that lacked immunoreactivity. Mean specific immunoreactivity was reported as the relative optical density.

Restraint Or Forced Swim Stress And Blood Withdrawal

Separate cohorts of neonatally injured and control adults (PD75-80) were subjected to 15 minutes of restraint or 5 minutes of forced swim stress to activate the HPA axis. Animals were habituated to the testing rooms for 60 minutes, daily for 3 days before and on the day of testing. Males and females were tested on separate days in randomized order.

Blood samples were collected (9:00-12:30) from the lateral saphenous vein directly into 1 mL EDTA-hematology tube (BD from Fisher Scientific, USA) using a 23-gauge needle. Four time points were tested for each stressor. In tests using restraint stress, blood was collected immediately before placement into a plastic restrainer (baseline; 0 min), immediately after 15 min of restraint (stress), 30 min after the onset of restraint (peak) and 75 min following onset of stress (recovery). Animals used in the forced swim test were first given a 5 min pre-swim 24 hrs prior to testing (Porsolt et al., 1977) as in our previous studies (Victoria et al., 2013b). On the day of forced swimming, blood was collected 60 min before swim to allow wound clotting and avoid water contamination (baseline; 0 min). Blood was also collected immediately after 5 min of swim in 25°C water (stress), 30 min after the onset of swim (peak) and 75 min post-swim (recovery). Blood samples were maintained at room temperature for ≥ 30 min and centrifuged at 4000 rcf at 4°C for 15 min. Plasma was stored at -80°C until radioimmunoassay for CORT (^{125}I Double Antibody Corticosterone kit, MP Biomedicals, USA). Concentration of CORT was determined against known standards ($R^2 > 0.98$) according to the manufacturer's instructions. The minimum limit of detection was 7.02 ng/mL. Intra-assay coefficient of variation was 8.4%.

Statistical Analysis

All values are presented as Mean \pm SEM. Significant main effects of neonatal treatment and sex were assessed using two-way analysis of variance (ANOVA) or Repeated Measures ANOVA for time. Area under the curve analyses relative to ground are reported for corticosterone data and were calculated using the formula,

$$AUC_{Ground} = \frac{(C_2 - C_1)(t_2 - t_1) + (C_3 - C_2)(t_3 - t_2) + (C_4 + C_2)(t_4 - t_3)}{2},$$

where C and t denote concentration of corticosterone and time point for sample collection, respectively (Pruessner et al., 2003). Percentages are reported as mean percent change from control. Tukey-Kramer was used for post-hoc analyses to determine differences between groups. Where applicable, values ≥ 2 standard deviations from the mean were eliminated as outliers. All comparisons were *a priori* specified. Confidence was set to $P < 0.05$ and considered statistically significant.

3.4 Results

The Impact Of A Single Neonatal Injury On The Adult CRFR System

Effect of neonatal injury on CRFR1 protein binding

Competitive receptor autoradiography was used to measure binding of CRFR1 in mPFC, LS, BNST, PVN, MeA, BLA and vIPAG. Two-way ANOVA revealed that neonatal injury significantly decreased CRFR1 protein binding in the BLA (overall mean decrease: 30%; $F_{(1,16)} = 6.57$; $P = 0.021$) and vIPAG (overall mean decrease: 31%; $F_{(1,16)} = 4.73$; $P = 0.045$) (Fig. 2A-B), regions important for activation of the HPA axis, autonomic control and processing of noxious stimuli. No change in CRFR1 binding was observed in the PVN ($F_{(1,16)} < 1.0$; $P = 0.60$), LS ($F_{(1,16)} < 1.0$; $P = 0.57$), MeA ($F_{(1,16)} < 1.0$; $P = 0.46$) and mPFC ($F_{(1,16)} < 1.0$; $P = 0.44$) (Supplemental Fig. 1). Independent of treatment, males had significantly greater CRFR1 binding in the BNST as compared with females (overall mean increase: 40%; $F_{(1,16)} = 11.34$; $P = 0.0039$) (Supplemental Fig. 1). Significant sex differences were not observed in any other regions examined; similarly, no interactions between treatment and sex were noted.

Effect of neonatal injury on CRFR2 protein binding

CRFR2 binding was measured in mPFC, LS, BNST, PVN, MeA, CoA, and vHPC using competitive receptor autoradiography. Two-way ANOVA revealed that neonatal injury signifi-

cantly increased binding of CRFR2 in the LS (overall mean increase: 42%; $F_{(1,16)} = 6.93$; $P = 0.018$) and CoA (overall mean increase: 58%; $F_{(1,16)} = 7.23$; $P = 0.016$) (Fig. 3 A-B). No change in CRFR2 binding was observed in the PVN ($F_{(1,16)} < 1.0$; $P = 0.96$), MeA ($F_{(1,16)} < 1$; $P = 0.82$), mPFC ($F_{(1,16)} < 1.0$; $P = 0.75$) or vHPC ($F_{(1,16)} < 1.0$; $P = 0.44$) (Supplemental Fig. 1). Independent of neonatal treatment, males showed significantly more CRFR2 binding in the BNST as compared to females (overall mean increase of 72%; $F_{(1,16)} = 12.61$; $P = 0.0027$) (Supplemental Fig. 1). In the LS, significantly more CRFR2 binding was observed in injured males (71% increase; Tukey's post hoc, $P < 0.05$) relative to control males, but not between injured and control females (Tukey's post hoc, $P > 0.05$). In contrast, significantly more CRFR2 binding in the CoA was observed in injured females (98% difference; Tukey's post hoc, $P < 0.05$) relative to control females, but not between injured and control males (Tukey's post hoc, $P > 0.05$). No significant interactions between treatment and sex were observed in any brain region examined.

The Impact Of A Single Neonatal Injury On Adult CORT/GR System

Neonatal injury alters GR mRNA and protein expression

In situ hybridization was used to examine the impact of neonatal injury on adult GR mRNA expression in the PVN, dCA1, vCA1, and CeA. Two-way ANOVA revealed that the density of GR mRNA was significantly increased in the PVN (overall mean increase: 94%; $F_{(1,16)} = 6.28$; $P = 0.023$) of neonatally injured adults relative to controls (Fig. 4A). In contrast, neonatal injury decreased adult GR mRNA in dCA1 (overall mean decrease: 42%; $F_{(1,16)} = 9.67$; $P = 0.0067$) and vCA1 (overall mean decrease: 38%; $F_{(1,16)} = 11.79$; $P = 0.0034$) as compared with controls (Fig. 4B-C). No change in GR mRNA was observed in the CeA ($F_{(1,16)} < 1$; $P = 0.58$) (Supplemental Fig. 2). No significant main effect of sex or sex by treatment interaction was noted for any region examined.

To confirm our observed injury-induced changes in GR mRNA expression, density of GR protein immunoreactivity (ir) was measured in the PVN, dCA1, vCA1 and the CeA (Fig. 5). Con-

sistent with GR mRNA data, two-way ANOVA revealed GR-ir was increased in the PVN (overall mean increase: 33%; $F_{(1,35)} = 11.37$; $P = 0.0018$) but decreased in both dCA1 (overall mean decrease: 24%; $F_{(1,36)} = 10.89$; $P = 0.0022$) and vCA1 (overall mean decrease: 26%; $F_{(1,34)} = 5.11$; $P = 0.030$) of neonatally injured adults as compared with controls (Fig. 5A-C). No significant change in GR protein expression was observed in the CeA ($F_{(1,35)} < 1.0$; $P = 0.60$) (Supplemental Fig. 2). As noted with the *in situ* data, no significant sex or sex by treatment effects were observed in any region examined.

Neonatal injury alters CORT negative feedback in response to restraint and swim stressors

We next tested if neonatal injury alters adult CORT negative feedback in response to stress. Serum CORT concentrations were assayed from blood drawn before, immediately after and during recovery from restraint or forced swim (Fig. 6A and 6B, respectively). Repeated measures ANOVA across time points revealed a significant impact of neonatal treatment on adult CORT responses to 15 min of restraint ($F_{(3,102)} = 6.55$; $P = 0.015$) (Fig. 6A) and 5 min of forced swim stress (time x sex x treatment interaction: $F_{(3,45)} = 3.65$; $P = 0.019$) (Fig. 6B). Area under the curve analysis revealed that CORT release was significantly attenuated in neonatally injured adults as compared with controls (restraint: $F_{(1,34)} = 5.78$; $P = 0.022$; swim: $F_{(1,15)} = 5.25$; $P = 0.037$). Further, neonatally injured males had significantly reduced CORT relative to controls at the 30 min peak (swim: Tukey's post hoc, $P < 0.01$) and during recovery from stress at 75 min (restraint: Tukey's post hoc, $P < 0.001$; swim: Tukey's post hoc, $P < 0.05$).

Independent of neonatal treatment, females had significantly higher CORT concentrations following restraint or swim as compared with males (restraint: $F_{(1,102)} = 36.90$; $P < 0.0001$; swim: $F_{(1,45)} = 17.31$; $P < 0.0008$). However, no effect of estrus was observed (restraint: $F_{(2,45)} < 1.0$; $P = 0.72$; swim: $F_{(2,12)} = 2.88$; $P = 0.17$). Consistent with the observed differences in CORT concentrations, adrenal glands of injured and control females comprised a larger percent of body weight relative to males; among males adrenal glands comprised a significantly larger per-

cent of body weight for injured relative to control males (sex x treatment interaction: $F_{(1,36)} = 4.28$; $P = 0.046$) (Supplemental Fig. 2).

3.5 Discussion

The present study examined the long-term impact of early life pain on mediators of stress reactivity. Our results demonstrate that neonatally injured adults have significantly decreased binding of CRFR1 in the BLA and vIPAG as compared with controls. In contrast, CRFR2 binding was significantly increased in the LS and CoA of neonatally injured adults relative to controls. As activation of the stress response is associated with CRFR1 (Vale et al., 1981), whereas CRFR2 promotes return to homeostasis following a perturbation (Bale et al., 2000; Coste et al., 2000), the present findings are consistent with models of acute early life stress showing long-term decreases in stress reactivity (Macri et al., 2011). These results are also consistent with our previous reports that early life pain blunts adult behavioral sensitivity to stress-, anxiety- and pain-provoking stimuli (LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b). In parallel with the changes in CRFR, GR mRNA and protein were increased in the PVN but decreased in the hippocampus of neonatally injured adults in comparison to controls. Further, neonatally injured males showed reduced CORT release following restraint or swim stress. Taken together with our previous studies, these data suggest that a single neonatal injury alters receptor systems and neural circuits that contribute to activation of the stress axis and neuroendocrine recovery from stress, and likely contribute to the blunted behavioral responses previously observed in response to stress- and anxiety-provoking stimuli.

A Single Neonatal Injury Decreases Extrahypothalamic CRFR1

CRFR1 in the BLA has been implicated previously in the stress response. For example, direct BLA administration of the CRFR1 agonist stressin-1 decreases percent time in the open arms of the elevated plus maze in mice (Bruchas et al., 2009), whereas lentiviral RNAi knock-

down increases time in the center of the open field (Sztainberg et al., 2010). Similarly, lesions specific to the BLA reduce adrenocorticotropin hormone (ACTH) and CORT following acute restraint (Bhatnagar et al., 2004), suggesting that reduced CRFR1 binding in the BLA may contribute to the decrease in CORT we observed following restraint stress. The decrease in BLA CRFR1 binding is also consistent with reports demonstrating that neonatally injured adults spend significantly more time in the center of the open field and support the hypothesis that early life pain dampens adult responses to anxiety-provoking stimuli (Anseloni et al., 2005; Victoria et al., 2013b).

The PAG has been linked to a variety of physiological changes that occur in parallel with the stress response. For example, chemical or electrical stimulation of the vIPAG results in a redistribution of peripheral blood flow, changes in cardiovascular and autonomic output, and decreases pain sensitivity (Lewis and Gebhart, 1977; Behbehani and Fields, 1979; Carrive and Bandler, 1991; Depaulis et al., 1994; Inui et al., 1994), suggesting that the vIPAG alters autonomic tone and promotes passive coping in response to stressors. In the context of early life pain, decreases in vIPAG CRFR1 binding suggest reduced perception of nociceptive information, consistent with previous studies showing that early life pain decreases adult pain sensitivity (Anseloni et al., 2005; LaPrairie and Murphy, 2007). Moreover, our findings support clinical data showing that as the number of skin breaking procedures increase in the NICU, autonomic and behavioral responses of preterm infants become significantly blunted (Grunau et al., 2005; Grunau et al., 2010), adaptations that are likely essential in an early life environment of repeated pain, inflammation and stress.

A Single Neonatal Injury Increases Extrahypothalamic CRFR2

In the present study, CRFR2 binding was significantly increased in the LS and CoA of adult rats injured on the day of birth. Numerous lines of evidence suggest that CRFR2 is necessary for reinstating homeostasis and dampening stress. CRFR2 knockout mice have significant-

ly accelerated ACTH and CORT release in response to brief restraint (Bale et al., 2000); CORT levels remain elevated relative to controls after 90 min (Coste et al., 2000), suggesting CRFR2 is necessary for post-stress modulation and hormone recovery. Further, administration of a CRFR2 agonist intracerebroventricularly decreases stress-induced anxiety in the elevated plus maze (Valdez et al., 2002), while pharmacological blockade or genetic knockdown increases anxiety in the elevated plus maze and immobility in the forced swim test (Liebsch et al., 1999; Kishimoto et al., 2000). Together, these data support the role of CRFR2 in dampening stress to promote coping and recovery, and suggest that the observed increase in binding of CRFR2 in the LS and CoA may serve to promote homeostasis and dampen responses to stress, adaptations that would promote survival following trauma early in life.

Neonatal Injury Accelerates Corticosterone Negative Feedback

Recovery from stress, defined as a return of CORT to basal levels, is achieved primarily through GR binding in the hippocampus (Sapolsky et al., 1984b; Herman and Cullinan, 1997; Ulrich-Lai and Herman, 2009). In the present study adult males injured on PD0 showed blunted CORT secretion between 30 and 75 minutes following stress (stress recovery), consistent with reports that inflammatory injury on P3 decreases release of CRF and ACTH 35 minutes after swim stress in adult male rats (Anseloni et al., 2005). In parallel, longitudinal studies measuring stress-reactivity of former preterm infants report blunted CORT secretion during recovery from pain-induced stress (Grunau et al., 2010). Collectively, these studies suggest a potentially permanent dysregulation of the glucocorticoid system as a consequence of early life stress in the form of injury.

While hypersecretion of glucocorticoids is often found in models of chronic stress and is associated with high anxiety and major depressive disorder (Heim and Nemeroff, 2001; Ulrich-Lai and Herman, 2009), we observed hyposcretion of CORT during recovery from both restraint and forced swim stressors in injured males, with similar trends observed in injured fe-

males. Clinically, low cortisol has been reported in groups suffering from severe trauma and/or PTSD, and is comorbid with rheumatoid arthritis, fibromyalgia, chronic fatigue and chronic pain syndromes (Heim et al., 2000). In the context of the present studies, early life pain may predispose preterm infants to inflammatory diseases (O'Reilly et al., 2013) and put them at greater risk for developing PTSD when faced with trauma later in life (Ward-Begnoche, 2007).

Neonatal Injury Results In Compensatory Changes In GR Expression

It is essential to consider the HPA axis as a functional unit, as we observed a significant increase in GR mRNA and protein immunoreactivity in the PVN, whereas GR expression decreased in both dorsal and ventral CA1. Recent studies in our lab indicate that induction of inflammatory pain in newborn rats elicits CORT release for at least 24 hours following injury (Victoria, et al., unpublished observations). As GR in the hippocampus is exquisitely sensitive to high levels of CORT, especially during the first postnatal week when binding affinity is increased (Sapolsky et al., 1984b; Sapolsky and Meaney, 1986; Vazquez et al., 1996), we hypothesize that sustained injury-induced CORT release downregulates hippocampal GR, thereby, reducing hippocampal ability to terminate stress responding. In response, GR is upregulated in the PVN as a compensatory change that ultimately results in hastened negative feedback. Although speculative, these data are consistent with previous studies testing the impact of early life metabolic perturbation in rodents reporting changes in hippocampal and hypothalamic GR and accelerated negative feedback (Proulx et al., 2001). Interestingly, rodent models of early life immune challenge show disparate alterations in this circuit. Specifically, neonatal endotoxin decreases adult GR expression in both the hippocampus and hypothalamus and can produce either dexamethasone resistance or blunted CORT reactivity (Shanks et al., 1995; Shanks et al., 2000; Bilbo et al., 2008; Walker et al., 2009a). Despite differences in the direction of change and type of early life stress, these data, together with the present study, support a common circuit whereby neonates adapt to, and program, responses to stress in order to maximize survival.

Working Hypothesis

Our working hypothesis is that early life pain experienced during the critical neurodevelopmental period (PD0-PD8; human equivalent 24-36 gestational weeks; (LaPrairie and Murphy, 2007; Workman et al., 2013)) increases afferent drive to brain regions responsive to noxious input. This increase in afferent nociceptive drive results in the activation of supraspinal circuits subserving pain and stress (Walker et al., 1986; Fitzgerald, 2005; LaPrairie and Murphy, 2009). Endogenous opioids, including met-enkephalin and β -endorphin, are released to dampen pain perception (Loh et al., 1976; Konig et al., 1996; Hurley and Hammond, 2001) and stress (Rossier et al., 1977; Rivier et al., 1982; Lightman and Young, 1987; Bilkei-Gorzo et al., 2008). Concurrently, neurohormones from the HPA axis, including CRF, ACTH and CORT, are released to mount appropriate physiological responses and promote recovery from the physical threat associated with inflammation (Vale et al., 1981; Dallman et al., 1987; Taylor et al., 1998). As the inflammation associated with intraplantar carrageenan persists for 24-72 hours, and release of CORT is sustained (Victoria, et al., unpublished observations), it is likely that sustained elevation of CRF downregulates CRFR1, while increasing CRFR2 (Bale and Vale, 2004) in regions mediating stress activation and perception of noxious stimuli to re-program circuits such that future insults are less potent or aversive. As CORT levels remain high and continue to feed up to the hippocampus, GR becomes downregulated and the organism's ability to terminate stress is impaired (Boyle et al., 2005); GR in the PVN becomes upregulated to compensate and promote HPA axis inhibition (Proulx et al., 2001) and CORT negative feedback becomes more efficient to facilitate recovery (Sapolsky and Meaney, 1986). As these perturbations occur during a highly plastic developmental period, and GR transcriptionally regulates numerous genes (Schoneveld et al., 2004), it is probable that methylation or chromatin profiles of GR (Weaver et al., 2004) and CRFR (Elliott et al., 2010) promoters are modified, such that this new receptor production profile becomes the basal state and persists throughout the life span.

Accelerated negative feedback may seem attractive. However, reducing the time over which glucocorticoids circulate in response to stress has physiological consequences, including decreased production and uptake of glucose, reduced breakdown of adipose tissue into free fatty acids, reduced protein synthesis and changes in immune system regulation (Bateman et al., 1989). In addition, we cannot rule out the potential contribution of cytokines to our observed changes in stress responsiveness, as neonatal administration of CGN results in upregulation of adult IL-10 (Ren et al., 2005).

While accelerated recovery from an acute stressor may have an immediate physiological or survival benefit, reduced ability to liberate and sequester appropriate glucose could have serious consequences for responses to repeated or chronic stressors and confer vulnerability to psychopathology. Indeed, for former preterm infants, altered cortisol reactivity is significantly associated with issues of internalization, emotional reactivity, anxiety, depression, inattention, and high rates of negative verbalization (Bagner et al., 2010).

Conclusion

Collectively, our findings demonstrate that a single inflammatory insult on the day of birth is associated with site-specific changes in circuits that contribute to stress activation, perception of noxious stimuli and neuroendocrine recovery from stress. Although issues surrounding prematurity are diverse and complex, strong reconsideration of inconsistent and infrequent analgesia in the NICU is necessary to reduce physical and mental health complications associated with preterm birth.

3.6 Chapter 3 Figures

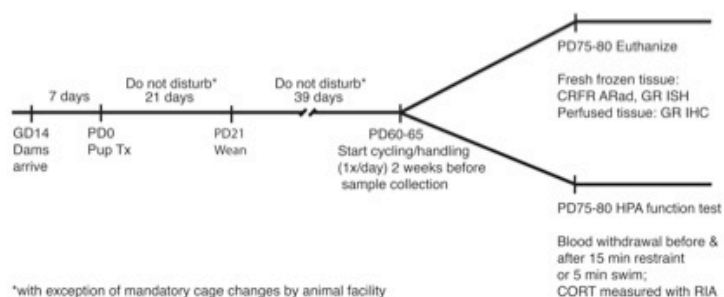
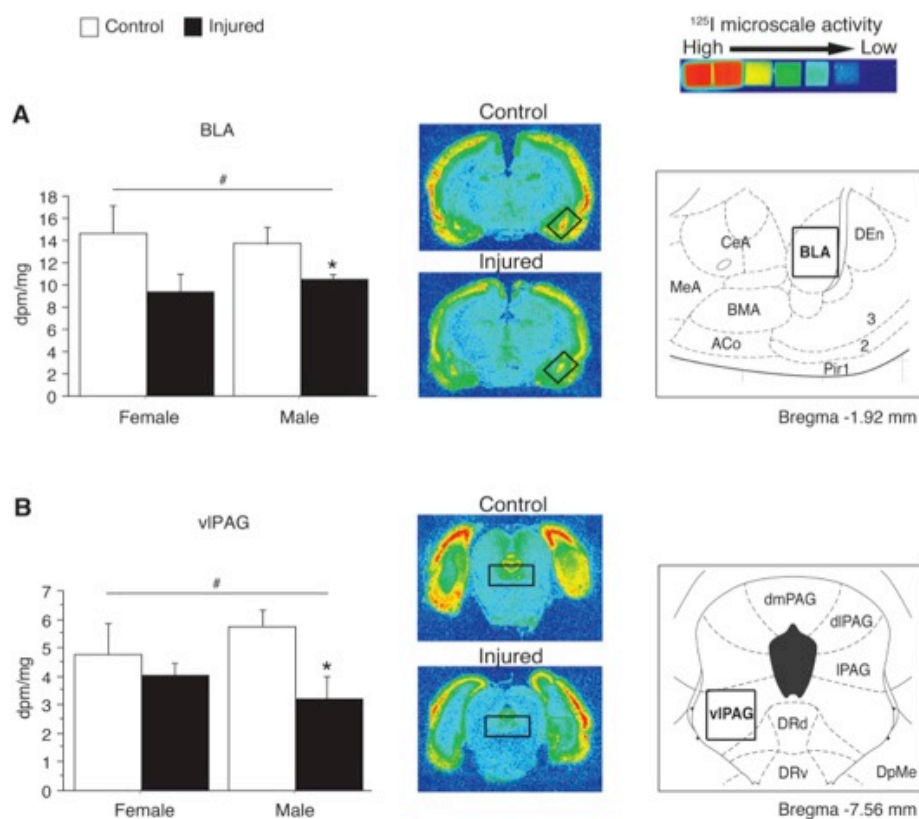


Figure 3.1 Experimental procedure

Time pregnant Sprague Dawley dams arrived in the animal facility on gestational day 14 (GD14). Within 24 hrs of birth on postnatal day 0 (PD0) pups were injured (intraplantar injection of 1% carrageenan) or handled identically. With the exception of mandatory cage changes and weaning on PD21, animals were not disturbed until adulthood (\geq PD60). Daily estrus cycling in females and handling in males (removal from cage for 10 s) began between PD60-65 and continued for 2 weeks until the day before sample collection (PD75-80). Between PD75-80 animals were either euthanized for receptor autoradiography (ARad), *in situ* hybridization (ISH), immunohistochemistry (IHC) or tested for HPA axis functioning in response to restraint or swim stress.



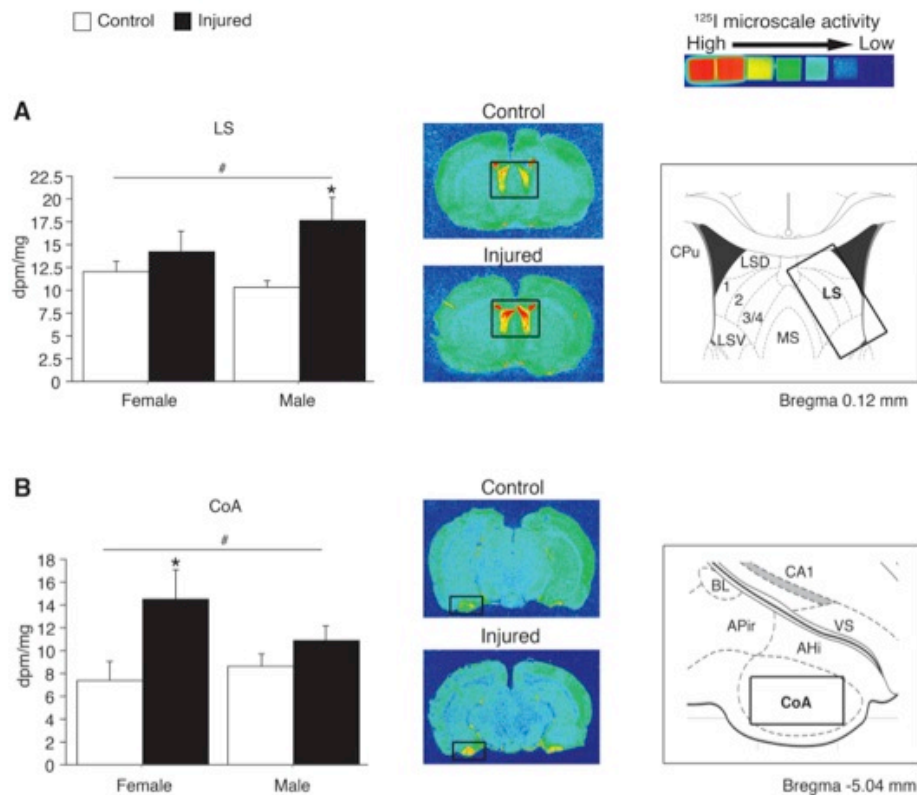


Figure 3.3 The impact of neonatal injury on adult CRFR2 binding

Neonatal injury significantly increased binding of ^{125}I sauvagine to CRFR2 in the (A) lateral septum (LS) and (B) cortical amygdala (CoA) relative to controls. Region of interest boxed in radio-graphs and atlas plates. Surrounding region abbreviations: caudate putamen (CPu), dorsal or ventral lateral septum (LSD, LSV, respectively), medial septum (MS), basal amygdala nucleus (BL), field CA1 hippocampus (CA1), amygdalopiriform transitional area (APir), amygdalohippocampal transition area (AHi), ventral subiculum (VS). Data are presented as Mean \pm SEM; $n = 5$ subjects per group. Specific binding measured in disintegrations per minute per milligram of tissue (dpm/mg). Significant main effect of treatment observed using 2-way ANOVA, $\#P < 0.05$ over bar. Significant between group differences measured post-hoc by Tukey-Kramer, $*P < 0.05$.

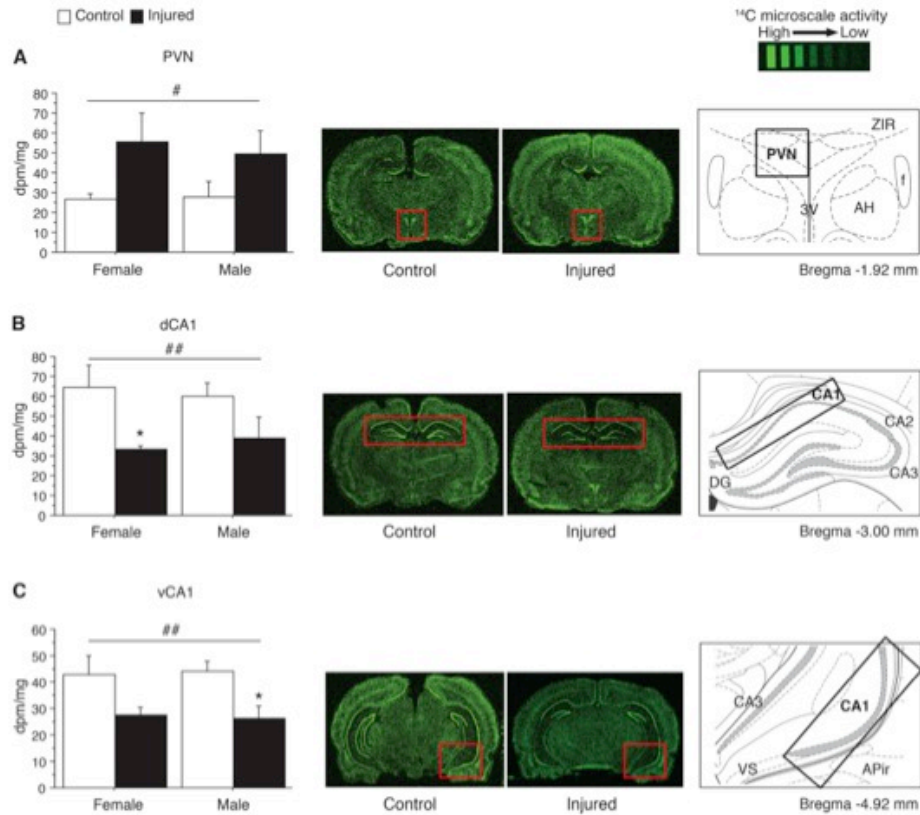


Figure 3.4 The impact of neonatal injury alters adult expression of GR mRNA

Neonatal injury significantly increased GR mRNA in the (A) paraventricular nucleus (PVN) relative to controls. (B-C) Decreased GR mRNA was observed in both dorsal and ventral CA1 of the hippocampus (dCA1 and vCA1, respectively) of neonatally injured adults. Region of interest boxed in radiographs and atlas plates. Surrounding region abbreviations: zona inserta (ZIR), fornix (f), anterior hypothalamus (AH), third ventricle (3V), dentate gyrus (DG), field CA2 and CA3 hippocampus (CA2 and CA3, respectively), amygdalopiriform transitional area (APir), ventral subiculum (VS). Data are presented as Mean \pm SEM; $n = 5$ subjects per group. Specific hybridization measured in disintegrations per minute per milligram of tissue (dpm/mg). Significant main effect of treatment observed using 2-way ANOVA, # $P < 0.05$, ## $P < 0.01$ over bar. Significant between group differences measured post-hoc by Tukey-Kramer, * $P < 0.05$.

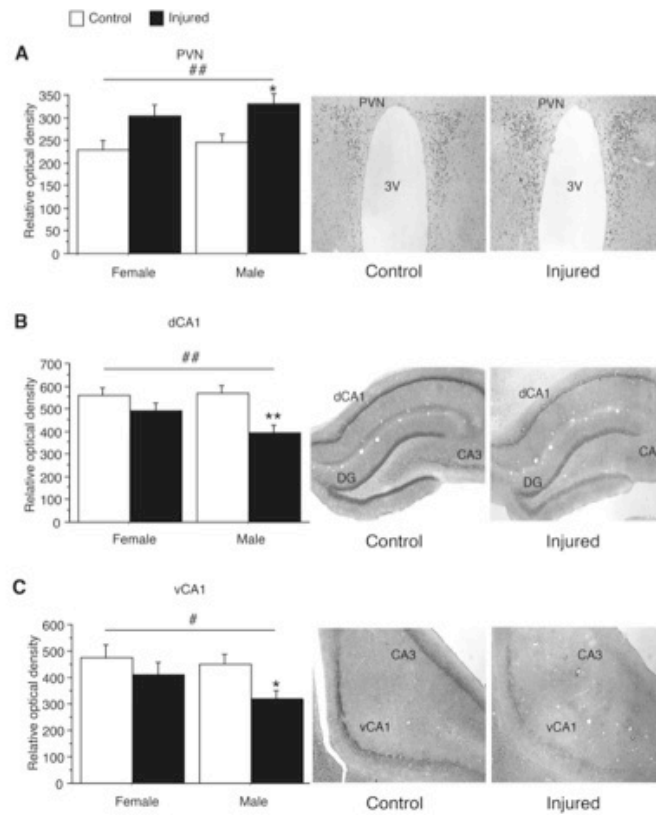


Figure 3.5 The impact of neonatal injury on adult GR protein immunoreactivity (ir)
 GR-ir was significantly increased in neonatally injured adults in the (A) paraventricular nucleus (PVN) relative to controls. (B-C) Neonatal injury significantly decreased GR-ir in dorsal and ventral CA1 of the hippocampus (dCA1, vCA1, respectively). Data are presented as Mean \pm SEM; n = 9-11 subjects per group. Significant main effect of treatment observed using 2-way ANOVA, #P < 0.05 or ##P < 0.01 over bar. Significant between group differences measured post-hoc by Tukey-Kramer, *P < 0.05, **P < 0.01.

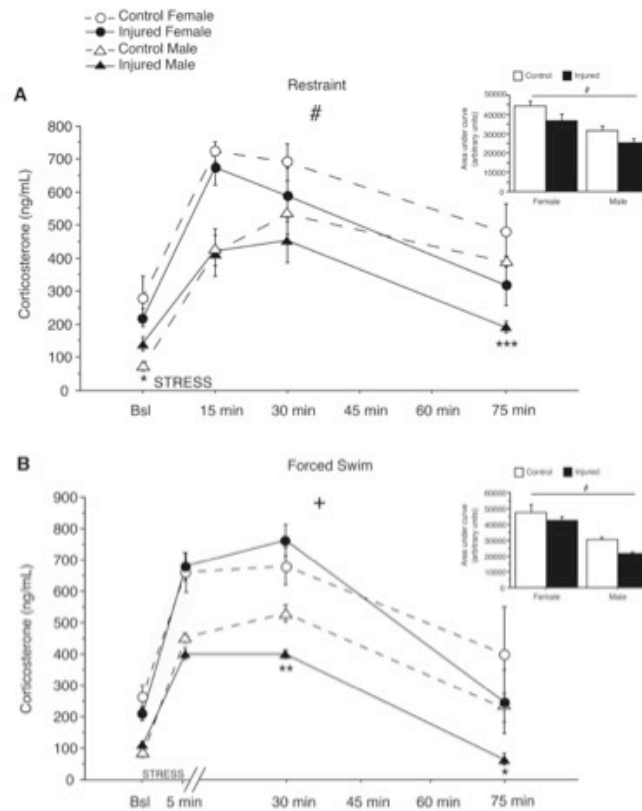


Figure 3.6 Plasma corticosterone (CORT) levels in response to restraint (A) or forced swim (B) stress

(A) Repeated Measures ANOVA for time revealed that neonatal injury significantly decreased CORT in males 75 min after the onset of restraint (recovery). (B) Following 5 min of swimming, CORT was significantly reduced at 30 min and 75 min in neonatally injured males relative to controls. Females had significantly higher CORT concentrations in response to restraint and swim independent of neonatal treatment as compared with males (A-B). Insets for (A-B) depict area under the curve. Data are presented as Mean \pm SEM; $n = 5-12$ subjects per group. Significant main effects of treatment, $^{\#}P < 0.05$. Significant time \times sex \times treatment interaction observed in (B), $^{\dagger}P < 0.05$. Significant between group differences measured post-hoc by Tukey-Kramer, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

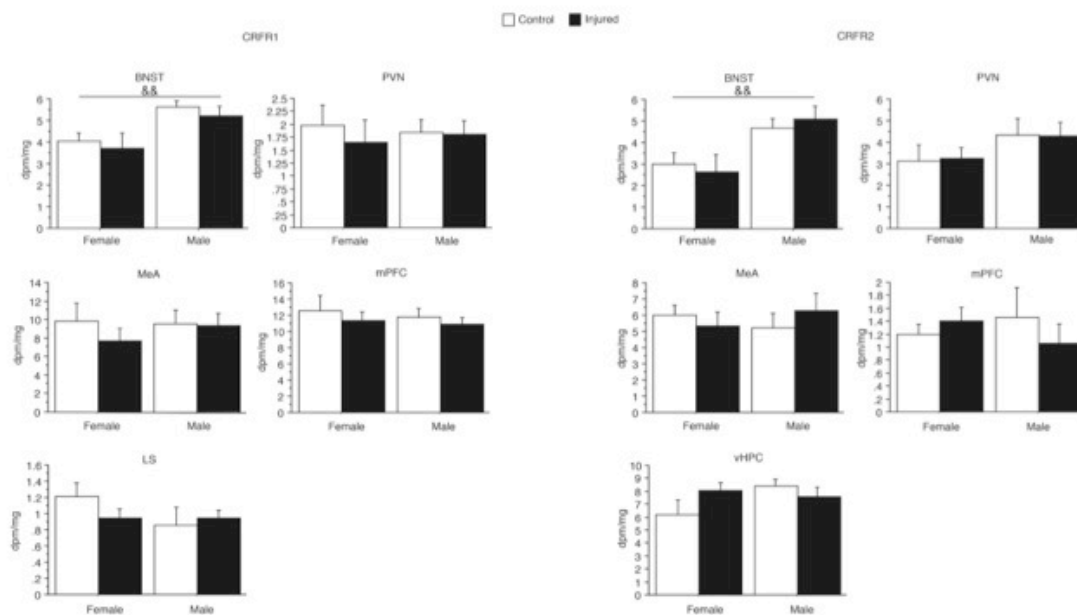


Figure 3.7 Supplemental 1

No significant change in competitive binding of ^{125}I Sauvagine to CRFR1 (left column) or CRFR2 (right column) was observed as a result of neonatal injury in a number of brain regions. Data are presented as Mean \pm SEM; $n = 5$ subjects per group. Specific binding measured in disintegrations per minute per milligram of tissue (dpm/mg). Region abbreviations: bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), medial amygdala (MeA), medial prefrontal cortex (mPFC), lateral septum (LS) and ventral hippocampus (vHPC). Significant main effect of sex observed using 2-way ANOVA $\&\&P < 0.01$ over bar.

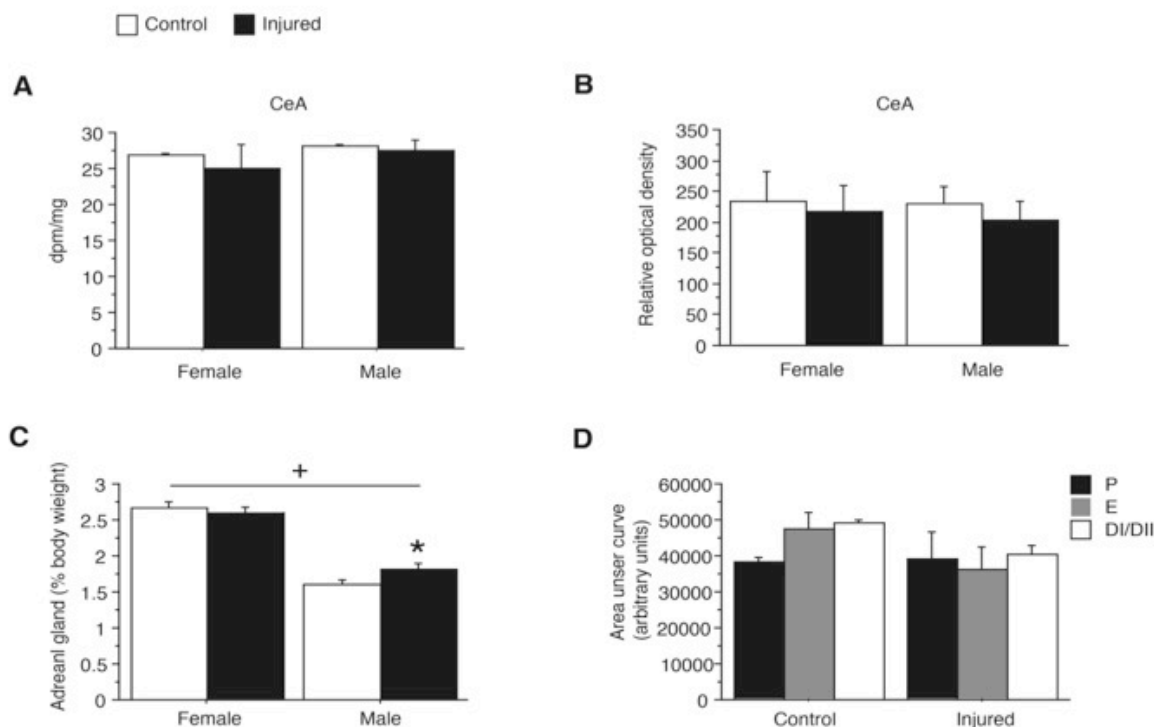


Figure 3.8 Supplemental 2

(A) Neonatal treatment did not significantly change GR mRNA expression and (B) immunoreactivity in the central amygdala (CeA) as measured with *in situ* hybridization (n = 5 subjects per group) and immunohistochemistry (n = 9-11 subjects per group), respectively. (C) Adrenal gland weight as a percent of total body weight was significantly increased in females in comparison to males (n = 8-11 subjects per group). Among males, adrenal glands comprised a significantly larger percent of body weight for injured relative to control males. (D) No effect of estrus on CORT concentrations was found in either restraint or swim tests, therefore data are collapsed across tests into AUC. Similarly, no significant main effects were observed on CORT AUC (estrus: $F_{(2,25)} < 1$; $P = 0.46$; treatment: $F_{(1,25)} = 2.71$; $P = 0.11$; estrus x treatment interaction: $F_{(2,25)} < 1$; $P = 0.46$) (n = 3-8 subjects per group). Abbreviations: Proestrus (P), Estrus (E), Diestrus (DI/II). Data are presented as Mean \pm SEM. Significant sex x treatment interaction observed using 2-way ANOVA, $^{\dagger}P < 0.05$ over bar. Significant group differences measured post-hoc by Tukey-Kramer, $*P < 0.05$.

4 CHAPTER FOUR: NEONATAL INJURY RAPIDLY ALTERS MARKERS OF PAIN AND STRESS IN RAT PUPS

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4.1 Abstract

Less than 60% of infants undergoing invasive procedures in the NICU receive analgesic therapy. These infants show long-term decreases in pain sensitivity and cortisol reactivity. In rats we have previously shown that inflammatory pain experienced on the day of birth significantly decreases adult somatosensory thresholds and responses to anxiety- and stress-provoking stimuli. These long-term changes in pain and stress responsiveness are accompanied by 2-fold increases in central met-enkephalin and β -endorphin expression. However, the time course over which these changes in central opioid peptide expression occur, relative to the time of injury, are not known. The present studies were conducted to determine if the observed changes in adult opioid peptide expression were present within the first postnatal week following injury. The impact of neonatal inflammation on plasma corticosterone, a marker for stress reactivity, was also determined. Brain, spinal cord and trunk blood were harvested at 24 hrs, 48 hrs and 7 days following intraplantar administration of the inflammatory agent carrageenan on the day of birth. Radioimmunoassay was used to determine plasma corticosterone and met-

enkephalin and β -endorphin levels within the forebrain, cortex, midbrain, and spinal cord. Within 24 hrs of injury met-enkephalin levels were significantly increased in the midbrain, but decreased in the spinal cord and cortex; forebrain β -endorphin levels were significantly increased as a result of early life pain. Corticosterone levels were also significantly increased. At 7 days post-injury, opioid peptides remained elevated relative to controls, suggesting a time point by which injury induced changes become programmed and permanent.

4.2 Introduction

Each year, 16.5% of infants worldwide and 12% of infants in the United States are born prior to 37 gestational weeks and are considered preterm (Martin et al., 2006). Preterm infants spend an average of 25 days in the Neonatal Intensive Care Unit (NICU) where they undergo 10-18 painful, inflammatory and invasive procedures each day, including repeated heel lance, endotracheal intubation, surgery, and respiratory and gastric suctioning (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008; PeriStats, 2011). While the majority of these procedures are painful and induce inflammation, analgesia or anesthesia is used in only 2-21% of the invasive procedures performed in the NICU (Simons et al., 2003; Carbajal et al., 2008). Although it was previously thought that the newborn sensory system was incapable of responding to noxious stimuli, it is now clear that preterm infants as young as 25 weeks gestation display evoked cortical activity (Bartocci et al., 2006; Slater et al., 2006), robust secretion of stress hormones (Anand et al., 1987b), and elevated heart rate and facial reactivity (Grunau et al., 2005; Grunau et al., 2010) in response to noxious stimulation. These neuroendocrine and autonomic responses become significantly blunted as the number of invasive procedures experienced increases (Grunau et al., 2005; Grunau et al., 2010), indicating changes in systems mediating pain and stress. Decreased responses to pain- and stress-evoking stimuli persist later in life, as former preterm infants have dampened cortisol reactivity as children and reduced pain sensitivity as

young adults in comparison to term controls (Grunau et al., 2005; Hermann et al., 2006; Grunau et al., 2007; Grunau et al., 2010).

Using a rat model for early life pain, we have previously reported that a single inflammatory insult (carrageenan 1% (CGN); hindpaw) occurring within the first postnatal week (P0-8) significantly dampens adult responses to pain- and stress-evoking stimuli (i.e. a hypo-responsive phenotype) (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009; Victoria et al., 2013b). As adults, neonatally injured rats show significant and bilateral increases in their response to noxious thermal and mechanical stimuli (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009), as well as blunted responses to anxiety- and stress-provoking stimuli (Anseloni et al., 2005; Victoria et al., 2013b). These behavioral changes are paralleled by increased expression of the endogenous opioid peptides met- and leu-enkephalin and β -endorphin in several brain regions, including the midbrain periaqueductal gray (PAG), central amygdala (CeA) and lateral septum (LS) (LaPrairie and Murphy, 2009; Victoria et al., 2013b). The injury-induced changes in pain and stress responsiveness are naloxone-reversible (Ren et al., 2004; LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b), suggesting that changes in central opioid tone contribute to the long-term consequences of early life pain.

Our overarching hypothesis is that changes in central opioid peptide expression occur neonatally as an immediate response to the injury, and that these changes are subsequently maintained into adulthood. However, the critical developmental time point when early life pain results in long-term changes in somatosensory thresholds (P0-P8) corresponds to the stress hypo-responsive period where plasma corticosterone levels are lower than normal and application of a noxious stimulus evokes minimal changes in glucocorticoid levels (Corbier and Roffi, 1978b, a; Henning, 1978; Meaney et al., 1985a; Walker et al., 1986). Therefore, the present study was conducted to determine if early life pain alters brain and spinal cord enkephalin and β -endorphin levels within the first postnatal week. The impact of early life pain on plasma corti-

costerone was also examined. Using radioimmunoassay we present evidence for the first time that a single inflammatory insult on the day of birth significantly increases plasma corticosterone and central met-enkephalin and β -endorphin 24 hrs after injury. By the end of the first postnatal week, these essential mediators of stress and pain remain elevated in comparison to controls, suggesting a time point by which injury induced changes become programmed and permanent.

4.3 Materials And Methods

Animals

Pregnant Sprague-Dawley rat dams were obtained on gestational day 14 (G14) (Charles River, USA). Dams were housed individually under 12:12 hr light:dark cycle with *ad libitum* access to food and water. On the day of birth (P0), pups were subjected to neonatal treatment. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP, and were approved by the Georgia State University Animal Care and Use Committee.

Neonatal Treatment

Acute neonatal inflammatory injury was induced as in our previous studies (LaPrairie and Murphy, 2007, 2009; Victoria et al., 2013b). Briefly, rat pups were injected with 5 μ L carrageenan (CGN; 1% dissolved in saline; Sigma, St. Louis, MO) into the intraplantar surface of the right hindpaw or handled identically within 24 hours of birth (P0). This well-established model causes acute, local inflammatory pain that persists for 24-72 hours and does not alter maternal behavior (Ren et al., 2004; LaPrairie and Murphy, 2007). Intraplantar saline alone was not used, as we have previously observed no difference from handled controls (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009). Pups were separated from their dam for <20 minutes and returned to the home cage as a group. Treatment of all litters occurred between 11:00-12:00. Each litter (n = 5-14 pups; approximately equal numbers males and fe-

males) received a single treatment as pups are indistinguishable after resolution of paw edema. Sex differences were beyond the scope of these studies due to disparate litter sizes.

Sample Harvest

Neonatally injured and control animals were decapitated with heavy scissors 24 hrs, 48 hrs and 7 days after neonatal treatment. Immediately thereafter, trunk blood, brains and spinal cord were collected. Blood samples were collected directly into 1 mL EDTA-hematology tubes (BD from Fisher Scientific), maintained at room temperature for ≥ 30 min and centrifuged at 4000 rcf at 4°C for 15 min. Plasma was pipetted into microcentrifuge tubes and stored at -80°C until time of assay. Brains and spinal cords were extracted, flash frozen in 2-methylbutane (VWR, USA) chilled on dry ice and stored at -80°C. Brains harvested at 24 hrs and 48 hrs after neonatal treatment were sectioned coronally with a razor blade on dry ice into forebrain (caudal to olfactory bulbs and rostral to superior colliculus; corresponding to coronal figures 3-17 in *Atlas of the Neonatal Rat Brain* (Ramachandra and Subramanian, 2011) and midbrain (caudal to superior colliculus and rostral to medulla; coronal figures 18-28 (Ramachandra and Subramanian, 2011)). Brains harvested on P7 were sectioned as above; forebrain and midbrain segments corresponded to coronal figures 47-61 and 61-69, respectively (Ramachandra and Subramanian, 2011). External anatomy and atlas plates were used to include the striatum, septum, thalamus, hippocampus, hypothalamus and amygdala in the forebrain and colliculi and periaqueductal gray in the midbrain. For all time points, cerebral cortex was dissected away caudal to olfactory bulbs and rostral to superior colliculus. For each region of interest, sections were pooled from 2-3 pups of the same treatment.

Corticosterone Concentrations

Plasma corticosterone was measured using I-125 labeled Double Antibody Corticosterone kit (MP Biomedicals, USA) according to the manufacturer's instructions. Concentra-

tion of corticosterone was determined against known standards ($R^2 > 0.98$) where the minimum limit of detection was 3.125 ng/mL. Intra-assay coefficient of variation was 3.6%.

Protein Extraction

Protein was extracted from brain sections and the entire spinal cord using the protocols of Tsang and Ng (1979) and Kim et al. (1999). Tissue samples were bathed in 0.1 M (1 mL/sample) acetic acid and boiled (95-100°C) for 10-15 min. Samples were cooled for 1 hr, then homogenized with an electric pestle and centrifuged at 13000 x g for 15 min at 4°C. Supernatant from each sample was divided into 300 µL aliquots, frozen to -80°C, and then lyophilized to isolate extracted peptides. Samples were reconstituted with assay specific buffers and analyzed for met-enkephalin, β-endorphin or total protein concentrations.

Opioid Peptide Concentrations

Met-enkephalin protein was measured using I-125 labeled Met-enkephalin RIA kit (Bachem, USA; Cat.# S-2119) according to the manufacturer's protocol. Concentration of met-enkephalin was determined against known standards ($R^2 > 0.99$), where the minimum limit of detection was 0.01 ng/mL. Intra-assay coefficient of variation was 2.4%.

β-endorphin protein was measured using I-125 labeled β-endorphin RIA kit (Phoenix Pharmaceuticals, Inc., USA; Cat.# RK-022-06) according to the manufacturer's protocol. Concentration of β-endorphin was determined against known standards ($R^2 > 0.99$), where the minimum limit of detection was 10.0 pg/mL. Intra-assay coefficient of variation was 1.8 %.

Total protein concentrations were determined using Quick Start™ Bradford Protein Assay (Bio Rad, USA) according to the manufacturer's protocol. Concentration of total protein was compared to known standards ($R^2 > 0.92$), where the minimum limit of detection was 2.5 µg/mL. Intra-assay coefficient of variation was 3.6%.

Statistical Analysis

Endogenous opioid concentrations are presented as total protein (ng/ μ g or pg/ μ g). Values are shown as Mean \pm SEM. Significant main effects of neonatal treatment (injured, handled) and time post-injury (24 hrs, 48 hrs, 7 days) were assessed using two-way ANOVA. Percentages are reported as mean percent change from control. Student's unpaired t-tests were used for post-hoc analyses to determine differences between treatments at specific time points. Where applicable, values ≥ 2 standard deviations from the mean were eliminated as outliers. All comparisons were *a priori* specified. Confidence was set to $p < 0.05$ and considered statistically significant.

4.4 Results

The Impact Of A Single Neonatal Injury On Corticosterone Over The First Postnatal Week

The impact of neonatal injury on plasma corticosterone levels was determined at 24 hrs, 48 hrs and 7 days post-treatment (Fig. 1). A two-way analysis of variance (ANOVA) revealed a significant interaction between injury and time ($F_{(2,55)} = 12.85$; $P < 0.0001$). Twenty-four hours after hindpaw inflammation, corticosterone was significantly higher relative to controls ($t_{(18)} = 4.07$; $P = 0.0007$), but precipitously decreased 48 hrs after injury ($t_{(20)} = 2.29$; $P = 0.033$). Seven days after inflammatory pain, corticosterone levels plateaued and remained 92% higher than controls ($t_{(17)} = -2.12$; $P = 0.049$).

The Impact Of A Single Neonatal Injury On Endogenous Opioid Concentrations During The First Postnatal Week

The impact of neonatal injury on brain and spinal cord met-enkephalin and β -endorphin levels was determined as a function of total protein at 24 hrs, 48 hrs and 7 days post-treatment. Two-way ANOVA revealed a significant interaction of injury and time in the spinal cord ($F_{(2,22)} = 5.07$; $P = 0.015$) such that met-enkephalin was 61% lower than controls at 24 hrs post-injury,

remained low at 48 hrs, after which it increased by 96% relative to controls at 7 days (Fig 2). Significant main effects of injury ($F_{(1,22)} = 5.84$; $P = 0.024$) and time ($F_{(2,22)} = 9.17$; $P = 0.0013$) on met-enkephalin protein levels were observed for the midbrain, where met-enkephalin was significantly increased by 62% at 24 hrs in injured pups ($t_{(7)} = -3.51$; $P = 0.0099$), was similar to control levels at 48 hrs, but remained 85% higher than controls 7 days after injury (Fig 2). A significant main effect of time ($F_{(2,22)} = 3.68$; $P = 0.042$) but not treatment ($F_{(1,22)} < 1$; $P = 0.36$) was observed in the forebrain, where met-enkephalin of injured pups was 69% higher than controls at 24 hrs, decreased below controls by 42% at 48 hrs, then increased above controls by 62% 7 days after injury (Fig 2). Similarly, a significant effect of time ($F_{(2,22)} = 5.75$; $P = 0.0098$) but not treatment ($F_{(1,22)} < 1$; $P = 0.89$) was observed in the cortex, where met-enkephalin was significantly decreased by 50% relative to controls at 24 hrs in injured pups ($t_{(7)} = 2.38$; $P = 0.049$), was similar to control levels at 48 hrs, but increased to 14% higher than controls 7 days after injury (Fig 2).

A significant main effect of injury on β -endorphin protein levels was observed in the cortex ($F_{(1,22)} = 5.83$; $P = 0.024$), independent of time ($F_{(2,22)} < 1$; $P = 0.42$). Pups injured on P0 showed a 51% increase in cortical β -endorphin at 24 hrs and a 188% increase at 7 days relative to controls ($t_{(7)} = -2.68$; $P = 0.031$) (Fig 3A). A significant interaction between injury and time ($F_{(2,22)} = 3.54$; $P = 0.046$) was observed in the forebrain such that after hindpaw inflammation β -endorphin was 504% higher than controls at 24 hrs ($t_{(7)} = -2.49$; $P = 0.041$), remained elevated by 107% above controls at 48 hrs, then decreased to control levels at 7 days (Fig 3B). A significant main effect of time ($F_{(2,22)} = 4.02$; $P = 0.033$) but not injury ($F_{(1,22)} = 2.84$; $P = 0.11$) on β -endorphin protein levels was observed for the midbrain, where β -endorphin was increased by 118% at 24 hrs in injured pups, was similar to control levels at 48 hrs, but remained 400% higher than controls 7 days after injury (Fig 3C). No significant main effect of time ($F_{(2,22)} < 1$; $P = 0.58$) or treatment ($F_{(1,22)} < 1$; $P = 0.66$) on β -endorphin was observed in the spinal cord (Fig 3D).

4.5 Discussion

The present studies were conducted to determine the time course for change in glucocorticoid and endogenous opioid concentrations over the first postnatal week following a single inflammatory injury at birth. Our results show that 24 hrs post-treatment, injured pups had significantly increased levels of met-enkephalin and β -endorphin in the midbrain and forebrain. By contrast, significant decreases in met-enkephalin levels were observed in the cortex and spinal cord at this time point. Plasma corticosterone levels were elevated in injured pups relative to controls indicating that intraplantar CGN resulted in the activation of the hypothalamic pituitary adrenal axis (HPA) axis. Seven days after treatment, met-enkephalin levels remained elevated relative to controls in all brain regions examined, while β -endorphin remained significantly higher in the cortex and midbrain. Plasma corticosterone also remained significantly higher in injured pups. Together, these data suggest that early life pain impacts both glucocorticoid release and endogenous opioid concentration in a site- and time-specific manner during early postnatal development.

Our working hypothesis is that neonatal pain experienced during a critical neurodevelopmental period (P0-P8 (LaPrairie and Murphy, 2007)) increases afferent nociceptive drive to brain regions responsive to noxious input, including the thalamus and the periaqueductal gray. This increase in afferent drive triggers the activation of descending pain modulatory circuits, resulting in the release of endogenous opioids (met-enkephalin and β -endorphin) to dampen pain perception and produce analgesia (Walker et al., 1986; Fitzgerald, 2005; LaPrairie and Murphy, 2009). As pain is a potent stressor, neurohormones from the HPA axis are also released to mount appropriate physiological responses that promote recovery from the physical perturbation of inflammatory insult (Vale et al., 1981; Iny et al., 1987; Lightman and Young, 1989; Taylor et al., 1998). As inflammation associated with intraplantar carrageenan persists for 24-72 hours, the release of endogenous opioids, as well as corticotrophin releasing factor (CRF) and corticosterone, is sustained. In adulthood, both met-enkephalin and β -endorphin levels remain ele-

vated within the PAG, CeA and LS (LaPrairie and Murphy, 2009; Victoria et al., 2013b). These regions have been previously implicated in pain and stress, and suggest that early life pain results in the permanent transcriptional modulation of promoters regulating opioid peptide expression (Schoneveld et al., 2004).

One Neonatal Injury Disrupts Gradual Reduction Of Corticosterone Within The First Postnatal Week

Systemic corticosterone levels are typically elevated shortly after birth, then gradually decrease to undetectable levels within the first postnatal week (Corbier and Roffi, 1978b, a; Henning, 1978; Meaney et al., 1985a; Walker et al., 1986). This reduction marks the stress hyporesponsive period (SHRP), which spans approximately P2-P14 in rat pups (Sapolsky and Meaney, 1986; Walker et al., 1986). During the SHRP, low glucocorticoid levels promote neurogenesis, axonal outgrowth, synaptogenesis and myelination (Sapolsky and Meaney, 1986; Walker et al., 1986; Baud et al., 2005; Antonow-Schlorke et al., 2009; Du et al., 2009; Liston and Gan, 2011). In the present study, corticosterone was significantly elevated 24 hrs after injury, precipitously decreased at 48 hrs, but remained significantly higher than controls on P7. These results parallel clinical data reporting that preterm infants undergoing surgery in the absence of anesthesia experience significantly elevated corticosterone levels post-operatively (Anand et al., 1987b).

Persistently high corticosterone during the SHRP is known to negatively impact postnatal development. For example, treatment with the corticosteroid dexamethasone on P3-P4 increases the density of cortical GABAergic interneurons by 50% and decreases cortical thickness by P5 in mouse pups (Baud et al., 2005). Similarly, hydrocortisone injections between P1-P4 decrease hippocampal volume of rat pups (Bohn, 1980). Moreover, high concentrations of glucocorticoids early in life accelerate dendritic spine formation and elimination in the somatosensory (S1) cortex (Liston and Gan, 2011), suggesting variations in corticosterone may have

a sustained impact on circuit formation. Finally, high doses of corticosterone increase cell death, decrease Bcl2-glucocorticoid receptor (GR) complexes and promote excitotoxicity in cortical neurons (Du et al., 2009).

We have recently reported that early life pain decreases behavioral sensitivity to stress- and anxiety-provoking stimuli (Victoria et al., 2013b). Adult male and female rats who were injured on the day of birth spend significantly more time in the inner area of the open field and have increased latencies to immobility in the forced swim test (Victoria et al., 2013b). We have further reported that neonatal injury results in significant changes to adult neurocircuits underlying the activation and termination of the stress response. For example, CRF receptor 1 (CRFR1) is significantly decreased in the basolateral amygdala and PAG, sites that activate stress (Smith et al., 1998; Bhatnagar et al., 2004) and mediate autonomic tone (Lewis and Gebhart, 1977; Behbehani and Fields, 1979; Carrive and Bandler, 1991; Depaulis et al., 1994; Inui et al., 1994), respectively (Victoria et al, 2013, unpublished data). These rats also have significantly increased levels of CRF receptor 2 (CRFR2) in regions that promote stress recovery (Bale et al., 2000; Coste et al., 2000), including the LS (Victoria et al, 2013, unpublished data). Given that corticosterone-GR complexes transcriptionally regulate the central expression of CRFRs (Schoneveld et al., 2004), such changes are expected.

Injury On P0 Changes Met-Enkephalin And β -Endorphin Concentration With Regional And Temporal Specificity

Early life pain induced large time- and region-dependent changes in met-enkephalin concentration over the first postnatal week. In the cortex, met-enkephalin was reduced for injured pups 24 hrs after treatment, then increased slightly above controls at 48 hrs and 7 days. Spinal cord met-enkephalin levels decreased 24 hrs after treatment then increased to 96% greater than controls by P7. This result is consistent with previous studies demonstrating significant reduction in spinal cord preproenkephalin mRNA within 24 hrs of hindpaw inflammation

(Noguchi et al., 1989), suggesting a period during which enkephalin must be sufficiently replenished to combat persistent inflammatory pain. In the midbrain and forebrain, met-enkephalin levels were elevated in injured pups at 24 hrs post-treatment and remained elevated 7 days after hindpaw inflammation. This increased level of expression is maintained into adulthood, where significantly increased met-enkephalin mRNA and protein is observed in the midbrain PAG, CeA and LS (LaPrairie and Murphy, 2009; Victoria et al., 2013b), and suggests that neonatal pain permanently upregulates the expression of central enkephalin in regions essential for processing pain- and stress-associated information.

As with met-enkephalin, large time-dependent changes in β -endorphin were observed over the first postnatal week. In the cortex, forebrain and midbrain, β -endorphin levels were increased at 24 hrs post-injury, and remained elevated in the cortex and midbrain one week later. These data are consistent with our previous report that early life pain increases β -endorphin protein in the PAG of adults (LaPrairie and Murphy, 2009). Although no change in β -endorphin levels was noted in the spinal cord, spinal cord β -endorphin expression is known to be low and present primarily as POMC, its unprocessed precursor (Gutstein et al., 1992).

In the present study, changes in endogenous met-enkephalin and β -endorphin cannot be localized to specific nuclei within the midbrain and forebrain. However, these data support the hypothesis that neonatal pain rapidly impacts neural circuits and support our previous reports that early pain permanently upregulates central endogenous opioid tone (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009; Victoria et al., 2013b). Early life changes in enkephalin and β -endorphin have implications for brain development and function. In the absence of pain, acute subcutaneous administration of met-enkephalin reduces proliferation of neurons and glia in the cerebellum, and decreases DNA synthesis in P6 rat pups (Zagon and McLaughlin, 1991). Such changes are reversible with concurrent application of naloxone (Zagon and McLaughlin, 1991) or naltrexone (Hammer et al., 1989). Together these find-

ings suggest that aberrantly high levels of opioids early in life may have deleterious effects on brain development.

Conversely, blocking endogenous opioids during postnatal life also has developmental consequences. For example, blockade of μ - and δ -opioid receptors from P0-P10 increases neuronal maturation, spine number and dendrite length of pyramidal cells in the cerebral cortex, hippocampus and cerebellum (Hauser et al., 1989). Moreover, brain size, number of neurons and glia, and thickness of S1 cortex are significantly increased in juvenile rats receiving naltrexone between P0-P21 (Zagon and McLaughlin, 1983). Collectively, these findings suggest that either excessive or insufficient opioid levels in the brain during postnatal life disrupt sensitive developmental processes. Further, they support the hypothesis that development is regulated by finely tuned concentrations of neuropeptide early in life.

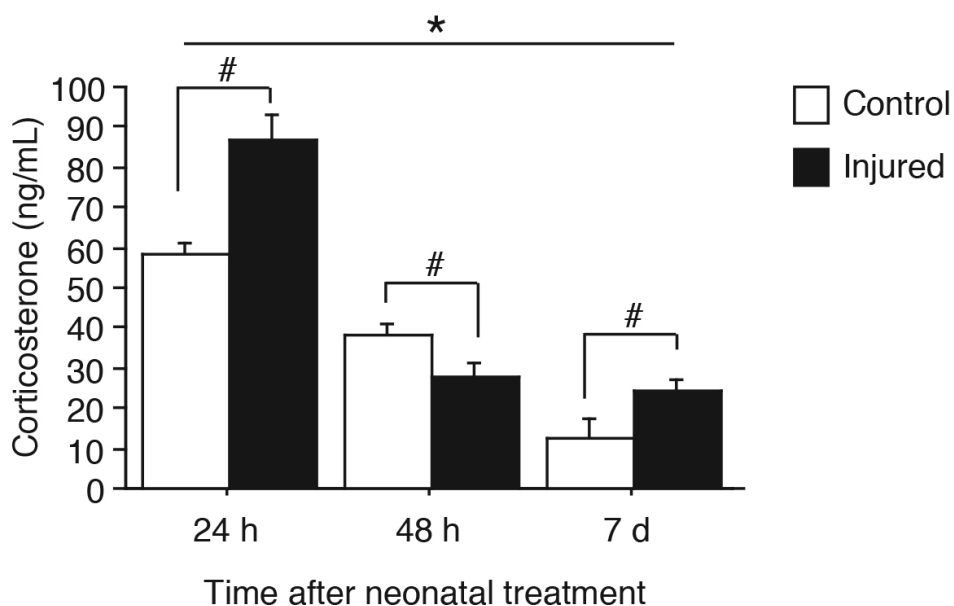
Our results showing that early life pain results in the immediate and long-term release of endogenous opioid peptides (LaPrairie and Murphy, 2009; Victoria et al., 201bc) are consistent with growing clinical data suggesting that exposure to repeated tissue damaging procedures in neonates, with limited analgesic therapy, induces lasting changes in the brain and spinal cord, which have profound consequences for subsequent nociceptive processing (Anand, 2000; Whitfield and Grunau, 2000; Lidow, 2002; Walker et al., 2003; Grunau et al., 2005; Hermann et al., 2006; Hohmeister et al., 2010; Wollgarten-Hadamek et al., 2011). For example, infants with previous NICU experience display decreased facial and cardiovascular responses to heel lance compared to age matched full-term infants (Johnston et al., 1996). Moreover, decreased facial responsiveness to immunization at 4 and 8 months (Oberlander et al., 2000), and blunted nociceptive sensitivity have been reported in 18 month old former preterm neonates compared to full term peers (Grunau et al., 1994a). Former NICU toddlers are also rated by parents as less sensitive to pain compared to term-born controls, with a higher frequency of procedural pain exposure associated with more dampened nociceptive responsiveness (Grunau et al., 1994a). Similarly, 9-12 year olds that had previously undergone infant cardiac surgery with limited pain ther-

apy display global alterations in both mechanical and thermal somatosensory processing (Schmelzle-Lubiecki et al., 2007). Collectively, these studies strongly indicate that early life pain in humans induces centrally-mediated changes in nociceptive pathways resulting in a long-term, if not permanent, attenuation in pain sensitivity. Our data demonstrating increased endogenous opioid peptide levels in immediate response to a traumatic injury that are maintained into adulthood provide a mechanism whereby early life pain permanently attenuates subsequent nociceptive processing.

Conclusions

The current studies demonstrate that a single inflammatory insult on the day of birth significantly alters central met-enkephalin and β -endorphin concentrations over the first postnatal week. Our data further suggest that injury disrupts the SHRP, a period of quiescence that facilitates postnatal maturation (Sapolsky and Meaney, 1986). Together, our findings suggest a time course over which injury-induced changes become programmed and permanent. Collectively, these data have implications for the importance of analgesic intervention during painful and inflammatory procedures in the NICU.

4.6 Chapter 4 Figures

**Figure 4.1 Injury on P0 alters corticosterone over the first postnatal week**

Concentrations of corticosterone were measured from trunk blood collected 24 hrs, 48 hrs, and 7 days after injury on P0. Corticosterone was significantly increased 24 hrs and 7 days after hindpaw inflammation on P0, but significantly decreased at 48 hrs in comparison to controls. Data are shown as 2-way ANOVA (Mean \pm SEM); n = 5-14 subjects per group. Significant effect of time was observed. (*) Denotes significant effect of injury. (#) Denotes significant between group differences as measured post-hoc by Student's t-test. P < 0.05.

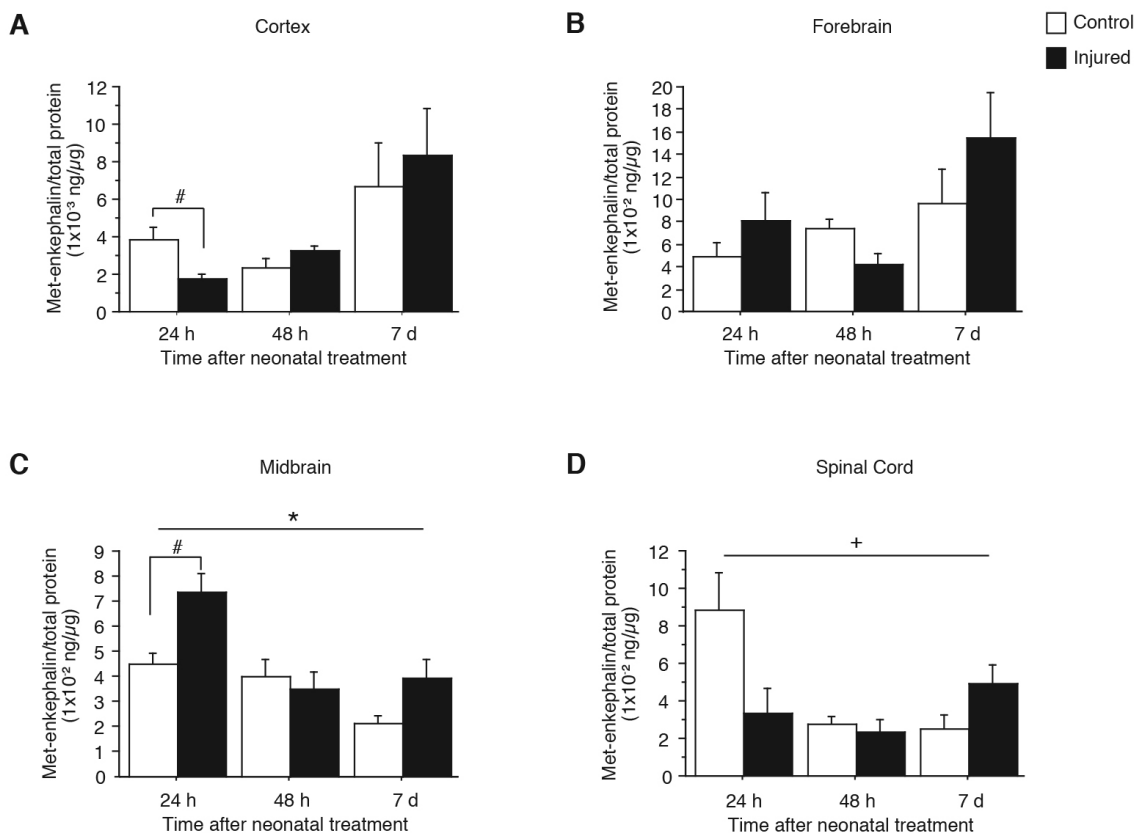


Figure 4.2 Neonatal injury changes central met-enkephalin over the first postnatal week

Met-enkephalin protein was measured relative to total protein in brain and spinal cord harvested 24 hrs, 48 hrs, and 7 days after injury on P0. Met-enkephalin was significantly decreased in the cortex and spinal cord (A and D, respectively), but significantly increased in the midbrain (C) 24 hrs after injury. Increases in met-enkephalin were observed in the cortex, forebrain, midbrain and spinal cord of injured pups relative to controls 7 days after treatment (A-D). Data are shown as 2-way ANOVA (Mean \pm SEM); $n = 3 - 6$ pooled samples per group. Significant main effect of time was observed in (A, B, C). (*) Denotes significant main effect of treatment (C). (+) Denotes significant interaction of injury and time (D). (#) Denotes significant between group differences as measured post-hoc by Student's t-test. $P < 0.05$.

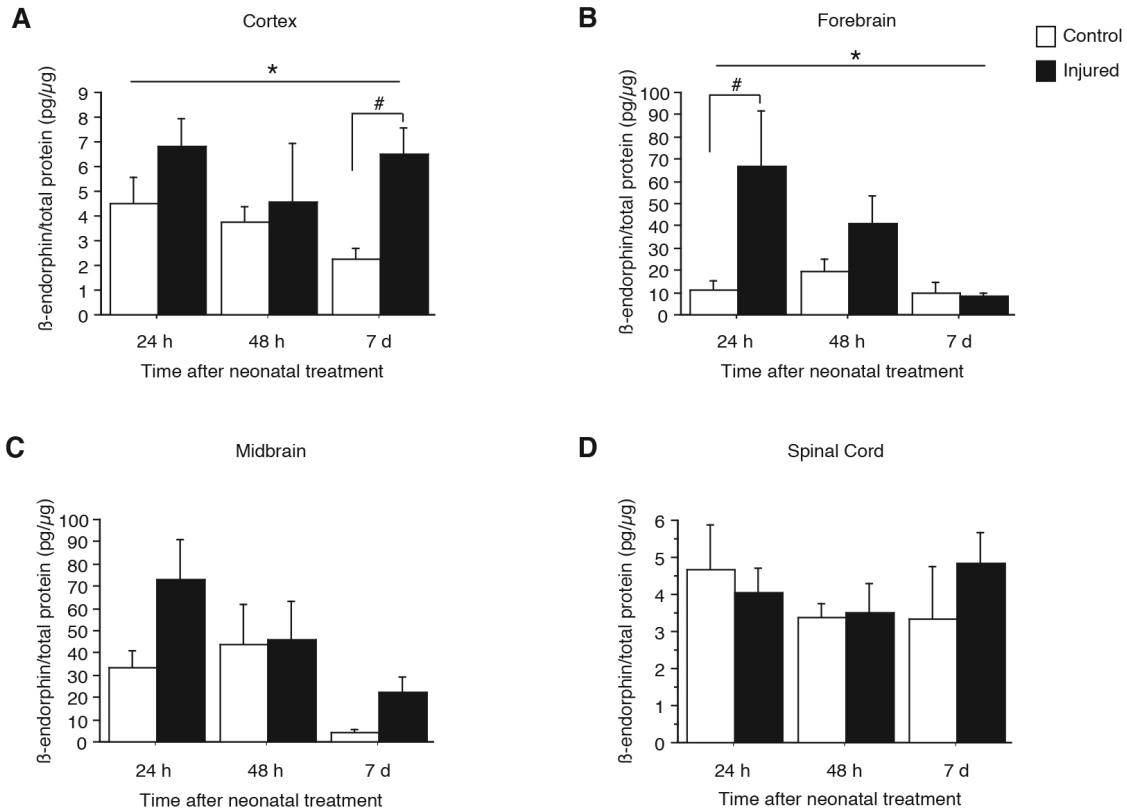


Figure 4.3 Neonatal injury rapidly changes β -endorphin in the brain

Neonatal injury rapidly changes β -endorphin in the brain. β -endorphin protein was measured relative to total protein in brains harvested 24 hrs, 48 hrs, and 7 days after injury on P0. β -endorphin was increased in the cortex (A), forebrain (B) and midbrain (C) 24 hrs after injury. β -endorphin concentrations returned to control levels in the forebrain but remained elevated in the cortex (A) and midbrain (C) 7 days after hindpaw inflammation. Data are shown as 2-way ANOVA (Mean \pm SEM); n = 3 - 6 pooled samples per group. Significant main effect of time was observed in (A and C). (*) Denotes significant main effect of treatment (A and B). (#) Denotes significant between group differences as measured post-hoc by Student's t-test. P < 0.05.

5 CHAPTER FIVE: ANALGESIA FOR EARLY LIFE PAIN PREVENTS DEFICITS IN ADULT ANXIETY AND STRESS IN RATS

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5.1 Abstract

Worldwide approximately 1 in 6 infants are born prematurely each year. Typically, these infants spend several weeks in the Neonatal Intensive Care Unit where they experience 10-18 painful, inflammatory procedures each day. However, more than 70% of these procedures are conducted in the absence of pain therapy. Early life pain is associated with decreases in pain sensitivity, blunted cortisol responses and high rates of neuropsychiatric disorders later in life. In rats we have previously reported that a single inflammatory pain experience on the day of birth (P0) results in adult hypoalgesia, hyposensitivity to anxiety- and stress-provoking stimuli and blunts corticosterone following acute stress. Here, we asked whether morphine treatment for early life pain prevents changes in adult behavioral and hormonal sensitivity. On P0 male and female Sprague-Dawley rat pups were given an intraplantar injection of 1% carrageenan in the presence or absence of (+/-) morphine. In adulthood, neonatal injury significantly increased time in the inner area of the open field, increased latency to immobility and decreased time immobile in the forced swim test, and accelerated return of corticosterone to baseline relative to controls.

Following 7 days of chronic variable stress, injured animals initiated immobility, spent significantly more time floating and had significantly elevated corticosterone. Responses to acute and chronic stress were significantly attenuated in animals that received morphine at the time of inflammation. These data suggest analgesia for early life pain prevents adult hyposensitivity to acute anxiety- and stress-provoking stimuli, vulnerability to chronic stress and have important clinical implications.

5.2 Introduction

Each year, 16.5% of infants worldwide and 12% of infants in the United States are born premature, defined as birth prior to 37 gestational weeks (Martin et al., 2006). The majority of these infants spend an average of 25 days in the Neonatal Intensive Care Unit (NICU) (PeriStats, 2011), where they undergo 10-18 invasive procedures each day, including repeated heel lance, endotracheal intubation, surgery, and respiratory and gastric suctioning (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008; PeriStats, 2011). Although the majority of these NICU procedures result in pain and inflammation, 79-98% are performed in the absence of analgesia or anesthesia (Carbajal et al., 2008).

It was previously believed that infants born prematurely were incapable of responding to noxious stimuli (Rodkey and Pillai Riddell, 2013). However, recent studies show that preterm infants as young as 25 gestational weeks display evoked cortical activity (Bartocci et al., 2006; Slater et al., 2006), robust secretion of stress hormones (Anand et al., 1987b), and elevated heart rate and facial activity (Grunau et al., 2005; Grunau et al., 2010) in response to noxious stimulation. As the number of invasive procedures experienced increases, behavioral, neuroendocrine and autonomic responses become significantly blunted. These changes in response to pain and stress may be permanent; clinical studies report former preterm infants have decreased sensitivity to noxious stimuli and altered cortisol reactivity later in life as teenagers and young adults (Botting et al., 1997; Hack et al., 2004; Grunau et al., 2005; Hermann et al., 2006;

Hayes and Sharif, 2009; Walker et al., 2009b; Grunau et al., 2010; Sullivan et al., 2012). Whether these permanent changes can be prevented by analgesia at the time of injury is not known.

Using a rat model of early life pain, we have previously reported that hindpaw inflammation (carrageenan 1%) during the first postnatal week (P0-8) results in decreased sensitivity to acute pain-provoking stimuli, but exaggerated responses to chronic inflammatory pain in adult male and female rats (LaPrairie and Murphy, 2007). Morphine treatment at the time of injury prevented both effects in adulthood (LaPrairie et al., 2008). Most recently, we reported that neonatally injured adults show behavioral hyposensitivity to acute anxiety- and stress-provoking stimuli (Victoria et al., 2013b) and blunted corticosterone release (Victoria et al., 2013c), indicating dysregulation of the hypothalamic pituitary adrenal axis (Chrousos, 2009). These changes in response to pain- and stress-provoking stimuli are accompanied by site-specific changes in enkephalin, as well as glucocorticoid receptor (GR) and CRF receptors 1 and 2 (Victoria et al., 2013c; Victoria et al., 2013b).

While early life pain clearly alters adult responses to acute anxiety- and stress-provoking stimuli, to date, its impact on behavioral and hormonal responses to chronic stress is not known. Also, it is unknown whether morphine administration at the time of injury attenuates these long-term changes in response to acute or chronic stressors. For the first time, we present evidence that morphine treatment for early life pain prevents hyposensitivity to acute anxiety- and stress-provoking stimuli, vulnerability to chronic stress, and alters corticosterone release following acute and chronic stressors similarly to controls.

5.3 Materials And Methods

Animals

Pregnant Sprague-Dawley rat dams were obtained on gestational day 14 (G14) (Charles River). Dams were housed individually under 12:12 hr light:dark cycle with *ad libitum* access to

food and water. On the day of birth (P0), pups were sexed by examination of anogenital distance and subjected to neonatal treatment. All litters were reared identically, weaned on P21 and housed with same sex littermates in groups of 2-3. Male and female rats were used in all experiments and tested on separate days. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP, and were approved by the Georgia State University Animal Care and Use Committee.

Neonatal Treatment

On the day of birth (P0), male and female rat pups received an injection of carrageenan (5 μ L, CGN 1%, dissolved in saline; Sigma, USA) into the intraplantar surface of the right hind-paw or were handled in an identical manner (LaPrairie et al., 2008; Victoria et al., 2013b). Intraplantar CGN results in acute, local inflammatory pain that persists for 24-72 hours and does not alter maternal behavior (Ren et al., 2004; LaPrairie and Murphy, 2007). All animals received morphine sulfate (2 mg/kg, i.p.) or equivolume saline (0.9%, i.p.) 15 min prior to intraplantar CGN or handling. At peak paw inflammation (5 hrs post-CGN), a second dose of morphine or saline was administered (LaPrairie et al., 2008). This resulted in a total of 4 groups: Injury + Saline, Handled + Saline, Injury + Morphine, Handled + Morphine. Pups were separated from their dam for 15 minutes, maintained on a warm surface and returned to the home cage as a group. All pups within a litter received the same neonatal treatment. Animals (n = 102) were undisturbed until adulthood (P60) except for cage changes and weaning (P21).

Test Of Anxiety-Like Behavior

Adult anxiety-like behavior was assessed using the Open Field (OF), a well-established test sensitive to detecting the effects of early life manipulations on anxiety (Joffe et al., 1973). Animals (P60-80; n = 4-8/treatment/sex) were habituated to the testing room daily for 60 minutes, 2 days before and on the day of testing. Adults were gently placed in the OF (gridded

Plexiglas box 120 cm x 120 cm x 30 cm) facing the same direction in randomized order by an experimenter blinded to neonatal treatment. Testing occurred under red light (10:00 – 14:00). Each animal experienced the OF one time for 5 minutes. Behaviors were recorded digitally with Noldus Observer 5.0 (Noldus, USA) and observed remotely with a video monitor. The testing apparatus was cleaned thoroughly with 70% ETOH between each animal; vapors were allowed to evaporate completely before the next session commenced. Scoring of anxiogenic (OF: duration in outer perimeter), anxiolytic behaviors (duration in inner area) and locomotor behavior (number of lines crossed) occurred post-hoc by an experimenter blinded to neonatal treatment. Data were expressed as duration or frequency.

Forced Swim Test And Blood Withdrawal

Adult stress-related behavior was assessed with the Forced Swim Test (FST). Animals (P70-90) were habituated to the testing room daily for 60 minutes, 3 days before and on the day of testing. Testing occurred during the light phase (9:00-12:30). Water was maintained at 25°C and filled to height such that animals could neither escape nor could the tail touch the bottom (63.5 cm) (Porsolt et al., 1977; Porsolt et al., 1978). On day one of the FST, adults (n = 4-8/treatment/sex) were placed in a circular swim tank (71.2 cm x 62.5 cm x 56 cm) for a 5 minute pre-swim to elicit “behavioral despair”(Armario et al., 1988). On day two, animals were placed in the swim tank for a 5-minute FST; all behaviors were digitally recorded. Following the FST, animals were dried with a clean towel and placed in clean cages. Fecal boli were counted and removed from the tank between each test. The tank was cleaned with detergent and ETOH between tests. The following behaviors were scored post-hoc: (1) latency to immobility, defined as the first cessation of swimming with arched-back floating (Porsolt et al., 1978); (2) duration of immobility, characterized by arched-backed floating and movement only necessary to keep the head above water or prevent drowning (Porsolt et al., 1978). Data are expressed as frequency and duration.

HPA output in response to swim stress was tested before and after 5 minutes of forced swimming. Blood samples were collected (9:00-12:30) from the lateral saphenous vein directly into 1 mL EDTA-hematology tube (BD from Fisher Scientific) using a 23-gauge needle 60 min before forced swimming to allow wound clotting and avoid water contamination (baseline; 0 min), immediately after 5 min of swim in 25°C water (stress), 30 min after the onset of swim (peak) and 75 min post-swim (recovery). Blood samples were maintained at room temperature for ≥ 30 min and centrifuged at 4000 rcf at 4°C for 15 min. Plasma was pipetted into microcentrifuge tubes and stored at -80°C until radioimmunoassay for CORT (I-125 Double Antibody Corticosterone kit, MP Biomedicals, USA). Concentration of CORT was determined against known standards ($R^2 > 0.98$) according to the manufacturer's instructions. The minimum limit of detection was 3.125 ng/mL. Intra-assay and inter-assay coefficients of variation were 4.3% and 5.6%, respectively.

Mild Chronic Variable Stress

To test the impact of neonatal injury on coping with unpredictable and repeated stress, adult rats (P85-P105) were exposed to 7 consecutive days of mild chronic variable stress (mCVS). A different cohort of neonatally-injured adult male and female rats ($n = 5-9$ /treatment/sex) were used in these experiments to avoid potential carryover effects from acute OF and FST testing. Animals were single housed 9-14 days prior to mCVS exposure. Stressors consisted of (1) water saturated bedding, (2) restraint in acrylic cylinder (30 min), (3) fox odor in cage (30 min; 1:5000 2,4,5-trimethylthiazole; Sigma, USA), (4) hypothermia stress (4 hrs; 4-6°C), (5) 6 cage changes in 24 hrs, (6) insufficient bedding (1:2), (7) white noise exposure (100 dB), (8) novel objects in cage (7 white golf and ping pong balls), and (9) 36 hrs of constant light (Mueller and Bale, 2008; Morgan and Bale, 2011). Stressors were presented in random order and spanned the entire AM (7:00-13:00), PM (13:00-19:00), or overnight (19:00-7:00) period unless otherwise specified. All stressors were experienced 2-3 times by each animal. On day 7,

all animals experienced 30 min of restraint in the AM, 5 min of pre-swim in the PM and novel objects + white noise overnight. On day 8, adults were given FST and blood withdrawal (9:00-12:30) as stated above to measure coping behavior and corticosterone following mCVS.

Statistical Analysis

Significant main effects of neonatal treatment and sex were assessed using two-way ANOVA or Repeated Measures ANOVA. Fisher PLSD was used for post-hoc analyses to determine differences between groups. Area under the curve analyses relative to ground are reported for corticosterone data and were calculated using the formula, $AUC_{Ground} = \frac{(C_2 - C_1)(t_2 - t_1) + (C_3 - C_2)(t_3 - t_2) + (C_4 + C_3)(t_4 - t_3)}{2}$, where C and t denote concentration of corticosterone and time point for sample collection, respectively (Pruessner et al., 2003). Percentages are reported as mean percent change from control. Where applicable, values ≥ 2 standard deviations from the mean were eliminated as outliers. All comparisons were *apriori* specified. Confidence was set to $P < 0.05$ and considered statistically significant.

5.4 Results

Impact Of Morphine Treatment On Adult Anxiety Responses

To determine if morphine reversed the impact of early life pain on adult responses to acute anxiety-provoking stimuli, injured and control male and female rats (+/- morphine) were tested in the Open Field (OF) apparatus. A two-way ANOVA revealed a significant main effect of treatment ($F_{(3,42)} = 24.68$; $P < 0.0001$) on time spent in the inner area; no significant effect of sex ($F_{(1,42)} = 1.28$; $P = 0.26$), and no significant interaction ($F_{(3,42)} < 1$; $P = 0.78$) was observed. Consistent with our previous report (Victoria et al., 2013b), neonatally injured adults spent significantly more time in the inner area than non-injured controls (Injury + Saline versus Handled + Saline, Fisher's PLSD $P < 0.0001$) (Fig. 1A). This effect was reversed by morphine treatment

(Injury + Morphine versus Injury + Saline; Fisher's PLSD post hoc, $P < 0.0001$). Indeed, no significant differences were noted between morphine injured animals and handled controls (Injury + Morphine versus Handled + Saline; Fisher's PLSD, $P > 0.05$). Interestingly, animals given morphine in the absence of pain spent significantly more time in center of the OF as compared to handled controls (Handled + Morphine versus Handled + Saline; Fisher's PLSD, $P < 0.001$) and injured animals treated with morphine (Fisher's PLSD, $P < 0.01$).

A two-way ANOVA was used to assess the impact of early life pain on the number of line crosses, and indicator of locomotor behavior. A significant main effect of sex ($F_{(1,42)} = 26.81$; $P < 0.0001$) was noted, with females crossing significantly more lines than males independent of treatment ($F_{(3,42)} = 1.13$; $P = 0.35$). Together, these data suggest that dampened behavioral responses to anxiety-provoking stimuli induced by early life pain are reversed by morphine treatment. In addition, they indicate that administration of morphine in the absence of pain results in moderate anxiolysis.

Morphine Treatment For Neonatal Injury And Adult Behavioral Responses To Acute Stress

We have previously reported that early life pain increases the latency to immobility in the forced swim test (FST) and reduces FST-induced CORT release in adulthood (Victoria et al., 2013c; Victoria et al., 2013b). To determine if morphine treatment reverses this effect, neonatally injured or handled adult male and female rats (+/- morphine) were exposed to the FST for 5 min. A two-way ANOVA revealed a significant main effect of treatment ($F_{(3,42)} = 57.54$; $P < 0.0001$), with no effect of sex ($F_{(1,42)} = 1.31$; $P = 0.26$) or a sex by treatment interaction ($F_{(3,42)} < 1$; $P = 0.96$). Consistent with our recent report (Victoria et al., 2013b), latency to immobility was significantly increased 6-fold in neonatally injured adults as compared with non-injured controls (Injury + Saline versus Handled + Saline, Fisher's PLSD, $P < 0.0001$; Fig. 2A). Administration of morphine at the time of injury completely reversed this effect (Injury + Morphine versus Handled + Saline, Fisher's PLSD, $P > 0.05$). Both male and female rats that received morphine in the

absence of pain also had significantly longer latencies to immobility than non-injured control animals (Handled + Morphine versus Handled + Saline, Fisher's PLSD, $P < 0.0001$).

A significant main effect of treatment was also observed for duration of immobility ($F_{(3,42)} = 15.03$; $P < 0.0001$), with no effect of sex ($F_{(1,42)} = 4.00$; $P = 0.062$) or sex by treatment interaction ($F_{(3,42)} < 1$; $P = 0.99$). Neonatally injured male and female rats spent significantly less time immobile than handled controls (Injury + Saline versus Handled + Saline, Fisher's PLSD, $P < 0.0001$). This effect was reversed by morphine treatment at the time of injury (Injury + Morphine versus Handled + Saline, Fisher's PLSD, $P > 0.05$). Administration of morphine in the absence of pain significantly reduced duration of immobility in both males and females (Handled + Morphine versus Handled + Saline, Fisher's PLSD, $P < 0.05$). As in our previous reports (Victoria et al., 2013b), neonatally injured male and female rats excreted significantly less fecal boli in comparison to handled controls (Injury + Saline versus Handled + Saline, Fisher's PLSD, $P < 0.01$), an effect that was also reversed by morphine treatment. Together these data suggest that pain on the day of birth decreases adult sensitivity to acute stress, an effect that is prevented by treatment with morphine at the time of injury.

Morphine Treatment For Neonatal Injury And Adult Corticosterone Responses To Acute Stress

To determine if morphine treatment at the time of injury alters adult corticosterone release in response to acute stress, plasma concentrations were assayed from blood drawn before, immediately after and during recovery from 5 min of forced swimming (Fig. 3). Repeated measures ANOVA revealed a significant treatment by sex by time interaction ($F_{(9,126)} = 2.65$; $P = 0.0077$). For females, a significant treatment by time interaction was observed ($F_{(9,63)} = 3.54$; $P = 0.0013$). Pain on the day of birth resulted in significantly higher basal corticosterone levels in comparison to morphine treated animals (overall mean increase: 142%; Injury + Saline versus Handled + Morphine, Fisher's PLSD, $P < 0.05$; overall mean increase: 86%; Injury + Saline versus Injury + Morphine, Fisher's PLSD, $P < 0.05$). No other group differences in baseline corti-

corticosterone levels were observed. Immediately after swimming (0 min) neonatally injured females had significantly lower corticosterone relative to those treated with morphine for neonatal pain (overall mean decrease: 26%; Injury + Saline versus Injury + Morphine, Fisher's PLSD, $P < 0.05$). Thirty minutes after stress, corticosterone levels were significantly elevated in injured females treated with morphine as compared with handled controls (overall mean increase: 33%; Injury + Morphine versus Handled + Saline, Fisher's PLSD, $P < 0.05$). During recovery from swim stress (75 min) females that experienced neonatal pain had significantly reduced corticosterone levels relative to handled controls (overall mean decrease: 46 %; Injury + Saline versus Handled + Saline, Fisher's $P < 0.05$). Morphine treatment at the time of injury reversed and augmented this effect (overall mean increase: 258%; Injury + Morphine versus Injury + Saline; Fisher's PLSD, $P < 0.01$; overall mean increase: 92%; Injury + Morphine versus Handled + Saline, Fisher's PLSD, $P < 0.05$).

A significant treatment by time interaction was also observed for male corticosterone levels ($F_{(9,63)} = 2.07$; $P = 0.046$) (Fig 3B). Although significant changes were not observed in basal corticosterone concentrations, the pattern exhibited was similar to females. At 0 min no differences in corticosterone concentrations were observed for handled controls versus injured animals or those that were treated with morphine (Handled + Saline versus Injury + Saline; versus Injury + Morphine; versus Handled + Morphine, Fisher's PLSD, $P > 0.05$). Thirty minutes after stress, corticosterone levels were significantly decreased in injured males as compared with injured animals treated with morphine (overall mean decrease: 24%; Injury + Saline versus Injury + Morphine, Fisher's PLSD, $P < 0.01$). At 75 min, corticosterone levels in animals given morphine before neonatal injury or those given morphine in the absence of pain had significantly reduced corticosterone levels relative to handled controls (overall mean decrease: 49%; Handled + Saline versus Injury + Morphine; overall mean decrease: 54%; Handled + Saline versus Handled + Morphine, Fisher's $P < 0.05$). Corticosterone levels of injured males tended to decrease by 40% relative to handled controls.

Morphine Treatment For Neonatal Injury And Adult Behavioral Responses To Mild Chronic Variable Stress (mCVS)

To determine the impact of neonatal injury on adult responses to chronic stress, male and female rats were exposed to 7 days of mCVS. On the 8th day, latency to immobility and total duration of immobility in response to a 5 min forced swim test were measured (Fig. 4). A two-way ANOVA on latencies to immobility revealed significant main effects of treatment ($F_{(3,44)} = 22.71$; $P < 0.0001$) and sex ($F_{(1,44)} = 6.22$; $P = 0.016$), but no significant interaction ($F_{(3,44)} < 1$; $P = 0.62$). In contrast to responses following acute stress, 7 days of mCVS significantly reduced latency to immobility in neonatally injured adult males and females as compared with controls (Injured + Saline versus Handled + Saline, Fisher's PLSD, females: $P < 0.0001$; males: $P < 0.0001$, Fig. 4A), with injured animals stopping swimming 2-3 times faster than handled controls. Administration of morphine at the time of injury reversed this effect, with no significant differences noted for Injured + Morphine versus Handled + Saline adults (Fisher's PLSD, females: $P > 0.05$; males: $P > 0.05$). Administration of morphine in the absence of pain (Handled + Morphine) significantly affected adult phenotype, as latency to immobility for both males and females were significantly decreased relative to Handled + Saline controls (Fisher's PLSD, females: $P < 0.01$; males: $P < 0.05$).

Adult duration of immobility in the FST was also affected by mCVS, with significant effects of treatment ($F_{(3,44)} = 8.50$; $P = 0.0001$) and sex ($F_{(1,44)} = 6.20$; $P = 0.016$) but no significant interaction ($F_{(3,44)} = 1.51$; $P = 0.23$) (Fig 4B). Injured adults that did not receive morphine (Injury + Saline) floated significantly longer than Handled + Saline controls (Fisher's PLSD, females: $P < 0.01$; males: $P < 0.01$). Morphine treatment at the time of injury reversed this effect in both males and females with no significant differences noted in latency to immobility for Injury + Morphine versus Handled + Saline adults (Fisher's PLSD, females: $P > 0.05$; males: $P > 0.05$).

Neonatal treatment also had a significant impact on adult fecal boli excreted in response to FST following mCVS ($F_{(3,44)} = 3.67$; $P = 0.019$) with no noted effect of sex ($F_{(1,44)} < 1$; $P = 0.39$)

or sex by treatment interaction ($F_{(3,44)} < 1$; $P = 0.92$). Specifically, adults that experienced early life pain without analgesia (Injured + Saline) excreted significantly more fecal boli than Handled + Saline controls (Fisher's PLSD, $P < 0.01$) and Injury + Morphine treated animals (Fisher's PLSD, $P < 0.05$). No differences were noted in Injured + Morphine versus Handled + Saline controls (Fisher's PLSD, $P > 0.05$) (data not shown). Together, these data indicate that early life pain in the absence of analgesia decreases adult resilience to chronic stress, an effect that is preventable with analgesia at the time of injury.

Morphine treatment before neonatal injury and adult corticosterone responses after mCVS

We next determined whether 7 days of mCVS differentially affected corticosterone release in neonatally injured animals +/- morphine treatment. Repeated measures ANOVA revealed a significant treatment by sex by time interaction ($F_{(12,176)} = 2.73$; $P = 0.0020$). For females (Fig 5A), a significant treatment by time interaction was observed ($F_{(12,92)} = 2.49$; $P = 0.0072$). Females exposed to mCVS had significantly elevated corticosterone levels compared to females exposed to acute stress (Area under the curve: $F_{(7,44)} = 3.53$; $P = 0.0043$). Corticosterone was increased by 37% for Injured + Saline females (Fisher's PLSD, $P < 0.05$). For Handled + Saline females an increase of 34% was observed (Fisher's PLSD, $P < 0.0001$). Interestingly, no difference was observed between females treated with morphine (+/- pain) for acute versus chronic stress (Fisher's PLSD, $P > 0.05$). Corticosterone levels before mCVS (pre-mCVS) were similar across groups. After 7 days of mCVS, basal corticosterone levels of injured females treated with morphine were significantly elevated (122-241%) in comparison to handled controls (+/- morphine) and injured females (- morphine) (Fisher's PLSD, $P < 0.05$). No significant group differences were observed in corticosterone concentrations at 0, 30 or 75 min after swim stress. However, corticosterone levels of neonatally injured females at 75 min failed to return to baseline faster than handled controls and injured females treated with morphine (Injury + Saline versus Handled + Saline; versus Injury + Morphine, $P > 0.05$) as observed in response to

acute stress (Fig 3A), suggesting that mCVS heightened HPA activity for females injured as neonates without analgesia.

A significant time by treatment interaction was also observed for male corticosterone levels ($F_{(12,84)} = 2.25$; $P = 0.016$) (Fig 5B). Overall, males exposed to mCVS had significantly elevated corticosterone levels in comparison to males exposed to acute stress (Area under curve: $F_{(7,42)} = 3.66$; $P = 0.0036$). Specifically, corticosterone was significantly elevated for neonatally injured males that experienced mCVS in comparison to males of the same treatment that experienced acute stress (overall mean increase: 37%; acute Injured + Saline versus mCVS Injured + Saline, Fisher's PLSD, $P < 0.001$). No difference was observed between handled males or those treated with morphine (+/- pain) (Fisher's PLSD, $P > 0.05$). Similar to females, no significant changes were observed in pre-mCVS baseline between groups. After 7 days of mCVS, basal corticosterone was significantly increased in injured males as compared with injured males treated with morphine (overall mean increase: 245% Injury + Saline versus Injury + Morphine, Fisher's PLSD, $P < 0.05$); a similar trend was observed relative to handled controls (132%, Fisher's PLSD, $P = 0.05$), suggesting that mCVS heightened baseline functioning of the HPA axis for males that experienced pain without analgesia. No significant changes were observed in corticosterone concentrations at 0 and 30 min. At 75 min after swim stress, corticosterone levels of injured males were significantly elevated above handled controls (overall mean increase: 137%; Injury + Saline versus Handled + Saline, Fisher's PLSD, $P < 0.01$), males given morphine in the absence of pain (98%; Injury + Saline versus Handled + Morphine; Fisher's PLSD, $P < 0.01$) and injured males treated with morphine (240%; Injury + Saline versus Injury + Morphine; Fisher's PLSD, $P < 0.05$), suggesting that males injured without analgesia were vulnerable to the effects of chronic stress.

5.5 Discussion

The present studies tested the impact of neonatal injury in the presence and absence of pain therapy on adult responses to 7 days of mild chronic variable stress (mCVS). In contrast to behavioral responses to acute stress, neonatally injured adults initiated floating rapidly and spent significantly more time immobile in the FST after mCVS exposure, suggesting vulnerability to sequential, unpredictable perturbations and dysregulation of HPA activity. Morphine administration for neonatal inflammatory pain prevented adult behavioral vulnerability to chronic stress, as well as hyposensitivity to acute anxiety- and stress-provoking stimuli. This provides additional support for opioid-dependent changes in stress responding that result from early life pain (Victoria et al., 2013b), and is consistent with our previous report that neonatal morphine treatment prevents adult changes in pain sensitivity (LaPrairie et al., 2008).

Morphine Treatment For Neonatal Pain Rescues Deficits In Acute Anxiety And Stress Responding

Adults that experienced unalleviated pain as neonates spent significantly more time in the center of the OF. In the FST, P0 injury without analgesia resulted in a 5-6 fold increase in latency to immobility and decreased total time floating. Together, OF and FST data suggest that unalleviated pain decreases sensitivity to acute anxiety- and stress-provoking stimuli in adulthood (Victoria et al., 2013b). In support of the role of the endogenous opioid system in the observed behavioral changes, others have shown that overexpression of preproenkephalin or enkephalin administration potentiates the anxiolytic effects of benzodiazepines, increases time in the open arms and blocks swim-stress induced anxiety in the elevated plus maze (EPM) in adult rodents (Kang et al., 2000; Randall-Thompson et al., 2010). By contrast, animals that received morphine for pain on the day of birth spent the same amount of time in the inner area of the OF, initiated floating (after approximately 10-12 seconds) and spent a comparable amount of time immobile in the FST as handled controls, suggesting that appropriate analgesia at the

time of injury prevents adult hyposensitivity to anxiety- and stress-provoking stimuli. This finding is consistent with previous studies showing that morphine treatment for neonatal pain prevents adult hypoalgesia (Bhutta et al., 2001; LaPrairie et al., 2008). In addition, chronic perinatal blockade of opioid signaling through μ - and δ -opioid receptor systems, or selective knockout of preproenkephalin increases anxiety-like behavior in the EPM and OF, and increases aggression (de Cabo de la Vega et al., 1995; Konig et al., 1996; Bilkei-Gorzo et al., 2008). Together these behavioral data suggest that blocking early life pain with opioid analgesia prevents hyposensitivity to acute anxiety- and stress-provoking stimuli, in turn allowing for normal stress coping.

Morphine treatment rescues stress coping following chronic stress

Early life perturbations have been shown to result in long-term changes in response to anxiety- and stress-provoking stimuli. In general, animals that have experienced either acute or mild perturbations during the perinatal period, including handling, licking, and grooming, show decreased responsiveness to stress-provoking stimuli and reduced HPA reactivity (Bhatnagar and Meaney, 1995; Caldji et al., 2000; Weaver et al., 2005; Bouffleur et al., 2013). By contrast, the opposite behavioral profile is observed in adults exposed to severe neonatal stressors such as maternal separation and maternal isolation (Coutinho et al., 2002; Marais et al., 2008). In the present study, animals injured neonatally without morphine responded to acute stress with excessive resilience, but were extremely passive and vulnerable after 7 days of chronic, unpredictable stress. In particular, mCVS significantly elevated corticosterone relative to acute stress, and promoted depressive-related behaviors in the FST for adults injured in the absence of morphine.

Similar disparities in acute versus chronic stimuli responding have been observed in other stress and early life perturbation models. For example, rats given acute restraint stress struggle 2-3 times longer than rats that are chronically restrained (Grissom et al., 2008). Adult rats injured as neonates exhibit hypoalgesia in response to a brief thermal stimulus, but severe

hyperalgesia in the presence of chronic inflammatory pain (Ren et al., 2004; LaPrairie and Murphy, 2007; LaPrairie et al., 2008). Neonatal endotoxin exposure on P4 results in increased sucrose preference and social interaction, and decreases corticosterone following acute tail shock (Bilbo et al., 2008). However, adult chronic stress exposure or LPS administration increases anxiogenic behavior in EPM and OF, increases acoustic startle amplitude and elevates corticosterone release (Bilbo et al., 2008; Walker et al., 2009a). Interestingly, a model of chronic maternal separation (3 hrs/day on P2-P14) shows basal hyperactivation of the HPA axis in response to acute air puff startle stress, but reduced ACTH and corticosterone following chronic stress (Ladd et al., 2005), suggesting that the HPA axis has flexibility for dichotomous dysregulation in both directions as a result of early life perturbations.

This hypo- versus hyper-reactive profile in response to acute versus chronic/severe stimuli is consistent with clinical findings in former preterm infants. For example, children, teens and young adults born prematurely are rated as less sensitive to pain by both their parents and physicians (Grunau et al., 1994b; Johnston et al., 1996; Oberlander et al., 2000; Hermann et al., 2006), display reduced stress-induced analgesia (Wollgarten-Hadamek et al., 2011) and show blunted cortisol reactivity to psychological stress testing (Buske-Kirschbaum et al., 2007). In contrast, a hyperalgesic response is observed following surgery in the same dermatome, as well as increased negative verbalizations, and a higher incidence of catastrophic, rather than solution-based, thoughts related to painful interventions (Peters et al., 2005; Bagner et al., 2010; Hohmeister et al., 2010). Collectively, these findings support the hypothesis that early life trauma confers physiological and psychological adaptations that result in extreme coping strategies, which are known risk factors for the manifestation of depression and post-traumatic stress disorder (PTSD) (Taylor and Stanton, 2007).

Indeed, parents and teachers report former NICU patients as having significantly more issues with internalizing and externalizing, reduced cognitive and behavioral flexibility, and higher rates of anxiety and depression than full-term peers (Levy-Shiff et al., 1994; Botting et al.,

1997; Hack et al., 2004; Aarnoudse-Moens et al., 2009; Hayes and Sharif, 2009). In this context, our data suggest that early life pain decreases the ability to cope with sequential and unpredictable challenges and may increase vulnerability to severe disorders of perception and stress. Notably, the present data show that behavioral and hormonal changes in adult responding to mCVS were prevented by treatment with morphine for early life pain. Similarly, administration of opioid analgesia to preterm infants for surgical or procedural pain reduces cortisol, norepinephrine, epinephrine and β -endorphin release, suggesting that at least some of the effects of early life pain on stress reactivity are mitigated (Anand et al., 1987b; Anand and Hickey, 1992). Together, these data suggest that appropriate analgesic intervention for painful NICU procedures may mitigate later-life vulnerability to neuropsychiatric disorders for former preterm infants.

Morphine Treatment In The Absence Of Pain

Interestingly, male and female rats that received morphine on the day of birth in the absence of pain displayed a phenotype intermediate to rats injured with and without morphine. This suggests that early activation of the opioid system, either endogenously by early noxious stimulation (Victoria et al., 2013d) or exogenously with morphine, produces long-term changes in adult responses to anxiety- and stress-provoking stimuli. Consistent with opioid regulation of anxiety-like behavior, adult rats given morphine exhibit significantly reduced fear-potentiated startle (Glover and Davis, 2008) and spend significantly more time in the open arms of the EPM (Anand et al., 2012). Similarly, perinatal morphine exposure in rats increases opioid release in response to acute psychological stressors and reduces adult anxiety-related behaviors (Buisman-Pijlman et al., 2009). Others have shown that morphine treatment of adult rats during 7 days of chronic variable stress increases immobility in the FST and decrease sucrose preference (Molina et al., 1994; Zurita et al., 2000). Daily naloxone or naltrexone administration before stressors rescues depressive-related behaviors (Molina et al., 1994; Zurita et al., 2000), sug-

gesting that chronic opioid signaling is necessary for FST behaviors in response to repeated stress. In addition, we observed basal corticosterone levels were significantly lower in females treated with morphine as neonates relative to injured or handled females given saline, with a similar trend observed in morphine treated males. Significant reductions in basal corticosterone have been observed in mice lacking preproenkephalin (Bilkei-Gorzo et al., 2008). Together, these data support the hypothesis that early life activation of the opioid system alters sensitivity to noxious or aversive stimuli later in life.

Conclusions

Our working hypothesis is that morphine treatment for early life pain mitigates the injury-induced increase in afferent nociceptive drive and reduces activation of supraspinal circuits subserving pain and stress (Walker et al., 1986; Tive and Barr, 1992; Abbott and Guy, 1995; Fitzgerald, 2005; LaPrairie and Murphy, 2009; Barr and Wang, 2013). In turn, release of endogenous opioids, such as met-enkephalin and β -endorphin, and neurohormones from the HPA axis, including CRF, ACTH and corticosterone, is reduced (McDonald et al., 1959; Stubbs et al., 1978; Anand et al., 1987b; Anand and Hickey, 1992; Van Bockstaele et al., 2000). Animals receiving morphine in the absence of pain also showed long-term changes in behavioral and hormonal responses to stress. Indeed, others have shown that morphine administration to rat pups twice daily from P3-7 in the absence of pain decreases adult immobility in the FST and basal corticosterone levels (McPherson et al., 2007) supporting the hypothesis that early life activation of the opioid system, either with injury-driven upregulation in endogenous opioid peptides (LaPrairie and Murphy, 2009; Victoria et al., 2013b; Victoria et al., 2013d) or administration of morphine in the absence of pain, permanently re-programs circuits sensitive to anxiety and stress.

Although evidence over the last 30 years has clarified that infants feel pain, and the vast majority of modern pediatric physicians acknowledge this (Purcell-Jones et al., 1988), less than

35% of NICU patients receive analgesia before the 10-18 invasive and painful procedures experienced each day in the NICU (Barker and Rutter, 1995; Simons et al., 2003; Carbajal et al., 2008). A variety of studies have aimed to test the efficacy and long-term impact of opioid analgesia in the NICU, but this has proven to be a difficult task, challenged by small sample sizes, inclusion of infants with confounding illness such as hypotension or pre-existing neurological impairment, or dosing that may be age inappropriate (MacGregor et al., 1998; Bouwmeester et al., 2001; Anand et al., 2004; Roze et al., 2008; de Graaf et al., 2011). However, a recent study reported that former preterm infants at 8-9 years old who received morphine for pain management in the NICU had improved executive functioning and reduced problems with externalization relative to infants that received placebo (de Graaf et al., 2013).

In our simplified, yet clinically relevant model (Workman et al., 2013) of early life pain, we show for the first time that specific, low-dose morphine treatment for inflammatory injury prevents hyposensitivity to acute anxiety- and stress-provoking stimuli in adulthood, and behavioral and hormonal vulnerability to chronic stress. These studies support the need for specific and appropriate analgesic regimes for human infants. The current absence and inconsistency of such treatment plans leaves former preterm infants at high risk for disorders of stress and perception such as anxiety, depression and PTSD throughout life.

5.6 Chapter 5 Figures

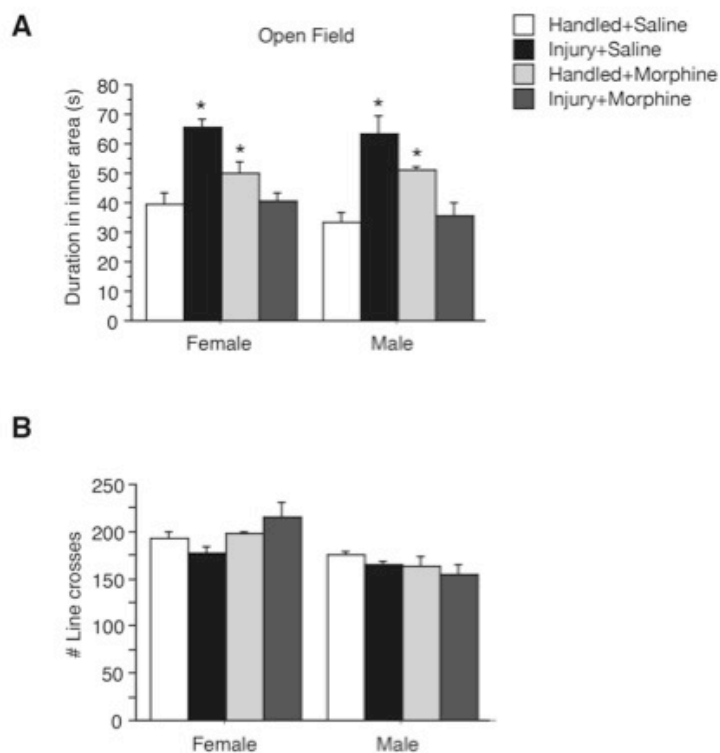


Figure 5.1 Morphine treatment reverses the impact of early life pain on adult anxiety responses

A. Adult male and female rats that received an intraplantar injection of 1% CGN on the day of birth (Injury+Saline: $n = 5$ male; $n = 7$ female) spent significantly more time in the inner area of the Open Field than handled controls (Handled+Saline: $n = 7$ male; $n = 8$ female). This effect was reversed by treatment with morphine at the time of injury (Injury+Morphine: $n = 7$ male; $n = 6$ female). A significant increase in time spent in inner area was also observed for animals that received morphine in the absence of pain (Handled+Morphine: $n = 6$ male; $n = 4$ female). **B.** Females crossed significantly more lines than males, independent of treatment. Data are shown as Mean \pm SEM. Main effects measured with two-way ANOVA for sex and treatment. Significant group differences ($*P < 0.05$) were measured post-hoc by Fisher's PLSD.

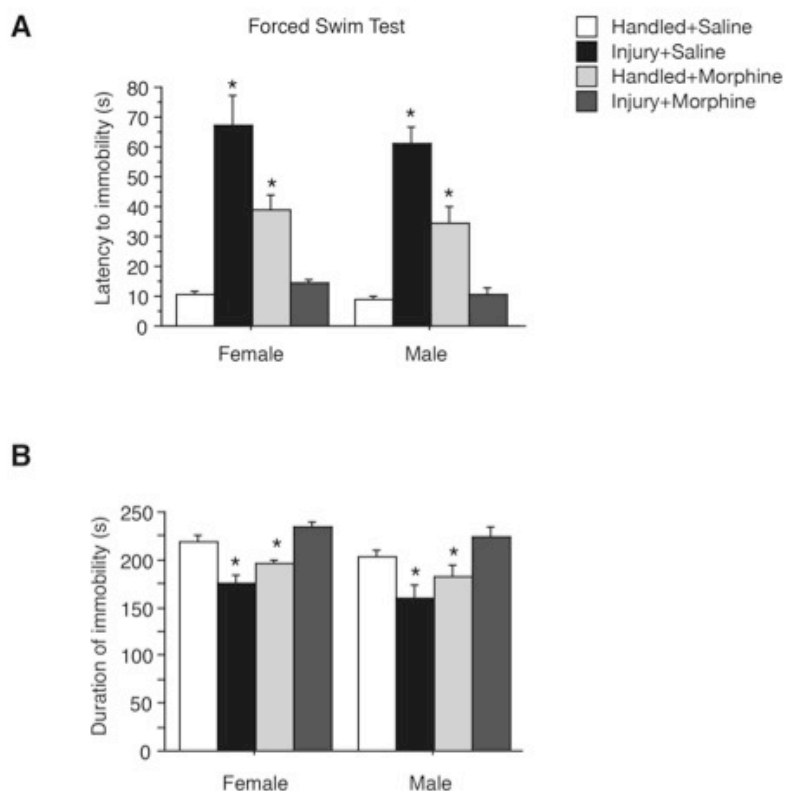


Figure 5.2 Morphine treatment reverses the impact of early life pain on adult responses to acute stress

A. Latency to immobility was significantly increased for male and female rats that received hind-paw inflammation in the absence of morphine on P0. Administration of morphine at the time of injury completely reversed this effect. **B.** Similar treatment effects were noted in the duration of immobility. Data are shown as Mean \pm SEM. Main effects measured with two-way ANOVA for sex and treatment. Significant group differences ($*P < 0.05$) were measured post-hoc by Fisher's PLSD; (Handled+Saline: $n = 7$ male; $n = 8$ female); (Injury+Saline: $n = 5$ male; $n = 7$ female); (Handled+Morphine: $n = 6$ male; $n = 4$ female) (Injury+Morphine: $n = 7$ male; $n = 6$ female).

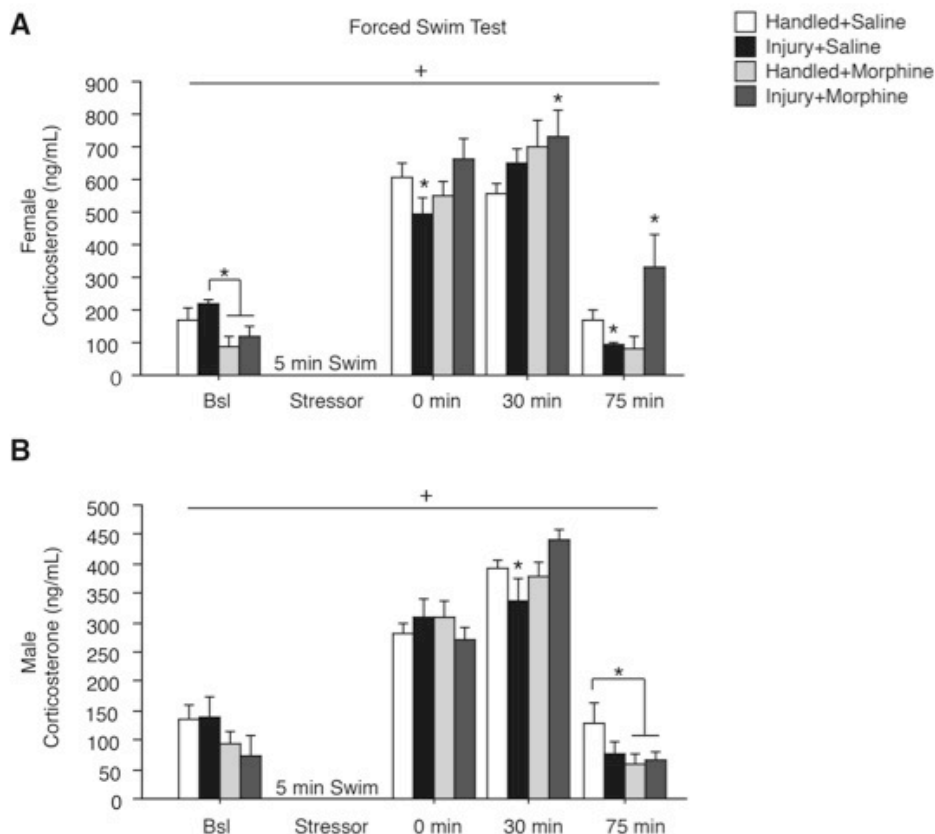


Figure 5.3 Morphine treatment for neonatal injury alters adult corticosterone responses to acute swim stress.

Plasma corticosterone was measured from blood drawn before (baseline; bsl), after (0 min, 30 min) and during recovery (75 min) from 5 min of swim stress in adult females (**A**) or males (**B**). Basal corticosterone was significantly decreased in adult females that were given morphine (+/- injury). Immediately after swimming (0 min), corticosterone was significantly reduced for injured females. At 30 min corticosterone was significantly increased for females and males treated morphine for injury. At 75 min corticosterone was elevated in handled controls of both sexes. For females, morphine for injury rescued and augmented corticosterone levels. For males, morphine treatment (+/- injury) reduced corticosterone. Data were analyzed using Repeated Measures ANOVA for time and are presented as Mean \pm SEM. Significant interaction between time and treatment, $^+P < 0.01$. Significant between group differences measured post-hoc by Fisher's PLSD. $*P < 0.05$; (Handled+Saline: $n = 7$ male; $n = 8$ female); (Injury+Saline: $n = 5$ male; $n = 7$ female); (Handled+Morphine: $n = 6$ male; $n = 4$ female) (Injury+Morphine: $n = 7$ male; $n = 6$ female).

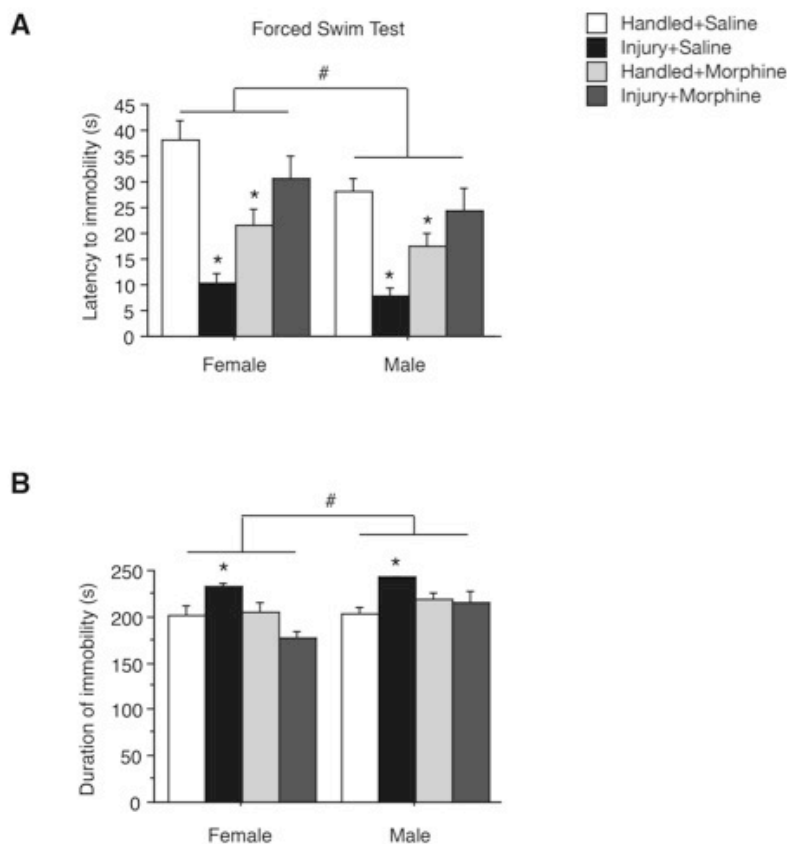


Figure 5.4 Neonatal injury significantly alters response to FST following 7 days of mCVS

A. Latency to immobility in the Forced Swim Test was significantly decreased in adult male and female rats that received hindpaw inflammation on P0 in the absence of morphine (Injury + Saline). This effect was reversed in animals that received morphine at the time of inflammation. Administration of morphine in the absence of pain also significantly reduced latency to immobility. **B.** Neonatal injury significantly increased duration of immobility during the 5 minute FST in comparison to handled controls, an effect reversed by morphine treatment at the time of injury. Data are shown as Mean \pm SEM. Main effects measured with two-way ANOVA for sex and treatment. Significant group differences ($*P < 0.05$) were measured post-hoc by Fisher's PLSD; (Handled+Saline: $n = 9$ male; $n = 9$ female); (Injury+Saline: $n = 5$ male; $n = 8$ female); (Handled+Morphine: $n = 5$ male; $n = 5$ female); (Injury+Morphine: $n = 6$ male; $n = 5$ female).

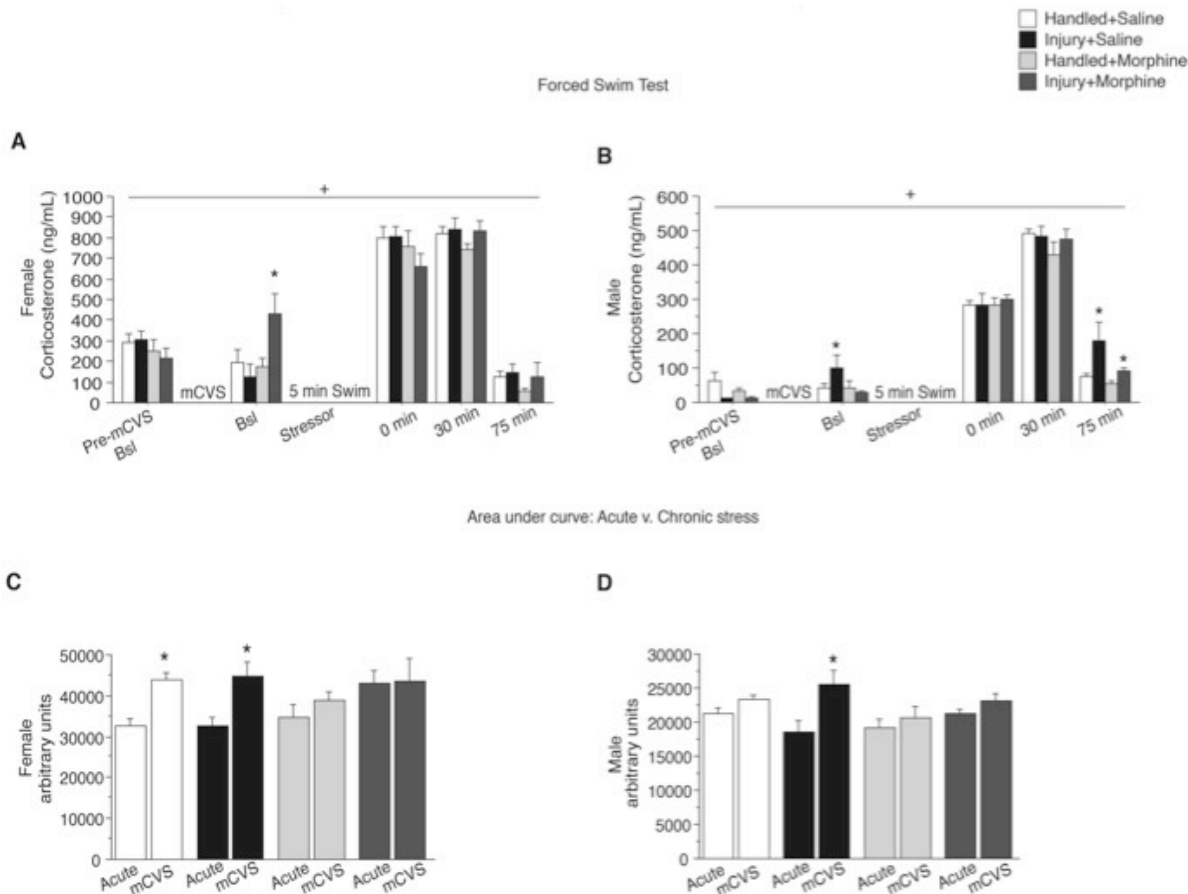


Figure 5.5 Morphine treatment for neonatal injury prevents hormonal dysregulation after mild chronic variable stress (mCVS) in females (A) and males (B)

On the day of the Forced Swim Test, basal corticosterone was significantly elevated for males injured (-) morphine. At 75 min after swim stress, corticosterone concentrations remained significantly elevated in adult males injured (-) morphine on P0. **C.** Chronic stress significantly elevated corticosterone area under the curve relative to acute stress for females injured (-) morphine and handled females. **D.** Chronic stress significantly increased corticosterone area under the curve relative to acute stress for males injured (-) morphine. mCVS corticosterone and area under the curve data were analyzed using Repeated Measures ANOVA or one-way ANOVA, respectively and are presented as Mean \pm SEM. Significant interaction between time and treatment, * $P < 0.01$. Significant between group differences measured post-hoc by Fisher's PLSD. * $P < 0.05$; (Handled+Saline: $n = 9$ male; $n = 9$ female); (Injury+Saline: $n = 5$ male; $n = 8$ female); (Handled+Morphine: $n = 5$ male; $n = 5$ female); (Injury+Morphine: $n = 6$ male; $n = 5$ female).

6 CHAPTER SIX: CONCLUSIONS

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6.1 Overview

Each year, 16.5% of infants worldwide and 12% of infants in the United States are born prior to 37 gestational weeks and are considered preterm (Martin et al., 2006). The etiologies underlying preterm birth are complex and not completely understood, but risk factors include maternal diabetes, hypertension, smoking, prenatal substance use, lack of prenatal care and assisted reproductive therapies (PeriStats, 2011). Advances in medical technology now allow for preterm infants born as young as 24 gestational weeks to survive *ex utero* (Qiu, 2006b). However, the costs of preterm birth are high, both economically and physiologically. Annually, an estimated \$26.2 billion is associated with the immediate and long-term impacts of prematurity on medical, education and work force costs (PeriStats, 2011).

Preterm infant spend approximately one month in the NICU (PeriStats, 2011), where they are exposed to hundreds of procedures in the absence of analgesia. The experience of unalleviated pain at this developmental time point results in immediate reductions in pain and stress reactivity (Grunau et al., 2005; Carbajal et al., 2008). Indeed, numerous clinical studies have reported that NICU practices are associated with long-term changes in sensory perception, neuroendocrine and autonomic responses to stress, brain maturation and cognitive processing and emotional profiles (Botting et al., 1997; Hack et al., 2004; Hermann et al., 2006; Buske-Kirschbaum et al., 2007; Grunau et al., 2007; Doesburg et al., 2008; Aarnoudse-Moens

et al., 2009; Hayes and Sharif, 2009; Walker et al., 2009b; Bagner et al., 2010; Grunau et al., 2010; Haley et al., 2010; Wollgarten-Hadamek et al., 2011; Brummelte et al., 2012; Sullivan et al., 2012). Unfortunately, clinical studies are limited in the time over which they follow former preterm infants, their ability to control frequency of injury, and their ability to discern mechanisms underlying changes associated with early life pain in the NICU. However studies in rodents using a variety of different methodologies have now clearly established that early life pain results in long-term changes in adult somatosensory thresholds (Shimada et al., 1990; Ruda et al., 2000; Bhutta et al., 2001; Ren et al., 2004; Sternberg et al., 2005; LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009). Surprisingly, very few animal studies have tested the impact of early life pain on adult stress-related behavior, including potential changes in the anatomical and physiological characteristics of stress-associated neural circuits (Anseloni et al., 2005). Similarly, whether administration of analgesia at the time of injury mitigates the potential long-term effects of early life pain on stress responsiveness is also not known. The studies comprising this dissertation research were designed to address these gaps in knowledge. In particular, experiments were conducted to delineate the specific impact of a single inflammatory injury on the day of birth on adult behavioral and hormonal responses to anxiety- and stress-provoking stimuli. Anatomical changes in neurocircuits regulating stress, anxiety and pain were also investigated, as well as the efficacy of neonatal analgesia for preventing any such changes in adulthood.

6.2 Early Postnatal Perturbations Result In Long-Term Adaptations In Later-Life Stress, Anxiety And Pain

Summary Of Behavioral Findings

One consistent finding we observed across all of our studies was that early life pain blunts adult sensitivity to acute anxiety- and stress-provoking stimuli (Victoria et al., 2013b; Victoria et al., 2013a). Neonatally injured adults spent significantly more time in the center of the

OF and took significantly longer to become immobile for the first time in the FST. We further showed that neonatally injured adults have a significantly attenuated stress-induced analgesia response (Victoria et al., 2013b). Blockade of endogenous opioid signaling with the broad-spectrum opiate antagonist naloxone reversed the injury induced changes in response to the FST and stress-induced analgesia, suggesting that the observed behavioral hyposensitivity to stress in neonatally injured adults was mediated by an opioid-dependent mechanism (Victoria et al., 2013b). Such findings are consistent with previous reports from our lab that neonatal injury decreases adult nociceptive sensitivity through an opioid-dependent mechanism (LaPrairie and Murphy, 2007, 2009).

In our final behavioral studies we tested the impact of injury on responses to 7 days of mild chronic variable stress (mCVS) (Victoria et al., 2013a). In contrast to the observed behavioral responses to acute stress (hyposensitivity), neonatally injured adults initiated floating more rapidly and spent significantly more time immobile in the FST after mCVS exposure, suggesting vulnerability to sequential, unpredictable perturbations and dysregulation of HPA activity. Morphine administration at the time of injury prevented adult behavioral vulnerability to chronic stress, as well as hyposensitivity to acute anxiety and stress-provoking stimuli. These data are consistent with our previous reports that neonatal morphine treatment prevents injury-induced changes in adult pain sensitivity (LaPrairie et al., 2008).

Summary Of Anatomical and Physiological Findings

Anatomically, we observed that neonatal injury significantly increased central expression of the opioids met-enkephalin and β -endorphin, as well as plasma corticosterone within 24 hrs of injury (Victoria et al., 2013d). These markers of both pain and stress remained elevated by the end of the first postnatal week as compared with controls, suggesting aberrant regulation of critical developmental processes, and potential consequences for the developing organism. In adulthood, increases in endogenous opioid-peptide expression persisted in regions underlying

responses to pain, anxiety and stress, such as the vIPAG, CeA and LS (Victoria et al., 2013b). Alterations in CRFR1 and CRFR2 binding were observed in the vIPAG, several amygdalar nuclei and the LS (Victoria et al., 2013c), suggesting a common circuit whereby injury alters adult perception and responding to noxious stimulation.

Lastly, neonatal injury altered adult HPA axis functionality, as evidenced by changes in GR expression in the PVN and hippocampus (Victoria et al., 2013b), key sites of HPA axis activation and termination (Dallman et al., 1987; Cullinan et al., 1993). In response to acute stressors, corticosterone was reduced and returned to baseline more rapidly, whereas repeated HPA activation by mCVS resulted in prolonged corticosterone reactivity (Victoria et al., 2013b; Victoria et al., 2013a). Together with our behavioral findings, these data support the hypothesis that injury confers resilience to acute stress but vulnerability to chronic stress. As with behavioral adaptations, morphine treatment for early life pain mitigated changes in corticosterone release in response to both acute and chronic stressors, suggesting that long-term changes resulting from neonatal injury were preventable with appropriate and specific pain therapy (Victoria et al., 2013a). As a whole, these findings provide valuable insight into the long-term consequences of early life injury and have the potential to influence pain treatment regimens for human infants in the NICU.

Neonatal Injury In Relation To Behavioral Findings From Models Of Postnatal Perturbations

Early life perturbations have been previously shown to result in long-term changes in response to anxiety- and stress-provoking stimuli. In general, animals who have experienced either acute or mild perturbations during the perinatal period, including handling, licking, and grooming, show decreased responsiveness to stress-provoking stimuli and reduced HPA reactivity (Bhatnagar and Meaney, 1995; Caldji et al., 2000; Weaver et al., 2005; Boufleur et al., 2013). By contrast, the opposite behavioral profile is observed in adults exposed to severe stress (e.g. maternal separation and maternal isolation) as neonates (Coutinho et al., 2002;

Marais et al., 2008)(for recent reviews of perinatal stress consequences: (Weinstock, 2008; LaPrairie and Murphy, 2010; Macri et al., 2011)).

In our behavioral studies we observed that a single painful experience early in life results in behavioral and hormonal hyposensitivity to acute anxiety- and stress-provoking stimuli, but hypersensitivity upon exposure to chronic perturbations. Other studies testing the long-term consequences of postnatal perturbation have observed a similar dichotomy in adult responses to acute versus chronic/severe stressors. For example, early life inflammatory pain reduces basal pain sensitivity (hypoalgesia) in adult rats exposed to acute thermal or mechanical noxious stimuli. However, following exposure to a chronic and more intense noxious stimulus, these rats display severe hypersensitivity (hyperalgesia) (Ren et al., 2004; LaPrairie and Murphy, 2007). Similarly, neonatal endotoxin exposure on P4 results in increased sucrose preference and social interaction, and decreased CORT following acute tail shock (Bilbo et al., 2008). However, adult chronic stress exposure or LPS administration increased anxiogenic behavior in EPM and OF, increased acoustic startle amplitude and elevated CORT release (Bilbo et al., 2008; Walker et al., 2009a). Interestingly, some models of chronic maternal separation (3 hrs/day on P2-P14) have shown basal hyperactivation of the HPA axis in response to acute air puff startle stress, but reduced ACTH and CORT following chronic stress (Ladd et al., 2005), suggesting that HPA axis has flexibility for dichotomous dysregulation in both directions.

This hypo- versus hyper-reactive response profile in response to acute versus chronic/severe stimuli is consistent with clinical findings in former preterm infants. For example, children, teens and young adults born prematurely are rated as less sensitive to pain by both their parents and physicians, (Grunau et al., 1994b; Johnston et al., 1996; Oberlander et al., 2000; Hermann et al., 2006), display reduced stress-induced analgesia (Wollgarten-Hadamek et al., 2011) and show blunted cortisol reactivity to psychological stress testing (Buske-Kirschbaum et al., 2007). In contrast, a hyperalgesic response is observed following surgery in the same dermatome, as well as increased negative verbalization, and increased catastrophizing related to

painful interventions. These former preterm infants also have higher rates of anxiety, depression and emotional reactivity following a more pronounced stressor (Hack et al., 2004; Peters et al., 2005; Aarnoudse-Moens et al., 2009; Hayes and Sharif, 2009; Bagner et al., 2010; Sullivan et al., 2012). Together these data suggest that this hypo-/hyper-response profile may be a common adaptation that results from early life trauma. However, such extreme physiological and psychological coping strategies increase the risk for manifestation of disorders such as post-traumatic stress (PTSD) (Taylor and Stanton, 2007).

Neonatal Perturbations Affect Common Neurocircuits And Neurotransmitters Systems

Animal models of early life perturbations have shown that the amygdala, septum, hypothalamus and hippocampus are common sites where long-term changes in expression and function occur for the CRFR and GR systems (Bhatnagar and Meaney, 1995; Shanks et al., 1995; Proulx et al., 2001; Ladd et al., 2005). Indeed, we observed changes in these regions and transmitter systems, as well as novel changes in the vIPAG and endogenous opioid system (Victoria et al., 2013c; Victoria et al., 2013b; Victoria et al., 2013d). For example, our initial anatomical studies showed early life pain significantly increased adult enkephalin expression in the LS, CeA and vIPAG (Victoria et al., 2013b). In support, a similar model of neonatal intraplantar CGN reports upregulation of preproenkephalin in the adult CNS (Ren et al., 2005). Our subsequent studies showed that neonatal injury decreased CRFR1 binding in the BLA and vIPAG, and increased CRFR2 in LS and CoA in adults (Victoria et al., 2013c). In support of a common circuit for postnatal adaptations, our findings overlap with observations in adult rats that experienced maternal separation, showing decreases in CRFR1 binding in the BLA and stress-induced increases in CRFR2 binding in the LS (Ladd et al., 2005).

In our studies, GR expression was significantly increased in the PVN but decreased in both dCA1 and vCA1. Lack of neonatal handling and exposure to adult chronic stress results in decreased GR mRNA expression in the septum and hippocampus of rats (Bhatnagar and

Meaney, 1995). Further, metabolic perturbation with leptin on P2-P9 in rodents decreases GR expression in the hippocampus, increases GR expression in the PVN and accelerates dexamethasone suppression of CORT (Proulx et al., 2001). Lastly, early life immune challenge in rats (P3-P5) decreases adult GR expression in both the hippocampus and hypothalamus (Shanks et al., 1995).

Changes in GR are associated with early life trauma in humans as well. For example, suicide victims that experienced childhood abuse show decreases in GR mRNA expression in post-mortem hippocampal samples (McGowan et al., 2009). Individuals that suffer from depression or PTSD show alterations in CRF from plasma and CSF (Bremner et al., 1997; Catalan et al., 1998; McEwen, 2002). Former NICU patients suffer from a higher incidence of anxiety and depression (Levy-Shiff et al., 1994; Botting et al., 1997; Hack et al., 2004; Aarnoudse-Moens et al., 2009; Hayes and Sharif, 2009; Sullivan et al., 2012), suggesting changes in GR and CRF systems for former preterm infants. Together, these findings support the hypothesis that traumatic early life experience impacts later-life susceptibility to affective dysfunction, which is associated with changes in CRFR and GR systems in the septum, amygdala, hippocampus, hypothalamus. In addition, our results suggest a role for the endogenous opioid system, as well as the PAG, in this susceptibility.

Sexually Dimorphic Findings

In all of our studies, the impact of neonatal injury on adult behavioral and hormonal responses to stress was assessed in both males and females, with sex differences observed in select measures. For example, the decrease in pain sensitivity observed following neonatal injury is exacerbated in females in comparison to males (LaPrairie and Murphy, 2007). Similarly, neonatally injured females show a significantly greater hyperalgesic response following a traumatic painful stimulus in comparison to males. Both effects are independent of estrus stage. In the present studies, sex differences were not observed in response to either acute anxiety- and

stress-provoking stimuli, or in early life pain-induced changes in enkephalin expression (Victoria et al., 2013b). Independent of treatment, acute stress induced significantly higher CORT release in females in comparison to males, and adrenal glands comprised a significantly larger proportion of body weight as compared with males (Victoria et al., 2013c; Victoria et al., 2013a). This is consistent with previous studies showing females have higher HPA stress reactivity in comparison to males (Seeman et al., 2001).

In contrast to studies of acute stress, males and females were affected differentially by chronic stress (Victoria et al., 2013a). Independent of neonatal treatment, females had significantly longer latencies than males to become immobile in the FST after chronic stress exposure. However, males spent more time immobile in the FST. These data suggest that divergent behavioral sex differences (McCarthy et al., 2012) may result from neonatal injury. Potentially, these differences remained latent (Shors et al., 2001) in response to acute stressors but manifested in the presence of stress that is strong, persistent, and of unpredictable intensity.

In many of our studies we observed trends towards effects of sex. This was the case for GR protein immunoreactivity in the PVN and CA1 of the hippocampus, as effects appeared to be more pronounced in males in comparison to females (Victoria et al., 2013c). Similarly, binding of CRFR2 was significantly higher in males than females in the BNST, a region known to be sexually dimorphic (del Abril et al., 1987). The observed injury-induced increase in septal CRFR2 was more pronounced in the males, whereas females exhibited a larger increase in the CoA, suggesting a potential sex-dependent adaptation through which responses to stress are mediated as a result of early life pain. As the animals used in our anatomy studies were behaviorally naïve, our observed trends in sex differences represent the basal state. Therefore, it is possible that adult exposure to stressful perturbations, such as chronic stress, amplify these trends in binding or expression profiles in opioid and stress receptor systems (Ladd et al., 2005; McCarthy et al., 2012).

6.3 Potential Mechanisms Underlying Long-Term Changes In Stress-, Anxiety- And Pain-Related Responding

Opioids For Behavior And Anatomical Support

Our pharmacological manipulations show that neonatal injury changes adult behaviors through an opioid-dependent mechanism. In particular, we observed naloxone- changes in FST, stress-induced analgesia behaviors, suggesting opioids are necessary for injury-induced behavioral changes. As our data collectively suggest neonatal injury dampens basal perception of noxious or aversive stimuli, but exacerbates responses to chronic perturbations, the following sections provide behavioral evidence for the role of the opioid system in these coping strategies. Our data also suggest contribution of upregulated enkephalin mRNA and protein, therefore the role of this peptide in stress-, anxiety- and pain-related anatomy is discussed as well.

Animal studies: Analgesia

In our previous and current studies we observed increases in basal pain thresholds and impaired stress-induced analgesia (LaPrairie and Murphy, 2007; Victoria et al., 2013b). Our anatomical and pharmacological data suggest this is supported by increased endogenous opioid-tone in pain- and stress-related brain regions (LaPrairie and Murphy, 2007; LaPrairie et al., 2008; LaPrairie and Murphy, 2009; Victoria et al., 2013b; Victoria et al., 2013d; Victoria et al., 2013a). In particular, we showed previously that enkephalin and endorphin immunoreactivity were significantly increased in vIPAG of adults that were injured on the day of birth (LaPrairie and Murphy, 2009). Systemic blockade of opioid receptors with naloxone, or vIPAG-specific antagonization of μ - and δ -, but not κ -receptor attenuated the injury-induced increase in basal pain thresholds. In the current studies, neonatally injured adults showed significant upregulation of met-enkephalin mRNA and protein CeA and LS in addition to the vIPAG. As neonates, met-enkephalin and β -endorphin protein concentrations were elevated in the spinal cord, midbrain,

forebrain and cortex within 24 hrs of neonatal injury (Victoria et al., 2013d). By P7 opioids remained elevated in the midbrain.

Impaired stress-induced analgesia was observed in both male and female neonatally injured animals (Victoria et al., 2013b). In particular, 30 min of restraint stress significantly increased paw withdrawal latencies in control, but not neonatally injured adults. Naloxone administration before restraint completely blocked stress-induced analgesia in controls, implicating an opioid dependent mechanism. Others have shown that stress-induced analgesia increases nociceptive thresholds through μ -opioid receptor signaling in the amygdala and PAG (Stein et al., 1992), and is prevented by pretreatment with naloxone (Lewis et al., 1980). In our studies, the inability of prolonged restraint (30 min) to alter sensory thresholds in injured rats suggests that enkephalin or endorphin could not increase beyond the 2-fold elevations observed for basal levels (Victoria et al., 2013c; Victoria et al., 2013b). Notably, stress-induced CORT levels peak at 30 min making it possible that any opioid surge above baseline was mitigated by CORT activated GR, which negatively regulates enkephalin and β -endorphin expression (Schoneveld et al., 2004). Impaired stress-induced analgesia suggested opioid-dependent vulnerability to more severe stressors, as we later observed in the FST following chronic variable stress (Victoria et al., 2013a). Our data are consistent with clinical reports hypothesizing that changes in the endogenous opioid system mediate elevations in thermal pain sensitivity and impairments in stress-induced analgesia observed in adolescence and teens burned early in infancy (Peters et al., 2005; Hermann et al., 2006; Goffaux et al., 2008).

In support of behavioral adaptations via the intersection of pain and stress systems, administration of the enkephalin analogue, DAMGO, into the BLA, a nucleus known to regulate responses to stress, produces hypoalgesia that is blocked by lidocaine or electric lesion of the PAG or RVM (Helmstetter et al., 1998). Administration of enkephalinase inhibitor, RB101, produces analgesia similar to endogenous enkephalin or morphine (Valverde et al., 1996). In particular, infusion of RB101 into the CeA, thalamus, vPAG or raphe results in antinociception in

response to electrical tail stimulation and decreases stimulation-evoked vocalizations, all of which are blocked by μ -opioid receptor antagonization. Enkephalinase inhibitor, thiophan, increases jump latency and potentiates tail flick latency produced by inescapable foot shock; effects are preventable with naloxone (Chipkin et al., 1982). Evidence also supports the contribution of β -endorphin, as plasma levels increase while pain reactivity decreases (Loh et al., 1976; Tordjman et al., 2009). Together these data support the role for the μ -opioid system in blunting sensitivity to noxious stimuli.

Animal studies: Social behavior

A number of studies have tested the impact of opioids on social and affective behaviors in animal models. While we did not test social behavior directly, opioid regulation of defeat or predator-threat has implications for our observed injury-induced behavioral coping strategies and sites of enkephalin upregulation. Brief 10 min exposure of adult mice to predator odor increases anxiety-like behaviors such as defensive burying, rearing, stretch-attending, freezing and time in the dark portion of the light-dark chamber. Shortly after odor exposure, activated enkephalin protein levels (co-labeled with fos-related antigen) increase in the CeA and BLA, suggesting dynamic changes in the enkephalin system in response to noxious, aversive perturbations (Hebb et al., 2004). Interestingly, mice that displayed the lowest levels of anxiety showed the largest increases in enkephalin expression (Hebb et al., 2004). This finding is in agreement with our injury-induced decrease in anxiety-like behavior in the OF and upregulation of enkephalin in the amygdala (Victoria et al., 2013b). In support of a dynamic role in coping for enkephalin, enkephalinase activity is decreased in the amygdala of adult rats immediately following 1 hr of immobilization stress (Hernandez et al., 2009). In addition, electrical stimulation of enkephalinergic neurons projecting from the CeA to the PAG have been implicated in reducing defensive rage in cats, whereas the opposite role is observed in glutamatergic neurons connecting the BLA and PAG (Siegel et al., 1997). Together, these data suggest that brain regions me-

diating aversive experiences recruit and utilize the opioid system and support our hypothesis that the amygdala and PAG work in concert to mediate the dampened behavioral responding we observed in response to acute anxiety- and stress-provoking stimuli.

Animal studies: Anxiety- and stress-related behavior and anatomy

Based on our early studies, we hypothesized that the neonatal injury-induced increase in endogenous opioid-tone decreases adult anxiogenesis in the OF and sensitivity to forced swim stress (Victoria et al., 2013b). Indeed, overexpression in enkephalin reduces anxiety-like behavior in the EPM and OF, whereas loss of enkephalin has the opposite effect (Konig et al., 1996; Kang et al., 2000; Randall-Thompson et al., 2010). Similarly, perinatal morphine exposure reduces adult anxiety-related behaviors and increases opioid release in response to psychological stressors in rats (Buisman-Pijlman et al., 2009). We observed that morphine treatment for early life pain prevented the injury-induced increase in duration in the inner area of the OF, suggesting that elevation of endogenous opioids and over activation of opioid receptors was prevented. In response to acute FST, neonatal injury significantly increases adult LTI and duration of swimming (Victoria et al., 2013b); naloxone reversed adult behaviors and neonatal morphine treatment prevented them from manifesting in adulthood (Victoria et al., 2013a). In response to mCVS, neonatally injured adults initiated floating rapidly and spent significantly more time immobile relative to controls, suggest that high levels of opioids interact with chronic stress to result in depression-related behavior (Victoria et al., 2013a). Indeed, other have shown that morphine treatment of adult rats during 7 days of chronic variable stress increases immobility in the FST and decreases sucrose preference (Molina et al., 1994; Zurita et al., 2000). Daily naloxone or naltrexone administration before stressors rescues these behaviors (Molina et al., 1994; Zurita et al., 2000), suggesting that chronic opioid signaling is necessary for FST behaviors in response to repeated stress. They further suggest that opioid blockade prevents vulnerability to

chronic stress. This is similar to what we observed in adults given morphine for neonatal pain (Victoria et al., 2013a).

In our studies documenting the impact of injury on endogenous opioids profiles during the first postnatal week, we observed significant and time dependent increases in enkephalin and endorphin (Victoria et al., 2013d). We observed immediate changes in opioid concentrations in the spinal cord, midbrain, forebrain and cortex. By the end of the first postnatal week, levels remained elevated in a site- and peptide-specific manner. Of note, both enkephalin and endorphin were upregulated in the midbrain. While the contribution of other endogenous opioids, such as β -endorphin and leu-enkephalin, to our adult phenotype cannot be ruled out, we reported previously that adult β -endorphin fiber number was low and distribution sparse in the adult vIPAG (LaPrairie and Murphy, 2009). Injury also increases leu-enkephalin (LaPrairie and Murphy, 2009), however, in comparison preproenkephalin yields four times more met-enkephalin protein (Yoshikawa et al., 1984). Therefore, we hypothesize the anatomical basis for our opioid-dependent behaviors are primarily due to the increase in endogenous enkephalin. In adulthood we observed significant elevation of enkephalin mRNA expression and protein in the LS, CeA and vIPAG, regions known to mediate responses to stress- anxiety- and pain-provoking stimuli. The specific contribution of each region to our behavioral phenotype would be an interesting point of study in the future.

Importantly, the CeA and vIPAG are reciprocally connected and work together to cope with noxious or aversive stimuli (Rizvi et al., 1991; Behbehani, 1995; Manning and Mayer, 1995; Zubieta et al., 2001). The CeA is essential for processing and applying affective valence to stimuli including affective components of pain (Davis and Whalen, 2001; Neugebauer, 2007; Fu and Neugebauer, 2008; Ulrich-Lai and Herman, 2009). The vIPAG is an important integrator of noxious stimuli (Behbehani and Fields, 1979) and autonomic tone (Bandler and Shipley, 1994; Floyd et al., 1996) to promote coping and survival (Bernard and Bandler, 1998). The CeA and vIPAG are both highly responsive to inflammatory pain and communicate to produce analgesia

in rat pups and adult rodents (Wiedenmayer et al., 2002) (Rizvi et al., 1991; Behbehani, 1995; Fu and Neugebauer, 2008). Electrical stimulation of the PAG, thalamus or CeA in adult rats decreases sensitivity to pressure, tail shock, burning, cold or laparotomy (Ribeiro et al., 2005). In rats, bilateral excitotoxic lesions of the CeA, but not BLA or MeA, prevents morphine antinociception suggesting that the CeA is an important contributor to analgesia (Manning and Mayer, 1995). In addition, electrical stimulation of the CeA results in increased endogenous opioid concentration in the PAG (Nakamura et al., 2013), supporting the hypothesis that the CeA and PAG work in concert to block perception of noxious stimuli.

Neuronally, the CeA and PAG are rich sources of GABAergic/enkephalinergic signaling (Akil et al., 1984; Behbehani, 1995; Poulin et al., 2006). Agonization of the GABAergic cells in these regions results in increased exploration of the open arm in the EPM and decreased response to aggression, suggesting a neural circuit for dampening anxiety and stress (Graeff et al., 1993). Enkephalin is known to dampen perception of noxious or aversive stimuli directly through postsynaptic inhibition or indirectly by presynaptic modulation of glutamatergic and GABAergic transmission (Sugita and North, 1993; Zhu and Pan, 2004, 2005).

We also observed an injury-induced increase in enkephalin in the LS. The LS is primarily composed of GABAergic neurons that inhibit HPA axis activation (Herman, 2010). Benzodiazepine administration into the LS increases the frequency of open arm exploration in the EPM and decreases burying of an electrified probe (Menard and Treit, 1999). Conversely, lesions of the LS increase anxiety-like behaviors and produce “septal rage” (Dobráková et al., 1982; Herman, 2010). When considered in the context of our behavioral observations, this evidence implicates the LS as an important region for decreasing anxiety to facilitate recovery from aversive stimuli. Potentially, the LS collaborates with the CeA and vPAG to dampen the noxious perception associated with neonatal pain. Increases in adult enkephalin protein and mRNA provides support for injury-induced programming of the enkephalin gene within the LS making it a probable contributor to the impairment of adult anxiety and stress-related behavior as we have

observed. Together, the LS, CeA and PAG are a potential circuit whereby early in life neonates are protected from noxious stimulation, and in adulthood sensitivity to anxiety-, stress- and pain-provoking stimuli is reduced. Whether morphine treatment for early life pain prevents enkephalin upregulation in these regions remains to be tested in future studies.

In the PVN, enkephalin mRNA is expressed rapidly in response to inflammation but is not detectable in the absence of stress (Lightman and Young, 1989). Therefore, it is not surprising that changes in enkephalin protein or mRNA were absent in the adult PVN. It is possible that PVN excitability is influenced by enkephalin upregulation in the CeA and LS, as both regions provide HPA-inhibition through their projections to the BNST (Jakab and Leranath, 1991; Chalmers et al., 1995; Choi et al., 2007; Choi et al., 2008a; Choi et al., 2008b). Together, our behavioral and anatomical data suggest that neonatal injury selectively acts on extrahypothalamic circuits controlling pain, stress and anxiety to permanently alter responses to noxious or aversive stimuli.

Human studies: Analgesia, affect, stress-related anatomy and physiology

While our model of early life pain employs rodents, the effects of opioids on analgesia, affect, stress and underlying anatomy are similar in humans. Opioid-dependent hyposensitivity to pain is observed in individuals that have low social support, high levels of social obstruction, and people suffering from PTSD (McCubbin, 1993). In PTSD-diagnosed veterans, exposure to combat videos produces naloxone-reversible decreases in pain sensitivity (McCubbin, 1993). Morphine administration at the time of trauma, during resuscitation or early during treatment protects veterans from developing PTSD (Holbrook et al., 2010). Notable, these effects were specific to morphine, as benzodiazepines or serotonin reuptake inhibitors were not effective. Consistent with this finding, children given morphine for burn injuries are significantly less likely to show signs of PTSD in a 6 month follow up assessment (Saxe et al., 2001). Together these data suggest that morphine administration is effective for preventing long-term psychological

consequences associated with trauma, potentially through decreasing sensory-affective perception of pain and injury severity. In addition, these studies support our findings that morphine treatment for neonatal injury confers protection against extreme behavioral responses to acute or chronic perturbations.

In our studies, early life pain resulted in significant changes in endogenous opioids in stress-, anxiety- and pain-related brain regions. Human imaging studies support the role of anxiety- and stress-related brain regions in sensory-affective perception. For example, intramuscular hypertonic saline injections produce persistent pain in people (Zubieta et al., 2001), which is associated with increased activity of a radiolabel fentanyl derivative in the PAG, amygdala, hypothalamus, as well as the PFC (anterior cingulate, anterior insula, lateral cortex), thalamus and ventral basal ganglia (NAcc and VP). In the absence of painful stimulation, sad autobiographical recollections are significantly associated with decreases in opioid signaling in these same regions, with the exception of the hypothalamus and PAG (Zubieta et al., 2003). Interestingly, only opioid activity in the amygdala and ventral basal ganglia were significantly associated with an increase in negative affect and decrease in positive affect (Zubieta et al., 2003).

Changes in the endogenous opioid system directly impact human HPA physiology. For example, morphine treatment dampens CORT output from the HPA axis (McDonald et al., 1959; Zis et al., 1984). Adult men with the μ -opioid receptor gene A118G polymorphism show significant increases in plasma ACTH and CORT in response to naloxone, suggesting tighter endogenous opioid regulation over HPA functioning (Wand et al., 2002). The A-G substitution has been linked to a 3-fold increase in β -endorphin binding to μ -opioid receptor and is significantly associated with decreases in personality factors related to planning, task completion and organization (Wand et al., 2002). Adult females suffering from major depressive disorder show general decreases for *in vivo* μ -receptor binding potential in the amygdala, thalamus and PFC, in addition to decreases in ACTH and CORT following autobiographical recall of a neutral or sad story relative to healthy controls (Kennedy et al., 2006). While these studies were not performed

in former preterm infants, their findings support the hypothesis that dysregulation of the endogenous opioid and stress systems are significantly related, and impact responding to environmentally relevant stimuli that can culminate in affective dysfunction.

Injury Is Sufficient To Create A New HPA Profile In Presence Of Acute And Chronic Stress

Functionally, stressful stimuli, including pain and tissue inflammation, result in robust release of CRF from the hypothalamus, ACTH from the pituitary gland and CORT from the adrenal cortex (Vale et al., 1981; Dallman et al., 1987; Taylor et al., 1998). CORT then feeds back to the hypothalamus and pituitary where it binds to GR to inhibit further peptide release (Rivier et al., 1982; Dallman et al., 1987). Collectively, we observed that injury is sufficient to create a new HPA profile in response to acute or chronic stress (Victoria et al., 2013c; Victoria et al., 2013a). Following injury at P0, a significant elevation in CORT was observed that persisted over the first postnatal week (Victoria et al., 2013d). As adults, no differences in basal CORT were observed. However, in response to acute restraint or swim stress, CORT concentrations of neonatally injured adults returned to basal levels more rapidly than controls (Victoria et al., 2013c; Victoria et al., 2013a).

Importantly, altering the time over which glucocorticoids circulate in response to stress has physiological consequences on production of new glucose from the liver, breakdown of adipose tissue into free fatty acids, protein synthesis, glucose and amino acid uptake into cells, and immune system regulation (Bateman et al., 1989). Although the observed accelerated recovery from an acute stress may have an immediate physiological or survival benefit, reduced ability to liberate and sequester appropriate glucose could have serious consequences for responses to repeated or chronic stressors. Interestingly, individuals at risk for depression have more rapid CORT recovery times in response to a clinical stress test as compared with clinically depressed people (Dienes et al 2012). In addition, it is well documented that individuals who experienced early life trauma show alterations in HPA activity and are at significantly higher risk for the de-

velopment of affective disorders. This includes former preterm infants, whom also display blunted HPA in response to clinical stress tests (Buske-Kirschbaum et al., 2007) and experience affective disorders at a significantly higher rate than full term peers (Botting et al., 1997; Hack et al., 2004; Hayes and Sharif, 2009; Sullivan et al., 2012).

Prolonged pain and inflammation during the first postnatal week provides the opportunity for sustained activation and reprogramming of the HPA axis. Our studies show that in adulthood, GR mRNA and protein levels are significantly increased in the PVN but decreased in the hippocampus, suggesting compensatory adaptations to facilitate termination of the HPA axis. As affinity for GR is elevated in the first postnatal weeks, high levels of CORT likely resulted in more frequent negative feedback, and therefore more efficient stress recovery (Sapolsky and Meaney, 1986). Based on early studies examining the development of the GR system and HPA axis functioning (Meaney et al., 1985b; Sapolsky and Meaney, 1986; Walker et al., 1986), we hypothesize that as CORT continues to feed up to the hippocampus, GR becomes downregulated (Victoria et al., 2013c) and less able to terminate stress. To compensate, GR in the PVN becomes upregulated to promote HPA axis inhibition. Indeed, models of early life stress, immune challenge and metabolic perturbations have also observed long-term changes in hypothalamic and hippocampal GR expression, supporting the hypothesis that this system is malleable early in life (Sapolsky and Meaney, 1986; Proulx et al., 2001; Ladd et al., 2005; Bilbo et al., 2008). This is further supported by human studies showing that postmortem hippocampal GR expression is significantly decreased in severely depressed adults who were abused as children (McGowan 2009).

In addition to the HPA axis-specific changes we observed in the GR system, we reported that neonatal injury significantly decreases adult CRFR1 binding only in the BLA and vPAG. This suggests that injury on the day of birth selectively impacts circuits underlying the activation of stress (Bhatnagar et al., 2004), autonomic tone (Bandler and Shipley, 1994; Floyd et al., 1996) and processing of noxious stimuli (Behbehani and Fields, 1979).

The BLA has long been implicated in the activation of the stress axis, as acute stressors like restraint, swim stress or foot shock evoke BLA *cfos* expression (Cullinan et al., 1995; Sawchenko et al., 1996; Dayas et al., 2001). Acute administration of CRF into the BLA increases local neuronal excitability (Rainnie et al., 1992), suggesting this region is responsive to acute stress- or anxiety-provoking stimuli. BLA specific lesions reduce ACTH and CORT reactivity following acute restraint (Bhatnagar et al., 2004), suggesting that reduced CRFR1 binding in the BLA may contribute to the decrease in CORT we observed following restraint stress.

In addition, we observed that CRFR2 binding was significantly increased in the LS and CoA of adult rats injured on the day of birth. Although little is known about the role of the CoA in stress, it is highly connected to the BLA (Canteras et al., 1992; McDonald et al., 1999), where we observed significantly decreased CRFR1 binding as a result of early life pain. Lesions of the LS increase stress-induced ACTH, CORT and the time spent immobile in the forced swim test (Singewald et al., 2011). Further, LS receives excitatory drive from CoA, along with other amygdalar regions and the hippocampus (Joels and Urban, 1984; Gallagher et al., 2008) to modulate HPA activity (Chalmers et al., 1995; Reul and Holsboer, 2002; Singewald et al., 2011). Together, these data suggest that the observed increase in binding of CRFR2 in the LS and CoA may serve to promote homeostasis and dampen responses to stress, adaptations that would promote survival following trauma early in life.

Importantly, the intra and extrahypothalamic changes in the GR and CRFR systems that result from neonatal injury were observed in behaviorally naïve animals (Victoria et al., 2013c). Therefore, while this receptor profile likely mediates acute responses to stress, it is possible that dynamic changes in expression or binding occur as a result of chronic stress exposure. Although speculative, it is a possibility, as previous studies in adult rats that were maternally separated as pups show opposite CORT, GR and CRFR profiles in response to acute and chronic stressors (Ladd et al., 2005). In addition, we observed that morphine treatment for early life pain had sex-specific effects on CORT reactivity in response to acute and chronic stress. Specific-

ly, females treated with morphine had elevated CORT responses to acute stress relative to injured and handled females, suggesting morphine treatment augmented acute stress responding and possibly changed either GR receptor expression or binding. Males treated with morphine recovered as quickly as injured males in response to acute swim stress, suggesting no change in the basal GR. In response to chronic stress, females treated with morphine before injury had higher basal CORT but recovered as quickly as controls. In contrast, CORT responses of males treated with morphine mirrored control males, suggesting complete protection from the effects of early life injury (Victoria et al., 2013a).

Collectively, these findings suggest that while morphine treatment for early life pain was effective in rescuing injury-induced behavioral changes, reversal of HPA physiology may be more complicated. Similarly, environmental enrichment in peripubertal rats that were maternally separated as pups, rescues behavior and HPA reactivity, but does not return hippocampal GR mRNA or PVN CRF mRNA to control levels, suggesting compensatory mechanisms offset the impact of early life stress (Francis et al., 2002). In addition, contributions from the endogenous opioid system are also likely. Morphine, enkephalin or β -endorphin all dampen HPA activity (McDonald et al., 1959; Zis et al., 1984; Wand et al., 2002). In addition, endogenous opioids co-localize and co-express with CRFR and are negatively regulated by GR making it difficult to assume specific and localized neuroanatomical changes as a result of neonatal morphine treatment.

Factors In Addition To Pain May Change Adult HPA Reactivity

In addition to pain, neonatal injury results in inflammation, therefore the role of immune factors on HPA activity cannot be ruled out. In response to inflammation, peripheral CRF and cytokine IL-1 β are released and act on immune cells to stimulate the release of endogenous opioids, β -endorphin or met-enkephalin, and result in antinociception (Schafer et al., 1994; Schafer et al., 1997). Centrally, IL-1 β is known to stimulate norepinephrine (NE) and activate

the HPA along with CRF (Brunton et al., 2005). While IL-1 β may contribute to the injury-induced changes in HPA reactivity, it is worth noting that enkephalin rapidly reduces stress-induced release of NE (Tanaka et al., 1989), suggesting that the injury-induced surge in enkephalin would mitigate the effects of IL-1 β on HPA activity. In addition, peripheral application of enkephalin mimics the anti-inflammatory effects of CRF (Schafer et al., 1994; Schafer et al., 1997), suggesting neonatal CGN may instead upregulate *anti*-inflammatory factors. In fact, a similar model of early life inflammation results in adult upregulation of IL-10 and proenkephalin gene expression in the spinal cord with no observed changes in pro-inflammatory markers (Ren et al., 2005). Certainly, this does not rule out the contribution of other immune system mediators, such as TNF α , which can stimulate the HPA axis and is also negatively regulated by GR (Tsigos and Chrousos, 2002).

Working Hypothesis

Our working hypothesis is that neonatal pain experienced during a critical neurodevelopmental period (P0-P8 (LaPrairie and Murphy, 2007)) increases afferent nociceptive drive to supraspinal brain regions responsive to noxious input, including the septum, thalamus, hypothalamus, amygdala and periaqueductal gray (Walker et al., 1986; Fitzgerald, 2005; LaPrairie and Murphy, 2009; Victoria et al., 2013b). Endogenous opioids, including met-enkephalin and β -endorphin, are released to dampen pain perception (Loh et al., 1976; Konig et al., 1996; Hurley and Hammond, 2001) and stress (Rossier et al., 1977; Rivier et al., 1982; Lightman and Young, 1987; Bilkei-Gorzo et al., 2008). Concurrently, neurohormones from the HPA axis, including CRF, ACTH and CORT, are released to mount appropriate physiological responses and promote recovery from the physical threat associated with inflammation (Vale et al., 1981; Dallman et al., 1987; Taylor et al., 1998). As the inflammation associated with intraplantar carrageenan persists for 24-72 hours, endogenous opioids are released in regions mediating descending pain inhibition, perception of pain and HPA regulation (e.g. vIPAG, CeA, LS). Sustained activa-

tion of the HPA axis observed in the first postnatal week following injury likely results in sustained elevation of CRF, which in turn downregulates CRFR1, while increasing CRFR2 in regions mediating stress activation and perception of noxious stimuli (e.g. vIPAG, amygdala, LS) to program circuits such that future insults are less potent or aversive. As CORT levels remain high and continue to feed up to the hippocampus, GR becomes downregulated and the organism's ability to terminate stress is impaired (Boyle et al., 2005); GR in the PVN becomes upregulated to compensate and promote HPA axis inhibition (Proulx et al., 2001). In turn, CORT negative feedback becomes more efficient to facilitate recovery (Sapolsky and Meaney, 1986). As these perturbations occur during a highly plastic developmental period, and GR transcriptionally regulates itself, CRFRs and endogenous opioids (Schoneveld et al., 2004), it is probable that this new production profile becomes epigenetically programmed as the basal state and persists throughout the life span. Notably, opioids and CRFRs regulate inhibition and excitation, respectively and changes in their expression and function occur in regions that regulate HPA-tone and responses to stress-, anxiety- and pain-provoking stimuli. As such, the neuroanatomical changes we observed likely interact to produce the behavioral phenotypes that results from injury. As adults, sensitivity to acute stress-, anxiety- and pain-provoking stimuli are dampened. However, chronic, unpredictable stressors induce extreme behavioral hypersensitivity, suggesting repeated dynamic demands (Figure 6.1). Morphine treatment for early life pain rescued the injury-induced behavioral changes, suggesting pain therapy mitigates activation of circuits responsive to pain and stress. We hypothesize that morphine reverses the site-specific changes in neurohormone and receptor profiles. However, partial rescue or additional compensatory changes are possible, and are a potential starting point for future studies.

6.4 Environmental Context, Adaptation, Programming And Permanency

Epigenetics are changes in gene expression in the absence of DNA sequence alterations. The two primary mechanisms of epigenetics are DNA methylation and post-translational

modification of histones (Clark et al., 2006; Qiu, 2006a; Berger, 2007). In addition, secondary epigenetic mechanisms modify gene expression through differential recruitment of transcription factors (e.g. CREB), alternative splicing of mRNA, RNA interference (RNAi) or differential processing of protein products (Klug et al., 2006).

It is possible that the long-term changes in opioid peptide and GR expression we observed are the result of epigenetic modifications, and is a hypothesis that remains to be tested in future studies. Indeed, a number of studies have shown early life perturbations result in long-term changes in gene expression in the stress system, as well as the opioid system (Weaver et al., 2004; Mueller and Bale, 2008; McGowan et al., 2009; Morgan and Bale, 2011; Vucetic et al., 2011). Typically, such studies show the maintenance of adaptations in gene expression occur through primary or primary-dependent epigenetic phenomenon, specifically via changes in DNA methylation at CpG sites in gene promoters, chromatin remodeling via histone modifications, and more recently through miRNA regulation (Weaver et al., 2004; Hwang et al., 2007; Mueller and Bale, 2008; Wu et al., 2008; McGowan et al., 2009; Plagemann et al., 2009; Morgan and Bale, 2011; Vucetic et al., 2011).

From an evolutionary perspective, adaptation of an organism to its early environment is essential for survival. The coincidence of developmental plasticity and early life perturbations provide a unique opportunity for such adaptations to become programmed and persist later in life. However, adaptations to one's environment may result in extreme response strategies, and in turn, vulnerability and dysfunctional responding in another. For example, childhood adaptations to survive abuse may result in extreme anxiety, fear, aggression or stress in non-threatening situations later in life. Indeed, this is observed in people with PTSD, and is associated with changes in gene methylation profiles as a function of total life stress (Smith et al., 2011).

The results of many early life manipulation studies can easily be interpreted to mean that the long-term consequences in physiology and behavior are permanent. However, evidence exists to the contrary, suggesting that early life programming is not a life sentence. For example,

rodent models of maternal separation show that the changes in behavior and HPA reactivity can be prevented by environmental enrichment given during the peripubertal period (Francis et al., 2002). Modifications of methylation and histone profiles of the GR promoter, mRNA expression, protein levels and HPA responsivity that result from maternal care are also reversible with pharmacological treatment in adulthood (Weaver et al., 2005). Further chronic stress induces social avoidance behavior and demethylates the CRF promoter in the PVN of *adult* mice. Behavioral resilience and CRF promoter remethylation are rescued after only 3 weeks of treatment with the antidepressant, imipramine (Elliott et al., 2010), suggesting that programming of gene expression is not necessarily permanent if appropriate intervention is provided.

In the case of preterm birth and NICU treatment, evidence suggests that maternal presence and educational level are significant factors that can attenuate and even prevent long-term changes in pain sensitivity, cognitive performance and potential risk of affective dysfunction (Levy-Shiff et al., 1994; Hayes and Sharif, 2009; Hohmeister et al., 2010; Sullivan et al., 2012; Lowe et al., 2013)). Therefore, while changes in pharmacological pain treatment for NICU patients may take time, alternative steps can be taken to prevent deficits in sensory and affective functioning that are associated with preterm birth.

6.5 Final Remarks

Numerous clinical studies have shown that exposure to unalleviated pain and stress in the NICU has immediate and long-lasting consequences for sensory perception, neuroendocrine stress responses and emotional health in former preterm infants. In humans, the mechanism(s) by which these long-term changes in stress and pain behavior and physiology occur, and whether such changes can be prevented by analgesic intervention at the time of injury, is not known. The goal of these dissertation studies was to delineate the specific impact of a single injury early in life on adult behavioral responses to anxiety- and stress-provoking stimuli, stress axis functioning, and associated changes in underlying neuroanatomical circuits. In addition,

these studies tested whether blockade of inflammatory pain with morphine administration attenuated the previously observed behavioral and hormonal responses. Our studies show for the first time that a single injury on the day of birth significantly reduces sensitivity to acute anxiety- and stress-provoking stimuli. Injury upregulated endogenous enkephalin in brain regions responsive to stress, anxiety and pain, and blockade of opioid signaling prevented injury-induced changes in stress-related behavior. These findings suggest that opioid dysregulation underlies blunted behavior sensitivity, which is consistent with clinical reports in former preterm infants. Our studies further showed that neonatal injury significantly altered classic stress-receptor systems in regions modulating the HPA axis. In turn, HPA functioning was dampened in response to acute stressors, consistent with dampened hormonal response to stress observed in former preterm infants. Our subsequent studies established that injury-induced increases in endogenous opioids occurred within the first 24 hrs post-injury, and remained elevated at the end of the first postnatal week, suggesting a time point by which changes in the opioid system become programmed. In addition, HPA activity was significantly elevated following injury, suggesting disruption of developmentally sensitive HPA maturation, and establishing a mechanism supporting the observed adult changes in brain receptor systems mediating stress responses. Our final studies showed that neonatally injured adults were behaviorally and hormonally hypersensitive to the effects of chronic stress, suggesting vulnerability to affective dysfunction. However, morphine analgesia for early life pain rescued adult behavioral hyposensitivity to acute stress, adult behavioral hypersensitivity to chronic stress, and attenuated associated hormonal changes. These data argue strongly that the experience of pain associated with early life injury is necessary for the long-term changes in stress-related behavior and hormone responses. Collectively, these dissertation studies are the first to provide evidence that dysregulation of the opioid system alters adult stress-related responding, and show that such changes are preventable with analgesic treatment. As former preterm infants are at risk for disorders of stress and perception,

such as anxiety, depression and PTSD, these studies provide imperative evidence for the development and use of specific and appropriate analgesic regimes for human infants.

6.6 Chapter 6 Figures

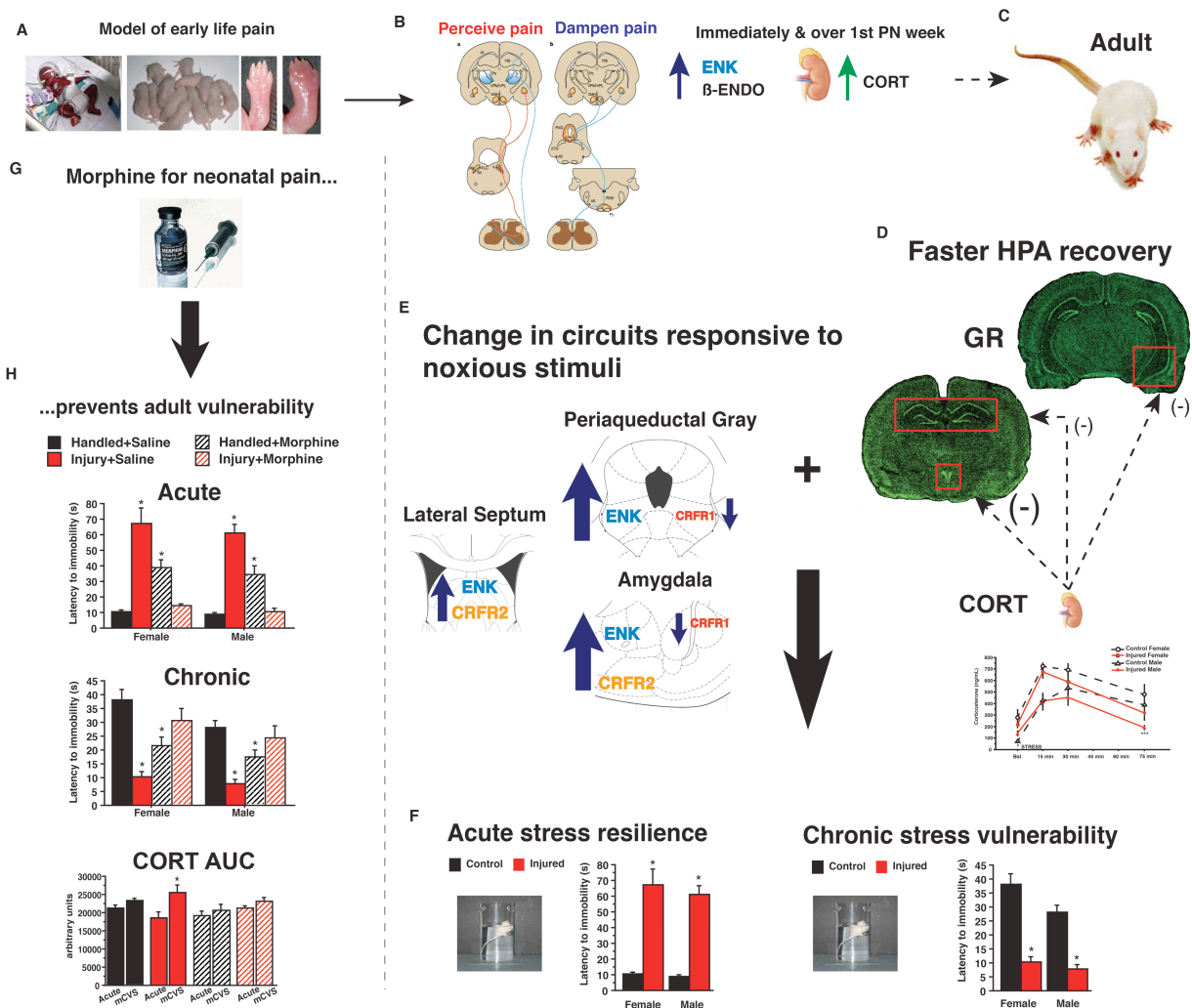


Figure 6.1 The lasting impact of a single neonatal injury on anxiety and stress responding.

A. Preterm infants experience numerous painful, inflammatory procedures in the Neonatal Intensive Care Unit often in the absence of pain therapy. To model a *single* painful experience on the day of birth, rat pups receive an injection of the inflammatory agent carrageenan (CGN; 1%) into the intraplantar surface of the right hindpaw. **B.** Inflammatory pain increases afferent nociceptive drive to supraspinal brain regions responsive to noxious stimuli (e.g. septum, thalamus, hypothalamus, amygdala and periaqueductal gray (PAG)). Met-enkephalin (ENK), β -endorphin (β -ENDO) and corticosterone (CORT) are released to dampen pain perception and stress associated with inflammation. ENK and plasma CORT remain elevated at the end of the first postnatal week, suggesting permanent changes in the endogenous opioid and stress systems. **C.** In neonatally injured adults (**D**) CORT levels return to baseline more rapidly following acute stress stimulation of the hypothalamic pituitary adrenal (HPA) axis. In parallel, glucocorticoid receptor (GR) mRNA and protein are increased in the paraventricular nucleus of the hypothalamus but decreased in the dorsal and ventral hippocampus, suggesting support for accelerated negative feedback. **E.** Anatomical changes in corticotrophin releasing factor receptor (CRFR) 1 and 2,

and ENK are observed in circuits that process anxiety-, stress-, and pain-provoking stimuli, contribute to stimulation of the HPA axis and homeostasis. CRFR1 binding is significantly decreased in the amygdala and ventrolateral (vl) PAG. CRFR2 binding was increased in the amygdala and lateral septum (LS). ENK mRNA and protein expression are significantly increased in the vlPAG, amygdala and LS. **F**. In response to acute stressors, neonatally injured adults take significantly longer to initiate floating. By contrast, adults injured early in life float rapidly after exposure to 7 days of mild chronic variable stress. **G, H**. Hypo-sensitivity to acute stress-provoking stimuli and hypersensitivity to sequential, unpredictable stress is rescued if male and female rats are given morphine for early life pain, suggesting that 1) injury-induced behavioral and hormonal vulnerability are preventable, 2) neonatal pain is necessary for the long-term changes in stress responding.

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APPENDIX**Curriculum Vitae****Nicole C. Victoria, Ph.D.**

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 Atlanta, GA 30303
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Education

- 2002-2006 *Degree:* Bachelor of Science
 Major and Advisor: Psychology, Karen Berkley Ph.D.
 Institution: Florida State University, Tallahassee, FL
- 2008-2011 *Degree:* Master of Science
 Major and Advisor: Neuroscience, Anne Z. Murphy Ph.D.
 Institution: Georgia State University, Atlanta, GA
- 2008-2013 *Degree:* Doctor of Philosophy
 Major and Advisor: Neuroscience, Anne Z. Murphy Ph.D.
 Institution: Georgia State University, Atlanta, GA

Professional Experience

- 1997 *Position:* NASA Sharp Plus Apprentice
 Institution and Sponsor: Morgan State University, Biology Department, Baltimore, MD, Christine Hohmann Ph.D.
- 1997-1999 *Position:* Research Apprentice
 Institution and Sponsor: Florida Game and Fresh Water Fish Commission & Boca Raton High School, Boca Raton, FL, Hector Graz-Lopez, JoAnne Weise M.S., and Howard Weise M.S.
- 2004 *Position:* Research Assistant
 Institution and Sponsor: Florida State University, Psychology Department, Tallahassee, FL, Michael Kaschak Ph.D.
- Position:* Research Assistant
 Institution and Sponsor: Florida State University, Psychology Department, Tallahassee, FL, Jon Bailey Ph.D. and C. Baker Wright Ph.D.
- 2007-2008 *Position:* Research Assistant
 Institution and Sponsor: Florida State University, Psychology Department, Tallahassee, FL, Karen Berkley Ph.D.

2008-2013 *Position:* Graduate Student, June 2008-2013
Institution and Sponsor: Georgia State University, Neuroscience Institute, Atlanta, GA, Anne Z. Murphy Ph.D.

Research Interests

- Impact of early life experience on adult pain, stress and anxiety behaviors, physiology, and neuroanatomy
- Sex differences in central opioid analgesia and stress responsivity

Publications

- (1) **Victoria N.C.**, Inoue K., Young L.J., Murphy A.Z. A Single Neonatal Injury Induces Life-Long Deficits In Responses To Stress, *Developmental Neuroscience* (2013) DOI: 10.1159/000351121
- (2) **Victoria N.C.**, Inoue K., Young L.J., Murphy A.Z. Long-Term Dysregulation Of Brain Corticotrophin And Glucocorticoid Receptors And Stress Reactivity By Single Early-Life Pain Experience In Male And Female Rats (*In Press, Psychoneuroendocrinology, July 2013*)
- (3) **Victoria N.C.**, Karom M.C., Eichenbaum H., Murphy A.Z. Neonatal Injury Rapidly Alters Markers Of Pain And Stress in Rats (*In Press, Developmental Neurobiology, August 2013*)
- (4) **Victoria N.C.**, Karom M.C., Murphy A.Z. Analgesia For Early Life Pain Prevents Deficits In Adult Anxiety And Stress (*Manuscript in preparation for Journal of Neuroscience, Summer 2013*)
- (5) Ogawa Y., **Victoria N.C.**, Parent M.B., Murphy A.Z. Impact of Neonatal Pain and Inflammation on Hippocampal-dependent Memory in Middle-aged Rats (*Manuscript in preparation for Journal of Neuroscience, Summer 2013*)
- (6) Ogawa Y., **Victoria N.C.**, Murphy A.Z., Parent M.B. Neonatal Inflammatory Pain Increases Food Intake and Body Weight in Adulthood (*In progress*)
- (7) Cooke B.M., Bradford M., **Victoria N.C.**, Murphy A.Z. Neonatal Injury Increases Juvenile Play (*In progress*)

Honors, Awards and Grants

- 1997 U.S. Air Force Earth and Space Science Category Award-First place, International Science and Engineering Fair - \$3000
- 1998 Boeing Achievement Award, International Science and Engineering Fair
 Indiana University Scholarship-Second place, International Science and Engineering Fair –\$2000 annually
 Rensselaer Medal Scholarship-First place, International Science and Engineering Fair - \$40,000
 Baylor University Scholarship-First place, International Science and Engineering Fair - \$2000 annually
 NASA Achievement Award-First place, International Science and Engineering Fair

- U.S. Global Change Award-Second place, International Science and Engineering Fair - \$3000
Earth and Space Science Category Award-Second place, International Science and Engineering Fair
- 1999 United States Department of Agriculture-Honorable Mention, Annual Science and Engineering Fair of Florida
Bausch & Lomb Science Award
Earth and Space Science Category Award-Third place, International Science and Engineering Fair
Florida Junior Science, Engineering, and Humanities Symposium-Outstanding Speaker, University of Florida
- 2009 **Early Life Programming Conference Travel Award**, Early Life Programming Conference, University of Pennsylvania, June 2009
- 2010 **Neuroscience Graduate Student Association FY2011** (July 1, 2010-June 30, 2011) **Funding Grant**, College of Arts and Sciences Student Activity Committee, Georgia State University, July 2010, \$1800
- 2011 **Pediatric Technology and Surgery Translational Science Award**, Pediatric Technology and Surgery Conference, Emory University, June 2011
- 2011 **Atlanta Chapter Society for Neuroscience Poster Award-Second place**, Atlanta Chapter Society for Neuroscience Poster Preview, Emory University, November 2011
- 2012 **Neurobiology of Stress Workshop National Science Foundation Travel Award**, Neurobiology of Stress Workshop, University of Pennsylvania, June 2012, \$750
- 2012 **Neurogenomics Second Century Initiative Provost-appointed Fellow**, Georgia State University, June 2012
- 2012 **Society for Neuroscience 2012 Press Room "Hot Topic"**, Society for Neuroscience Meeting, New Orleans, October 2012
- 2012 **Atlanta Chapter Society for Neuroscience Poster Award-Third place**, Atlanta Chapter Society for Neuroscience Poster Preview, Emory University, October 2012
- 2012 **Acknowledged for technical contributions** in Weathington J.M. and Cooke B.M., Corticotropin-Releasing Factor Receptor Binding in the Amygdala Changes Across Puberty in a Sex-Specific Manner, *Endocrinology*, electronic publication ahead of print, November 2012 doi: 10.1210/en.2012-1815.

Oral Presentations

- (1) Novel Oral Dosing Method for Experimental Neurological Medications in Mice, Morgan State University, Baltimore, MD, August 1997
- (2) Growth of a Cyanobacteria under Simulated Martian Conditions, Florida Junior Science, Engineering, and Humanities Symposium, University of Florida, Gainesville, FL, February 1999
- (3) Comprehensive School-Based Behavioral Assessment of the Effects of Methylphenidate, Florida State University, Tallahassee, FL, December 2004
- (4) Stress: The Role of the HPA axis in Neonatal Trauma, Georgia State University, Atlanta, GA, June 2008
- (5) Impact of Neonatal Injury on Adulthood Anxiety-like and Stress-related Behaviors, **McNair Program**, Georgia State University, Atlanta, GA, July 2009

- (6) Impact of Neonatal Injury on Long-term Stress Responsivity, **Early Life Programming Conference**, University of Pennsylvania, Philadelphia, PA, July 2009
- (7) Impact of Neonatal Injury on Long-term Stress Responsivity, **Neuroscience Institute Brown Bag Lunch Series**, Georgia State University, Atlanta, GA, February 2010
- (8) Impact of Neonatal Injury on Long-term Stress Responsivity, **Early Life Experience Meeting**, Georgia State University, Atlanta, GA, February 2010, March 2010
- (9) Future Directions for Impact of Neonatal Injury on Long-term Stress Responsivity: Maternal Deprivation and Chronic Early Life Stress, **Early Life Experience Meeting**, Georgia State University, Atlanta, GA, March 2010
- (10) The Role of Epigenetics in the Maintenance of Neonatal Injury-induced Pain Insensitivity in Adulthood, **Qualifying Exam Proposal**, Georgia State University, Atlanta, GA, April 2011
- (11) Impact of Neonatal Inflammatory Injury on Stress-related Behavior and Circuits in Adult Male and Female Rats, **Pediatric Surgery and Technology Conference**, Emory University, Atlanta, GA, June 2011
- (12) Impact of Acute Neonatal Inflammatory Injury on Stress Behavior and Circuitry in Adult Male and Female Rats, **Dissertation Proposal**, Georgia State University, Atlanta, GA, January 2012
- (13) A single neonatal injury induces life-long deficits in pain and stress responsiveness and increases brain enkephalin expression, **Neuroscience Institute Breakfast and Lecture Series**, Georgia State University, Atlanta, GA, September 2012

Abstract and Poster Presentations

- (1) **Victoria N.C.** and Weise J. Growth of Cyanobacteria under Simulated Martian Conditions (Phases I-III), International Science and Engineering Fair, 1997-1999
- (2) Dumas L.D., **Victoria N.C.**, and Murphy A.Z. Impact of Neonatal Injury on Adulthood Anxiety-like and Stress-related Behaviors, Georgia State University, **McNair Program**, Atlanta, GA, July 2009
- (3) **Victoria N.C.** and Murphy A.Z. Sex Differences in the Impact of Neonatal Inflammatory Injury on Long-term Stress Responsivity in Rats, **Atlanta Chapter Society for Neuroscience Poster Preview**, Emory University, Atlanta, GA, October 2009
- (4) **Victoria N.C.** and Murphy A.Z. Sex Differences in the Impact of Neonatal Inflammatory Injury on Long-term Stress Responsivity in Rats, **Society for Neuroscience** annual meeting, Chicago, IL, October 2009
- (5) **Victoria N.C.**, Huguelet W., and Murphy A.Z. Sex Differences in the Impact of Neonatal Inflammatory Injury on Long-term Stress Responsivity in Rats, **South Eastern Nerve Net and Georgia/South Carolina Neuroscience Consortium**, Emory University, Atlanta, GA, March 2010
- (6) **Victoria N.C.** and Murphy A.Z. Sex Differences in the Impact of Neonatal Inflammatory Injury on Long-term Stress Responsivity in Rats, **Brains and Behavior Retreat**, Georgia State University, Atlanta, GA, April 2010
- (7) **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Circuits in Adult Male and Female Rats, **Atlanta Chapter Society for Neuroscience Poster Preview**, Emory University, Atlanta, GA, November 2010
- (8) **Victoria N.C.**, Travis B.T., and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Circuits in Adult Male and Female Rats, **Society for Neuroscience** annual meeting, San Diego, CA, November 2010
- (9) **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Behavior and Circuits in Adult Male and Female Rats, **Atlanta Chapter Society for Neuroscience Poster Preview**, Emory University, Atlanta, GA, November 2011

- (10) Parent M.B., Ogawa Y., **Victoria N.C.**, and Murphy A.Z. Impact of Neonatal Pain and Inflammation on Hippocampal-dependent Memory in Middle-aged Rats, **Atlanta Chapter Society for Neuroscience Poster Preview**, Emory University, Atlanta, GA, November 2011
- (11) **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Behavior and Circuits in Adult Male and Female Rats, **Society for Neuroscience** annual meeting, Washington D.C., MD, November 2011
- (12) Parent M.B., Ogawa Y., **Victoria N.C.**, and Murphy A.Z. Impact of Neonatal Pain and Inflammation on Hippocampal-dependent Memory in Middle-aged Rats, **Society for Neuroscience** annual meeting, Washington D.C., MD, November 2011
- (13) **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Behavior and Circuits in Adult Male and Female Rats, **Pediatric Research Retreat** for Children's Hospital of Atlanta Pediatric Research Center, Emory University, Atlanta, GA, January 2012
- (14) **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Stress-related Behavior and Circuits in Adult Male and Female Rats, **Brains and Behavior** annual retreat, Georgia State University, Atlanta, GA, April 2012
- (15) Shukla D., **Victoria N.C.**, Murphy A.Z., and Cooke B.M. Sex Specific Effects of Neonatal Injury on Juvenile Play, **Brains and Behavior** annual retreat, Georgia State University, Atlanta, GA, April 2012
- (16) Seo B., **Victoria N.C.** and Murphy A.Z. The Impact of Neonatal Inflammatory Injury on Expression of Enkephalin Protein in Adult Male and Female Rats, **Senior Capstone Apprenticeship Program**, Gwinnet School of Mathematics, Science and Technology, Lawrenceville, GA, April 2012
- (17) **Victoria N.C.**, Inoue K., Young L.J., Murphy A.Z. A Single Neonatal Injury Impairs Adult Stress Responsiveness: An Opioid Story, **Neurobiology of Stress Workshop**, University of Pennsylvania, Philadelphia, PA, June 2012
- (18) **Victoria N.C.**, Inoue K., Young L.J., Murphy A.Z. A Single Neonatal Injury Impairs Adult Stress Responsiveness: An Opioid Story, **Society for Neuroscience** annual meeting, New Orleans, LA, October 2012
- (19) Ogawa Y., **Victoria N.C.**, Murphy A.Z., and Parent M.B. Neonatal Inflammatory Pain Increases Food Intake and Body Weight in Adulthood and Impairs Hippocampal-dependent Memory, **Society for Neuroscience** annual meeting, New Orleans, LA, October 2012
- (20) Nagar N., Ogawa Y., **Victoria N.C.**, Murphy A.Z., and Parent M.B. Neonatal Pain and Inflammation Impairs Hippocampal-Dependent Memory in Middle-Aged Rats, Georgia State University **Undergraduate Research Conference**, Atlanta, GA, March 2013
- (21) Schmuck L.M., Ogawa Y., **Victoria N.C.**, Murphy A.Z., and Parent M.B. Neonatal Pain Accelerates Meal Onset and Increase Body Mass in Adult Female Rats with Poor Spatial Memory, Georgia State University, **Undergraduate Research Conference**, Atlanta, GA March 2013
- (22) Parent M.B, Henderson Y.O., **Victoria N.C.** and Murphy A.Z. Pre-emptive morphine analgesia prevents the impairing effects of neonatal inflammatory pain on adult hippocampal dependent memory and produces memory deficits in non-injured rats, **Society for Neuroscience** annual meeting, San Diego, CA, November 2013

Laboratory and Research Skills

- Rodent early life intervention with inflammatory and pharmacological agents
- Rodent behavioral videography and analysis

- Rodent behavioral pharmacology
- Rodent survival surgery
- Rodent vaginal lavage
- Rodent blood withdrawal
- Rodent perfusion and dissection
- Histology and immunohistochemistry
- Microscopy and densitometry
- Molecular biology
- Radioimmunoassay
- Receptor autoradiography
- *in situ* hybridization experience
- Experimental design, data analysis and presentation

Junior Graduate Students Mentored

- 2008-2009 Amanda Arnold, Department of Biology, Biology Georgia State University, Atlanta, GA
- 2009-2010 Elizabeth Jeffress, Department of Biology, Georgia State University, Atlanta, GA
- 2011-2012 John McNeill, Neuroscience Institute, Georgia State University, Atlanta, GA

Undergraduate Students Mentored

- 2008-2009 Vincent Laufer, Georgia State University, Atlanta, GA
- 2009 Latrenda Dumas, Ronald E. McNair Scholar, Georgia State University, Atlanta, GA
- 2009-2010 Whitney Huguelet, Georgia State University, Atlanta, GA
- 2009-2011 Brittany Grandy, Presidential Scholar, Georgia State University, Atlanta, GA
- 2010-2011 Brynn Travis, Georgia State University, Atlanta, GA
- 2010-2011 Hila Eichenbaum, Honors Scholar, Georgia State University, Atlanta, GA
- 2011-2012 Ronak Shah, Georgia State University, Atlanta, GA
- 2011-2012 Preston Girardot, Georgia State University, Atlanta, GA
- 2011-2012 Jean-Marc Sauzier, Honors Scholar, Georgia State University, Atlanta, GA
- 2012 Sierra Moore, Presidential Scholar, Georgia State University, Atlanta, GA
- 2012 Ozaer Faroqui, Georgia State University, Atlanta, GA
- 2012-2013 Laura Butkovich, Honors Scholar, Georgia State University, Atlanta, GA

High School Students Mentored

- 2011-2012 Bonnie Seo, Senior Capstone Fellow, Gwinnett School of Mathematics, Science and Technology, Gwinnett, GA

Professional Societies

- 2008-present Society for Neuroscience, National and Atlanta Chapters
- 2008-present Center for Behavioral Neuroscience, Atlanta, GA
- 2009-present Society for Behavioral Neuroendocrinology
- 2009-present American Association for Advancement of Science

Professional Service

- 2008-2013 High School, Undergraduate and Graduate **Student Mentor**, Georgia State University, Atlanta, GA
- 2009 Brains Rule **Volunteer**, Atlanta, GA
Ronald E. **McNair Program Mentor**, Georgia State University, Atlanta, GA
- 2009-2010 Neuroscience Graduate Student Association **Co-President**, Georgia State University, Atlanta, GA
Neuroscience Graduate Student Association **Undergraduate Placement Program (UP) Co-founder**, Georgia State University, Atlanta, GA
- 2010-2013 Neuroscience Graduate Student Association, **Student Recruitment Organizer** (2010) and **Participant** (2011, 2012, 2013) Georgia State University, Atlanta, GA
- 2010-2013 Henderson Middle School **Science Fair Judge**, Decatur, GA
- 2011 Psi Chi National Honors Society, **Panel Member**: Paths to Graduate School, Georgia State University, Atlanta, GA
- 2011 BRAIN Research **Symposium Judge**, Georgia State University, Atlanta, GA
- 2011-2012 **College of Arts and Sciences Graduate Council, Elected Graduate Student Representative**, Georgia State University, Atlanta, GA
*Graduate Student Fee Subcommittee member. Committee enacted payment plan for graduate student fees and increased availability of technical software to graduate students.
**Proposed and working with Dean's Office to develop program for "Doctoral Training and Education in Careers outside of Academia"

Course Instruction

- 2006 Hardman Reading Technique for Dyslexia and Attention Deficit Hyperactivity, Woodland Hall Academy, Tallahassee, FL
- 2006-2007 English as a Second Language, Brighton Junior English School, Busan, S. Korea
- 2009-2010 BIO 3850 Animal Biology Lab, Georgia State University, Atlanta, GA
- 2011-2012 BIO 2108 Principles of Biology Lab, Georgia State University, Atlanta, GA