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The Impact of Neonatal Inflammatory Insult on Adult Somatosensory Processing: The Role of the Descending Nociceptive Circuit

Jamie L. LaPrairie

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

ABSTRACT

The neonatal period represents a critical window of increased neurodevelopmental plasticity in the immature nervous system. Unlike other sensory modalities, which require appropriate stimulation for proper development, maturation of nociceptive circuitry in neonates typically occurs in the absence of noxious stimulation. Premature infants, however, are routinely exposed to multiple invasive medical procedures during neonatal intensive care treatment, which are largely performed in the absence of anesthetics or analgesics. To date, it is largely unknown how exposure to early noxious insult during this time of increased plasticity alters the development of the CNS and influences future nociceptive responses. As previous studies examining the impact of neonatal inflammatory insult on adult nociceptive responses have been conducted primarily in males, the potential adverse effects in females are unknown. Furthermore, the biological mechanisms underlying neonatal insult-induced deficits in nociceptive processing have yet to be elucidated. Therefore, this dissertation addressed the

following questions: (1) Does neonatal inflammatory insult differentially alter male and female baseline somatosensory thresholds and response to re-inflammation in adulthood?; (2) Are neonatal inflammation-induced deficits in nociceptive responsiveness mediated by a potentiation in endogenous opioid tone?; and (3) Does pre-emptive morphine analgesia attenuate the behavioral consequences of neonatal inflammatory insult? Collectively, these studies will provide valuable information about the long-term consequences of neonatal noxious stimulation in males and females, which may lead to improved understanding and prevention of the lasting effects of repeated invasive interventions in premature infants in the NICU.

INDEX WORDS: Pain, Neonate, Periaqueductal Gray, Nociception, Inflammation, Endogenous Opioids, Preterm Infant

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by

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DEDICATION

To my amazing parents, ***Dennis and Phyllis***, for the unconditional love, support and sacrifice that has enabled me to achieve my dreams. To my loving husband, ***Cary***, for bestowing on me an abundance of love, humor, perspective, balance, patience and pure goodness. To my beautiful son, ***Jonah***, for coming into my life and reminding me every day of what really matters-thank you for filling my soul with giggles and allowing me to experience the kind of love that I had only read about in fairy tales. I love you all dearly, xoxo.

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CHAPTER ONE

The Impact Of Neonatal Noxious Stimulation On Developing Nociceptive Circuits-Introductory Overview

Submitted for Publication as a Review in Frontiers in Neuroscience

1.1 The Impact Of Neonatal Noxious Stimulation On Developing Nociceptive Circuits

Over the last several decades, the relative contribution of early life events to individual disease susceptibility has been explored extensively. Only fairly recently, however, has it become evident that abnormal or excessive nociceptive activity experienced neonatally may permanently alter the normal development of the CNS and influence future responses to somatosensory input. Given the significant rise in the number of premature infants receiving high-technology intensive care over the last ten years, ex-preterm neonates may be exceedingly vulnerable to the long-term effects of repeated invasive interventions. The present review summarizes available clinical and laboratory findings on the lasting impact of exposure to noxious stimulation during early development, with a focus on the structural and functional alterations in nociceptive circuits.

1.2 Premature Birth

Overview

Premature birth, defined as birth prior to 37 weeks gestation, occurs at alarmingly high rates worldwide. According to the World Health Organization, 16.5% of all infants are born premature, with over 500,000 preterm babies born each year in the United States alone (Martin et al., 2006). Indeed, the rates of premature births in the United States have been escalating steadily to nearly 35% of all live births within the last two decades

(Martin et al., 2006). While the underlying causes of prematurity are diverse and not completely understood, several factors are known to contribute to the increased prevalence of preterm births. Assisted reproduction, which often produces multi-fetal pregnancies (twins, triplets, etc.), is one of the most significant risk factors, with nearly 40% of all multiples born premature (Martin et al., 2006). Moreover, tobacco, alcohol and illicit drug use during pregnancy contribute to a heightened risk of premature delivery (Shiono et al., 1995). Although nearly 50% of all premature births have no known etiology, high maternal blood pressure, diabetes and obesity are also contributing factors (Mathews and MacDorman, 2006). Finally, there is an increasing prevalence of deliberately induced premature deliveries and caesarean sections due to maternal or infant distress, and physician convenience.

During the last 25 years, advances in perinatal care have substantially increased the survival of infants born extremely premature. As part of these life-saving interventions, preterm neonates are routinely exposed to numerous noxious and invasive procedures in the Neonatal Intensive Care Unit (NICU), which are frequently accompanied by local inflammation and tissue damage lasting for several hours to days (Anand, 1998). Intrinsic to their care in the NICU, preterm infants undergo more than 300 noxious procedures per week including repeated heel lances, circumcision, endotracheal intubation, surgery, and respiratory and gastric suctioning (Barker and Rutter, 1995, Simons et al., 2003). Mounting evidence indicates that nociceptive circuitry is both intact and functional during late gestation, and that premature infants are indeed capable of mounting developmentally specific and distinct responses to noxious and non-noxious stimuli (Anand and Carr, 1989, Giannakoulopoulos et al., 1999). Furthermore, recent

studies report cortical activation in response to acute noxious stimulation in preterm neonates at 25 weeks, suggesting the potential for higher-level processing of pain (Bartocci et al., 2006, Slater et al., 2006b). Thus, the lasting effect of these procedures on the developing somatosensory nervous system is a subject of considerable importance.

1.3 The Impact Of Neonatal Noxious Insult

Long-Term Effects of Early Noxious Insult on Developing Nociceptive Systems-Clinical Studies

The neonatal period is a time of increased neurodevelopmental plasticity. Unlike other sensory systems which require proper stimulation for appropriate development, the maturation of nociceptive circuitry typically occurs in the absence of noxious stimuli (Lidow, 2002, Fitzgerald, 2004). Accumulating clinical evidence in humans indicates that exposure to invasive procedures during the neonatal period can lead to long-term alterations in subsequent physiological and behavioral responses to innocuous and noxious somatosensory stimulation (Porter et al., 1999, Anand and Scalzo, 2000, Whitfield and Grunau, 2000, Grunau et al., 2005).

Early pioneering studies on the lasting impact of early life noxious stimulation in human infants reported that heel lance elicits decreased facial and enhanced cardiovascular responses in preterm infants with prior NICU experience compared to age matched full-term infants (Johnston et al., 1996). Subsequent studies revealed that a higher frequency of invasive procedures in preterm infants is significantly associated with

dampened nociceptive responses at 32 weeks of age compared to controls (Grunau et al., 2001). 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 pain sensitive compared to term-born controls, with a higher frequency of procedural pain exposure associated with more dampened nociceptive responsiveness to noxious stimulation at 18 months of age (Grunau et al., 1994a).

In contrast to the reduced nociceptive responsiveness following *acute* neonatal noxious stimulation, early surgery and tissue damage in infancy lead to prolonged sensitization of nociceptive responses. Hypersensitivity to tissue damage is observed in human infants, in that a decrease in sensory thresholds is observed for days or weeks in the presence of local or deep visceral tissue injury (Andrews and Fitzgerald, 1999, Andrews and Fitzgerald, 2002). Furthermore in premature infants, the withdrawal reflex threshold in an area of local tissue damage following repeated heel lances is half the value of that on the intact contralateral heel for several months following the initial insult (Fitzgerald et al., 1988a, Fitzgerald et al., 1988b). Interestingly, this response is not restricted to the site of injury, as former NICU infants also display secondary hyperalgesia in the intact, contralateral limb (Andrews and Fitzgerald, 1994). Similarly, infants that experienced surgery within the first three months of life display enhanced hypersensitivity to subsequent surgery performed in the same dermatome that persists for more than one year (Peters et al., 2005). This hypersensitivity is also not restricted to the site of tissue damage, as neonates demonstrate greater sensitivity to mechanical stimulation both in

the area of incision and on the contralateral side of the body following corrective unilateral abdominal surgery (Andrews et al., 2002). Moreover, term-born males that experienced un-anesthetized neonatal circumcision respond more intensely to routine inoculation at 4-6 months in comparison to uncircumcised infants; this effect is partially attenuated by pre-treatment with a local anesthetic (Taddio et al., 1995).

Interestingly, alterations in nociception do not appear to be transient in nature, whereby both full- and preterm infants with prior NICU experience display an increased threshold for acute thermal stimuli (i.e. decreased sensitivity), but enhanced perceptual sensitization to a prolonged heat stimulus (i.e. hyper-sensitivity) up to 14 years of age (Hermann et al., 2006). Former preterm adolescents also display significantly greater tenderness in response to pressure (Buskila et al., 2003), are more prone to lasting clinical somatization (Grunau et al., 1994b), and report earlier onset of pediatric migraine (Maneyapanda and Venkatasubramanian, 2005) compared to full-term peers. Indeed, 10-year old children with former NICU experience also rate pictures of medical events as more intense than pictures of psychosocial pain events, unlike term-born children (Grunau et al., 1998b).

Long-Term Effects of Early Noxious Insult on Developing Nociceptive Systems-Experimental Animal Studies

There is considerable parallel evidence in non-human animal models that neonatal noxious stimulation induces persistent alterations in somatosensory structure and function which last into adult life (Ruda et al., 2000, Torsney and Fitzgerald, 2003, Ren et al., 2004). Data collected to date suggest, however, that the type of noxious

stimulation (acute versus tonic) is critical to the long-term impact. Early pioneering studies reported that persistent neonatal inflammation induced by unilateral intraplantar application of Complete Freund's adjuvant results in enhanced nociceptive sensitivity, as well as increased primary afferent nerve fiber innervation of the spinal cord that extend into adulthood (Ruda et al., 2000). Similarly, local hindpaw skin wounds induced during the first week of life result in long-lasting cutaneous hypersensitivity, expanded dorsal horn receptive fields, and profound sprouting of local sensory nerve terminals in adulthood (Ruda et al., 2000, Torsney and Fitzgerald, 2003). This hyperinnervation is associated with a long-lasting decrease in mechanical threshold in the wounded region, as well as a substantial up-regulation of growth factors including NGF and BDNF (Whitby and Ferguson, 1991, Constantinou et al., 1994, Reynolds and Fitzgerald, 1995). Finally, early life chronic intra-colonic irritation following repeated applications of mustard oil results in lasting visceral hyperalgesia (Al-Chaer et al., 2000).

In contrast, a generalized *decrease* in nociceptive sensitivity as a consequence of *acute* stimulation such as repetitive foot shock and intraplantar formalin injections has been demonstrated (Shimada et al., 1990, Bhutta et al., 2001). Likewise, neonatal laparotomy results in decreased nociceptive responses to tail-withdrawal and acidic acid abdominal constriction tests compared to control subjects in adulthood (Sternberg et al., 2005). Furthermore, a long-term global elevation of nociceptive thresholds in response to noxious thermal and mechanical stimulation following short-lasting local neonatal inflammation with intraplantar carrageenan has been reported (Ren et al., 2004). This response is also associated with excessive hyperalgesia in the presence of on-going inflammation following a subsequent inflammatory insult in adulthood (Ren et al., 2004).

Together, these data suggest that early life exposure to acute versus persistent noxious stimulation may differentially affect developing nociceptive circuitry, whereby producing distinct long-term effects.

Interestingly, all of the previous studies examining the impact of neonatal inflammation on adult somatosensory processing and dorsal horn physiology have been conducted exclusively in males (Anand et al., 1999, Ruda et al., 2000, Ren et al., 2004). Therefore, the lasting effects of early noxious insult in females are unknown. Furthermore, although the aforementioned animal studies demonstrate a causal effect of noxious sensory stimulation during the neonatal period on subsequent nociceptive sensitivity, the mechanisms underlying these deleterious neonatal insult-induced deficits have yet to be elucidated.

1.4 Sex Differences In Response to Neonatal Noxious Stimulation

Sex steroid hormones such as estrogens and androgens modulate prenatal and postnatal functional development and have potent influences on pain thresholds in male and female rats (Liu and Gintzler, 2000, Aloisi et al., 2003). Prenatally, males experience a significant surge of testicular testosterone that is centrally aromatized to estradiol and ultimately results in the masculinization of the male brain (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006). In females, the ovaries are quiescent and intracerebral estradiol remains low at birth (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006). Similar differences in hormone levels may also be present in peripheral tissues

as well. Given that estrogens have been shown to exert neuromodulatory and neuroprotective effects following acute and chronic central injuries, increased perinatal central estradiol in males may contribute to lasting sexually dimorphic responses to early life noxious stimulation (Garcia-Segura et al., 2001, Amateau et al., 2004, Maggi et al., 2004).

Estrogen also influences the expression of a number of pro-inflammatory as well as pro-nociceptive agents that may contribute to sex differences in nociceptive responses. For example, prostaglandins (which are pro-inflammatory) are released peripherally in response to injury, and estrogen has been shown to modulate both prostaglandin and COX-1 and COX-2 expression in peripheral tissues (Zhang et al., 1997). In adults, estrogen also modulates vascular tone in a tissue specific manner (vasodilation, vasoconstriction), which may lead to differences in inflammation-induced edema (Maggi et al., 2004). Peripheral injury also results in increased BDNF that is thought to promote neuronal survival and healing. As estrogen increases BDNF expression centrally, this may also attenuate the adverse effect of peripheral injury (Allen and McCarson, 2005).

In addition, activational gonadal hormones can alter the processing of nociceptive information. Sex-steroids influence endogenous opioid systems (Berglund et al., 1988, Smith et al., 1998), as well as the activity of other neuromodulators involved in nociceptive processing; including substance P, gamma-aminobutyric acid (GABA), glutamate, dopamine, serotonin and norepinephrine (Smith, 1994, Duval et al., 1996). Moreover, gonadal hormones have been shown to have a marked influence on estrous cycle effects on nociceptive and analgesic sensitivity in rodents (Fillingim and Ness, 2000, Craft et al., 2004), as well as menstrual cycle variability in chronic pain conditions

such as migraine headache (MacGregor, 1997), temporomandibular disorders (Warren and Fried, 2001), and fibromyalgia (Anderberg et al., 1998).

Thus, several mechanisms may contribute to a sexually dimorphic effect of neonatal noxious insult, including sex differences in the neuroendocrine environment at the time of injury and/or at the time of testing. While there are no reported sex differences in response to early life noxious stimulation in premature infants, the aforementioned studies suggest that the lasting impact of procedural pain experienced in the NICU may indeed be sexually dimorphic. In addition, all of the previous experimental rodent studies that have examined the lasting consequences of neonatal noxious insult on developing nociceptive circuits have been conducted exclusively in males. Hence, the inclusion of female subjects in future studies examining this topic is warranted, as premature females may be at considerably increased risk for long-term consequences of early life trauma.

1.5 Potential Mechanism Underlying The Neonatal Noxious Insult-Induced Deficits In Nociceptive Responsiveness

While the impact of neonatal noxious stimulation on developing nociceptive circuitry and subsequent pain processing and perception has been the focus of a significant amount of research within the last decade, clinical and experimental studies have failed to elucidate the mechanisms underlying the reported lasting alterations in nociceptive responsiveness. Several candidate mechanisms have been proposed to account for the

long-term alterations in nociceptive behavior following exposure to neonatal noxious insult, including an elevation in basal endogenous opioid tone.

The modulation of nociception is mediated in part by a descending inhibitory pathway that includes the midbrain periaqueductal gray (PAG), the brainstem rostral ventromedial medulla (RVM) and the spinal cord dorsal horn (Basbaum and Fields, 1978, Basbaum and Fields, 1984). In adults, this circuit is activated in the presence of persistent noxious stimulation, and functions to inhibit nociceptive transmission via endogenous opioids (Dubner and Ren, 1999). At birth, however, descending inhibitory controls are developmentally plastic and inherently vulnerable to noxious sensory stimulation (Fitzgerald and Koltzenburg, 1986, Fitzgerald, 1991). Evidence that early life trauma can shape the maturation of descending inhibitory circuits has previously been reported in both human and non-human animal studies. Preterm infants exposed to numerous interventions in the NICU display significantly altered descending inhibitory responses to noxious thermal stimulation, as well as heightened cardiac reactivity compared to both term-born and premature infants exposed to few noxious procedures (Goffaux et al., 2008), suggesting that the central nervous system may adapt to early noxious insult with a compensatory up-regulation of tonic endogenous opioid inhibition. Similarly, tonic descending inhibition of dorsal horn neurons is substantially altered in adult rats treated at birth with capsaicin (Cervero and Plenderleith, 1985). Non-nociceptive related perinatal manipulations, such as prenatal stress, prenatal ethanol exposure and post-natal handling have been reported to affect opioid-mediated processes in adulthood (Nelson et al., 1985, Nelson et al., 1986, Kinsley et al., 1988, Pieretti et al., 1991). Finally, a deficit in descending inhibitory systems is implicated in

several chronic pain conditions, including fibromyalgia (Henriksson, 1994, Julien et al., 2005), irritable bowel syndrome (Wilder-Smith et al., 2004), and tension-type headache (Pielsticker et al., 2005).

Therefore, the inherent developmental plasticity of the descending inhibitory circuit at birth may have significant implications, as noxious sensory input experienced neonatally may indeed alter the functional integrity of endogenous pain modulatory systems as an adaptive response to early insult, whereby leading to lasting alterations in nociceptive processing.

1.6 Effects Of Analgesia On Developing Nociceptive Circuitry

Despite the current knowledge that preterm infants are responsive to noxious stimulation (Giannakoulopoulos et al., 1999, Smith et al., 2000, Vanhatalo and van Nieuwenhuizen, 2000) and the accumulating evidence that invasive procedures can have lasting effects on developing nociceptive circuitry, neonatal pain remains an under-recognized and under-treated condition in the NICU (Porter et al., 1997, Kahn et al., 1998, Simons et al., 2003). Indeed, many life-saving intensive care interventions are performed in the absence of analgesics, (Johnston et al., 1997, Porter et al., 1997, Kahn et al., 1998, Simons et al., 2003) as recent studies have reported that neonates experience an average of 14 noxious procedures per day, with fewer than 35% receiving appropriate analgesic therapy (Anand and Craig, 1996, Simons et al., 2003).

Accumulating data suggests that premature infants undergoing invasive procedures, however, benefit from the use of opioid analgesics (Yaster and Deshpande, 1988,

Yaster et al., 1989, Fitzgerald, 1991, Anand and Hickey, 1992, Bhat et al., 1992). For example, altered pain responses in former preterm neonates can be predicted by the number of previous painful procedures and are normalized by the early use of morphine as an analgesic (Grunau et al., 2001). In addition, post-operative morphine analgesia in preterm and full-term infants reduces behavioral and hormonal stress responses (Farrington et al., 1993, Bouwmeester et al., 2001, Bouwmeester et al., 2003) and is associated with decreased mortality (Anand et al., 1987, Anand and Hickey, 1992). Furthermore, 45 month-old children that experienced operations following pre-emptive analgesia during early life respond to immunization pain in a similar manner as non-operated age-matched controls (Peters et al., 2003). Lastly, long-term assessments of formerly preterm children exposed to continuous morphine infusions during NICU care indicate no adverse effects of morphine on intelligence, motor function, or behavior at 6 years of age (MacGregor et al., 1998).

Clearly, the aforementioned studies imply the importance of analgesic therapy for neonates undergoing NICU and hospital treatment as a means to prevent lasting alterations in nociceptive processing. Further studies examining the effects of pre-emptive analgesics in the NICU are challenging, however, as the humane care of infants requires physicians to treat those perceived to be in distress. As such, the gaps in our knowledge of the long-term risks and benefits of analgesic therapy in newborns would greatly benefit from experimental animal models. Although there is significant evidence that opioid analgesics are efficacious in neonatal rodents (Abbott and Guy, 1995), few studies have examined whether opioid analgesics can be used to prevent the long-term sequelae associated with neonatal noxious insult (Sternberg et al., 2005).

1.7 Dissertation Goals

The overall aim of this dissertation was to determine the impact of early life noxious stimulation on developing nociceptive circuitry and somatosensory processing. Our overarching hypothesis was that neonatal inflammatory insult produces lasting deficits in nociceptive responsiveness mediated by specific neuroanatomical and neurochemical alterations in endogenous pain modulatory systems, and that these effects were differentially expressed in males and females. The dissertation research presented herein tested this hypothesis via the following questions: (1) Does neonatal inflammatory insult differentially alter male and female baseline somatosensory thresholds and response to re-inflammation in adulthood? (2) Are the neonatal inflammation-induced deficits in nociceptive responsiveness mediated by a potentiation in endogenous opioid tone? (3) Does pre-emptive morphine analgesia attenuate the behavioral consequences of neonatal inflammatory insult? Collectively, these studies provide valuable information about the long-term consequences of neonatal noxious stimulation in males and females, which may lead to improved understanding and treatment of the lasting effects of repeated invasive interventions in premature infants in the NICU.

CHAPTER TWO

Female Rats are More Vulnerable to the Long-Term Consequences of Neonatal Inflammatory Injury

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2.1 Abstract

Premature infants are routinely exposed to invasive medical procedures during neonatal intensive care treatment that are largely performed in the absence of anesthetics or analgesics. Data collected to date suggest that exposure to early insult during this time of increased plasticity alters the development of the CNS and influences future pain responses. As previous studies examining the impact of neonatal injury on nociception have been conducted primarily in males, the potential adverse effects on females is not known. Therefore, the present studies were conducted to determine whether neonatal injury differentially impacts male and female sensory thresholds in adulthood. A short lasting inflammatory response was evoked in male and female rats on the day of birth with an injection of carrageenan (CGN; 1% or 2%) into the right hind paw. Nociceptive thresholds were assessed using a noxious thermal stimulus at both adolescence (P40) and adulthood (P60). A more persistent inflammation was subsequently evoked in adult rats with an intraplantar injection of Complete Freund's adjuvant (CFA). Neonatally injured females exhibited significantly greater hypoalgesia at P60, and displayed enhanced inflammatory hyperalgesia following re-injury in adulthood compared to neonatally injured males and controls. These results demonstrate that the long-term adverse effects of neonatal injury are exacerbated in females, and may contribute to the higher prevalence, severity and duration of pain syndromes noted in women compared to men.

2.2 Introduction

The neonatal period is a time of increased neurodevelopmental plasticity. Unlike other sensory systems, which require proper stimulation for appropriate development, maturation of nociceptive circuitry typically occurs in the absence of adequate stimuli (Lidow, 2002, Fitzgerald, 2004). Premature infants, however, are routinely exposed to invasive medical procedures during neonatal intensive care treatment (Anand, 1998, Grunau et al., 2005, Peters et al., 2005). Hundreds of thousands of infants are born premature each year, and as a result of major medical and technological advances in neonatal care, infants born after 23 weeks' gestation are routinely kept alive (Qiu, 2006). In order to increase their chances of survival these newborns experience an average of 14 noxious procedures per day in the Neonatal Intensive Care Unit (NICU), including heel lances, endotracheal intubation, respiratory and gastric suctioning, and catheter insertion (Simons et al., 2003).

Recent evidence indicates that nociceptive circuitry is both intact and functional during late gestation, and that premature infants are indeed responsive to noxious stimulation (Bartocci et al., 2006, Slater et al., 2006a). Furthermore, growing clinical data suggests that exposure to noxious stimulation in premature neonates can lead to long-term alterations in subsequent physiological and behavioral responses to innocuous and noxious somatosensory stimulation (Anand, 2000b, Whitfield and Grunau, 2000, Grunau et al., 2005). Several studies report that former preterm infants exposed to multiple invasive procedures during NICU care display dampened behavioral responses and enhanced physiological responses to subsequent pain (Anand, 2000b, Oberlander

et al., 2000, Whitfield and Grunau, 2000, Grunau et al., 2005). Parallel evidence in non-human animal models also suggests that neonatal noxious stimulation is associated with long-term changes in somatosensory structure and function (Bhutta et al., 2001, Lidow, 2002, Walker et al., 2003, Ren et al., 2004, Wang et al., 2004).

Previous studies examining the impact of neonatal inflammation on adult somatosensory processing and dorsal horn physiology were conducted exclusively in males (Ruda et al., 2000, Lidow, 2002, Ren et al., 2004). The neuroendocrine profile of the newborn laboratory rat is sexually dimorphic such that in males, there is a significant surge of testicular testosterone that is centrally aromatized to estrogen and ultimately results in the masculinization of the male brain (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006). In females, the ovaries are quiescent and intracerebral estradiol remains low (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006). Estrogens have been shown to exert neuromodulatory and neuroprotective effects following acute and chronic central injuries (Garcia-Segura et al., 2001, Maggi et al., 2004, Amantea et al., 2005). Therefore, increased central levels of estrogen in males may attenuate the long-term adverse effects of neonatal injury, whereas in females the consequences of neonatal injury may be exacerbated. The aim of the current study was (1) to determine whether neonatal inflammation differentially affects male and female sensory thresholds in adulthood and (2) to test whether the impact of neonatal injury on inflammation-induced hyperalgesia in adulthood is sexually dimorphic.

2.3 Materials and Methods

Animals

Time-pregnant Sprague-Dawley rats were obtained on the 14th day of gestation (E14) (Zivic Miller) and housed individually. Animals were maintained on a 12:12h light:dark cycle, with food and water available ad libitum. On the day of birth (P0), sexing of the pups was determined by examination of the anogenital distance. All litters were reared identically, weaned at P21, and housed with same-sex littermates in groups of 2-3. 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.

Early Life Manipulations

Acute neonatal injury was induced by unilateral hindpaw injection of carrageenan (CGN; 1% or 2% soln dissolved in sterile in saline; 5ul volume; Sigma, St. Louis MO) into the plantar surface of the right hindpaw within 12 hours of birth on P0, except for critical period experiments where neonatal injury was induced on P8 or P14. This inflammatory agent provides a well-established model of acute local inflammation that lasts for 12-72 hours (Lidow, 2002, Ren et al., 2004). Control animals received either an equivolume of sterile saline into the right hindpaw or were “handled” in a similar manner and returned to their home cage. All pups within a litter received the same neonatal treatment.

Maternal Behavior

Mother-pup interactions were observed for 1 hour following the neonatal injury, and daily at 18:00 hours (1 hour prior to lights off) for 60 minutes from P0-P21. Maternal

observations were conducted by both direct observations (in a manner so as not to disrupt the dam) and by videotape for later offline analysis. Specific maternal behaviors were recorded including pup licking/grooming, nursing posture (crouching), hovering over pups, pup retrieval, nest construction, eating/drinking, exploring, inactive/napping and self-grooming. Observations were conducted by an individual blind to the neonatal group assignment.

Baseline Nociceptive Behavior

On P40 and P60, baseline paw withdrawal latencies (PWL) in response to a noxious thermal stimulus were determined. Thermal testing was conducted using the Paw Thermal Stimulator (UCSD, San Diego, California). In this test, animals were placed in a clear plastic testing chamber on a glass surface and allowed to acclimate for a minimum of 30 minutes prior to testing. A radiant beam of light beneath the glass base was directed at the plantar surface of the each hindpaw and the withdrawal reflex latency was electronically measured (in seconds). Intact male and cycling female rats were tested separately. The average withdrawal latency of 3 trials was taken; all trials were separated by a 5-minute inter-trial interval. Application of the thermal stimulus to either paw was randomly determined. To avoid potential tissue damage, a 20-second automatic termination of the heat stimulus was imposed if a paw withdrawal did not occur. The testing apparatus was thoroughly cleaned between sessions. Body weight and paw diameter for right and left hindpaws were measured prior to baseline testing on P40 and P60.

To characterize the estrous status of female rats, vaginal smears (using the saline lavage technique) were taken daily beginning two weeks prior to testing and continuing to the end of the experimental session. Proestrus was defined by the presence of nucleated epithelial cells in >90% of the total cell population; estrus was defined by the presence of cornified epithelial cells; diestrus-1 was defined as the presence of both leukocytes and cornified epithelial cells; diestrus-2 was defined as the relative absence of all cell types (Wang et al., 2006). All animals were smeared in the morning, approximately 3-4 hours after lights on. Vaginal smears were conducted a minimum of three hours prior to testing to minimize the potential effects of vaginal stimulation-produced analgesia (Komisaruk, 1977).

Nociceptive Behavior Following Re-Inflammation

Following baseline PWL determination at P60, animals received an injection of Complete Freund's adjuvant (CFA; 1:1 CFA:saline soln; 200ul; Sigma) into the plantar surface of the right (neonatally injured) hindpaw. A separate group of animals received intraplantar CFA into the left hindpaw. CFA was used for re-injury as neonatally-injured animals may potentially develop antibodies against carrageenan (CGN), thereby limiting its potency for use as an inflammatory agent. Twenty-four hours following CFA-induced inflammation, paw diameter and PWLs were tested using the Paw Thermal Stimulator as described above.

Drugs

The opioid antagonist naloxone hydrochloride (1 mg/kg; Sigma; St. Louis, MO) was injected subcutaneously fifteen minutes prior to P60 testing. This dose of naloxone was

chosen based on our previous observations demonstrating that 1 mg/kg of naloxone was effective in reversing the effects of systemic morphine but had no effect on nociceptive thresholds alone (Ji et al., 2006). Control animals received an equivolume of saline.

Statistical Analysis

Data were expressed as raw withdrawal latencies or difference scores. A maximal PWL of 20 seconds was used to prevent excessive tissue damage due to repeated application of a noxious thermal stimulus. All values are reported as Mean $\pm$ S.E.M. Data were analyzed for significant main effects of neonatal treatment and sex using ANOVA; $p < 0.05$ was considered statistically significant. Post-hoc tests using the method of Sheffe were conducted as warranted to determine significant mean differences. Where multiple comparisons were made, p values were adjusted accordingly using the Bonferroni adjustment.

2.4 Results

Neonatal Injury Differentially Affects Male and Female Sensory Thresholds in Adulthood

Previous studies have reported that neonatally injured animals have significantly higher sensory thresholds in adulthood (Lidow, 2002, Ren et al., 2004). These studies, however, were conducted primarily in males. As central estradiol levels are significantly elevated in males at this time point (Amateau et al., 2004), and estrogens have been shown to confer neuroprotection (Garcia-Segura et al., 2001, Maggi et al., 2004, Amantea et al., 2005), the present studies were conducted to determine if the long-term

consequences of neonatal injury are exacerbated in females compared to males. Intraplantar administration of carrageenan (CGN; 1% or 2%) on the day of birth resulted in significantly longer paw withdrawal latencies (PWL) in comparison to control animals at postnatal day 60 (P60). Thermal hypoalgesia was present in both the previously injured (Figure 1A) and uninjured (Figure 1B) hindpaws. As shown in Figure 1, paw withdrawal latency for the injured paw increased over 50% in injured animals (1% and 2% CGN) compared with saline and handled controls ($p<.0001$). Furthermore, neonatally injured females displayed up to a 3 second longer latency in the injured paw compared to neonatally injured males ($p=.0078$) (Figure 1A). This increased hypoalgesia was also present in the uninjured paw ($p<.0001$) (Figure 1B). There were no sex differences noted for saline and handled controls ($p<.05$).

Neonatal Injury Alters Sensory Thresholds at P40

The above studies demonstrate that neonatal injury produces decreased responses to noxious thermal stimuli in adulthood and that this effect is exacerbated in females. We next tested whether these sexually dimorphic effects were also evident during the peri-adolescent period (P40). At P40, neonatally injured male and female rats (1% and 2% CGN) displayed significantly longer paw withdrawal latencies in response to a thermal stimulus for the injured paw compared to saline and handled controls ($p<.0001$) (Figure 1C). Neonatal injury-induced hypoalgesia was also observed in the contralateral paw ($p<.0001$) (Figure 1D). No significant main effect of sex was noted on any of the withdrawal latencies, although there was a trend for the female contralateral paw ($p=.0737$).

Neonatal Injury Does Not Affect Body Weight, Paw Diameter, or Estrous Cycle

No significant differences in body weight or paw diameter (right and left hindpaws) were noted in animals exposed to neonatal inflammation (1% and 2% CGN) compared to control animals (saline and handled) at either P40 or P60 (Table 1). No significant differences in estrous cycle were noted in neonatally injured females (1% and 2% CGN) compared to control females (saline and handled) (i.e. all animals displayed normal four day estrous cycles). As we have previously shown that estrous has no effect on baseline pain sensitivity or CFA-induced hyperalgesia (Wang et al., 2006), females were grouped together regardless of estrous stage.

Neonatal Injury Has No Impact On Maternal Care

Previous studies have shown that naturally-occurring variations in maternal behavior can have a profound impact on a variety of developmental endpoints, including stress responsiveness, reproductive behavior, pain, and learning and memory (Moore, 1992, Sternberg, 1999, Liu et al., 2000, Johnston and Walker, 2003, Sternberg and Ridgway, 2003, Weaver et al., 2004). Given the profound and permanent changes induced by alterations in maternal care, daily maternal observations were conducted to determine whether neonatal inflammatory injury alters the display of maternal behavior. There were no significant differences in maternal behavior between injured and non-injured litters in the amount of time the dam spent on/with pups including crouching (nursing posture), lying with pups, and pup retrieval ($p=.2231$) (Figure 3A). Similarly, no differences were noted in the amount of time the dam spent off/without pups including nesting, eating/drinking, exploring, napping, self-grooming ($p=.2087$) (Figure 3B); or in the amount of time spent licking/grooming pups ($p=.6939$) (Figure 3C). This indicates

that changes in adult sensory thresholds produced by neonatal injury are not due to differences in maternal behavior directed at injured versus non-injured pups.

The Long-Term Consequences of Neonatal Injury are Critical Period Dependent

The next experiment was conducted to test whether the long-term consequences of neonatal injury were dependent upon a critical period. Male and female rat pups received a unilateral intraplantar injection of 1% CGN on P0, P8, or P14. Only 1% CGN was used as no significant differences were noted in the previous studies following administration of 1% versus 2% CGN. On P60, paw withdrawal latencies in response to noxious thermal stimulation were determined. Animals that were neonatally injured on P0 and P8 displayed significantly increased PWLs in both the injured ($p=.0002$) (Figure 4A) and uninjured ($p=.0003$) (Figure 4B) paws compared to animals that were injured on P14. Furthermore, a significant effect of sex was noted for both paws in animals injured on P0 and P8, with females displaying greater hypoalgesia compared to males ($p=.0068$, $p=.0014$). No significant effect of intraplantar carrageenan or sex was noted for animals injured on P14. These results suggest that the impact of neonatal injury is dependent upon a sensitive period, and that noxious insult occurring outside of this critical window does not permanently alter thermal sensory thresholds.

Neonatal Injury Enhances Hyperalgesia Following Re-Inflammation with CFA

The next series of experiments were conducted to test whether neonatally injured animals respond differentially to a subsequent injury in adulthood. Following baseline PWL determination at P60, animals received an injection of Complete Freund's adjuvant (CFA; 1:1 CFA:saline soln; 200ul; Sigma) into the plantar surface of either the right (P0

injured) or left (P0 uninjured) hindpaw. Twenty-four hours following CFA-induced inflammation, PWLs were tested in response to a noxious thermal stimulus.

The effect of neonatal injury on CFA induced hyperalgesia was quite profound. In response to noxious thermal stimulation, the paw withdrawal latencies of CFA treated adult control animals (handled and saline) decreased from approximately 8-9 seconds at baseline to 4-5 seconds following intraplantar CFA (mean difference score of 5.5 sec), a typical hyperalgesic response (Wang et al., 2006). By contrast, latencies for injured animals (1% CGN) decreased significantly from baseline PWLs of 10-12 seconds to 1-3 seconds (Figure 4A). This increased hyperalgesic response following CFA re-injury was significantly greater in neonatally injured females compared to neonatally injured males (mean difference scores of 12 for females versus 9 sec for males). There was no significant effect of neonatal treatment on the degree of edema produced by intraplantar CFA [$F(3,118)=1.32$, $p=.2705$].

Intraplantar CFA was administered in a separate group of animals into the left paw to determine whether the increased hyperalgesia following re-injury would be observed following adult re-inflammation of the neonatally uninjured paw. Similar to the previous results, neonatally injured animals (1% CGN) displayed enhanced hyperalgesia following intraplantar CFA compared to saline and handled controls ($p=.0176$) (Figure 5B). This effect was, again, exacerbated in females compared to males ($p=.0108$).

Neonatal Injury Induced Hypoalgesia is Attenuated By Systemic Naloxone

Neonatal injury results in significant long-term hypoalgesia that is present in both the previously injured and uninjured paw. This bilateral response suggests a global, injury-

induced change in basal nociceptive sensitivity. The next series of experiments were conducted to determine whether the observed hypoalgesia was a result of altered endogenous opioid tone. At P60, animals received either systemic administration of the opioid antagonist naloxone hydrochloride (NAL; 1 mg/kg) or equivolume saline (SAL) fifteen minutes prior to testing. As shown in Figure 5, administration of NAL significantly attenuated carrageenan-induced increases in paw withdrawal latencies, with no significant differences noted between neonatally-injured/naloxone treated animals and handled ($p=.1576$) or saline treated controls ($p=.3665$). Administration of naloxone alone had no effect on paw withdrawal latencies for P0 saline or handled animals.

2.5 Discussion

Our principal findings are as follows: (1) neonatal inflammatory injury produces bilateral basal hypoalgesia that is present in both adolescence and adulthood; (2) neonatally injured animals display enhanced hyperalgesia in response to a subsequent injury in adulthood; the effects of neonatal inflammatory injury on both baseline and re-injury induced changes in nociception are sexually dimorphic, with significantly greater effects present in females compared to males; (4) the long-term consequences of neonatal injury are critical period dependent; (5) injury-induced hypoalgesia is reversed by administration of the opioid antagonist naloxone.

Neonatal Inflammatory Injury Produces Thermal Hypoalgesia in Adulthood

Neonatal injury in rodents produces persistent and dramatic alterations in thermal baseline sensory thresholds (Anand et al., 1999, Bhutta et al., 2001, Lidow, 2002, Ren

et al., 2004). Previous studies have reported intraplantar carrageenan administered on postnatal day 3 results in thermal and mechanical hypoalgesia in adult male rats (Lidow, 2002, Ren et al., 2004). Additionally, long-term visceral hypoalgesia has been reported in animals exposed to carrageenan-induced inflammation as neonates (Wang et al., 2004). Somatic hypoalgesia has also been reported following repeated intraplantar 10% formalin injections in males (Bhutta et al., 2001). Here we demonstrate for the first time that female rats also display thermal hypoalgesia in adulthood following neonatal hind paw inflammation with carrageenan, and this hypoalgesia is significantly greater than that observed in males. Thermal hypoalgesia was manifest at both P40 and P60 and was present in both the injured and uninjured paw.

The global nature of the observed hypoalgesia following neonatal inflammatory injury suggests that the underlying mechanisms are not manifested peripherally at the site of injury, but rather may involve alterations in higher central regulatory systems. Our finding that administration of the opioid antagonist naloxone reverses the injury-induced hypoalgesia supports this theory. However, as there are currently no reliable and reproducible methods for assessing nociception in P0 rat pups, we cannot conclusively state that the observed hypoalgesia was due to the pain associated with inflammation. Indeed, it is also likely that our P0 manipulations induced changes in the developing hypothalamic-pituitary-adrenal axis. Nociception is one component in a broader context of stress reactivity (Anand et al., 1999, Sternberg and Ridgway, 2003, Grunau et al., 2005), and experimental studies have shown that exposure to early life stressors such as repetitive neonatal handling can permanently increase nociceptive thresholds in adult rats and decrease the behavioral and physiological responses to stress in adulthood

(Pieretti et al., 1991, Coutinho et al., 2002, Sternberg and Ridgway, 2003). Interestingly, a recent study demonstrating long-term thermal hypoalgesia in both sham operated and surgically-manipulated mice suggests that the stress of the neonatal procedure, and not necessarily the pain, contributes to the observed hypoalgesia (Sternberg et al., 2005).

Data from human preterm infants also suggests that neonatal exposure to noxious stimuli may alter the responses to subsequent painful or stressful experiences. Ex-preterm infants exposed to four weeks of NICU care display reduced behavioral pain behavior and enhanced cardiovascular responses following heel stick (Johnston and Stevens, 1996). In addition, stressful conditions at birth are associated with increased salivary cortisol in response to vaccination at 4 and 6 months of age (Peters et al., 2005). Furthermore, premature infants at 8 and 18 months of age display increased basal levels of stress hormones compared to their full-term counterparts (Grunau et al., 2007).

Administration of the broad-spectrum opioid antagonist naloxone completely reversed the hypoalgesia induced by neonatal injury, suggesting that the pain and stress associated with our neonatal manipulations resulted in a potentiation in descending endogenous opioid tone. Carrageenan-, formalin- and CFA-induced inflammation have all been shown to profoundly enhance pro-dynorphin and pro-enkephalin biosynthesis in spinal neurons in the dorsal horn (Iadarola et al., 1988, Noguchi et al., 1989). Inflammation–induced changes in endogenous opioid peptide expression and release have also been reported at several supraspinal sites, including the periaqueductal gray (PAG); this increase in opioid peptide expression is associated with hypoalgesia (Williams et al., 1995b). Pain-induced changes in opioid peptide expression are also

paralleled by an increase in mRNA expression (Iadarola et al., 1988). In the present study, naloxone was administered systemically; therefore, it is not known whether neonatal injury-induced changes in opioid tone are peripherally or centrally mediated. However, studies are currently underway using site-specific injections of naloxone to further identify the loci for injury-induced changes in endogenous opioid tone.

Long-Term Effects of Neonatal Injury are Sexually Dimorphic

The neuroendocrine profile of a newborn rat pup is sexually dimorphic, such that males have higher central levels of estradiol at birth compared to females, and similar differences in hormone levels may also be present in peripheral tissues (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006). Estrogens have been reported to exert neuroprotective effects following acute and chronic injuries in the adult CNS (Garcia-Segura et al., 2001, Maggi et al., 2004, Amantea et al., 2005). In the present study, neonatal injury resulted in significantly greater basal hypoalgesia at P60 in females in comparison to males. Indeed, the paw withdrawal latency of females injured with 1% CGN was 3 seconds longer in both the injured and uninjured paws compared to injured males. These results suggest that in males, estrogens may be acting as a neuroprotectant in response to early life injury, thereby leaving female rats with low to non-detectable levels of central estradiol increasingly vulnerable to the effects of neonatal noxious insult. Female rats injured at P14, when estradiol concentrations are comparable in males and females, displayed equivalent levels of baseline hypoalgesia as injured males, further suggesting that sex differences in the neonatal neuroendocrine environment contributed to the observed sexually dimorphic impact of neonatal injury. A potential neuroprotective effect of

androgens cannot be ruled out in the present study (Ramsden et al., 2003), and future studies directed at manipulating the neonatal neuroendocrine environment, including masculinizing females or castrating males, are necessary to more specifically implicate gonadal hormones as the primary factor contributing to the observed sex differences in the impact of neonatal inflammatory insult.

Estradiol has been shown to influence the expression of a number of pro-inflammatory and pro-nociceptive agents. For example, prostaglandins (which are pro-inflammatory) are released peripherally in response to injury, and estrogen has been shown to modulate both prostaglandin and COX-1 and COX-2 expression in peripheral tissues (Zhang et al., 1997). Furthermore, peripheral injury also results in increased BDNF that is thought to promote neuronal survival and healing (Price et al., 2005). As estradiol increases BDNF expression centrally, this may also attenuate the adverse effects of peripheral injury (Allen and McCarson, 2005).

Re-Injury with CFA Produces Hyperalgesia and the Effect is Sexually Dimorphic

Following re-injury in adulthood with CFA, neonatally injured male and female rats displayed significantly greater hyperalgesia than control animals. Furthermore, neonatally injured females exhibited significantly greater hyperalgesia in the inflamed paw than neonatally injured males, and male and female controls. This effect was observed in both the neonatally injured and uninjured paws, and is consistent with previous studies reporting long-term sensitization of afferent neurons and hyperalgesia following neonatal insult (Reynolds and Fitzgerald, 1995, Anand et al., 1999, Al-Chaer et al., 2000).

This increased *hyperalgesia* following re-injury in adulthood appears disparate with the observed basal *hypoalgesia*. Our preliminary anatomical studies, however, suggest that neonatal inflammatory injury results in bilateral alterations in primary afferent innervation of the dorsal horn (LaPrairie and Murphy, 2005), which may account for our observed hyperalgesia. In particular, neonatal injury increases primary afferent innervation in the L3-L5 spinal cord, as reflected by increased expression of both CGRP and substance P immunoreactivity. As both CGRP and substance P are pro-nociceptive, this increase in primary afferent innervation would be associated with a facilitated response to a noxious stimulus. Our working hypothesis is that the pain associated with intraplantar CGN on P0 results in a compensatory increase in descending inhibitory modulation as a mechanism of pain management. An increase in descending opioid tone is supported by our naloxone data, and would provide a direct mechanism for our observed hypoalgesia at baseline testing. By contrast, reinjury in adulthood with the inflammatory agent CFA results in an enhanced dorsal horn release of CGRP and/or substance P due to increased primary afferent input. Increased release of these pro-nociceptive peptides would be predicted to result in an enhanced hyperalgesic response. Increased primary afferent innervation of the spinal cord may also drive an enhanced descending facilitation in neonatally injured animals.

2.6 Chapter 2 Summary

Our findings clearly demonstrate that exposure to a single neonatal inflammatory insult is associated with long-term decreases in nociceptive sensitivity that are significantly

exacerbated in females. The presence of a sex difference in the response to early insult may contribute to the higher prevalence, severity, and duration of pain syndromes (i.e. migraine, temporomandibular joint disorder, fibromyalgia and irritable bowel syndrome) that are observed in women.

Chapter 2 Acknowledgements

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2.7 Chapter 2 Figures

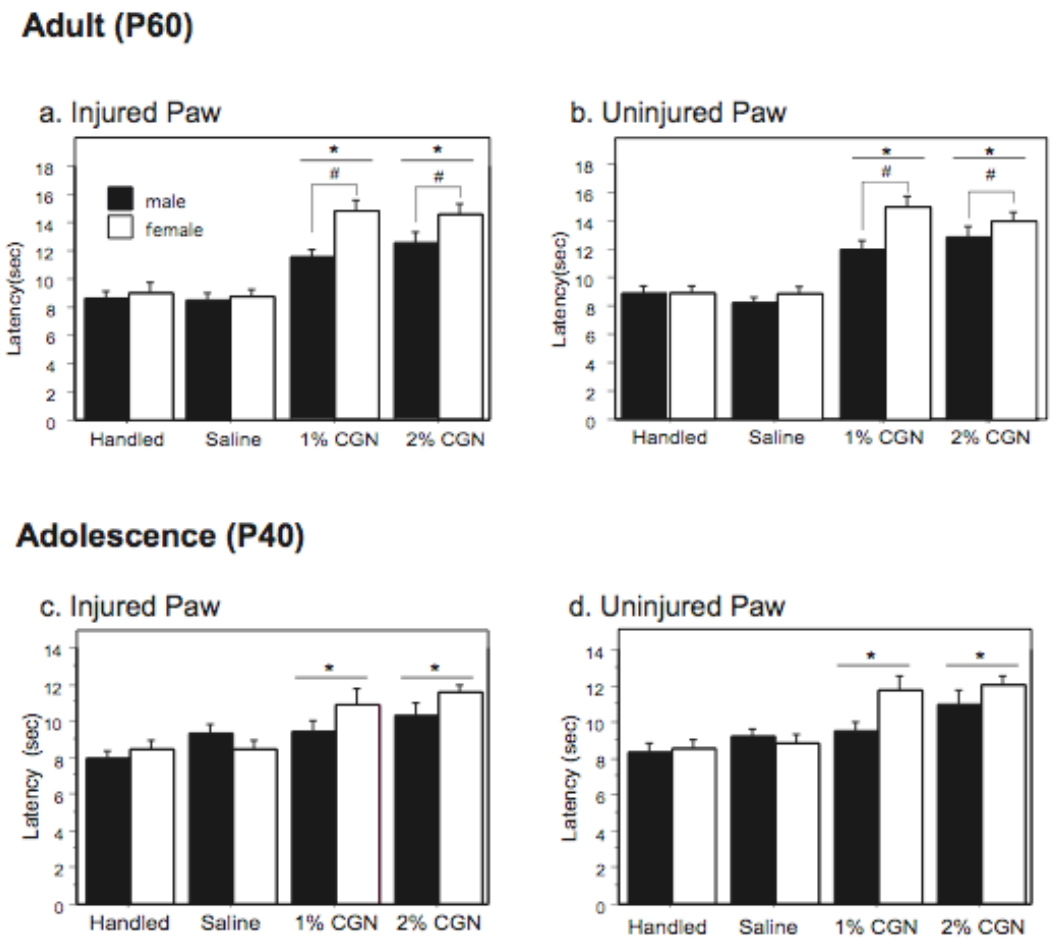

Figure 2.1 Neonatal injury differentially affects male and female sensory thresholds in adulthood (P60) and during adolescence (P40). At P60, neonatally injured animals display significantly longer withdrawal latencies compared to saline and handled controls in response to a noxious thermal stimulus applied to the (A) right (injured) paw [$F(3,114)=41.76$, $p<.0001$] and (B) (uninjured) paw [$F(3,114)=34.68$, $p<.0001$]. There was also a significant main effect of sex with injured females displaying significantly longer latencies in comparison to males: injured paw, $F(1,114)=7.34$, $p=.0078$; uninjured paw, $F(1,114)=11.31$, $p=.0011$. Significant hypoalgesia was also present at P40 in neonatally injured animals in both the (C) right (injured) paw [$F(3,114)=8.17$, $p< .0001$] and (D) contralateral (uninjured) paw [$F(3,114)=10.92$, $p<.0001$]. * denotes significant main effect compared to handled controls; # denotes a significant main effect of sex.

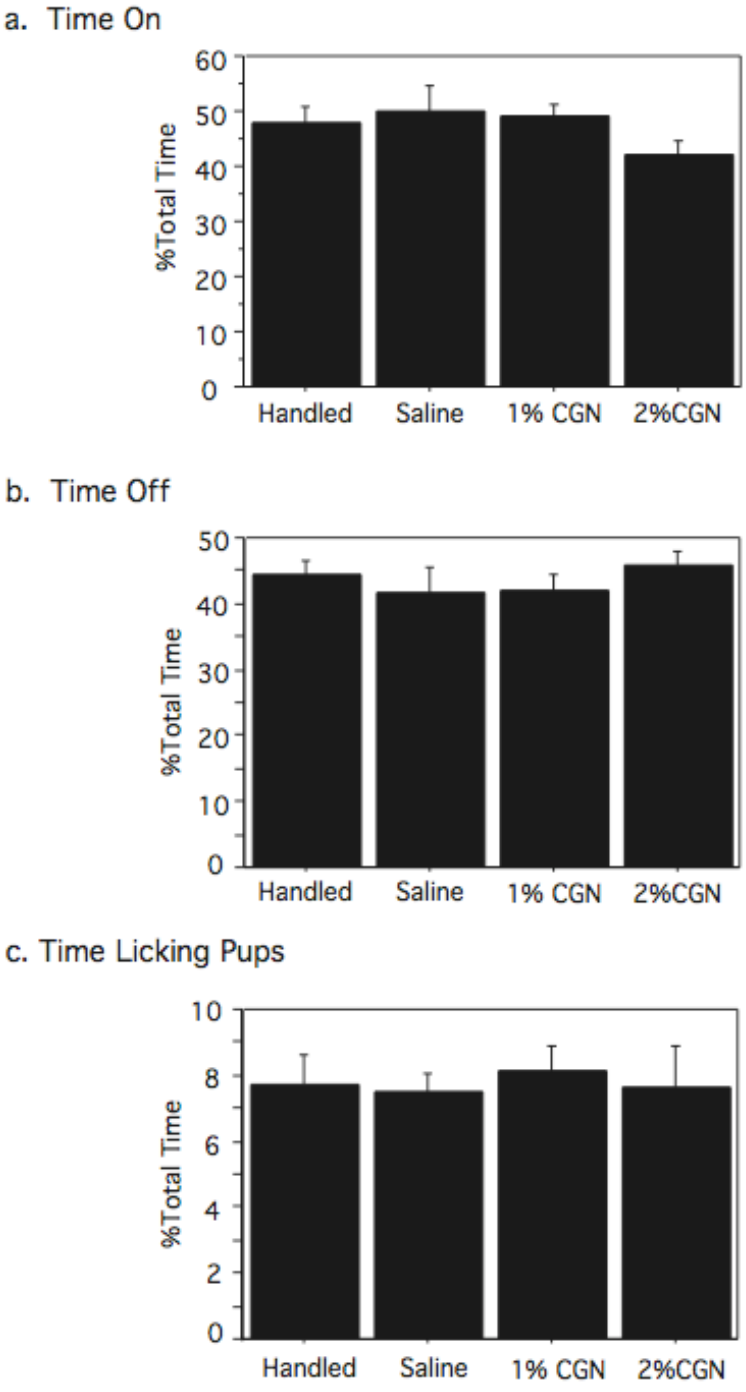

Figure 2.2. Neonatal Injury has no impact on maternal care. Neonatal injury had no effect on the (A) duration the dam spent on/with her litter [F(3,19)=.783, p=.5507], (B) amount of time the dam spent away from her litter [F(3,19)=.381, p=.7676], (C) duration of maternal licking and grooming behavior [F(3,19)=.128, p=.9425]. N=3-7 litters per group.

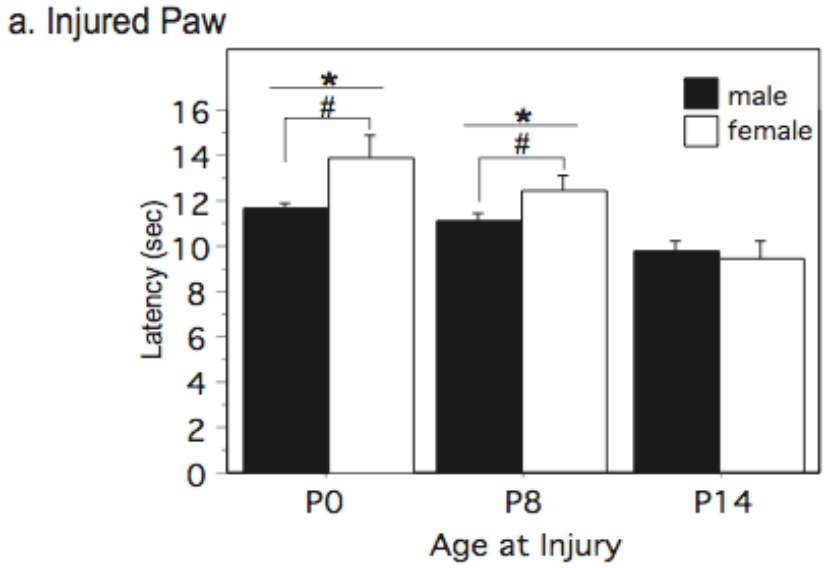

Figure 2.3. **The consequences of neonatal injury are critical period dependent.** Paw withdrawal latencies for the injured paw are significantly increased on P60 in animals neonatally injured with 1% CGN on P0 and P8 compared to animals injured on P14 [$F(2,39)=10.97$, $p=.0002$] . Neonatally injured females (P0 and P8) display significantly longer PWLs compared to neonatally injured males [$F(1,39)=3.85$, $p=.0068$]. (B) Similar results were noted for the uninjured paw [$F(2,39)=9.94$, $p=.0003$]. PWLs were significantly longer for neonatally injured females (P0 and P8) in comparison to injured males (P0) [$F(1,39)=7.09$, $p=.0014$]. N=6-12 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.

b. Uninjured Paw

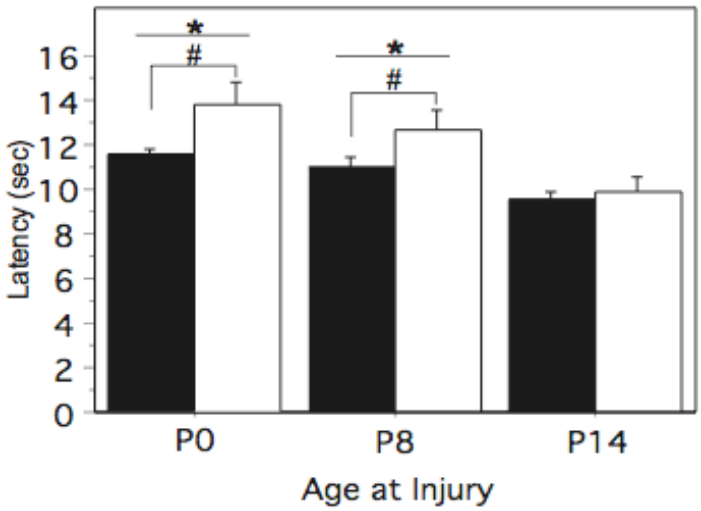

Figure 2.3 Continued

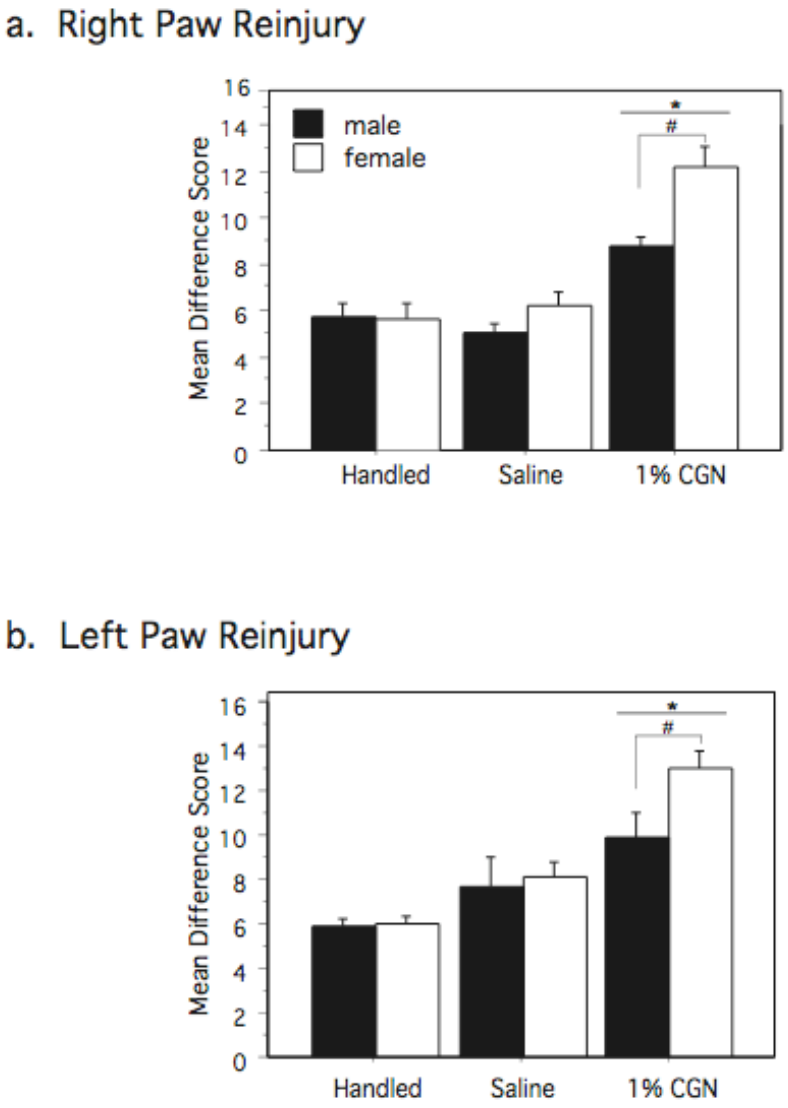

Figure 2.4. **Neonatal injury enhances hyperalgesia following re-inflammation with CFA.** Neonatally injured males and females (1% CGN) had significantly greater difference scores (Bsln PWL – CFA PWL) compared to saline and handled control animals in response to intraplantar CFA in adulthood. This effect was observed in both the (A) right (P0 injured) paw [$F(2,85)=43.54$, $p<.0001$] and (B) left (P0 uninjured) paw [$F(2,27)=19.08$, $p<.0001$]. Both effects were significantly exacerbated in neonatally injured females compared to neonatally injured males [$F(1,85)=3.96$, $p=.0499$] and [$F(1,27)=7.50$, $p=.0108$]. N=6-15 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.

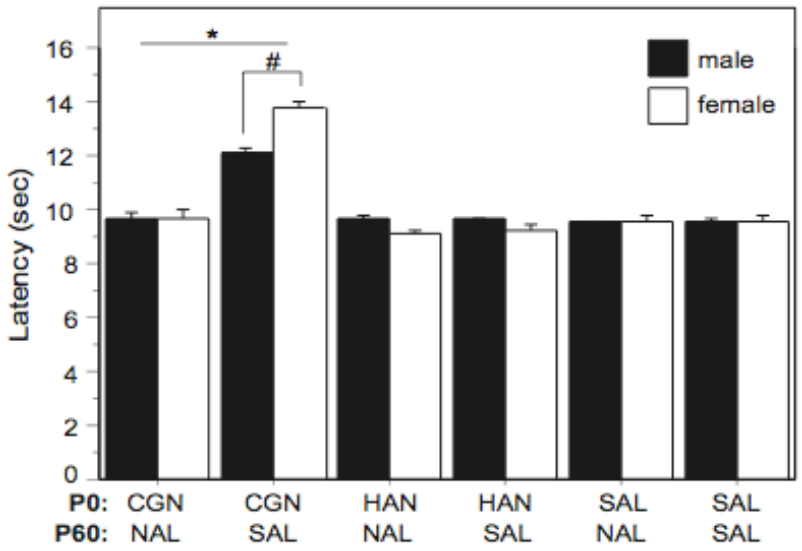

Figure 2.5. Hypoalgesia induced by neonatal injury is significantly attenuated by systemic naloxone. Neonatally injured males and females that received naloxone (CGN/NAL) prior to testing at P60 had significantly lower PWLs than injured animals that received saline control (CGN/SAL) [$F(5,38)=52.07$, $p<.0001$]. Administration of naloxone to saline (SAL/NAL) or handled (HAN/NAL) controls had no effect on PWLs. N=6-8 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.

Table 2.1. Average Body Weight and Average Paw Diameter. There is no significant difference in the average body weight across neonatal treatment groups on P40 and P60 in males and females. There is also no significant difference in the average paw diameter of the right and left hind paws across neonatal groups on P40 and P60 in males and females.

	MALE						FEMALE					
TREATMENT	P40			P60			P40			P60		
	WT	RP	LP	WT	RP	LP	WT	RP	LP	WT	RP	LP
HANDLED	298.8 ±36	4.88 ±.32	4.74 ±.27	456.9 ±38	5.55 ±.27	5.52 ±.28	213.8 ±10	4.39 ±.20	4.46 ±.21	272.9 ±19	4.67 ±.41	4.70 ±.40
SALINE	297.6 ±11	4.76 ±.29	4.76 ±.36	443.2 ±44	5.48 ±.21	5.49 ±.21	209.7 ±11	4.45 ±.14	4.36 ±.16	265.7 ±16	4.71 ±.38	4.68 ±.36
1% CGN	295.1 ±10	4.83 ±.15	4.82 ±.15	448.6 ±24	5.57 ±.14	5.57 ±.14	206.1 ±10	4.33 ±.21	4.32 ±.20	268.1 ±12	4.61 ±.12	4.61 ±.14
2% CGN	297.5 ±41	4.84 ±.30	4.77 ±.17	462.8 ±30	5.60 ±.28	5.60 ±.28	214.2 ±13	4.40 ±.23	4.35 ±.19	267.3 ±30	4.59 ±.15	4.56 ±.17

CHAPTER THREE

Neonatal Inflammatory Insult-Induced Hypoalgesia is Mediated by Increased Central Endogenous Opioid Tone: Role of Beta-Endorphin and Met/Leu-Enkephalin in the Periaqueductal Gray

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3.1 Abstract

We have previously reported that neonatal intraplantar inflammation results in significant long-term increases in nociceptive thresholds (hypoalgesia) in both male and female rats. The mechanisms underlying these long-term deficits, however, have yet to be elucidated. The present studies tested the hypothesis that the observed basal hypoalgesia is due to an injury-induced increase in endogenous opioid tone. On the day of birth, male and female Sprague Dawley rats received an intraplantar injection of the inflammatory agent carrageenan (CGN; 1%). Control animals received an equivolume of saline or were handled only. On postnatal day 60, paw withdrawal latencies to a noxious thermal stimulus were determined. Systemic administration of the opioid antagonist naloxone HCl, but not the peripherally-acting naloxone methiodide, completely reversed the neonatal injury-induced hypoalgesia, suggesting altered endogenous opioid tone. Subsequent behavioral and anatomical studies were conducted to determine the loci for injury-induced effects and the opioid receptor subtypes involved. Results from these studies demonstrated that neonatal inflammatory injury results in a significant increase in beta-endorphin and met/leu-enkephalin immunoreactivity in the PAG, as well as decreased mu- and delta- opioid receptor expression. Microinjection of the selective antagonist CTOP into the PAG reversed the injury-induced hypoalgesia. Together, the data presented herein constitute a compelling case that early noxious insult produces significant long-term alterations in endogenous descending inhibitory mechanisms, thereby profoundly impacting nociceptive responsiveness.

3.2 Introduction

The impact of neonatal noxious stimulation on developing nociceptive circuitry and subsequent pain processing and perception has been the focus of a significant amount of research within the last decade. Evidence from clinical and animal research studies have reported that noxious stimulation experienced early in life can result in lasting alterations in sensory processing (Grunau et al., 1994a; Anand et al., 1999; Ruda et al., 2000; Lidow et al., 2001; Bhutta et al., 2002; Ren et al., 2004; LaPrairie and Murphy, 2007). In preterm infants, a higher frequency of invasive procedures is associated with significantly dampened pain responses at 32 weeks of age (Grunau et al., 2001). Moreover, premature neonates with NICU experience display decreased behavioral responses to heel-lance and inoculation at 8 months (Johnston and Stevens, 1996; Oberlander et al., 2000), and are rated by parents as less sensitive to pain compared with full-term peers at 18 months of age (Grunau et al., 1994a; Grunau et al., 1994b). Interestingly, alterations in nociception do not appear to be transient in nature, such that both full- and preterm infants with prior NICU experience display increased thresholds for acute thermal stimuli up to 14 years of age (Hermann et al., 2006).

Non-human animal studies have also reported that neonatal noxious stimulation induces persistent alterations in somatosensory structure and function which last into adulthood (Ruda et al., 2000; Torsney and Fitzgerald, 2003; Ren et al., 2004; LaPrairie and Murphy, 2007). For example, our laboratory and others have established that neonatal intraplantar inflammation induces significant hypoalgesia in both the previously injured and uninjured paws in adulthood, as well as excessive hyperalgesia and significant reductions in the rate of recovery following a subsequent insult (Ren et al., 2004; LaPrairie and Murphy, 2007). A generalized decrease in nociceptive sensitivity as

a consequence of repetitive foot shock and intraplantar formalin injections has also been demonstrated (Shimada et al., 1990; Bhutta et al., 2001). To date, however, the mechanisms underlying these neonatal injury-induced deficits in sensory thresholds have yet to be elucidated.

Nociceptive information is modulated in part by a descending inhibitory pathway that includes the midbrain periaqueductal gray (PAG), the brainstem rostral ventromedial medulla (RVM) and the spinal cord dorsal horn. Anatomical and physiological studies have shown that the PAG contains endogenous opioid peptides, as well as opioid receptors (Spetea et al., 2002, Wang et al., 2002). Interestingly, while in rats the anatomical connections for nociceptive modulation are present at birth, descending inhibitory controls are functionally immature throughout the first three postnatal weeks (Fitzgerald and Koltzenburg, 1986; Boucher et al., 1998). The delayed maturation of descending inhibition may contribute to an increased vulnerability of the immature somatosensory system to excessive afferent input, whereby exposure to noxious stimulation during a critical window may alter the functional integrity of the endogenous descending inhibitory system.

The objective of the present study was to determine whether the observed deficits in nociceptive responsiveness are due to alterations in descending modulation. Behavioral and anatomical studies were conducted to test our hypothesis that neonatal injury-induced hypoalgesia is mediated by an increase in endogenous opioid tone resulting from facilitated activation of descending nociceptive inhibitory pathways.

3.3 Materials and Methods

Subjects

Time-pregnant Sprague-Dawley rats were obtained on the 14th day of gestation (E14) (Zivic Miller) and housed individually. Animals were maintained on a 12:12h light:dark cycle, with food and water available ad libitum. On the day of birth (P0), sexing of the pups was determined by examination of the anogenital distance. All litters were reared identically, weaned at P21, and housed with same-sex littermates in groups of 2-3. All experiments were approved by the Georgia State University Animal Care and Use Committee and were conducted in strict compliance with the guidelines for pain research established by the International Association for the Study of Pain (IASP).

Early Life Manipulations

Acute neonatal injury was induced by unilateral hindpaw injection of carrageenan (CGN; 1% soln dissolved in sterile in saline; 5ul volume; Sigma, St. Louis MO) into the plantar surface of the right hindpaw within 12 hours of birth on P0. This inflammatory agent provides a well-established model of acute local inflammatory pain that lasts for 12-72 hours (LaPrairie and Murphy, 2007). Control animals received either an equivolume of sterile saline into the right hindpaw or were “handled” in a similar manner and returned to their home cage. All pups within a litter received the same neonatal treatment.

Nociceptive Behavior Testing

Thermal testing was conducted using the Paw Thermal Stimulator (UCSD, San Diego, California). Briefly, animals were placed in a clear plastic testing chamber on a glass surface and allowed to acclimate for a minimum of 30 minutes prior to testing. A radiant beam of light was directed at the plantar surface of the each hindpaw or 1.5 inches from the distal end of the tail, and the latency to withdrawal was electronically measured (in

seconds) (Hargreaves et al., 1988). Intact male and cycling female rats were tested separately. The average withdrawal latency of 3 trials was taken; all trials were separated by a 5-minute inter-trial interval. Application of the thermal stimulus to either paw was randomly determined. To avoid potential tissue damage, a 20-second automatic termination of the heat stimulus was imposed if a paw or tail withdrawal did not occur. The testing apparatus was thoroughly cleaned between sessions.

Systemic Injections

To determine whether neonatal injury-induced hypoalgesia was due to increased endogenous opioid tone, naloxone HCl (1mg/kg s.c.; Sigma, St. Louis, MO) was administered 10 minutes prior to paw withdrawal latency (PWL) determination on P60. To test whether alterations in opioid tone were peripherally mediated, the peripherally acting opioid antagonist, naloxone methiodide (1mg/kg s.c.; Sigma, St. Louis, MO), was administered 15 minutes prior to determination of paw withdrawal latencies in adulthood. All drugs were dissolved in physiological saline to desired concentration; doses used in the present experiments were selected based on our previous studies (Ji et al., 2006; LaPrairie and Murphy, 2007).

Intracerebroventricular and Intra-PAG Injections

Rats (P45-50) were anesthetized with a mixture of ketamine, xylazine and acepromazine (50mg/kg, 10mg/kg, 1mg/kg; s.c.) and placed in a stereotaxic apparatus. For intracerebroventricular injections (i.c.v.), a craniotomy was made dorsal to the lateral ventricle and a stainless steel guide cannula (22G, C313G, PlasticOne Inc, Roanoke, VA) was implanted at the following coordinates (mm) [Bregma: -1.0; Mediolateral: 1.4; Dorsoventral: 3.6]. For PAG injections, cannula were implanted at the

following coordinates (mm) [Lambda: +.50; Mediolateral: .65; Dorsoventral: 5.8]. Cannulae were flushed every other day with 1.0μl of saline over a 60 second period. On P60, naloxone HCl was injected into the ventricle (i.c.v.: 1.0μg/2.0μl; PAG: .5μg/.5μl; Sigma, St. Louis, MO) over a two-minute period. In subsequent experiments the following opioid antagonists were administered: mu receptor antagonist CTOP (i.c.v.: 1μg/2μl and 10μg/2μl; PAG: .5μg/.5μl and 7.5μg/.5μl), the delta receptor antagonist naltrindole (i.c.v.: 1μg/2μl and 10μg/2μl; PAG: .5μg/.5μl and 7.5μg/.5μl) and the kappa receptor antagonist nor-BNI (i.c.v.: 10μg/2μl; PAG: 7.5μg/.5μl). All antagonists were gifts from NIDA. Mu and delta receptor antagonists and doses were administered in random order 15 minutes prior to behavioral testing. As nor-BNI is a permanent antagonist, all animals received this antagonist last (Craft et al., 2001; Craft and McNiel, 2003). In contrast to the mu and delta antagonists, nor-BNI was administered and testing followed 24 hours later (Craft et al., 2001). A minimum 72-hour washout period between all drugs and doses was imposed. Paw withdrawal latencies in response to noxious thermal stimulation were determined at 15, 30, 45, 60, 90 and 120 minutes post-injection. Injection sites were determined by administration of Pontamine Sky Blue (2.0μl; Sigma Aldrich, USA) at the completion of the experiment. Brains were removed, sectioned and mounted onto slides; all injections sites were verified histologically. Only animals with injections sites into the lateral ventricle (i.c.v. experiment) or into the ventrolateral PAG (Bregma: -7.64 to -8.30) were used for further analysis.

Immunohistochemistry

Perfusion Fixation

At P60, neonatally manipulated animals were given a euthanizing dose of Nembutal (160mg/kg; i.p.), and then perfused transcardially (descending aorta clamped) with 200ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator, followed by 250ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polysciences, Warrington PA). Following fixation, a final rinse with the sodium chloride/sodium nitrite solution was used to remove residual acrolein from the animal. Immediately after perfusion, the brains were removed and the tissue stored at 4°C in 30% sucrose solution until sectioned. The brains were cut into 30-μm coronal sections with a Leica 2000R freezing microtome (Leica Microsystems, Wetler Germany) and stored freely floating in cryoprotectant-antifreeze solution (Watson, 1986) at -20°C until immunohistochemical processing.

Immunohistochemistry

A 1:6 series through the rostrocaudal axis of each brain was processed for immunohistochemical localization of mu opioid receptor, beta-endorphin, met-enkephalin or leu-enkephalin. Briefly, sections were removed from the cryoprotectant-antifreeze solution, rinsed extensively in potassium phosphate-buffered saline, and then reacted for 20 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were then incubated in primary antibody solution in KPBS containing 0.1% Triton X for 1 hour at room temperature, followed by 48 hours at 4°C. Mu immunoreactivity was identified using the polyclonal rabbit anti-MOR antibody at a concentration of 1: 35,000 (Chemicon; Billerica, MA). Beta-endorphin immunoreactivity was recognized using the polyclonal rabbit anti-beta-endorphin antibody at a concentration of 1: 30,000 (Peninsula Labs; Torrance, CA). Met- and leu- enkephalin

immunoreactivity were identified using the polyclonal rabbit anti-met-enkephalin and anti-leu-enkephalin antibodies at concentrations of 1: 50,000 (Immunostar; Hudson, WI) and 1: 200,000 (Immunostar; Hudson, WI), respectively. After rinsing in KPBS, the tissue was incubated for 1 hour in biotinylated goat-anti-rabbit IgG (Jackson Immunoresearch, West Grove PA; 1:200), rinsed again, and incubated for 1 hour in avidin-biotin peroxidase complex (ABC Elite Kit, Vecor, Burlingame CA; 1:10). After rinsing in KPBS and sodium acetate (0.175M; pH 6.5), antigens were visualized using nickel sulfate-intensified 3,3'-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. The reaction product was terminated after 20-30 minutes by rinsing in sodium acetate buffer. Sections were mounted out of saline onto gelatin-subbed slides, air dried overnight, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped with Permount.

Densitometry

The PAG was divided into three representative rostrocaudal levels: (Rostral: Bregma -6.72 to -7.04; Mid: Bregma -7.30 to -8.00; Caudal: Bregma -8.30 to -8.80). Furthermore, dosomedial and lateral/ventrolateral subdivisions of the PAG were analyzed for each section. 12-bit grayscale images of each section were captured with a 2X, 4X and 20X objective on a Nikon Eclipse E800 microscope using a Sensys digital camera (Biovisions Technology; Exton, PA). Quantitative analysis of the tissue was performed using IP Spectrum software (Scanalytics; Fairfax, VA). The lateral/ventrolateral PAG was sampled three times and the average grayscale pixel value (ODU; optical density units) was recorded. Measures were corrected for nonspecific binding by subtraction of

background measures taken from areas lacking immunoreactivity adjacent to the lateral/ventrolateral PAG.

Receptor Autoradiography

Neonatally manipulated male and female rats were sacrificed on P60 by rapid decapitation. Brains were quickly removed, flash-frozen on dry ice and stored at -80°C. Next, brains were sectioned at 20 μm thickness with a Leica CM3050S cryostat, and immediately mounted onto Super-frost slides and stored at −80°C until the time of assay. For radiographical processing, sections were allowed to thaw to room temperature and then immersed in 0.1% paraformaldehyde for 2 minutes to optimize tissue integrity. Sections then were rinsed in 50mM Tris-NaCl (pH 7.4) at room temperature for 15 min followed by a 15-minute rinse in 50nM Tris (pH 7.4). Next, the tissue sections were incubated for 60 minutes at room temperature in a solution of 50 mM Tris (pH 7.4) with 10 mM MgCl2 and 0.1% bovine serum albumin. 3nm [3H]-Naltrindole (20 Ci/mmol; NIDA) was added to label delta receptor binding or 1nm [3H]-DAMGO (56.8 Ci/mmol; Perkin Elmer/NEN, MA) was added to label mu receptor binding. Following incubation, sections were washed 3 times at 10 minutes each in 50mM Tris-HCl (pH 7.4) with 10 mM MgCl2 at room temperature, followed by a final dip in cold dH2O. Sections were allowed to air-dry overnight and were then apposed to autoradiographic film with [3H]-microscale standards (Perkin-Elmer/NEN, MA) for 4 weeks. Film plates were processed using a FujiFilm BAS 5000. Autoradiographic [3H]-receptor binding was quantified from film using MultiGuage software (FujiFilm, USA). [3H]-standards were used to convert uncalibrated optical density to disintegrations per minute (DPM). For analysis, the PAG was divided into 3 rostrocaudal levels as

described above, and DPM were determined for the lateral/ventrolateral subdivision of each level. A mean for each level (rostral, mid, caudal) for each animal was then calculated.

Statistical Analysis and Data Presentation

All values are reported as Mean $\pm$ S.E.M. For behavioral analysis, data are expressed as raw withdrawal latencies. Data were analyzed for significant main effects of neonatal treatment (handled, saline, 1% carrageenan) and sex (male, female) using ANOVA or Repeated Measures ANOVA; $p < 0.05$ was considered statistically significant. Sheffe Post-hoc tests were conducted to determine significant mean differences between groups that were apriori specified. Where multiple comparisons were made, p values were adjusted accordingly using the Bonferroni method. For anatomical analysis, data are expressed either as optical density units (ODU; immunohistochemistry) or disinigrations per minute (DPM; autoradiography). ANOVA was used to test for significant main effects of neonatal treatment (handled, 1% carrageenan), sex (male, female) and PAG level (rostral, mid, caudal); $p<.05$ was considered significant for all analyses. For data presentation, a representative animal from each experimental group was selected. Photomicrographs were generated using a Synsys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software and finalized in Adobe Photoshop 7.0. Alterations to the images were strictly limited to enhancement of brightness and contrast.

3.4 Results

Neonatal injury-induced hypoalgesia is reversed with systemic naloxone

To test the hypothesis that neonatal injury results in the release of endogenous opioids, and that this upregulated opioid tone is maintained in adulthood, neonatally manipulated male and female rats received a systemic injection of the broad-ban opioid antagonist naloxone HCl or saline control 15 minutes prior to behavioral testing at P60. Neonatally injured (1% CGN) animals that were administered saline in adulthood displayed significantly increased paw withdrawal latencies compared to saline and handled control animals ($p<.05$; Figure 1a). This injury-induced hypoalgesia was significantly exacerbated in neonatally-injured females compared to males, as previously reported (LaPrairie and Murphy, 2007). In contrast, neonatally injured males and females that received systemic naloxone HCl on P60 displayed paw withdrawal latencies of approximately 9-10 seconds, consistent with withdrawal latencies in uninjured control animals (Figure 1a). Indeed, opioid receptor blockade by systemic naloxone completely reversed the neonatal injury-induced thermal hypoalgesia. Together, these data suggest that the long-term hypoalgesia observed in neonatally injured animals is due to altered endogenous opioid tone.

Alterations in opioid tone are not peripherally mediated

Opioid receptors are present both centrally and peripherally (Hiller et al., 1994). To determine whether the changes in opioid tone were restricted to the periphery, adult animals were administered the peripherally acting opioid antagonist naloxone methiodide prior to behavioral testing. Neonatally injured animals (1% CGN) that received saline prior to testing displayed significantly increased paw withdrawal

latencies compared to handled and saline controls ($p<.05$; Figure 1b). Again, neonatally injured females displayed significantly increased latencies compared to injured males ($p<.05$; Figure 1b). There were no significant differences in PWLs between neonatally injured animals administered saline versus naloxone methiodide (Figure 1b), suggesting that alterations in endogenous opioid tone are not peripherally mediated.

Injury-induced deficits in opioid tone are centrally mediated

To determine whether the injury-induced hypoalgesia is mediated by supraspinal alterations in endogenous opioid tone, naloxone HCl was administered into the lateral ventricle and paw withdrawal latencies were tested in response to noxious thermal stimulation over a 120-minute period. Neonatally injured males and females displayed significantly increased PWLs at baseline compared to handled and saline controls ($p<.05$; Figure 1c). Following central administration of naloxone (1.0μg/2.0μl, i.c.v; Sigma, St. Louis, MO), PWLs in neonatally injured animals significantly decreased and were comparable to control levels at 30-minutes post-naloxone. Moreover, withdrawal latencies in injured animals returned to pre-injection levels 120-minutes following administration of naloxone. In contrast, intracerebroventricular administration of naloxone at the dose used in the present experiment had no effect on withdrawal latencies in handled and saline control animals (Figure 1c). The transient reversal of the neonatal injury-induced hypoalgesia following central administration of naloxone HCl in adulthood suggests that alterations in endogenous opioid tone are mediated supraspinally.

Alterations in central opioid tone involve mu and delta opioid receptors

We next tested whether the alterations in central opioid tone were receptor-specific by administering mu, delta and kappa selective antagonists into the lateral ventricle prior to behavioral testing. Central administration of the mu receptor antagonist CTOP (10μg/2μl) significantly reduced PWLs in neonatally injured males and females to control levels at 15 minutes and 30 minutes post-injection, respectively ($p<.05$; Figure 2a). CTOP also blocked the injury-induced hypoalgesia in adult males and females at a lower dose (1μg/2μl; i.c.v.; $p<.05$; data not shown). The delta receptor antagonist naltrindole (10μg/2μl) reversed the hypoalgesia in neonatally injured males at 15 minutes post-naltrindole, whereas injured females displayed a significant reduction in withdrawal latencies at 15 minutes and reached control levels at 90 minutes following the injection ($p<.05$; Figure 2b). In contrast, low dose naltrindole (1μg/2μl) did not reduce PWLs to control levels in neonatally injured males or females (data not shown). Following administration of nor-BNI (10μg/2μl; Figure 2c), neonatally injured animals continued to display significantly increased PWLs compared to saline and handled control animals over the entire 120-minute testing period ($p<.05$; Figure 2c). Together, these results suggest that neonatal inflammation results in persistent changes in opioid tone that are centrally mediated, and involve both the mu and delta opioid receptor.

Alterations in opioid tone are mediated within the PAG

The PAG is an essential substrate for endogenous pain management (Basbaum and Fields, 1978; Fields et al., 1980; Dostrovsky et al., 1983; Basbaum and Fields, 1984; Fields et al., 1991; Williams et al., 1995). To test the hypothesis that neonatal injury-induced alterations in opioid tone were mediated within the PAG, we administered naloxone HCl directly into the mid ventrolateral PAG (VL-PAG) prior to behavioral

testing in neonatally injured and control animals. A representative PAG cannulation site is shown in Figure 3a. Intra-PAG naloxone significantly reduced PWLs in neonatally injured males and females at 15 minutes post-injection ($p<.05$; Figure 3b). The dose of naloxone HCl used in this experiment had no effect on PWLs in saline and handled control animals. The PAG contains mu, delta and kappa receptors (Mansour et al., 1995a; Mansour et al., 1995b); therefore, to determine which opioid receptor subtypes in the PAG contribute to alterations in opioid tone, we administered specific opioid antagonists directly into the mid VL-PAG. Intra-PAG administration of the mu receptor antagonist, CTOP, significantly reduced PWLs in males and females compared to control levels at 30 minutes post-injection ($p<.05$; Figure 4a). Administration of naltrindole into the PAG, significantly reversed the hypoalgesia in neonatally injured females, where PWLs were comparable or below control levels by 15 minutes post-injection and continued throughout the 120-minute testing period ($p<.05$; Figure 4b). Interestingly, intra-PAG naltrindole significantly attenuated the hypoalgesia in neonatally injured males (i.e. PWLs decreased 1-2 seconds) ($p<.05$; Figure 4b), but did not completely reduce PWLs to control levels. Not surprisingly based on our previous data, administration of nor-BNI into the VL-PAG did not significantly reduce withdrawal latencies in injured males or females (Figure 4c).

Injury-induced increase in beta-endorphin immunoreactivity in the PAG

The results from our above studies demonstrate that neonatal injury induces an increase in central endogenous opioid tone that is mediated within the PAG and involves both mu and delta opioid receptors. Therefore, the next series of experiments

were conducted to determine whether changes in endogenous opioid peptide and/or receptor expression within the PAG underlie our observed behavioral effects.

Beta-endorphin, an endogenous opioid neuropeptide that has the highest affinity for mu opioid receptors, was significantly increased in the lateral/ventrolateral region of the mid ($p<.05$; Figure 5d) and caudal ($p<.05$; Figure 5d) PAG in neonatally injured males and females compared to handled controls. No significant differences were observed in this region in the rostral PAG (Figure 5d). Moreover, no significant sex differences in beta-endorphin expression were detected at any level of the PAG (Figure 5d). Figures 5a-5c display representative examples of beta-endorphin immunoreactivity in the lateral/ventral lateral region of the mid-PAG in a handled and carrageenan-injected animal.

Increased met- and leu-enkephalin in PAG following neonatal injury

Immunohistochemical studies were also conducted to determine whether neonatal injury altered the expression of met- and leu-enkephalin in the PAG; these endogenous opioid peptides preferentially bind to delta opioid receptors. Neonatally injured males and females displayed significantly increased met-enkephalin immunoreactivity in the lateral/ventrolateral region of the mid ($p<.05$) and caudal ($p<.05$) levels of the PAG compared to control animals (Figure 6e). A significant effect of sex was noted in the lateral/ventrolateral region of the mid and caudal PAG, whereby injured females displayed significantly more immunoreactive fibers compared to injured males ($p<.05$). No significant differences in immunoreactivity were noted in the rostral level of the PAG (Figures 6e).

Similarly, neonatal inflammation resulted in significant increases in leu-enkephalin immunoreactivity in PAG. Specifically, in the lateral/ventrolateral region injured animals displayed significantly increased leu-enkephalin immunoreactive fibers compared to handled controls in the rostral ($p<.05$), mid ($p<.05$) and caudal ($p<.05$) PAG (Figure 6f). In contrast to met-enkephalin, no significant effects of sex on leu-enkephalin immunoreactivity was present at any level of the PAG. Representative examples of met-enkephalin and leu-enkephalin immunoreactivity at the mid level of the PAG in handled and carrageenan-injected animals are shown in Figures 6a-6d.

Injury-induced decrease in MOR-immunoreactivity and receptor binding in the PAG

Our final series of experiments used immunohistochemistry to determine if neonatal injury also resulted in changes in opioid receptor expression. Figures 7a-7c display representative examples of MOR-ir in the lateral/ventrolateral region of the mid-PAG in a handled and carrageenan-injected animal.

Neonatally injured animals displayed reduced mu opioid receptor immunoreactivity (MOR-ir) in the lateral/ventrolateral PAG in both the mid ($p<.05$) and caudal ($p<.05$) levels compared to handled control animals (Figure 7d). In contrast, no significant differences in MOR-ir were noted in the rostral PAG (Figure 7d). Furthermore, no significant effect of sex on MOR-ir was observed at any level of the PAG in contrast to our previous studies (Loyd and Murphy, 2008). However, in the present study females were not divided by stage of estrous.

Neonatally injured animals also displayed reduced lateral/ventrolateral MOR binding in the rostral ($p<.05$), mid ($p<.05$) and caudal ($p<.05$) levels of the PAG compared to

handled controls (Figure 8e). A representative example of MOR binding at the mid level of the PAG in a handled and carrageenan-injected male is shown in Figures 8a and 8b. No significant effect of sex on MOR binding was observed at any level or subdivision of the PAG.

Injury-induced decrease in DOR receptor binding in the PAG

A significant decrease in DOR binding was also observed at the mid level of the lateral/ventrolateral PAG in neonatally injured females compared to all other groups ($p<.05$; Figure 8c-8d, 8f). The sexually dimorphic expression of DOR binding in injured animals is consistent with our behavioral effects following intra-PAG administration of the delta opioid antagonist naltrindole (Figure 4b). No significant effects of neonatal treatment or sex on delta opioid receptor binding were noted in the lateral/ventrolateral area of the rostral and caudal levels of the PAG (Figure 8f).

3.5 Discussion

Our principle finding is that neonatal injury-induced hypoalgesia is reversed by systemic administration of naloxone HCl. The peripherally acting naloxone methiodide had no effect, while intracerebroventricular administration did, suggesting that injury-induced changes in opioid tone are centrally mediated. Parallel anatomical and behavioral experiments demonstrated that neonatal injury-induced alterations in opioid tone are mediated within the PAG, and involve up-regulation of the endogenous opioids beta-endorphin and met/leu-enkephalin, along with a concomitant decrease in mu and delta

opioid receptors. These studies, together, provide a biological mechanism for the hypoalgesia observed following neonatal injury.

Neonatal inflammation up-regulates endogenous opioid tone

In the present study, administration of the broad-spectrum opioid antagonist naloxone HCl completely reversed the hypoalgesia induced by neonatal inflammatory injury, suggesting that our neonatal manipulations resulted in a potentiation in endogenous opioid tone. It is well established that descending inhibitory pathways modulate nociceptive input and are activated in the presence of persistent pain in adulthood (Dubner and Ren, 1999). At birth, however, descending inhibitory controls are functionally immature (Fitzgerald, 1991), such that while the projections from the brainstem to the dorsal horn are present anatomically, electrical stimulation of the dorsolateral funiculus does not inhibit noxious-stimulus evoked activity in dorsal horn neurons until postnatal day 10 in the rat (Fitzgerald and Koltzenburg, 1986; Boucher et al., 1998). These early studies examining the physiological characteristics of the descending inhibitory pathway utilized a mild noxious stimulus (pinch). In the present studies a stronger nociceptive stimulus was used, which may have been more successful in activating the descending inhibitory circuit as a way of modulating the "pain". Certainly, previous studies in both human and rodents suggest that early life trauma can shape the maturation of descending inhibitory circuits. For example, preterm infants exposed to numerous interventions in the NICU display significantly altered inhibitory responses compared to both term-born and premature infants exposed to few noxious procedures, as indicated by reduced counterirritation-induced analgesia triggered using a cold pressor test (Goffaux et al., 2008). This suggests that inhibitory

circuits are very plastic in terms of increased or decreased afferent drive. Taken together with the present study, it appears that the central nervous system may adapt to early noxious insult with a compensatory up-regulation of tonic endogenous opioid inhibition.

Alterations in endogenous opioid tone are centrally, not peripherally, mediated

There is considerable evidence that the functional activity of central endogenous opioid systems in adults is enhanced following noxious stimulation. In dorsal horn spinal neurons, pro-dynorphin and pro-enkephalin biosynthesis is up-regulated in response to carrageenan-, formalin- and CFA-induced inflammation (Iadarola et al., 1988; Noguchi et al., 1989). Inflammation–induced changes in endogenous opioid peptide expression and release have also been reported at several supraspinal sites, including the periaqueductal gray (PAG) (Williams et al., 1995). Noxious stimulation-induced changes in opioid peptide expression are also paralleled by an increase in mRNA expression (Iadarola et al., 1988).

The periaqueductal gray (PAG), along with descending projections to the rostral ventromedial medulla (RVM) and the spinal cord dorsal horn, constitute a primary anatomical circuit involved in the descending modulation of pain and opioid analgesia. Here we report that site-specific administration of naloxone HCl into the ventrolateral PAG significantly reduced the persistent basal hypoalgesia associated with neonatal inflammation, suggesting that opioidergic systems are persistently and functionally altered in the PAG in response to neonatal inflammation. The PAG is rich in nerve terminals and fibers containing endogenous opioids (Reichling et al., 1988), and opioid receptors are localized throughout the rostral-caudal axis of the PAG, with the highest

level of expression in the mid to caudal ventrolateral PAG (Mansour et al., 1995b; Wang and Wessendorf, 2002). In the present study, we cannot exclude the involvement of additional loci including the RVM and spinal cord dorsal horn, as they are modulated by PAG activity and are also rich in opioid-containing nociceptive neurons (Mansour et al., 1995a; Mansour et al., 1995b; Budai and Fields, 1998). Indeed, changes in opioid tone induced by neontal injury are probably also evident in these regions.

Neonatal inflammation-induced anatomical alterations in endogenous opioidergic systems in the PAG

In the present study, a significant increase in beta-endorphin peptide expression was observed in the PAG of animals that were neonatally injured compared to controls. We also demonstrated that neonatal inflammation resulted in a significant increase in met- and leu-enkephalin immunoreactivity in the lateral/ventrolateral region of the mid and caudal PAG. Interestingly, a significantly greater increase in met-enkephalin was observed in neonatally injured females compared to injured males, and may provide the biological basis for the dimorphic response to neonatal injury observed in males and females. No significant sex difference in met-enkephalin immunoreactivity was present at baseline, indicating that sex differences in met-enkephalin were injury-induced. Although not investigated in the present study, endomorphins also play an important role in the modulation of descending controls, and possess partial agonist properties at mu-receptor sites in the PAG (Zadina et al., 1997; Horvath, 2000; Mizoguchi et al., 2001). Therefore, their contribution to alterations in mu-receptor mediated alterations in endogenous opioid tone cannot be ruled out.

It is notable that alterations in the two opioidergic systems were very well correlated across similar regions and levels of the PAG, thus suggesting that beta-endorphin and enkephalin systems are acting in parallel in response to neonatal inflammation and nociception. In addition to changes in opioid peptide expression, we also showed both immunohistochemically and autoradiographically a significant decrease in mu and delta opioid receptor expression in the PAG in neonatally injured animals compared to controls.

Thus, neonatal inflammatory insult induces a central up-regulation of endogenous opioid ligands paralleled by a compensatory down-regulation of cognate opioid receptors in the PAG. Similar findings have been reported in rodent models, where increased brain and spinal cord immunoreactive beta-endorphin, met-enkephalin and dynorphin is observed, as well as decreased opioid receptor binding following inflammatory insults in adult animals (Morris, 1993; Spetea et al., 2002) (Cesselin et al., 1980; Millan et al., 1986a; Millan et al., 1986b; Spetea et al., 2002).

Alternative mechanisms underlying the persistent hypoalgesia in animals subjected to neonatal inflammatory insult

Although the results of the present study strongly suggest that persistent alterations in baseline nociceptive thresholds associated with neonatal inflammatory insult are mediated by a central increase in endogenous opioid tone, additional mechanisms may also contribute to our observed results. Noxious neonatal experiences lead not only to decreased nociceptive sensitivity in adulthood, but also to significant alterations in the behavioral and neuroendocrine responses to stress (Bernardi et al., 1986; Anand et al., 1999; Bhutta et al., 2001; Schellinck et al., 2003; Sternberg and Ridgway, 2003;

Sternberg et al., 2005). Blunted emotionality, decreased anxiety, and reduced basal and stress induced plasma corticotropin releasing factor (CRF) and adrenocorticotropin hormone (ACTH) are displayed in adult rats following short-lasting, local inflammation experienced during the first week of life (Bhutta et al., 2001; Anseloni et al., 2005). Premature infants with extensive NICU care also exhibit low basal levels of stress hormones at 3 months of age compared to their full-term counterparts (Grunau et al., 2007). Recent studies have demonstrated increased serotonergic (5HT) receptor expression in the PAG following early life adverse experience (Anseloni et al., 2005). As stimulation of 5-HT receptors in the PAG produces reduced anxiety and global hypoalgesia in rodents (Jenck et al., 1990; Kishimoto et al., 2001; Griffiths and Lovick, 2002; Graeff, 2004), this midbrain region may be a critical site involved in the regulation of both anxiety and descending nociceptive inhibition (Bandler and Shipley, 1994; Behbehani, 1995).

In summary, alterations in the developing hypothalamic-pituitary-adrenal axis may also contribute to the long-term basal hypoalgesia observed following neonatal injury. Indeed, neonatal inflammatory insult may result in a generalized reduction in reactivity to aversive environmental stimuli due to parallel alterations in supraspinal nociceptive and stress modulatory circuits (Ren et al., 2004; Anseloni et al., 2005).

3.7 Chapter 3 Summary

Our findings clearly demonstrate that the profound elevation of nociceptive thresholds following neonatal inflammation reflects an experience-induced facilitation in

descending nociceptive pathways. The dynamic physiological and anatomical modification and modulation of opioidergic systems in the PAG is likely an adaptive response to early trauma. Clinically, alterations in descending inhibitory controls may have important implications, as the long-term impact of chronically elevated opioidergic tone in vulnerable preterm infants is currently unknown.

Chapter 3 Acknowledgements

This work was supported by National Institute of Health grants DA16272 and AR49555 awarded to Anne Z. Murphy, the Center for Behavioral Neuroscience (NSF: IBN 9876754), and the Georgia State University Brains and Behavior Program. Erica O. Famojure provided technical assistance for the immunohistochemical and autoradiography experiments for this manuscript.

3.7 Chapter 3 Figures

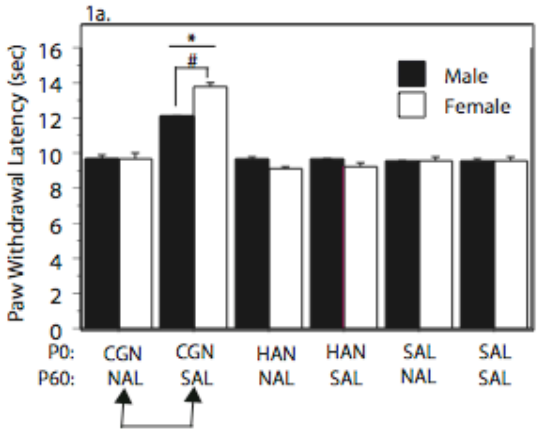
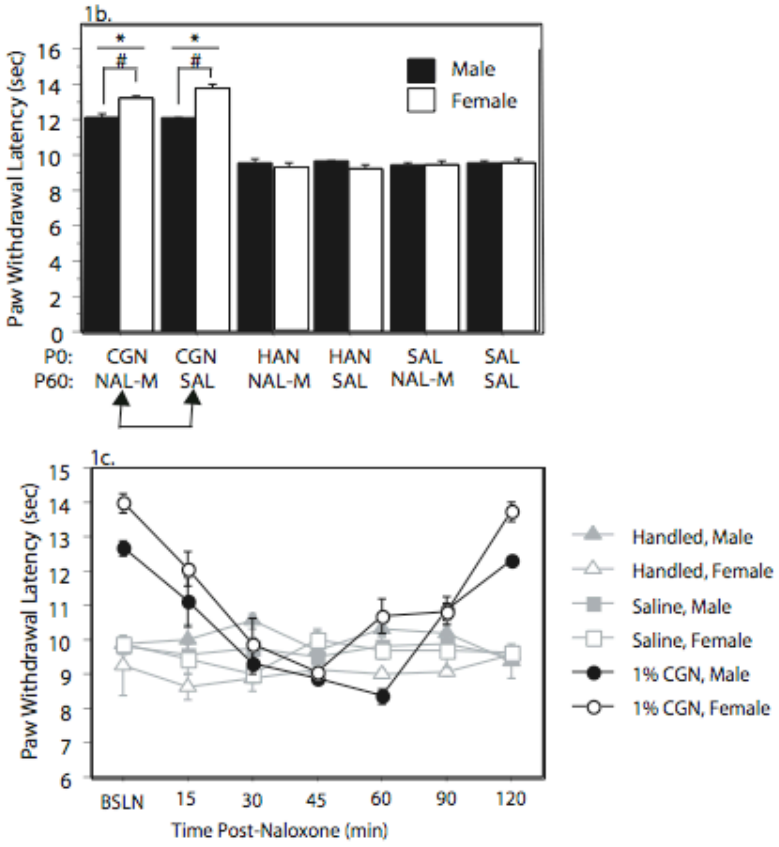


Figure 3.1. Systemic and ICV naloxone HCl significantly attenuated the hypoalgesia in neonatally injured animals, whereas naloxone methiodide had no effect. (A) Neonatally injured males and females that received naloxone (CGN/NAL) prior to testing at P60 had significantly lower PWLs than injured animals that received saline control (CGN/SAL) [$F(5,46)=32.56$, $p<.05$]. Administration of naloxone to saline (SAL/NAL) or handled (HAN/NAL) controls had no effect on PWLs. (B) Administration of naloxone methiodide to neonatally injured (CGN/NAL-M) males and females prior to testing at P60 had no significant effect on PWLs compared to injured animals that received saline control (CGN/SAL). A significant main effect of treatment was noted in that neonatally injured animals (CGN/NAL-M and CGN/SAL) displayed significantly higher PWLs compared to saline (SAL/NAL-M and SAL/SAL) and handled (HAN/NAL-M and HAN/SAL) controls [$F(5,35)=176.61$, $p<.05$]. (C) A significant effect of time post naloxone by neonatal treatment was noted following ICV administration of naloxone HCl, whereby neonatally injured males (●) and females (○) displayed significantly reduced PWLs during the 120 minutes post-naloxone compared to handled and saline controls [$F(12,132)=15.87$, $p<.05$]. Administration of naloxone to saline or handled controls had no effect on PWLs. N=6-8 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.



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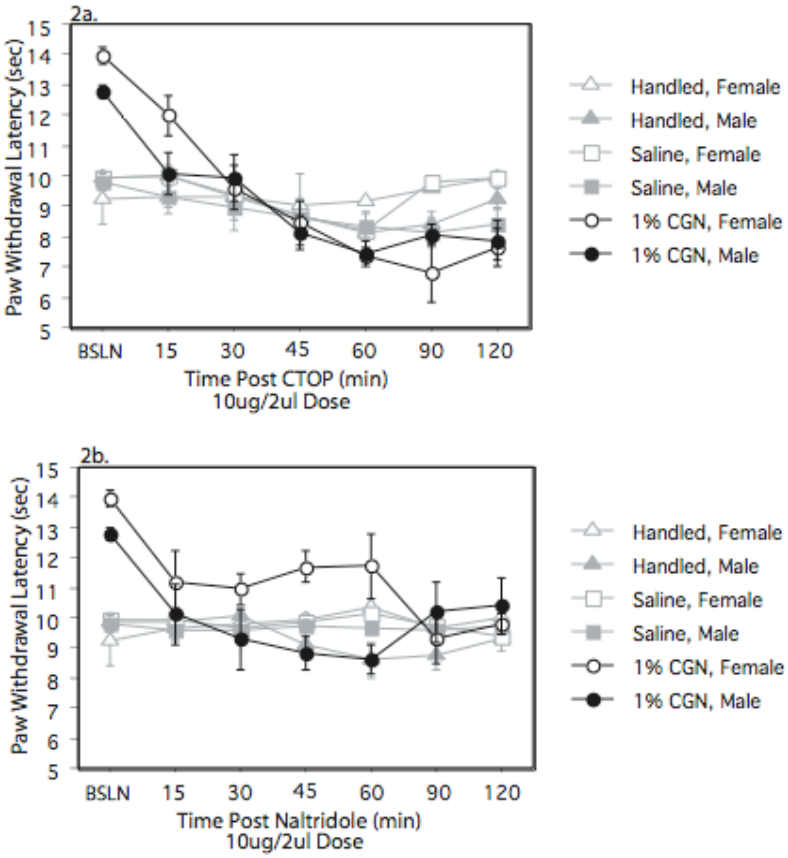
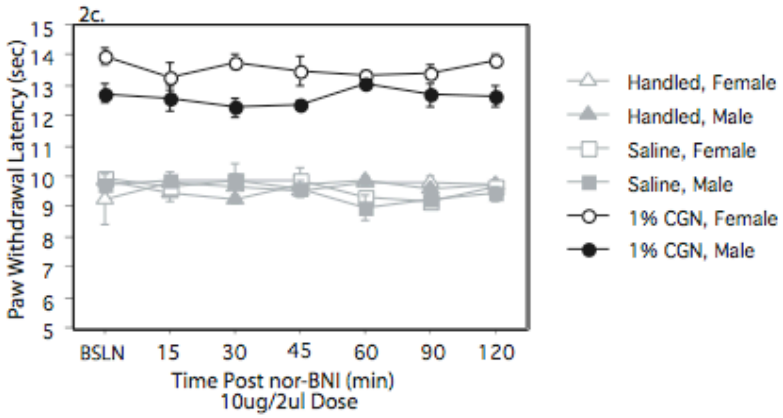


Figure 3.2. **Specific opioid antagonist ICV administration.** (A) A significant effect of time by neonatal treatment effect was observed following ICV administration of the mu receptor antagonist, CTOP, whereby neonatally injured males (●) and females (○) displayed significantly reduced PWLs during the 120-minute period compared handled and saline control animals [$F(12,108)=9.21$, $p<.05$]. Administration of CTOP to saline or handled controls had no effect on PWLs. (B) A significant effect of time post-naltrindole (delta antagonist) by neonatal treatment was indicated following ICV administration, whereby neonatally injured males (●) and females (○) displayed significantly reduced PWLs during the testing period compared to controls [$F(12,102)=.0009$, $p<.05$]. Administration of naltrindole to control animals had no effect on PWLs. (C) No significant effect of ICV administration of the kappa antagonist nor-BNI on PWLs was noted in neonatally injured animals or controls. A significant main effect of neonatal treatment was indicated, whereby neonatally injured animals displayed significantly elevated PWLs compared to saline and handled control animals [$F(2,14)=326.61$, $p<.05$). N=6-8 rats per group/per sex.



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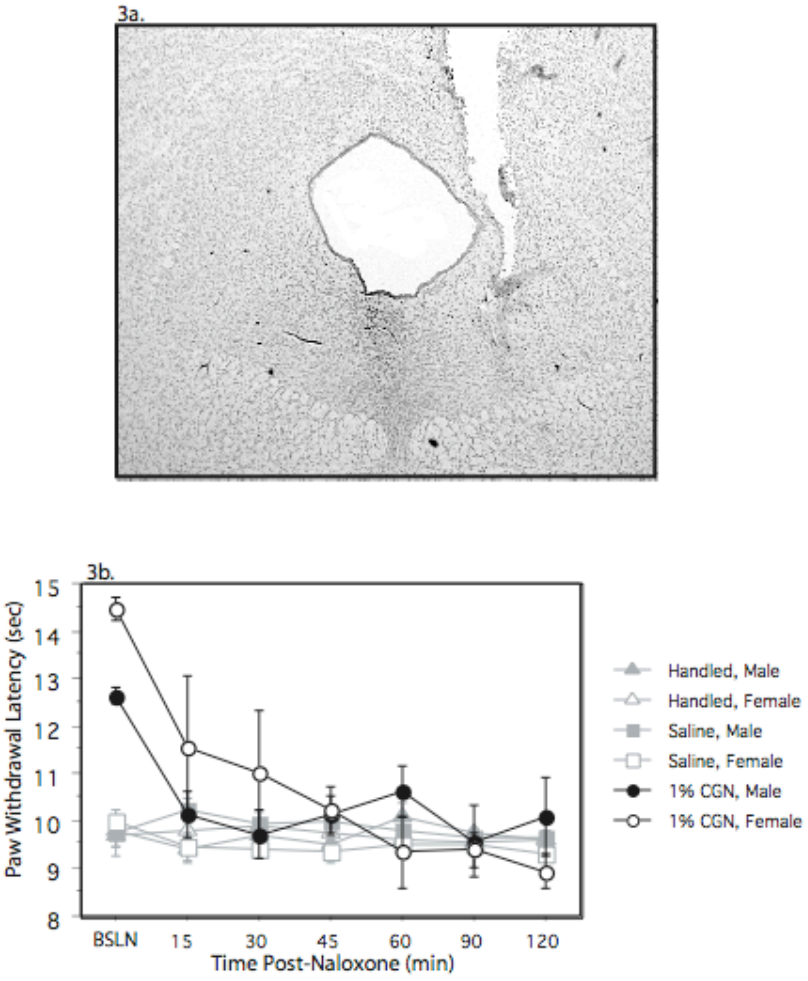


Figure 3.3. Intra-PAG naloxone significantly attenuated the persistent hypoalgesia following neonatal inflammatory insult. (A) A photomicrograph of a representative intra-PAG cannulation site in an adult rat (4X). (B) A significant effect of time post-naloxone by neonatal treatment was indicated following intra-PAG naloxone, whereby neonatally injured males (●) and females (○) displayed significantly reduced PWLs throughout the 120-minute testing period in comparison to saline and handled control animals [$F(12,144)=8.95$, $p<.05$]. Intra-PAG naloxone had no effect on PWLs in control animals. N=6-8 rats per group/per sex.

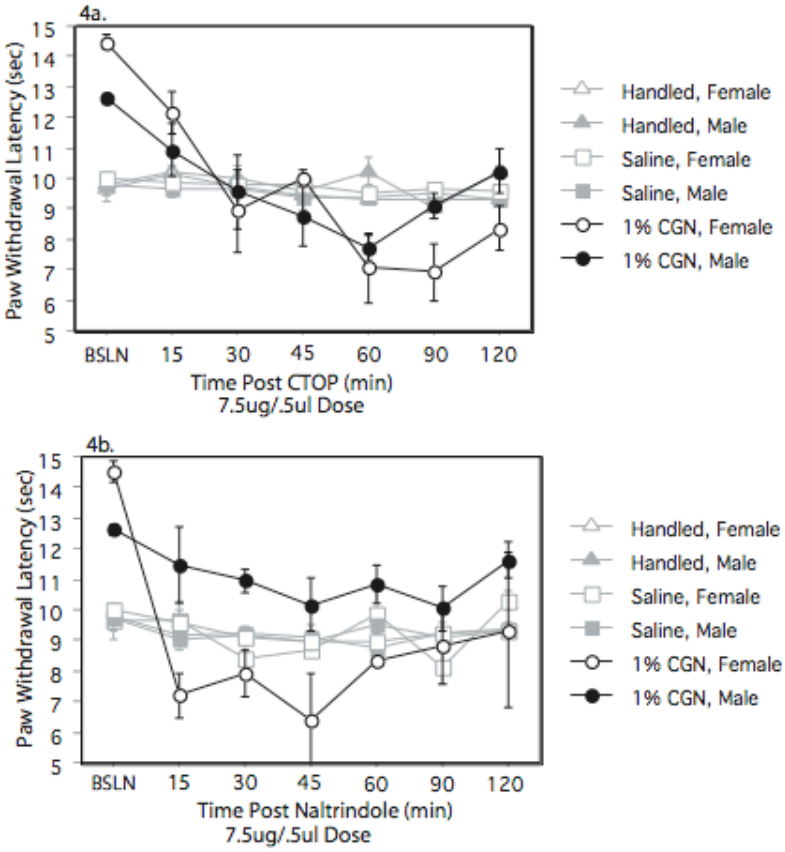
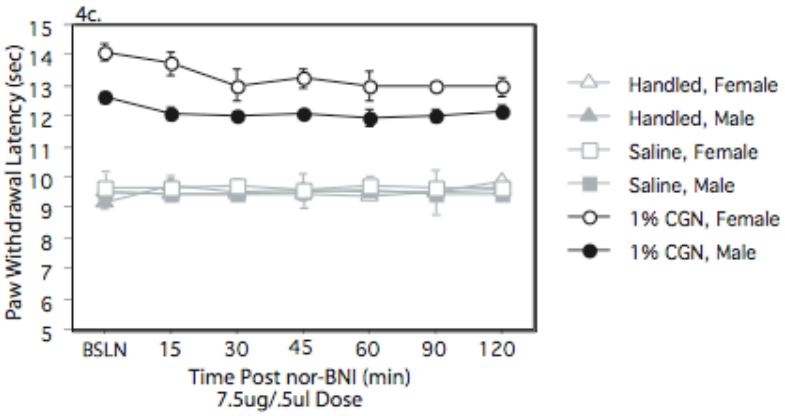


Figure 3.4. Specific opioid antagonist intra-PAG administration. (A) Intra-PAG CTOP administration resulted in a significant effect of time post-CTOP by neonatal treatment, whereby neonatally injured males (●) and females (○) displayed significantly reduced PWLs during the 120-minute testing period compared to handled and saline controls [$F(12,144)=13.593$, $p<.05$]. (B) Intra-PAG naltrindole produced a significant effect of time post-naltrindole by neonatal treatment by sex, whereby neonatally injured females (○) displayed significantly reduced PWLs during the 120-minute testing period in comparison to neonatally injured males and handled and saline controls [$F(12,144)=2.92$, $p<.05$]. N=6-8 rats per group/per sex. (C) Administration of nor-BNI into the PAG had no significant effect on PWLs in neonatally injured or control animals. A significant main effect of treatment was noted, whereby neonatally injured animals (males-●/ females-○) displayed significantly increased PWLs compared to controls [$F(2,42)=474.25$, $p<.05$]. Neonatally injured females also displayed significantly increased PWLs compared to injured males [$F(2,42)=13.65$, $p<.05$]. N=6-8 rats per group/per sex.



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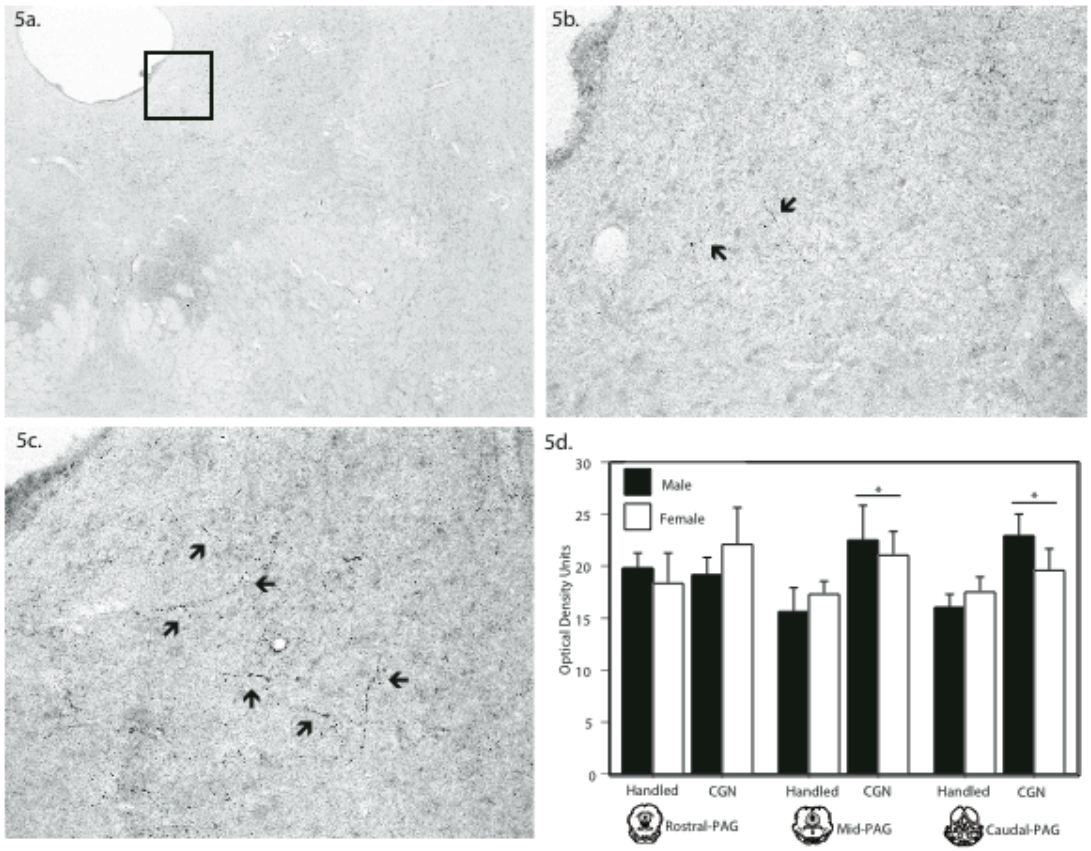


Figure 3.5. Neonatal injury results in a significant increase in beta-endorphin immunoreactivity in the PAG. Photomicrograph depicting representative examples of beta-endorphin-ir in the lateral/ventrolateral PAG in adult rats following neonatal treatment; (A) handled; (4X), (B) handled; (20X); (C) 1% carrageenan; (20X). (D) Neonatally injured (CGN) males and females display a significant increase in beta-endorphin immunoreactivity in the lateral/ventrolateral region of the PAG in the (D) mid level [$F(1,16)=4.62$, $p<.05$; and the (F) caudal level [$F(1,16)=6.57$, $p<.05$] compared to handled control animals. N=6-8 rats per group/per sex. * denotes significant main effect of treatment.

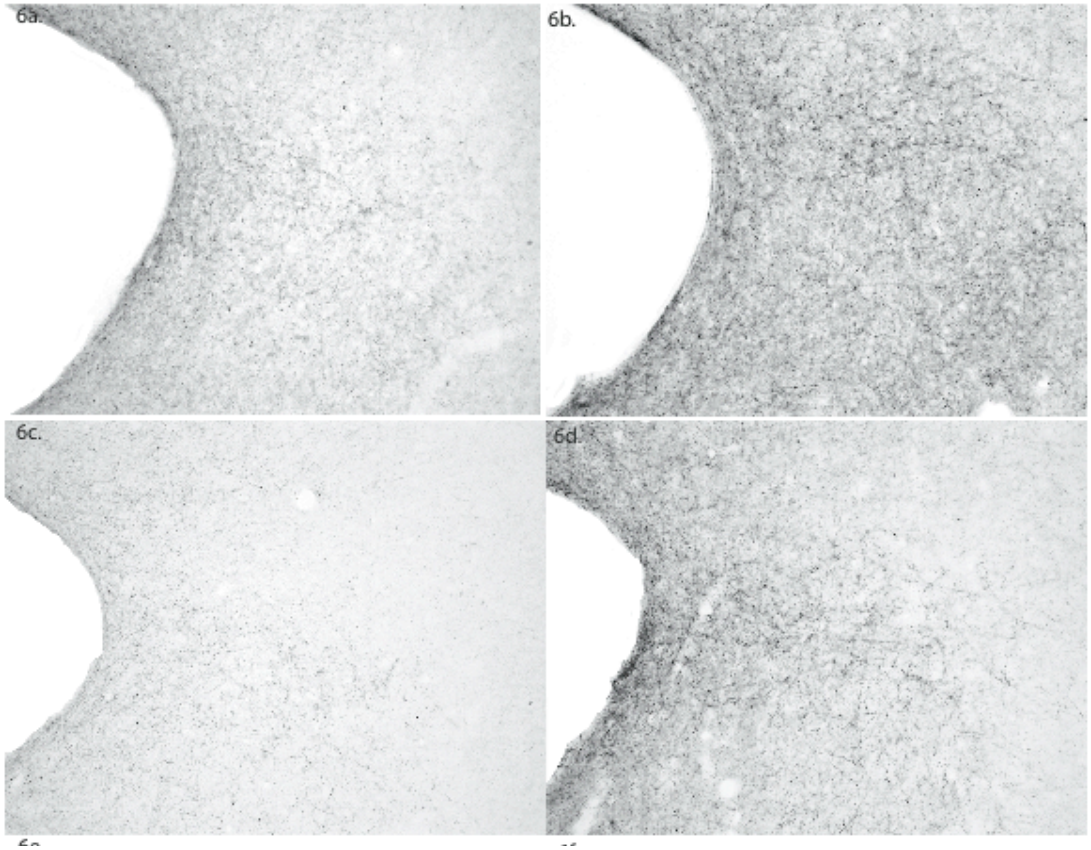
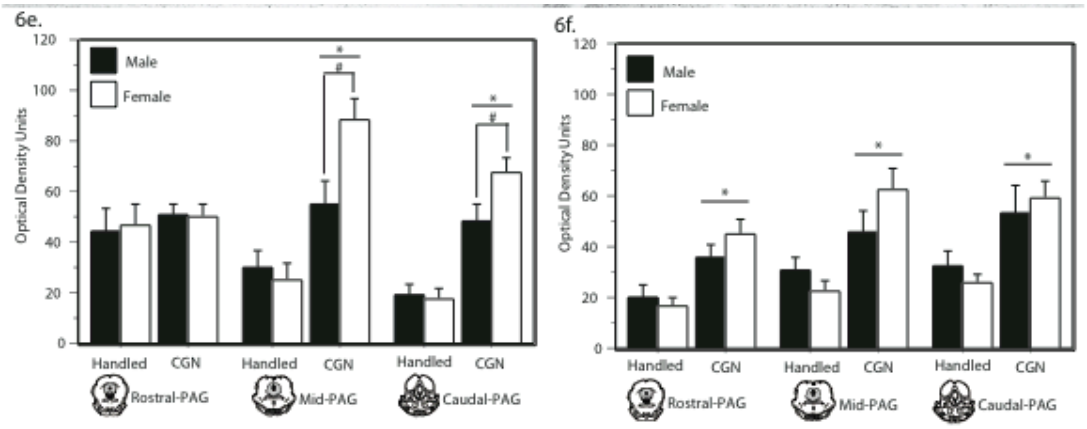


Figure 3.6. Neonatal injury results in a significant increase in met- and leu-enkephalin immunoreactivity in the PAG. Photomicrograph depicting representative examples of met-enkephalin-ir in the PAG in adult rats following neonatal treatment; (A) handled; (10X), (B) 1% carrageenan; (10X). Photomicrograph depicting representative examples of leu-enkephalin-ir in the PAG in adult rats following neonatal treatment; (C) handled; (10X), (D) 1% carrageenan; (10X). (E) Neonatally injured animals (CGN) display a significant increase in met-enkephalin immunoreactivity in the lateral/ventrolateral PAG in the mid [$F(1,20)=29.50$, $p<.05$] and caudal [$F(1,20)=50.88$, $p<.05$] in comparison to handled animals. Moreover, neonatally injured females displayed significantly increased met-enkephalin immunoreactivity compared to injured males in the mid [$F(1,20)=5.39$, $p<.05$] and caudal [$F(1,20)=4.72$, $p<.05$] levels of the PAG. (F) A significant increase in leu-enkephalin immunoreactivity was noted in CGN animals in comparison to handled controls in the rostral [$F(1,20)=18.46$, $p<.05$], mid [$F(1,20)=14.92$, $p<.05$], and caudal [$F(1,20)=13.23$, $p<.05$] levels of the PAG. N=6-8 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.



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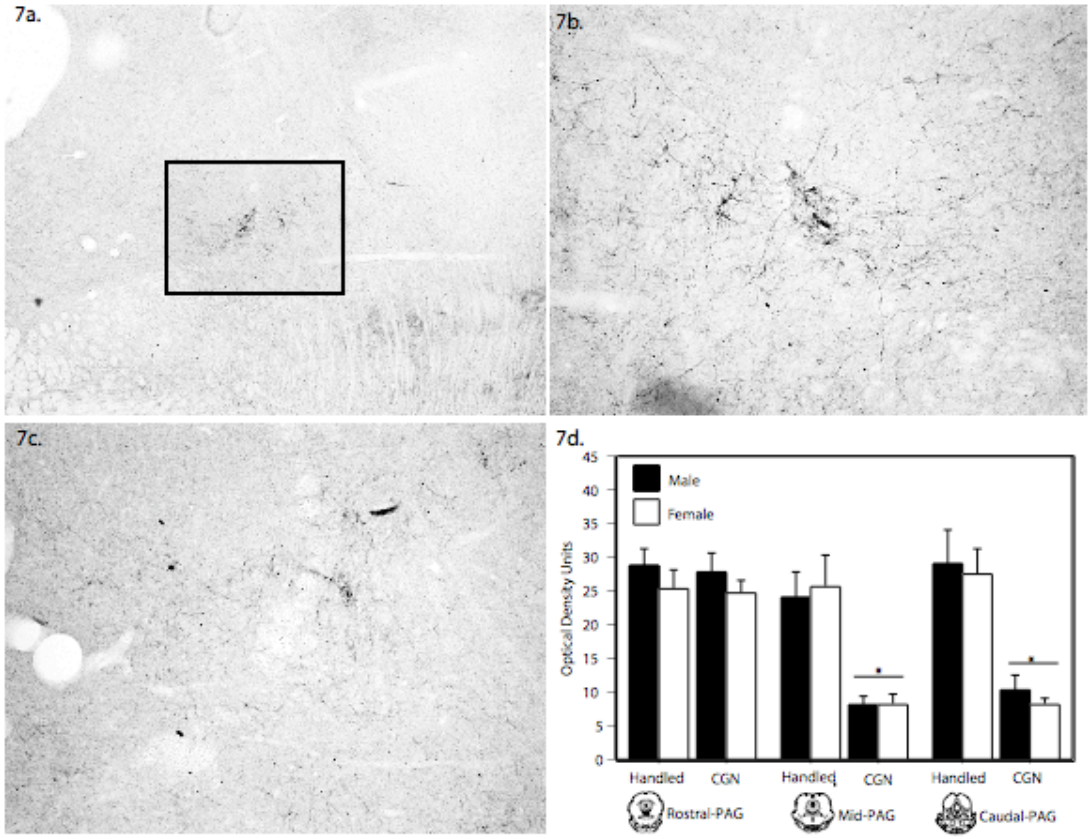

Figure 3.7. Neonatal injury results in decreased MOR-ir in the PAG. Photomicrograph depicting representative examples of MOR-ir in the lateral/ventrolateral PAG in adult rats following neonatal treatment; (A) handled; (4X), (B) handled; (10X), (C) 1% carrageenan; (10X). (D) A significant main effect of treatment on MOR-ir in the lateral/ventrolateral PAG was noted in the mid [$F(1,20)=26.34$, $p<.05$] and caudal [$F(1,20)=31.51$, $p<.05$] levels, where CGN animals displayed significantly reduced MOR-ir compared to handled animals. N=6-8 rats per group/per sex. * denotes significant main effect of treatment.

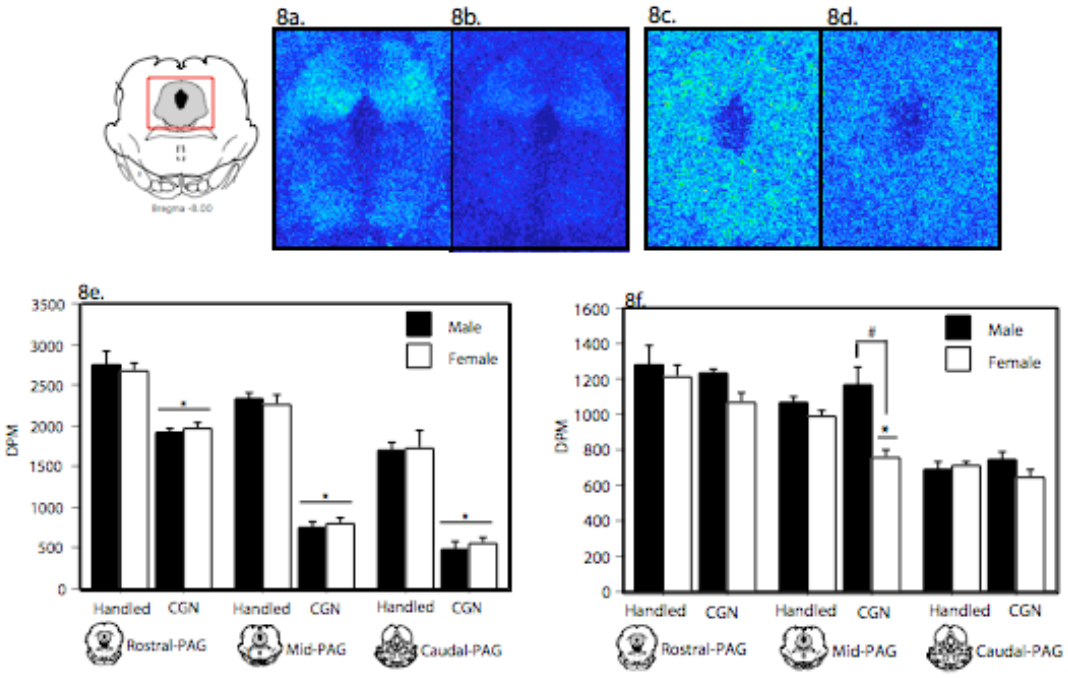

Figure 3.8. Neonatal inflammatory injury results in a significant decrease in mu and delta opioid receptor binding in the PAG. A representative example of mu opioid receptor binding distribution in the PAG in adult rats following neonatal treatment; (A) handled, (B)1% carrageenan. A representative example of delta opioid receptor binding distribution on the PAG in adult rats following neonatal treatment; (C) handled, (D) 1% carrageenan. (E) Neonatally injured (CGN) animals displayed a significant decrease in MOR-binding in the lateral/ventrolateral subdivision of the rostral [$F(1,20)=58.46$, $p<.05$], mid [$F(1,20)=286.50$, $p<.05$], and caudal [$F(1,20)=78.89$, $p<.05$] levels of the PAG compared to handled animals (F) A significant decrease in DOR-binding was noted in neonatally injured females in comparison to all other groups in the lateral/ventrolateral region of the mid-PAG [$F(1,20)=22.41$, $p<.05$]. A significant main effect of sex was also observed in this region [$F(1,20)=39.74$, $p<.05$]. N=6 rats per group/per sex. * denotes significant main effect of treatment; # denotes significant main effect of sex.

CHAPTER FOUR

Pre-Emptive Morphine Analgesia Attenuates the Long-Term Consequences of Neonatal Inflammation in Male and Female Rats

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4.1 Abstract

Despite mounting evidence on the importance of pain management in preterm infants, clinical use of analgesics in this population is limited. Our previous studies have shown that neonatal inflammatory injury results in long-term alterations in adult somatosensory thresholds, characterized by decreased baseline nociceptive sensitivity, and enhanced hyperalgesia following a subsequent inflammatory injury. The present studies were conducted to determine if pre-emptive morphine attenuates these negative consequences. At P0, pups received an injection of morphine sulfate (MOR) prior to an intraplantar injection of 1% carrageenan (CGN). Control pups received either saline (SAL) followed by intraplantar CGN, MOR followed by intraplantar SAL, or SAL followed by intraplantar SAL. Pre-emptive morphine significantly attenuated neonatal injury-induced hypoalgesia at P40 and P60. Similarly, morphine pre-treated animals displayed significantly less hyperalgesia and recovered faster from a subsequent injury compared to control animals. Neonatal morphine had no significant effect on morphine analgesia in adulthood. Interestingly, neonatally injured animals that did not receive morphine displayed a significant rightward shift in the morphine dose response curve in the absence of peripheral inflammation. Together, these results provide compelling justification for the use of analgesics prior to the initiation of noxious procedures performed on neonates.

4.2 Introduction

Advances in perinatal care have increased the survival of preterm infants in neonatal intensive care units (NICUs) worldwide. Despite the current knowledge that preterm infants are responsive to noxious stimuli (Giannakoulopoulos et al., 1999, Smith et al., 2000, Vanhatalo and van Nieuwenhuizen, 2000), many routine procedures in the NICU such as repeated heelsticks, circumcisions, endotracheal intubations, and minor surgeries are performed in the absence of analgesics (Johnston et al., 1997, Porter et al., 1997, Kahn et al., 1998, Simons et al., 2003). Indeed, recent studies have reported that neonates experience an average of 14 noxious procedures per day, with fewer than 35% receiving appropriate analgesic therapy (Anand and Craig, 1996, Simons et al., 2003).

Until the late 1980's, many in the medical community believed noxious stimuli had no lasting effects in neonates (Tibboel et al., 2005). It is now well established, however, that premature infants are indeed highly responsive to noxious stimulation (Andrews and Fitzgerald, 1999, Smith et al., 2000) and generate developmentally specific and distinct responses to noxious and non-noxious stimuli (Anand and Carr, 1989, Giannakoulopoulos et al., 1999). Nociceptive responses to noxious stimulation have been demonstrated in preterm neonates using an array of physiological, biochemical and behavioral measures (Franck et al., 2000, McNair et al., 2004). Cortical activation has also been reported in response to acute noxious stimulation in preterm neonates at 25 weeks, suggesting the potential for higher-level processing of pain (Bartocci et al., 2006, Slater et al., 2006a, Slater et al., 2006b).

Accumulating evidence in both human and non-human animal studies indicate that exposure to invasive procedures during the neonatal period leads to both short and long-term alterations in nociceptive processing (Grunau et al., 1994a, Anand et al., 1999, Porter et al., 1999, Anand, 2000b, Ruda et al., 2000, Lidow et al., 2001, Bhutta et al., 2002, LaPrairie and Murphy, 2007). For example, a higher frequency of painful invasive procedures in preterm infants has been significantly associated with dampened pain responses at 32 weeks of age compared to controls (Grunau et al., 2001). Decreased facial responsiveness to immunization at 4 and 8 months (Oberlander et al., 2000), and blunted pain sensitivity has also been reported in 18 month old former preterm neonates compared to full term peers (Grunau et al., 1994a).

Studies in animals have also reported that neonatal injury induces long-term alterations in basal somatic and visceral sensitivity (Lidow et al., 2001, Wang et al., 2004, LaPrairie and Murphy, 2007). In particular, animals that received an intraplantar injection of the inflammatory agent carrageenan on the day of birth display significant hypoalgesia in both the previously injured and uninjured paws when tested in adulthood (Lidow et al., 2001, LaPrairie and Murphy, 2007). Similar changes in nociceptive sensitivity have also been reported using repeated intraplantar 10% formalin injections (Bhutta et al., 2001). In addition, neonatal inflammatory injury results in enhanced hyperalgesia following a subsequent injury in adulthood and significantly decreases the rate of recovery (Lidow et al., 2001, LaPrairie and Murphy, 2007).

There is wide disagreement among neonatologists regarding the use of opioid analgesics in the treatment of neonatal pain, and adequate pain management in this population is hampered by a lack of knowledge regarding analgesic mechanisms in the immature nervous system. Accumulating data, however, suggests that premature infants undergoing invasive procedures benefit from the use of opioid analgesics (Yaster and Deshpande, 1988, Yaster et al., 1989, Fitzgerald, 1991, Anand and Hickey, 1992, Bhat et al., 1992). For example, altered nociceptive responses in former preterm neonates can be predicted by the number of previous noxious procedures and are normalized by the early use of morphine as an analgesic (Grunau et al., 2001). In addition, post-operative morphine analgesia in preterm and full-term infants reduces behavioral and hormonal stress responses (Farrington et al., 1993, Bouwmeester et al., 2001, Bouwmeester et al., 2003), and is associated with decreased mortality (Anand et al., 1987, Anand and Hickey, 1992).

There is significant evidence that opioid analgesics are efficacious in neonatal rodents (Abbott and Guy, 1995) Few studies, however, have examined whether opioid analgesics can be used to prevent the long-term consequences of neonatal injury (Sternberg et al., 2005). Therefore, the present studies were conducted to determine whether neonatal morphine pre-treatment 1) attenuates neonatal inflammatory injury-induced mechanical and thermal hypoalgesia in adulthood in male and female rats; 2) reduces neonatal re-injury induced hyperalgesia observed following adult inflammation and impacts the rate of recovery; and 3) alters the response to morphine in adulthood.

4.3 Materials and Methods

Animals

Time-pregnant Sprague-Dawley rats were obtained on the 14th day of gestation (E14) (Zivic Miller) and housed individually. Animals were maintained on a 12:12h light:dark cycle, with food and water available ad libitum. On the day of birth (P0), sexing of the pups was determined by examination of the anogenital distance. Offspring from the litters were combined on the day of birth, divided up equally, and randomly assigned to a neonatal treatment condition and dam. All litters were reared identically, weaned at P21, and housed with same-sex littermates in groups of 2-3. All experiments were approved by the Georgia State University Animal Care and Use Committee and were conducted in strict compliance with the guidelines for pain research established by the International Association for the Study of Pain (IASP).

Early Life Manipulations

On the day of birth (P0), male and female rat pups received an injection of morphine sulfate (MOR; 2 mg/kg, ip) or saline (SAL) fifteen minutes prior to a unilateral intraplantar injection of 1% carrageenan (CGN; 5ul) or SAL. This dose of morphine was chosen based on previous studies (Abbott and Guy, 1995, Nandi et al., 2004). In preliminary studies, we noted that paw inflammation peaked at five hours post-carrageenan. Therefore, at five hours post-CGN or SAL, a second dose of either MOR or SAL was administered. This resulted in a total of four groups: MOR+SAL, MOR+CGN, SAL+CGN, and SAL+SAL. All pups within a litter received the same neonatal treatment.

Maternal Behavior

Maternal behavior was observed for 1 hour following the neonatal manipulation on P0, and daily at 18:00 hours for 60 minutes from P0-P7. Maternal observations were conducted by both direct observations (in a manner so as not to disrupt the dam) and by videotape for later offline analysis. Specific maternal behaviors were recorded including pup licking/grooming, nursing posture (crouching), hovering over pups, pup retrieval, nest construction, eating/drinking, exploring, inactive/napping and self-grooming. Observations were conducted by an individual blind to the neonatal group assignment.

Baseline Nociceptive Behavior

On P40 and P60, baseline paw withdrawal (PWL) and tail flick (TFL) latencies in response to a noxious thermal and mechanical stimulus were determined. Thermal testing was conducted using the Paw Thermal Stimulator (UCSD, San Diego, California). In this test, animals were placed in a clear plastic testing chamber on a glass surface and allowed to acclimate for a minimum of 30 minutes prior to testing. A radiant beam of light beneath the glass base was directed at the plantar surface of the each hindpaw or 1.5 inches from the distal end of the tail, and the withdrawal latency was electronically measured (in seconds) (Hargreaves et al., 1988). Intact male and cycling female rats were tested separately. The testing apparatus was thoroughly cleaned between sessions. The average withdrawal latency of 3 trials was taken; all trials were separated by a 5-minute inter-trial interval. Application of the thermal stimulus to either paw or tail was randomly determined. To avoid potential tissue damage, a 20-second automatic termination of the heat stimulus was imposed if a paw or tail withdrawal did not occur. Mechanical testing was conducted using a Dynamic

Plantar Aesthesiometer (Ugo Basile, Italy). Animals were placed in Plexiglas chambers above a wire mesh, and allowed to acclimate for 30 minutes prior to testing. A metal probe was directed at the hind paw and the force applied electronically increased until paw withdrawal occurred. Both time and force were recorded, with an automatic cut off of 60 seconds and 50 grams, respectively. There was a 5-minute inter-trial interval, and the average time and force of 3 trials was taken. Body weight and paw diameter for right and left hind paws were measured prior to baseline testing on P40 and P60.

Nociceptive Behavior Following Re-Inflammation

Following baseline PWL determination at P60, animals received an injection of Complete Freund's adjuvant (CFA; 1:1 CFA:saline soln; 200ul; Sigma) into the plantar surface of the neonatally injured hind paw. CFA was used for re-injury as neonatally-injured animals may potentially develop antibodies against carrageenan (CGN), thereby limiting its potency for use as an inflammatory agent. Twenty-four hours following CFA-induced inflammation, paw diameter and PWLs were tested using the Paw Thermal Stimulator as described above.

Recovery Following Re-Inflammation

Paw diameter and paw withdrawal latencies (right and left paws) were determined in male and female Sprague-Dawley rats at 7, 14, and 21 days following CFA re-inflammation (P60) to determine the impact of morphine pre-treatment on the response to recovery from a subsequent insult in adulthood.

Response to Morphine in Adulthood Following Neonatal Morphine Exposure

The effect of neonatal morphine on adult morphine responses was assessed using a cumulative dosing paradigm. Animals were tested in the absence of inflammation. Following baseline PWL determination in response to a noxious thermal stimulus, animals were administered cumulative doses of morphine (1.8, 1.4, 2.4, 2.4, 2.0, and 8.0 mg/kg s.c. injections resulting in quarter log doses of 1.8, 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg; Sigma, USA) (Morgan et al., 2006). Right PWLs were determined using the paw thermal stimulator 15 minutes after each injection, followed by the administration of the subsequent injection 5 minutes later.

Mu Opioid Receptor Western Blot Analysis

Western blot analysis was conducted to confirm that mu opioid receptors are present supraspinally, spinally, and peripherally in male and female rat pups on the day of birth (P0). Five hours after birth on P0, rat pups were rapidly decapitated and brain, spinal cord, and right and left paw tissue was removed and collected on dry ice. Dams were also rapidly decapitated and maternal blood samples were taken. All tissue and blood was stored at -80 degrees until processing. Tissue samples were homogenized in Homogenization Buffer (50mM HEPES pH 7.4, 1mM EDTA, and 0.001% Protease Inhibitor Cocktail; Sigma USA) on ice. The samples then underwent freeze/thawing three times on dry ice and 37°C waterbath, respectively, and were subsequently stored at -80°C. Sample protein concentrations were determined with a BCA Protein Assay Kit (Pierce) against a BSA standard curve. Next, 20μg of each sample was run on 12% acrylamide gels, and transferred to PVDF membranes (Bio-Rad). PVDF membranes were blocked in 5% nonfat dry milk in TTBS (0.1% Tween in 20 mM Tris-HCl; pH 7.5,

175 mM NaCl) overnight at 4°C. PVDF membranes were washed 3 times with TTBS, 10 min each, then incubated with rabbit anti-MOR (1:50,000; Sigma) and rabbit anti-GAPDH (1:300,000; Covance) in 2% nonfat dry milk in TTBS overnight at 4°C. Membranes were then washed 3 times with TTBS, 10 min each, and incubated with anti-rabbit HRP conjugated antibodies (1:3000; Cell Signaling) for 1 h at room temperature. Membranes were then subsequently washed with TTBS 3 times, 10 min each, and washed in TBS (20 mM Tris-HCl pH 7.5, 175 mM NaCl) for 30 min. Bands were visualized via chemiluminescence using LumiGLO (KPL). To confirm that the mu opioid receptor protein observed in the tissue samples was not the result of mu receptors located on maternal immune cells, western blot analysis was also conducted on maternal blood samples. Both whole blood and serum were processed as described for neonatal tissue.

Statistical Analysis

Data are expressed as either raw withdrawal latencies or mean difference scores. All values are reported as Mean $\pm$ S.E.M. Data were analyzed for significant main effects of neonatal treatment and sex using ANOVA and repeated-measures ANOVA was used to analyze recovery post-CFA data; $p < 0.05$ was considered statistically significant. A priori specified post-hoc tests were conducted using the method of Sheffe as warranted to determine significant mean differences. Where multiple comparisons were made, p values were adjusted accordingly using the Bonferroni method. Morphine dose-response curves were plotted and the half maximal effective doses (ED50) were calculated using percent maximal possible effect (%MPE) data, defined as [(Dose

mg/kg-baseline PWL)/(20.0-baseline PWL)]100 (GraphPad, Prism). Analysis of variance was used to compare differences between ED50 values.

4.4 Results

Morphine Pre-Treatment Attenuates the Neonatal Injury-Induced Hypoalgesia in Adulthood

The present studies were conducted to determine whether neonatal morphine attenuated the long-term behavioral consequences associated with neonatal inflammatory injury. Pre-emptive morphine administration significantly attenuated both thermal (Figure 1) and mechanical (Figure 2) hypoalgesia induced by neonatal inflammation at P60. Animals that received SAL+CGN displayed significant thermal hypoalgesia in both the injured and uninjured paws, with females displaying PWLs of approximately 14 seconds and males 11.5 seconds ($p<.05$ treatment; $p<.05$ sex), versus PWLs of 10 seconds in SAL+SAL animals. In contrast, morphine pre-treated animals (MOR+CGN) were not significantly different from uninjured controls ($p<.05$). Consistent with our previous study (LaPrairie and Murphy, 2007), a significant main effect of sex on injury-induced thermal hypoalgesia was noted (Figure 1A and 1B). Morphine pre-treatment also reduced the injury-induced hypoalgesia present in the tail at P60 (Figure 1C) ($P<.05$). Furthermore, a significant reduction in the mechanical force threshold was present in morphine pre-treated animals in both paws in adulthood (P60) (Figure 2A and 2B) ($p<.05$). Surprisingly, no sex difference was noted in response to

noxious mechanical stimulation. Morphine reversal of the injury-induced hypoalgesia was also observed at P40 (data not shown).

Neonatal Morphine Alters the Response to Re-injury and Recovery

Our previous studies have shown that neonatal injury significantly exacerbates thermal hyperalgesia following re-inflammation with Complete Freund's adjuvant (CFA) in adulthood (LaPrairie and Murphy, 2007). Therefore, the next series of experiments were conducted to test whether neonatal morphine pre-treatment would attenuate the response to re-injury and recovery in adult animals. As shown in Figure 3A, neonatal morphine administration significantly attenuated CFA-induced thermal hyperalgesia at 24 hours compared to control animals ($p<.05$). At 24 hours post-CFA, PWLs for SAL+CGN animals were approximately 2 seconds in females (mean difference score, MDS, of 12 seconds), and approximately 3.5 seconds in males (MDS 8.5 seconds). Neonatally injured females displayed significantly enhanced thermal hyperalgesia (i.e. greater MDS) at 24 hours following intraplantar CFA compared to males as previously reported ($p<.05$; Figure 3A) (LaPrairie and Murphy, 2007). The rate of recovery was also significantly reduced in morphine pre-treated compared to control animals (Figure 3B). SAL+CGN animals continued to show signs of hyperalgesia at 14 days ($p<.05$; MDS of 1 second in males and 3 seconds in females). In contrast, MS+CGN animals had completely recovered at this time point (MDS of 0), similar to control animals ($p<.05$). Furthermore, a significantly decreased rate of recovery was noted in injured females compared to injured males; where females displayed greater MDS at 7 and 14 days post-CFA ($p<.05$).

Neonatal Morphine Does Not Affect Morphine Analgesia in Adulthood

Exposure to repetitive morphine in neonatal rat pups has been previously associated with increased TFLs and increased ethanol preference (Bhutta et al., 2001), as well as down-regulation of the mu opioid receptor in neonatal rat brain (Tempel, 1991). Therefore, the present study was conducted to determine whether morphine pre-treatment prior to intraplantar carrageenan on the day of birth alters the response to morphine in adulthood. Using a cumulative dosing paradigm, neonatal morphine administration did not significantly alter morphine's antinociceptive effects in adulthood in males or females (Figures 4A and 4B). As summarized in Table 1, morphine pre-treated females (MOR+CGN) displayed similar ED50 values as morphine (MOR+SAL) and saline (SAL+SAL) control females. Interestingly, a significant rightward shift in ED50 was noted in neonatally injured females (SAL+CGN) that did not receive morphine ($p<.05$). Similarly, neonatal morphine in injured males did not alter adult morphine responses (Figure 4B). Nevertheless, neonatally injured males that received pre-emptive saline (SAL+CGN) also displayed a significant rightward shift in ED50 compared to MOR+CGN and control males. Finally, a significant main effect of sex was noted, in that females had significantly higher ED50 values in comparison to males ($p<.05$; Table 1), as previously reported (Wang et al., 2006).

Mu Opioid Receptors are Present Peripherally, Spinally, and Supraspinally in Neonatal Rat Pups

The analgesic effects of morphine are mediated primarily by binding to mu opioid receptors. Previous studies have reported that mu opioid receptor binding is present in the spinal cord on P1 (Marsh et al., 1997, Fitzgerald and Beggs, 2001), and that

systemic morphine does indeed produce analgesia in response to an intraplantar injection of formalin from postnatal days one through twenty (McLaughlin et al., 1990, Abbott and Guy, 1995). The presence of brain and peripheral mu opioid receptors, however, has not been reported for P0 rat pups. Therefore, western blot analysis was conducted to determine whether mu opioid receptors (MOR) are present centrally and peripherally on the day of birth. As shown in Figure 5, MOR-like immunoreactivity (LI) is present on P0 in the brain, spinal cord, and right hindpaw tissue. To control for the presence of MOR on maternal immune cells, we assessed the presence of MOR-LI on maternal blood samples taken at 5 hours post parturition. MOR-LI was undetectable in maternal whole blood or serum (data not shown).

Neonatal Injury Has No Impact on Maternal Care

Previous studies have shown that naturally occurring variations in maternal care can induce profound and permanent changes in nociceptive responsiveness of offspring (Johnston and Walker, 2003). Therefore, daily maternal observations were conducted to determine whether neonatal morphine pre-treatment alters the display of maternal behavior. There were no significant differences in maternal behavior between morphine pre-treated and saline pre-treated control animals in the amount of time the dam spent on/with pups (Figure 6). Similarly, no differences were noted in the amount of time the dam spent off/without pups or in the amount of time spent licking/grooming pups (Figure 6). This indicates that morphine attenuation of the long-term consequences of neonatal injury are not due to differences in maternal behavior directed at pharmacologically-treated versus saline-treated pups.

4.5 Discussion

Our principal findings are as follows: (1) morphine pre-treatment attenuates neonatal injury-induced hypoalgesia in adolescent and adult male and female rats; (2) neonatal morphine reduces inflammatory hyperalgesia following a subsequent inflammation in adulthood; (3) neonatal morphine administration increases the rate of recovery following intraplantar CFA; (4) neonatal morphine does not affect morphine analgesia in adulthood; (5) neonatal injury significantly reduces morphine potency in adulthood.

Pre-emptive Morphine Analgesia Attenuates Long-Term Behavioral Consequences of Neonatal Inflammatory Injury

The present studies are the first to demonstrate that pre-emptive morphine attenuates the long-term behavioral consequences associated with neonatal intraplantar carrageenan in rodents. Morphine administration blocked neonatal injury induced thermal and mechanical hypoalgesia in both the injured and uninjured paws in adolescence (P40) and adulthood (P60) in males and females. These results are consistent with previous studies in rodents that report daily morphine administration prior to intraplantar formalin during the first week of life significantly reduces the long-term effects of repetitive pain (Abbott and Guy, 1995). Similarly, previous studies in humans have reported that morphine therapy ameliorates the effects of early repetitive noxious stimuli in extremely low birth weight infants at 4 months of age (Grunau et al., 2001). In addition, children who had minor neonatal operations and received pre-emptive analgesia responded to immunization pain in a similar manner as non-operated age-matched controls (Peters et al., 2003).

There is ample evidence that opioid receptors and endogenous opioid peptides appear early in the development of the CNS, and that the endogenous opioid system is able to contribute to analgesia during the neonatal period (Anand and Hickey, 1987, Marsh et al., 1997, Fitzgerald, 2005). Opioids produce potent analgesia by acting directly on opioid receptors (Anand et al., 2005). The analgesic effects of morphine, in particular, occur by activation of mu opioid receptors (MOR), which we have shown are present both centrally and peripherally on P0. In the present study morphine was given systemically and therefore may be acting on central or peripheral MOR to inhibit neonatal inflammatory pain.

There is ample evidence that the hypoalgesia observed following neonatal inflammation may be mediated by a potentiation in descending endogenous opioid tone. Studies have shown that the functional activity of endogenous opioid systems is enhanced following noxious inflammatory stimulation (Iadarola et al., 1988, Noguchi et al., 1989). Furthermore, increased endogenous opioid peptide expression and release in the periaqueductal gray (PAG) following peripheral inflammation has been reported, and is associated with decreased nociceptive sensitivity (Williams et al., 1995a). Moreover, we have previously reported that systemic administration of the opioid antagonist naloxone HCl results in decreased baseline hypoalgesia in neonatally injured animals (LaPrairie and Murphy, 2007), suggesting that alterations in opioid tone underlie the observed hypoalgesia (LaPrairie and Murphy, 2007). Our recent studies suggest that neonatal injury-induced alterations in opioid tone primarily involve mu and delta opioid receptors located in the periaqueductal gray (LaPrairie J 2007 SFN poster). These results are consistent with findings in both former pre-term infants (Buskila et al., 2003, Hermann et

al., 2006, Goffaux et al., 2008) and rodents treated neonatally with capsaicin (Cervero and Plenderleith, 1985) that early life trauma may impair the maturation and proper development of endogenous inhibitory systems in the CNS. In the present study, the ability of pre-emptive morphine to block the hypoalgesia may indeed occur through direct modulation of primary afferent drive into the spinal cord, thereby inhibiting the central relay of inflammatory pain and preventing the subsequent increase in descending endogenous opioid tone.

We cannot rule out alterations in the HPA axis as a contributing factor to the observed hypoalgesia. Nociception is one element in a broader context of stress reactivity (Sternberg and Ridgway, 2003, Grunau et al., 2005), and experimental studies have shown that exposure to early life stressors can permanently increase nociceptive thresholds and decrease the behavioral and physiological responses to stress in adult rodents (Pieretti et al., 1991, Coutinho et al., 2002, Sternberg and Ridgway, 2003). Data from human preterm infants also suggest that neonatal exposure to noxious stimuli may alter future pain responses. Ex-preterm infants exposed to four weeks of NICU care display reduced pain responsiveness following heel lance (Johnston and Stevens, 1996). In addition, pre-term birth is associated with increased salivary cortisol in response to vaccination at 6 months of age (Peters et al., 2005). Therefore, it is possible that the effects of morphine on neonatal noxious stimulation in our study are partly a consequence of modification of the stress response associated with neonatal inflammation (Stein et al., 1989, Adamson et al., 1991).

Neonatal Morphine Reduces Inflammatory Hyperalgesia And Increases The Rate Of Recovery Following Re-injury In Adulthood

In the present study, morphine pre-treatment significantly attenuated CFA- induced hyperalgesia and increased the rate of recovery, such that both males and females recovered 7 days faster than non- pre-treated animals. Interestingly, preliminary anatomical studies in our laboratory suggest that neonatal inflammation results in increased primary afferent innervation of the dorsal horn reflected by increased expression of CGRP and substance P immunoreactivity. (LaPrairie JL, Murphy AZ, SfN abstract, 2005), which may account for our observed hyperalgesia. Our working hypothesis is that re-inflammation in adulthood results in an enhanced dorsal horn release of CGRP and/or substance P due to increased primary afferent input. Increased release of these pro-nociceptive peptides would be predicted to result in an enhanced hyperalgesic response.

Clinical reports demonstrate that at 32 weeks, preterm infants experience a reduced rate of recovery to skin breaking procedures (Morison et al., 2003), and exhibit subtle differences in ability to recover from finger lance at 4 months compared to full term controls (Oberlander et al., 2000). There are no reports on the impact of pre-emptive morphine on recovery rates in premature neonates, however, our data suggest that morphine analgesia may in fact significantly increase the rate of recovery following procedural pain in NICU infants. Again, neonatal pre-treatment with morphine may inhibit injury-induced alterations in the descending nociceptive circuit, thereby blocking the susceptibility of a subsequent injury to produce enhanced hyperalgesia and increasing the rate of recovery.

Neonatal Morphine Does Not Affect Morphine Analgesia In Adulthood

Our original hypothesis was that exposure to morphine during neonatal development may alter opioid receptor number and/or affinity, thereby altering morphine's antinociceptive effects in adulthood. This hypothesis was based on previous studies that report a 60-fold shift in the morphine dose response curve in adult rats that received repeated neonatal morphine in the absence of pain (Rahman et al., 1998, Bhutta et al., 2001), and opioid receptor down regulation in the brains of neonatal rats chronically exposed to morphine (Tempel, 1991). Nevertheless, we show that neonatal morphine did not affect morphine analgesia in adult animals (i.e. no significant shift in ED50 values). In contrast to the previous reports, however, neonatal morphine administration was associated with peripheral inflammation and was only provided on P0 in the present study.

Interestingly, neonatal injury alone resulted in a significant decrease in morphine potency in both males and females in adulthood. These results have serious clinical implications. Previous studies have reported that pre-term infants that experience surgery during the first three months of life have significantly higher peri- and post-operative analgesic requirements in response to surgery in the same or different dermatome compared to control infants (Bouwmeester et al., 2003, Peters et al., 2005). Similarly, mice exposed to chronic noxious stimulation display increased tail flick latencies compared to control animals, and a significant two-fold increase in the ED50 of morphine in response to abdominal constriction (Christie et al., 1982). As noxious stimulation during the neonatal period leads to increased activation of opioid systems in a manner analogous to the repeated application of exogenous opiates, these studies

provide evidence that neonatal injury produces cross-tolerance to the analgesic effects of morphine thereby decreasing the subsequent effectiveness of morphine (Lett et al., 2002, Stoller et al., 2002, Stoller et al., 2007). Again interestingly, exposure to *morphine* neonatally did not result in a significant shift in ED50 values. Therefore, we believe that opioid cross tolerance is associated with neonatal injury-induced chronic exposure to endogenous opioids resulting from a potentiation of the descending inhibitory circuit, and not a result of exposure to morphine on P0. Alternatively, neonatal stress associated with maternal separation and repeated handling is also associated with reduced opioid analgesia (Coutinho et al., 2002, Sternberg and Ridgway, 2003). This suggests that alterations in opioid analgesia may reflect a combined effect of neonatal nociceptive experience as well as early life stress, and therefore may involve altered responsiveness of endogenous analgesia circuits and the hypothalamic-pituitary-adrenal axis (Grunau et al., 2005).

4.6 Chapter 4 Summary

It is well established that the physiological and neurobehavioral systems of preterm infants are unstable and immature, rendering them more vulnerable to the effects of invasive procedures. Here we show that pre-emptive morphine ameliorates long-term effects of neonatal inflammatory injury on adult nociception. Together, our data have important implications for the analgesic treatment of human infants exposed to invasive neonatal intensive care.

Chapter 4 Acknowledgments

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4.7 Chapter 4 Figures

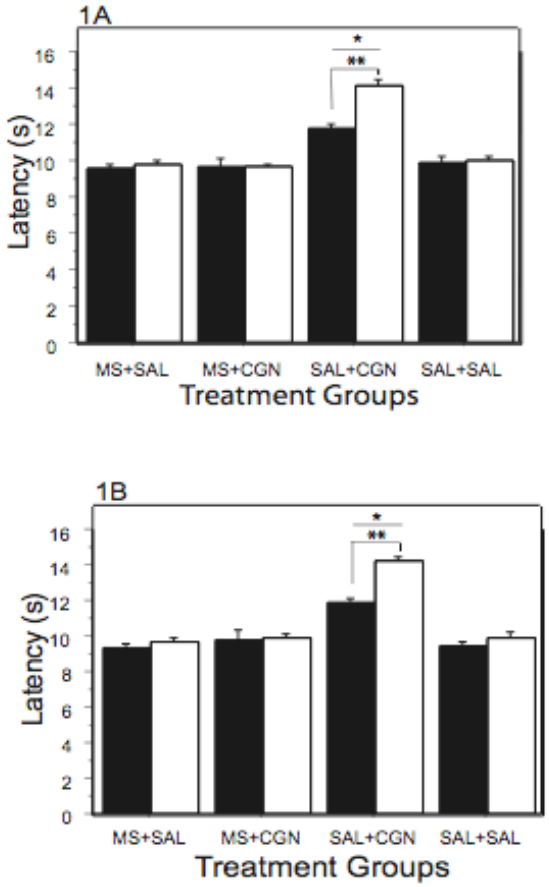


Figure 4.1. Morphine attenuates thermal hypoalgesia in adulthood. MS+CGN animals displayed significantly shorter PWLs compared to SAL+CGN in response to thermal stimulation applied to the (A) uninjured paw [$F(3,73)=61.330, p<.0001$]; (B) injured paw [$F(3,73)=80.066, p<.0001$]. (C) MS+CGN animals displayed significantly reduced TFLs compared to SAL+CGN animals [$F(3,73)=5.879, p=.0012$]. Neonatally injured females displayed significantly longer PWLs compared to males; uninjured paw [$F(1,73)=8.912, p=.0039$] and injured paw [$F(1,73)=16.914, p<.0001$]. N=5-15 rats per group/per sex; * significant main effect of treatment; ** significant main effect of sex; male: ■; female: □.

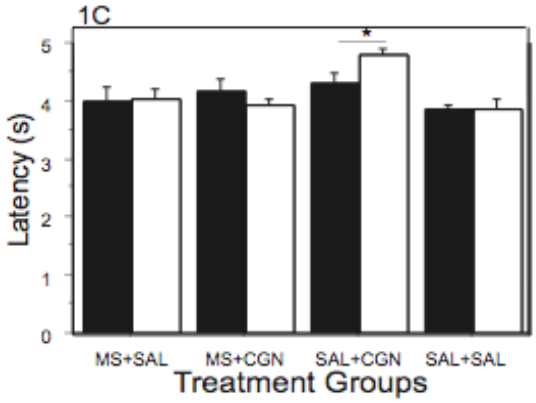
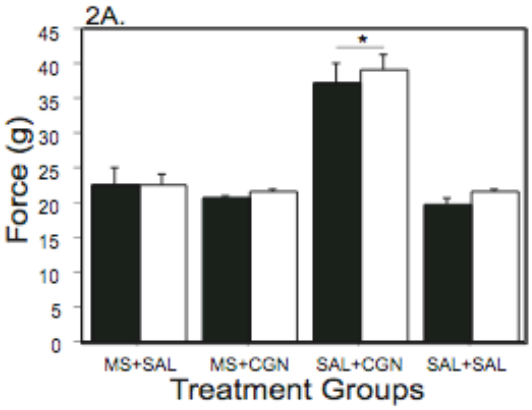


Figure 4.1. Continued



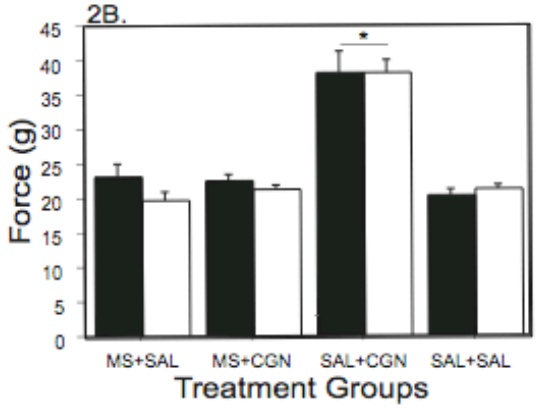


Figure 4.2. Morphine attenuates mechanical hypoalgesia in adulthood. MS+CGN displayed significantly reduced force (g) to withdrawal compared to SAL+CGN in response to mechanical stimulation applied to the (A) uninjured paw [$F(3,40)=50.604, p<.0001$] ; (B) injured paw [$F(3,40)=56.898, p<.0001$]. N=5-15 rats per group/per sex; * significant main effect of treatment; male: ■; female: □.

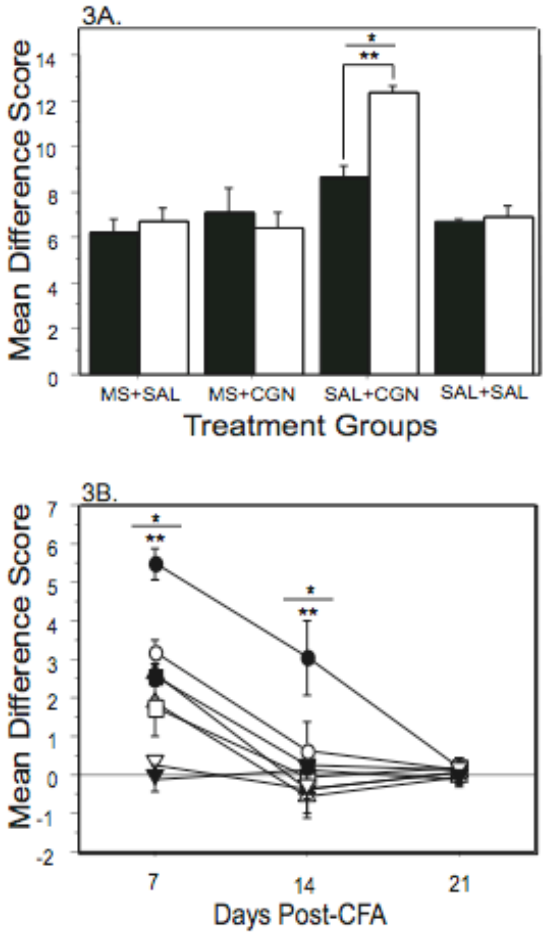


Figure 4.3. Morphine attenuates the hyperalgesia following re-inflammation and increases the rate of recovery. MS+CGN animals displayed significantly lower mean difference scores (MDS) (i.e. reduced hyperalgesia) compared to SAL+CGN animals (A) 24 hours post-CFA [$F(3,36)=22.524, p<.0001$]; male: ■; female: □. (B) At 7, 14 and 21 days post-CFA, SAL+CGN animals displayed a significantly higher MDS (i.e. reduced rate of recovery) compared to controls [$F(6,76)=10.644, p<.0001$]. Females displayed significantly higher MDS (i.e. recovered slower) compared to males [$F(2,76)=3.240, p<.05$]. N=5-7 rats per group/per sex; * significant main effect of treatment; ** significant main effect of sex; male: ■; female: □ (MS+SAL-Δ; MS+CGN-□; SAL+CGN-○; SAL+SAL-∇).

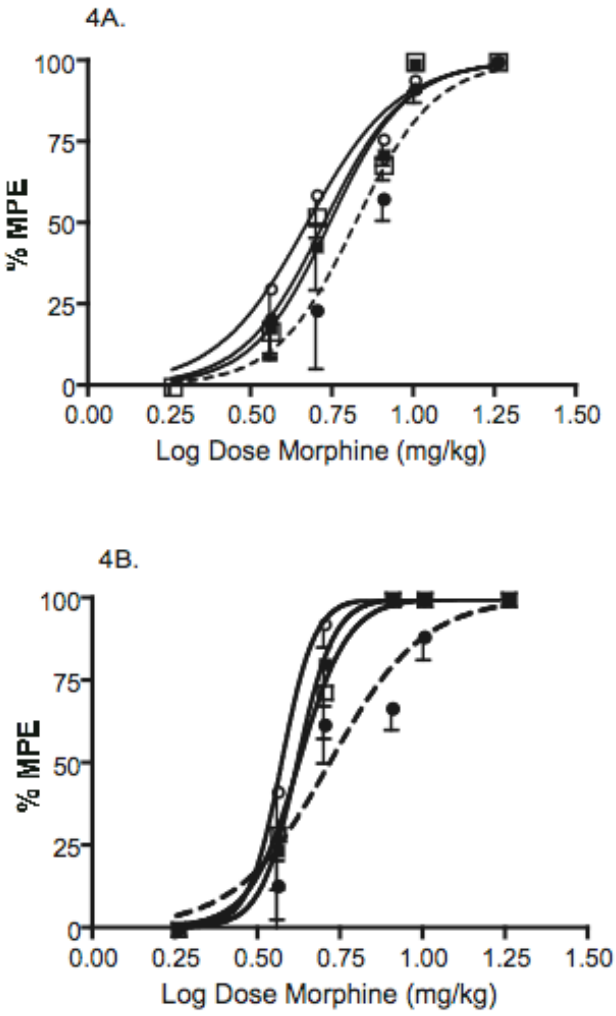


Figure 4.4. Neonatal morphine has no effect on morphine analgesia. Neonatal morphine had no effect on morphine potency in (A) females; or (B) males in adulthood. Neonatal injury (SAL+CGN) (- - -) produced a significant shift in the morphine dose-response curve in (A) females [F(3,250)=2.982,p=0.03]; and (B) males [F(3,208)=2.979,p=0.03] compared to control animals (-----). N=6-13 rats per group/per sex; MS+CGN-■; SAL+SAL-○; MS+SAL-□; SAL+CGN-●.

Figure 4.5. MOR is present centrally and peripherally on P0. Following western blot analysis, mu opioid receptor protein was present in the (A) brain; (B) spinal cord; (C) hind paw on P0.

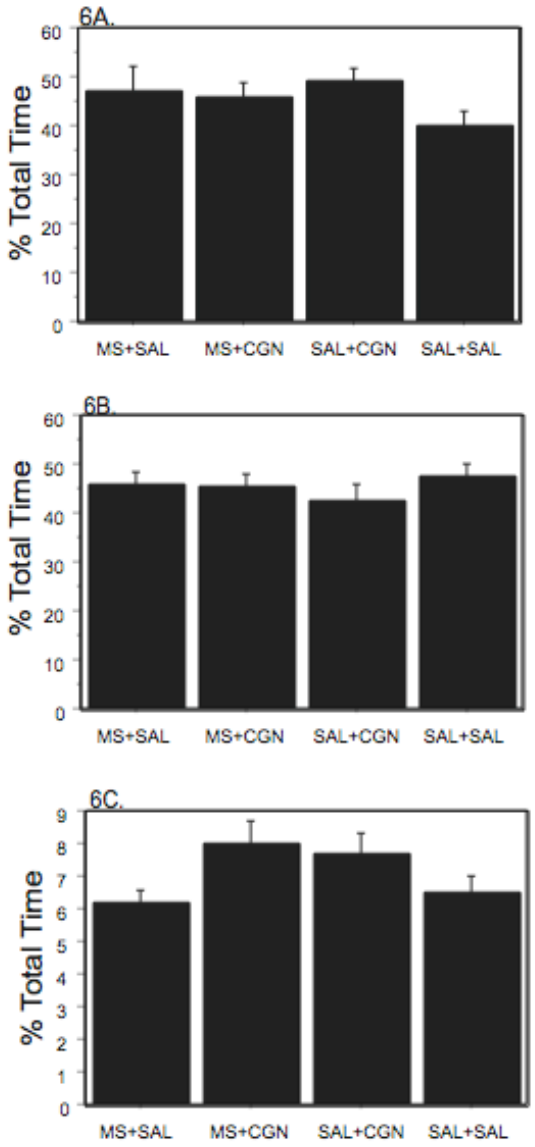


Figure 4.6. Neonatal injury has no impact on maternal care. Neonatal injury had no effect on the duration of time the dam spent (A) on/with her litter $F(3,13)=.734, p=.5503$, (B) away from her litter $F(3,13)=.393, p=.7600$, (C) licking and grooming pups $F(3,13)=1.883, p=.1822$. N= 3-6 litters per group.

Table 4.1. Morphine ED50 Values. A significant effect of sex was present in all four treatment groups, where males displayed significantly reduced ED50 values compared to females ($p<.05$). SAL+CGN males and females displayed significantly greater ED50 values compared to SAL+SAL control animals ($p<.05$).

Table 1. Morphine ED50 Values

Treatment	Female	95% C.I.	Male	95% C.I.
MS+CGN	5.49	4.82-6.26	4.17**	3.70-4.71
SAL+SAL	4.69	4.16-5.29	3.74**	3.41-4.11
MS+SAL	5.28	4.77-5.84	4.22**	3.76-4.74
SAL+CGN	6.60*	5.54-7.86	5.24**/*	4.35-6.32

*p<.05 significant effect of treatment; **p<.05 significant effect of sex; C.I.=Confidence Interval

CHAPTER FIVE

Conclusions

5.1 Conclusions-Overview

Advances in perinatal medical care over the last two decades have substantially increased the survival of infants born premature (Grunau et al., 2006). As part of this life-saving care, however, preterm neonates are exposed to multiple invasive procedures in the neonatal intensive care unit (NICU), including heel lances, circumcision, endotracheal intubation, and respiratory and gastric suctioning. Growing clinical and basic science data suggests that exposure to repeated tissue damaging interventions in neonates with limited analgesic therapy may induce lasting changes in the CNS and have profound consequences for subsequent nociceptive processing (Anand, 2000b, Whitfield and Grunau, 2000, Grunau et al., 2005) (Bhutta et al., 2001, Lidow, 2002, Walker et al., 2003, Ren et al., 2004, Wang et al., 2004). The previous studies examining the impact of early life noxious insult on adult nociceptive responses, however, have failed to provide insight into several important factors, including the existence of potential sex differences in response to the lasting effects of neonatal noxious stimulation, the biological mechanisms underlying insult-induced deficits in nociceptive responsiveness, and the attenuation of the long-term consequences of early noxious stimulation with pre-emptive analgesia. Collectively, the studies included in this dissertation aimed to address these considerable gaps in the knowledge and contribute to improved understanding and treatment of the lasting effects of repeated invasive interventions in premature infants in the NICU.

5.2 The Lasting Impact of Neonatal Noxious Stimulation

Pain is unique amongst sensory modalities. While olfactory, auditory, and tactile stimulation are plentiful after birth, the newborn mammalian CNS is rarely exposed to nociceptive input. During the last two decades, however, this situation has changed dramatically due to the wide application of intensive care interventions in high-risk preterm neonates (Lidow, 2002, Fitzgerald, 2004). As the neonatal period is a sensitive window for experience-induced plasticity due to the ongoing maturation of nociceptive systems (Fitzgerald and Jennings, 1999), recent evidence from clinical and animal research studies has reported that noxious stimulation, experienced early in life, can leave a legacy of altered CNS processing (Grunau et al., 1994a, Anand et al., 1999, Ruda et al., 2000, Lidow et al., 2001, Bhutta et al., 2002, Ren et al., 2004, LaPrairie and Murphy, 2007).

While a variety of experimental animal models have been employed to address the long-term consequences of neonatal noxious stimulation, including colonic irritation (Al-Chaer et al., 2000), physical tissue damage (Reynolds and Fitzgerald, 1995), sensory nerve ligation (De Lima et al., 1999), bladder distention (DeBerry et al., 2007) and recurring noxious stimulation (Anand et al., 1999), many fail to accurately characterize the common human neonatal experience. During routine medical procedures in the NICU, preterm infants often experience acute local inflammation that lasts for several hours to days induced by repeated heel lances, surgical wounds, skin burns, and other forms of tissue injury (Anand, 1998, Anand, 2000a). Our model of short-lasting neonatal local inflammation, produced by a single hindpaw injection of carrageenan on the day of

birth has been designed to simulate the acute, local inflammatory response frequently experienced by preterm infants in the NICU (LaPrairie and Murphy, 2007).

Neonatal Inflammation-Induced Baseline Hypoalgesia

In our initial studies, we demonstrated that intraplantar inflammation induced on the day of birth results in significant long-lasting hypoalgesia, as indicated by significantly longer paw withdrawal latencies in response to a noxious thermal stimulus. Remarkably, this hypoalgesia was not only present in the neonatally injected hindpaw but also in the intact contralateral paw. The degree of hypoalgesia produced by our P0 insult is not trivial; paw withdrawal latencies were more than 40% greater in neonatally inflamed adults compared to handled and saline control animals and was manifest at both adolescence (P40) and adulthood (P60). Furthermore, the increase in nociceptive thresholds following neonatal inflammatory insult was significantly greater in neonatally injured females compared to injured males (LaPrairie and Murphy, 2007). Our results parallel previous studies in males that similarly report increased sensory thresholds in both injected and intact hindpaws, as well as increased tail-flick responses and enhanced visceral hypoalgesia that persist into maturity in animals that experienced early life hindpaw inflammation (Lidow et al., 2001, Ren et al., 2004, Wang et al., 2004). Taken together, these results strongly indicate a neonatal inflammation-induced globally-driven deficit in nociceptive responsiveness.

Acute Versus Tonic Models of Noxious Insult

The ability of early-life noxious stimulation to induce persistent alterations in nociceptive processing is clear. Data collected to date, however, suggest that the specific direction of effects appears to be dependent on the type of insult (acute versus tonic). (Ruda et

al., 2000, Torsney and Fitzgerald, 2003, Ren et al., 2004). For example, a single injection of intraplantar carrageenan administered neonatally results in profound somatic and visceral hypoalgesia in adult rats in response to noxious thermal and mechanical stimulation (Ren et al., 2004, Wang et al., 2004, LaPrairie and Murphy, 2007). Similar results in somatic nociceptive sensitivity have been reported following other acute stimuli, including intraplantar formalin, foot shock and neonatal laparotomy (Shimada et al., 1990, Bhutta et al., 2001, Sternberg et al., 2005). In contrast, chronic inflammation induced by administration of intraplantar Complete Freund's adjuvant, as well as local hindpaw skin wounds, result in persistent hyperalgesia (Fitzgerald et al., 1989, Ruda et al., 2000). Moreover, *repeated* intraplantar carrageenan administration over the first three postnatal weeks results in enhanced nociceptive sensitivity (Lidow et al., 2001). Thermal hyperalgesia following exposure to repetitive needle pricks, and lasting visceral hyperalgesia associated with neonatal chronic chemical irritation of the colon have also been reported (Anand et al., 1999, Al-Chaer et al., 2000).

Interestingly, the aforementioned results suggest that long-term impact of neonatal noxious insult mirrors the developmental consequences of early life stress. Compelling evidence in experimental animal models has revealed that perinatal stressful experiences result in profound and permanent consequences on the behavioral and neuroendocrine response to stress stimuli in adulthood. Specifically, exposure to a potent stressor such as repeated maternal separation results in lasting hyperactivation of the hypothalamic pituitary adrenal (HPA) axis, while brief bouts of handling (i.e. mild stressor in rodents) during the perinatal period result in a stress hypo-responsive phenotype (Plotsky and Meaney, 1993, Liu et al., 1997, Caldji et al., 1998). Thus, given

that the lasting impact of early life stress on the HPA axis is dependent upon the degree (mild versus severe) of perinatal stress exposure, the type of noxious stimulation (acute versus tonic), similarly, appears to be critical to the long-term bivalent effects of neonatal insult on baseline nociceptive thresholds.

Critical Period

The ability of early life experience to alter the organization of the CNS and subsequent behavior is a major focus of neuroscientific research. Previous research in both human and non-human animal models suggests that there are periods during nervous system development within which perturbations have long-lasting, if not permanent consequences. This is in contrast to the relatively transient effects associated with the same perturbations at times outside these periods (Bishop, 1982, Rabinowicz et al., 1996). Our results indicate that there is indeed a critical period for the long-term consequences of neonatal inflammatory insult on adult sensory thresholds. Animals that experienced unilateral neonatal hindpaw inflammation on both postnatal days zero and eight (P0 and P8) displayed a significant decrease in sensitivity to noxious stimuli (hypoalgesia) in adulthood, compared to animals injured at two weeks of age (P14) (LaPrairie and Murphy, 2007). Together, these results suggest that the impact of neonatal inflammation is dependent upon a sensitive period, and that noxious insult occurring outside of this critical window does not permanently alter thermal sensory thresholds. Our results are consistent with previous studies that have also reported that neonatal injury permanently alters visceral and somatic sensory processing, however, only when induced during the first week of life (Ren et al., 2004, Wang et al., 2004).

Sex Differences in Response to Neonatal Noxious Stimulation

Given the sizable body of literature that indicates that males and females experience pain differently (Berkley et al., 1988, Unruh, 1996, Berkley, 1997), it is surprising that the majority of previous studies examining the impact of neonatal noxious insult have been conducted exclusively in male rodents (Ruda et al., 2000, Lidow et al., 2001, Ren et al., 2004). We originally hypothesized that sexually dimorphic organizational hormones contribute to significant sex differences in response to noxious inflammatory insult (Weisz and Ward, 1980, Amateau et al., 2004, Balthazart and Ball, 2006, Cornil et al., 2006), such that elevated central levels of estradiol may act as a "neuroprotectant" in males, leaving females with low to non-detectable levels of estradiol increasingly vulnerable to the lasting effects of early life insult (Garcia-Segura et al., 2001, Maggi et al., 2004, Amantea et al., 2005). To our knowledge, we reported for the first time that neonatal inflammatory insult was indeed sexually dimorphic, with females displaying significantly greater basal hypoalgesia in adulthood in comparison to males. The paw withdrawal latency of females injured with 1% CGN was more than 3 seconds longer in both the inflamed and intact hindpaws compared to injured males (LaPrairie and Murphy, 2007). Moreover, we showed that female rats injured at P14, when estradiol concentrations are comparable in males and females, displayed equivalent levels of baseline hypoalgesia as injured males. This further suggests that sex differences in the neonatal neuroendocrine environment contribute to the observed sexually dimorphic impact of neonatal inflammatory insult (LaPrairie and Murphy, 2007).

Interestingly, however, an observed sex differences in baseline paw withdrawal latencies was present only at P60 and not at P40 (LaPrairie and Murphy, 2007). This

observation lends support that activational hormones at puberty may also play a key role in the sexually dimorphic response to neonatal inflammation. Activational gonadal hormones have been shown alter the processing of nociceptive information by influencing endogenous opioid systems (Berglund et al., 1988, Smith et al., 1998), as well as the activity of other neuromodulators involved in nociceptive processing, including substance P, gamma-aminobutyric acid (GABA), glutamate, dopamine, serotonin and norepinephrine. In response to tissue injury, estradiol further modulates the expression of pro-inflammatory prostaglandins released peripherally (Zhang et al., 1997), and increases central BDNF expression thereby promoting neuronal survival and healing (Price et al., 2005). Finally, gonadal hormones have been shown to have a marked influence on nociceptive and analgesic sensitivity in rodents (Fillingim and Ness, 2000, Craft et al., 2004).

Future studies directed at manipulating the neonatal neuroendocrine environment, including masculinizing females or castrating males, as well as pre-pubertal gonadectomy, are necessary to more specifically implicate gonadal hormones as the primary factor contributing to the observed sex differences in the impact of neonatal inflammatory insult. As sex differences in nociceptive behavior likely result from a confluence of genetic, anatomical, physiological, and hormonal factors (Berkley, 1997), other key variables may indeed contribute to the sexually dimorphic response to neonatal inflammatory insult.

Response to a Subsequent Inflammatory Insult in Adulthood

An additional series of experiments was conducted to test whether neonatally injured animals respond differentially to a subsequent inflammatory insult in adulthood.

Following baseline behavioral testing at P60, animals received an injection of Complete Freund's adjuvant into the plantar surface of either the neonatally injured or previously intact hindpaw. 24 hours following CFA-induced inflammation, we demonstrated that male and female rats that experienced neonatal inflammation displayed significantly greater hyperalgesia than control animals (LaPrairie and Murphy, 2007). Furthermore, neonatally injured females exhibited significantly greater hyperalgesia in the inflamed paw than neonatally injured males, and male and female controls. This effect was observed in both the neonatally injured and uninjured paws, and is consistent with previous studies reporting long-lasting sensitization of afferent neurons and hyperalgesia following neonatal insult (Reynolds and Fitzgerald, 1995, Anand et al., 1999, Al-Chaer et al., 2000).

This increased *hyperalgesia* following re-inflammation in adulthood may appear disparate with the observed basal *hypoalgesia*. Our preliminary anatomical studies, however, suggest that neonatal inflammatory insult results in alterations in primary afferent innervation of the dorsal horn (LaPrairie and Murphy, 2005), which may account for our observed hyperalgesia. In particular, neonatal inflammatory insult increases primary afferent innervation in the L3-L5 spinal cord, as reflected by increased expression of both CGRP and substance P immunoreactivity. Parallel changes were not observed in CGRP expression in the thoracic spinal cord of injured animals. Similar findings of an increase in substance P levels in laminae I and II of the dorsal horn have been reported following chronic inflammation in rodents (Honor et al., 1999). As both CGRP and substance P are pro-nociceptive, enhanced dorsal horn release of these peptides due to increased primary afferent input would be associated with an enhanced

response to noxious stimulation, and may provide the biological basis for the observed increased hyperalgesia following intraplantar CFA in adulthood.

Our dual findings of baseline hypoalgesia and enhanced hyperalgesia following a subsequent insult are surprisingly consistent with previous reports in former premature children. Grunau and colleagues found that ex-preterm neonates are rated by parents as less reactive to everyday bumps and scrapes, while concurrently these children rate medical procedural pain as more intense (Grunau et al., 1994a, Grunau et al., 1998a). Similarly, adolescents with prior NICU experience display an increased threshold for acute thermal stimuli (i.e. decreased sensitivity), but enhanced perceptual sensitization to a prolonged heat stimulus (i.e. hyper-sensitivity) (Hermann et al., 2006).

5.3 Increased Endogenous Opioid Tone: A Potential Mechanism for the Neonatal Inflammation-Induced Deficits in Nociceptive Responsiveness

A substantial amount of research within the last decade has focused on the impact of neonatal noxious stimulation on developing nociceptive circuitry and subsequent pain processing. The mechanisms underlying the long-term deficits associated with neonatal noxious insult, however, have not been elucidated. Our laboratory has demonstrated that mild neonatal intraplantar inflammation results in significant long-term hypoalgesia that is present in both the previously injured and uninjured paws- indicating global, injury-induced alterations in basal nociceptive sensitivity. Therefore, behavioral and anatomical studies were conducted to test the hypothesis that our observed

hypoalgesia is mediated by an increase in endogenous opioid tone in descending inhibitory pathways.

Physiological Impact

In our initial studies, we found that administration of the broad-spectrum opioid antagonist naloxone HCl completely reversed the hypoalgesia induced by neonatal inflammatory injury, suggesting an increase in tonic endogenous opioid tone. Evidence that early life trauma can shape the maturation of descending inhibitory circuits has previously been reported in both human and non-human animal studies. Preterm infants exposed to numerous interventions in the NICU display significantly altered endogenous inhibitory responses to noxious thermal stimulation, as well as heightened cardiac reactivity compared to both term-born and premature infants exposed to few noxious procedures (Goffaux et al., 2008). A deficit in descending inhibitory systems has also been implicated in several chronic pain conditions, including fibromyalgia (Henriksson, 1994, Julien et al., 2005), irritable bowel syndrome (Wilder-Smith et al., 2004), and tension-type headache (Pielsticker et al., 2005). Lastly, tonic descending inhibition of dorsal horn neurons is substantially decreased in adult rats treated at birth with capsaicin, a neurotoxin that acts at unmyelinated c-fibers (Cervero and Plenderleith, 1985). Therefore, an increase in dorsal horn c-fiber innervation, as evidenced in adult animals following neonatal intraplanar carrageenan (LaPrairie and Murphy, 2005), may indeed result in a compensatory up-regulation of tonic endogenous opioid inhibition.

In subsequent experiments, we investigated the involvement of central versus peripheral opioid receptors in the mediation of neonatal inflammation-induced increase in opioid tone. Administration of naloxone methiodide, a peripherally-acting opioid

antagonist (Ji et al., 2006), had no effect on the behavioral deficits in nociception associated with early inflammatory insult. In contrast, intracerebroventricular administration of naloxone HCl significantly attenuated the neonatal injury-induced hypoalgesia, implicating central not peripheral, endogenous opioidergic pathways as mediating the observed hypoalgesia in neonatally injured animals.

There is considerable evidence that the functional activity of central endogenous opioid systems is enhanced following noxious stimulation. In spinal dorsal horn neurons, pro-dynorphin and pro-enkephalin biosynthesis is up-regulated in response to carrageenan-, formalin- and CFA-induced inflammation (Iadarola et al., 1988, Noguchi et al., 1989). Inflammation–induced changes in endogenous opioid peptide expression and release have also been reported at several supraspinal sites, including the periaqueductal gray (PAG) (Williams et al., 1995a). Noxious stimulation-induced changes in opioid peptide expression are also paralleled by an increase in mRNA expression (Iadarola et al., 1988). While these studies were conducted in adult males, it still provides supporting evidence that a peripheral insult can induce changes in central opioid peptide expression.

The PAG has been shown to a principal mediator of descending anti-nociceptive mechanisms (Budai and Fields, 1998). In addition, the PAG is rich in nerve terminals and fibers containing endogenous opioids (Reichling et al., 1988) and opioid receptors are localized throughout the rostral-caudal axis of the PAG (Duncan and Murphy, 2005). Therefore, we hypothesized that neonatally injury-induced alterations in endogenous opioid tone are mediated within the PAG. Site-specific administration of naloxone HCl into the ventrolateral PAG significantly reduced the basal hypoalgesia associated with

neonatal inflammation in both male and female rats. These results suggest that opioidergic systems are persistently and functionally altered in the PAG in response to neonatal inflammation, although the involvement of additional loci including the RVM and spinal cord cannot be ruled out (Mansour et al., 1995a, Mansour et al., 1995b, Budai and Fields, 1998).

We next examined the role of specific opioid receptor subtypes in the PAG. Intra-PAG administration of the highly selective mu and delta opioid receptor antagonists, CTOP and naltrindole, attenuated the observed hypoalgesia in neonatally injured animals, albeit to differing degrees in males and females. In contrast, the kappa receptor antagonist, Nor-BNI, did not reduce the hypoalgesia in neonatally-injured animals, indicating that mu and delta opioid receptor systems, but not kappa, contribute to alterations in endogenous opioid tone in the PAG induced by neonatal inflammation.

Anatomical Impact

Our next series of experiments was conducted to determine whether neonatal inflammatory insult induced anatomical alterations in opioidergic systems within the PAG. As site-specific antagonist injections indicated injury-induced alterations in mu opioidergic systems, immunohistochemical studies were conducted to examine the expression of beta-endorphin in the PAG. A significant increase in beta-endorphin peptide expression was noted in neonatally injured animals; this increase was restricted to mid and caudal levels of the lateral/ventrolateral PAG. Beta-endorphin was mainly concentrated in cell bodies of the hypothalamic arcuate nucleus with neuronal innervation of the PAG, as previously reported (Mansour et al., 1995a, Mansour et al., 1995b, Millan, 2002). According to previous studies, beta-endorphin containing

pathways projecting from the arcuate nucleus to the PAG moderate nociception via mu opioid receptors in response to tissue inflammation (Porro et al., 1988, Facchinetti et al., 1992).

Met- and leu-enkephalin via action at delta-opioid receptors exert an important role in contributing to the expression of descending inhibition in the dorsal horn. The significance of supraspinal populations of enkephalin-containing neurons in the response to noxious stimuli and their mediation of nociception has been widely discussed (Basbaum and Fields, 1978, Millan, 1982, Millan, 2002). We demonstrated that neonatal inflammation results in a significant elevation in enkephalin immunoreactivity in the lateral/ventrolateral region of the mid and caudal PAG.

Immunohistochemical and autoradiographical studies also revealed a significant decrease in mu and delta opioid receptor expression in the PAG in neonatally injured animals compared to controls. Specifically, neonatally injured animals displayed a significant reduction in mu opioid receptor (MOR) immunoreactivity and binding affinity in the lateral/ventrolateral PAG. Injury-induced alterations were mainly restricted to the mid and caudal levels of the PAG, whereas the rostral PAG appeared mostly unaffected. The mid and caudal levels of the PAG have dense projections to the RVM and are preferentially involved in the descending modulation of pain (Beitz, 1982, Beitz et al., 1983). Similarly, neonatal injury also resulted in a significant decrease in delta opioid receptor binding in the lateral/ventrolateral mid-PAG. Thus, neonatal inflammatory insult induces a central up-regulation of endogenous opioid ligands that is paralleled by a compensatory down-regulation of cognate opioid receptors in the PAG. Similar findings have been reported in rodent models in response to chronic arthritis

(Morris, 1993, Spetea et al., 2002). Future investigations directed at establishing a developmental time-course of the neonatal insult-induced anatomical alterations, as well as radioimmunoassay studies verifying increased basal endogenous opioid levels in the PAG are necessary to provide further insight into the lasting effects of early inflammation on descending inhibitory circuits.

The data presented herein constitute a compelling case that early noxious insult produces significant long-term alterations in endogenous descending inhibitory mechanisms, thereby profoundly impacting nociceptive responsiveness. Our laboratory has preliminary evidence to suggest that neonatal intraplantar carrageenan induces a substantial increase in primary afferent innervation of the dorsal horn of the lumbar spinal cord. Our working hypothesis is that the CNS responds to neonatal injury with a compensatory increase in endogenous opioid tone- characterized by a significant increase in beta-endorphin and met/leu-enkephalin in an effort to mask the sensitization of dorsal horn cells. This increased regulation of opioid peptide results in a parallel decrease in mu- and delta- opioid receptor expression in the PAG, probably due to receptor internalization or ligand-induced changes in receptor availability similar to that observed following long-term drug use (Zhang et al., 2002, Haberstock-Debic et al., 2003, Tong et al., 2003). This increase in opioid tone contributes to the observed hypoalgesia at baseline testing. However, in the presence of a subsequent major noxious insult, neonatally injured animals display enhanced hyperalgesia. This may be the result of either increased release of pro-nociceptive peptides (i.e. CGRP and substance P) from primary afferent neurons or reduced strength *dynamic* inhibition due

to a ceiling effect following the chronic elevation of *tonic* inhibitory mechanisms (Figure 5.1).

5.4 Neonatal Morphine Blocks the Lasting Consequences of Neonatal Inflammatory Insult

The survival rates of preterm infants have increased substantially around the world due to advances in NICU care. Despite the current knowledge that preterm infants are responsive to noxious stimuli (Giannakoulopoulos et al., 1999, Smith et al., 2000, Vanhatalo and van Nieuwenhuizen, 2000), many routine procedures in the NICU are performed in the absence of analgesics (Johnston et al., 1997, Porter et al., 1997, Kahn et al., 1998, Simons et al., 2003). Nevertheless, accumulating data suggests that premature infants undergoing invasive procedures benefit from the use of opioid analgesics (Yaster and Deshpande, 1988, Yaster et al., 1989, Fitzgerald, 1991, Anand and Hickey, 1992, Bhat et al., 1992). Therefore in our final series of experiments, we examined whether administration of pre-emptive morphine would attenuate the long-term consequences associated with neonatal inflammatory insult, and given the recent reports that morphine produces a greater degree of analgesia in males than females (Loyd et al., 1997), we also tested whether morphine was equally efficacious in both sexes.

Morphine administration blocked neonatal injury induced thermal and mechanical hypoalgesia in both the injured and uninjured paws in adolescence (P40) and adulthood (P60). Morphine attenuation of the injury-induced hypoalgesia was comparable in males

and females. These results are consistent with previous studies in rodents that report daily morphine administration prior to intraplantar formalin during the first week of life significantly reduces the long-term effects of repetitive pain (Abbott and Guy, 1995). Previous studies in humans have also reported that morphine therapy ameliorates the effects of early repetitive noxious stimuli in extremely low birth weight infants at 4 months of age (Grunau et al., 2001). Similarly, children who had minor neonatal operations and received pre-emptive analgesia responded to immunization pain in a similar manner as non-operated age-matched controls (Peters et al., 2003).

In the present study, the ability of pre-emptive morphine to block the hypoalgesia may indeed occur through direct modulation of primary afferent drive into the spinal cord, thereby inhibiting the central relay of inflammatory pain and preventing the subsequent increase in descending endogenous opioid tone. The effects of morphine in our study may partly be a consequence of modification of the inflammatory response and/or the stress response to neonatal inflammatory insult (Stein et al., 1989, Adamson et al., 1991).

Neonatal morphine also significantly attenuated CFA- induced hyperalgesia and increased the rate of recovery, such that both males and females recovered 7 days faster than saline treated injured controls. As previously stated, increased primary afferent innervation of the spinal cord dorsal horn following neonatal inflammatory insult may account for our observed hyperalgesia in adulthood (LaPrairie and Murphy, 2005). Administration of morphine at the time of injury would be expected to inhibit this increase in primary afferent input, thereby preventing the entire cascade of behavioral, physiological and anatomical deficits associated with neonatal inflammation. In regard

to recovery, clinical reports demonstrate that at 32 weeks of age, preterm infants experience a reduced rate of recovery to skin breaking procedures (Morison et al., 2003), and exhibit subtle differences in ability to recover from finger lance at 4 months compared to full term controls (Oberlander et al., 2000). There are no reports on the impact of pre-emptive morphine on recovery rates in premature neonates, however, our data suggest that morphine analgesia may in fact significantly increase the rate of recovery following procedural pain in NICU infants.

Initially, we hypothesized that exposure to morphine during neonatal development may alter opioid receptor number and/or affinity, thereby altering morphine's antinociceptive effects in adulthood. This hypothesis was based on previous studies that report a 60-fold shift in the morphine dose response curve in adult rats that received repeated neonatal morphine in the absence of pain (Rahman et al., 1998, Bhutta et al., 2001). Opioid receptor down regulation in the brains of neonatal rats chronically exposed to morphine has also been reported (Tempel, 1991). However in our studies using a cumulative dosing paradigm, we demonstrated that neonatal morphine administration did not significantly alter morphine's antinociceptive effects in adulthood in males or females (i.e. no significant shift in ED50 values). Interestingly, a significant rightward shift in ED50 was noted in neonatally injured animals that did *not* receive neonatal morphine.

These results have serious clinical implications. Previous studies have reported that pre-term infants that experience surgery during the first three months of life have significantly higher peri- and post-operative analgesic requirements in response to surgery in the same or different dermatome compared to control infants (Bouwmeester

et al., 2003, Peters et al., 2005). Similarly, mice exposed to chronic noxious stimulation display increased tail flick latencies compared to control animals, and a significant two-fold increase in the ED50 of morphine in response to abdominal constriction (Christie et al., 1982). As noxious stimulation during the neonatal period leads to increased activation of opioid systems in a manner analogous to the repeated application of exogenous opiates, these studies suggest that neonatal injury produces cross-tolerance to the analgesic effects of morphine thereby decreasing the subsequent effectiveness of morphine (Lett et al., 2002, Stoller et al., 2002, Stoller et al., 2007). Again interestingly, exposure to *morphine* neonatally did not result in a significant shift in ED50 values. Therefore, we believe that opioid cross tolerance is associated with neonatal injury-induced chronic exposure to *endogenous* opioids resulting from a potentiation of the descending inhibitory circuit, and not a result of exposure to morphine on P0. Alternatively, neonatal stress associated with maternal separation and repeated handling has also been suggested to reduced opioid analgesia (Coutinho et al., 2002, Sternberg and Ridgway, 2003). This suggests that alterations in opioid analgesia may reflect a combined effect of neonatal nociceptive experience as well as early life stress, and may involve altered responsiveness of endogenous analgesia circuits as well as the hypothalamic-pituitary-adrenal axis (Grunau et al., 2005).

5.5 Evolutionary Perspective

Critical Period

A critical period is a developmental time point in which heightened brain plasticity allows neural circuits to be shaped by experience (Hensch et al., 2004). We have demonstrated that the lasting consequences of neonatal noxious stimulation are critical period dependent-where animals injured at P0 and P8, but not P14 display significant hypoalgesia in adulthood (LaPrairie and Murphy, 2007). Our laboratory and others have demonstrated that a *mild* noxious insult during this critical period results in decreased nociceptive sensitivity in adulthood (LaPrairie and Murphy, 2007, Ren et al., 2006, Bhutta et al., 2004), whereas a *ma*jor noxious insult results in long-lasting cutaneous hypersensitivity (Fitzgerald et al., 1989, Ruda et al., 2000), suggesting that the direction of the response is mediated by the intensity of the noxious stimulus.

From an evolutionary prospective, natural selection may have shaped offspring to respond to subtle variations in their early environments during developmental critical periods as a forecast of the environmental conditions they will ultimately face in adulthood (Badyaev et al., 2005, Crespi et al., 2005). This phenotypic plasticity permits organisms to “mold” their form to prevailing circumstances during early development, ultimately leading to stable variations in phenotype that serve to increase the capacity of the offspring throughout its lifetime (Fishet al., 2004, Meaney et al., 2007). For example in rats, naturally occurring variations in the quality of mother-pup interactions program the development of individual differences in the behavioral and endocrine response to stress through epigenetic modification of the glucocorticoid receptor gene (Zhang et al.,

2004, Meaney et al., 2005). Similarly, water fleas (*Daphnia*) form helmet-like neck growths and tail spines in response to chemosignals from aquatic predators, thereby reducing the likelihood of capture and successful ingestion (Agrawal et al., 1999). Exposure of females to predator chemosignals is sufficient to alter the morphology (i.e. significantly larger helmets) of predator chemosignal-naïve offspring (Agrawal et al., 1999, Tollrian et al., 1995). Therefore, variations in early environmental exposure to noxious stimulation may alter the development of nociceptive systems during an early postnatal critical period, which lead to sustained variations in nociceptive responsiveness (i.e. hyper versus hypo). Under conditions of increased adversity and nociceptive demand (i.e. high threat and predation, prevalent intra-group competition), it would be advantageous for an animal to *enhance* its behavioral and physiological responsiveness to nociceptive stimulation. These responses would be essential under the increased demands of repeated exposure to noxious stimuli that are potentially life threatening. *Decreased* nociceptive reactivity resulting in reduced responsiveness to "irrelevant" or "background" stimuli, would increase the capacity of offspring under favorable environmental conditions with few threats of endangerment.

While lasting alterations in gene expression induced by environmental factors during critical periods have been demonstrated in a wide variety of species (Reik et al., 2007, Chandler et al., 2007, Casadesus et al., 2006), a critical period for the effects of neonatal noxious stimulation on developing nociceptive circuitry has only been demonstrated in rats (LaPrairie and Murphy, 2007, Ren et al., 2004). One may speculate that these results would indeed extend to other species that may experience a noxious insult during a critical period of *postnatal plasticity* in nociceptive circuits (i.e.

rat, mouse, zebra finch). In contrast, these effects would be unlikely in species where development of nociceptive circuitry primarily occurs during gestation (i.e. guinea pigs, sheep, rhesus macaques), when the likelihood of noxious insult is low. In summary, critical periods for sensory systems may be selected for evolutionarily to program advantages in the developing neural circuits that adaptively match the environments the neonates will ultimately face in adulthood, whereby differing degrees of environmental demand require sustained differences in nociceptive reactivity dependent upon noxious stimulation during early life.

Sex Differences

Our studies have demonstrated that an inflammatory insult induced on the day of birth results in long-lasting hypoalgesia, as indicated by significantly longer paw withdrawal latencies in response to noxious thermal stimulation. Increased sensory thresholds (i.e. decreased nociceptive sensitivity) are present in the both neonatally injured hindpaw as well as the intact contralateral paw in adulthood, and are significantly greater in neonatally injured females compared to injured males (LaPrairie and Murphy, 2007).

ADAPTIVE EXPLANATION: A major contribution of Charles Darwin was the observation that evolutionary pressures often differ for males and females, and that many of these differences are centered around the dynamics of reproduction (Darwin, 1871). Sexually selective pressures typically result in male-male competition for social status, resources, or territory in an effort to attract mates, and female choice of mating partners (Andersson et al., 1994). Although the dynamics of male intrasex competition can vary across species, one common result is the evolution of physical and behavioral sex differences. Male-male competition and female mate choice are most evident in

species in which males devote most of their reproductive energies to attracting mates and female provide the majority of parental care (Trivers et al., 1972), characteristics observed in most mammalian species (Clutton-Brock et al., 1991). Therefore, sex differences in nociceptive responsiveness following early noxious insult may have evolved in response to the differing reproductive demands of adult males and females. For example in an environment with few threats of endangerment, significantly reduced nociceptive responsiveness would be highly advantageous in females, as their primary role involves the successfully care of offspring. In males, this degree of reduced nociceptive reactivity would be disadvantageous. A moderate degree of reduced behavioral responsiveness may be selected for in males, whereby still allowing for increased vigilance in noxious bouts of intrasex competition for resources. Sex differences in response to neonatal noxious insult would likely be evident in all species that demonstrate characteristic sexually dimorphic reproductive roles.

NONADAPTIVE EXPLANATION (Biological Spandrel): Alternatively, the observed sex differences following neonatal noxious insult may be similar to Stephen J. Gould's Spandrels of San Marco-a byproduct of the sexual differentiation process (Gould et al., 1979). "Spandrel" is a term used to describe a phenotypic characteristic that is considered to have developed during evolution as a side effect of a true adaptation (Gould et al., 1979, Gould et al., 1997). Sexual differentiation of the rat brain occurs from E18-P10 (MacLusky et al., 1981, Arnold et al., 1984), whereby males experience a significant surge of testicular testosterone that is centrally aromatized to estradiol, ultimately resulting in masculinization of the nervous system (Amateu et al., 2004, Cornil et al., 2006). During this period of sexual differentiation, steroids affect a wide variety of

cellular mechanisms, including neurogenesis, cell death, cell migration, synapse formation and synapse elimination throughout the CNS (Cooke et al., 1998). Therefore, sexual differentiation of the nervous system may be adaptive, whereas its impact on nociceptive circuitry and development of a sexually dimorphic response to neonatal noxious insult may be a secondary consequence, albeit with a subsequent exaptive utility. While this physiological adaptation may not be heritable, the capacity to develop the adaptation presumably is. Our laboratory is the first to demonstrate a sex difference in response to neonatal injury (LaPrairie and Murphy, 2007), and therefore, it is unknown whether these observations would extend to other species. One may speculate that this effect would be present in species where noxious insult and sexual differentiation of the nervous system could theoretically overlap (i.e. late prenatal or postnatal sexual differentiation).

5.6 Final Remarks

Although research into the long-term consequences of noxious stimulation during the neonatal period have spanned over two decades, our understanding of neonatal pain is literally still in its infancy. The studies in this dissertation aimed to fill some of the critical gaps of knowledge in this field. First, we established that exposure to a single neonatal inflammatory insult is associated with a lasting decrease in basal nociceptive sensitivity and enhanced sensitivity following a subsequent inflammatory injury that is significantly exacerbated in females. The clear presence of a sex difference in the response to early insult may indeed contribute to the higher prevalence, severity and duration of pain

syndromes in women than men. Second, we were the first to demonstrate that the profound elevation of nociceptive thresholds following neonatal inflammation is mediated by an experience-induced facilitated activation of descending nociceptive pathways, characterized by dynamic physiological and anatomical modification and modulation of opioidergic systems in the PAG. Finally, we showed that pre-emptive morphine ameliorates the long-term effects of neonatal inflammatory injury on adult nociception, which provides compelling justification for the use of analgesics prior to the initiation of noxious procedures performed on neonates. Collectively, these studies present valuable information about the long-term consequences of neonatal noxious stimulation in males and females, which may ultimately lead to improved understanding and treatment of the lasting effects of repeated invasive interventions in premature infants in the NICU.

5.7 Chapter 5 Figures

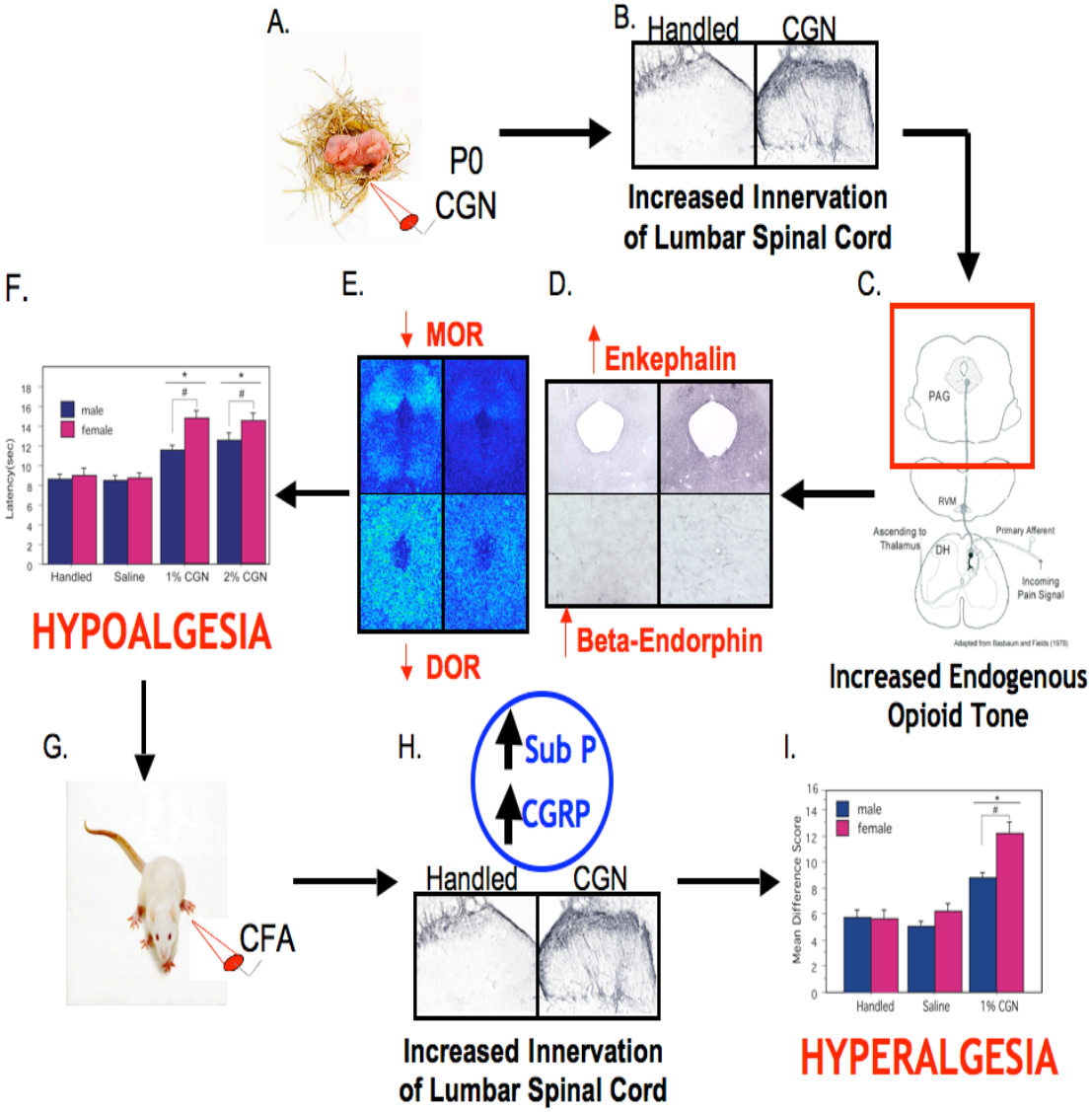


Figure 5.1 The Lasting Impact of Neonatal Inflammatory Insult: A Summary. (A) Intraplantar carrageenan (CGN) on the day of birth (P0) results in (B: left-handled; right-1% CGN) a lasting increase in primary afferent innervation of the dorsal horn of the lumbar spinal cord, ultimately leading to (C) an increase in endogenous opioid tone which is characterized by (D: top left-met enkephalin handled; top right-met enkephalin 1% CGN; bottom right-beta endorphin 1% CGN; bottom left-beta endorphin handled) a significant increase in enkephalin and beta endorphin immunoreactivity and (E: top left-MOR handled; top right-MOR 1% CGN; bottom right-DOR 1% CGN; bottom left-DOR handled) a significant decrease in mu and delta opioid receptor binding in the PAG. This increase in opioid tone contributes to the (F) observed hypoalgesia at baseline testing. (G) In the presence of a subsequent major noxious insult in adulthood, (H: left-handled; right-1% CGN) neonatally injured animals have increased release of pro-nociceptive peptides (i.e. CGRP and substance P) compared to handled animals, resulting in (I) enhanced hyperalgesia following intraplantar CFA.

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APPENDIX

A.1

CURRICULUM VITAE

Jamie L. LaPrairie Ph.D.
1615 Rivers Edge Trail
Atlanta, Georgia 30324

EDUCATION AND EXPERIENCE

DEGREE: Bachelor of Science
MAJOR: Biopsychology (GPA 3.6)
ADVISOR: Bryan Pfingst, Ph.D.
INSTITUTION: University of Michigan, Ann Arbor, MI (1994-1998)

DEGREE: Master of Science
MAJOR: Biomedical Sciences (GPA 3.7)
ADVISOR: James Rillema, Ph.D.
INSTITUTION: Wayne State University School of Medicine, Detroit, MI (1998-2000)

DEGREE: Master of Science
SPECIALIZATION: Neurobiology and Behavior (GPA 4.0)
ADVISOR: Anne Murphy, Ph.D.
INSTITUTION: Georgia State University, Atlanta, GA (2003-2006)
INSTITUTION: Transferred from Michigan State University, Lansing, MI (2004)

DEGREE: Doctor of Philosophy
SPECIALIZATION: Neurobiology and Behavior (GPA 4.0)
ADVISOR: Anne Murphy, Ph.D.
INSTITUTION: Georgia State University, Atlanta, GA (2003 -2008)

FELLOWSHIPS AND AWARDS

Undergraduate Research Fellowship, University of Michigan (1996-1997)
Neuroscience Training Grant Fellowship, Michigan State University (2003-2004)
Center for Behavioral Neuroscience Scholar, Georgia State University (2004-2008)
Brain and Behavior Fellowship, Georgia State University (2005-2008)
Georgia State University Dissertation Research Grant (2007-2008)
Biology Department Graduate Award for Outstanding Instruction (2008)
FIRST Postdoctoral Fellowship, Emory University (2008)

PROFESSIONAL ORGANIZATIONS

Society for Neuroscience
The American Association for the Advancement of Science
American Pain Society
International Association for the Study of Pain
Organization for the Study of Sex Differences

PROFESSIONAL EMPLOYMENT AND SERVICE

Yerkes Primate Research Center Research Associate, Emory University (2001-2003)

Institute on Neuroscience High School Mentor, Emory University (2004)

Brain Awareness Educator (2005 - 2007)

Presidential Scholar Mentor, Georgia State University (2005-2008)

BRAIN Program Research Mentor, Georgia State University (2006-2008)

GRADUATE TEACHING EXPERIENCE

Graduate Teaching Assistant (2006, 2007, 2008)
Human Anatomy and Physiology 1120
Head Graduate Teaching Assistant (2007)
Human Anatomy and Physiology 1120

PEER-REVIEWED PRIMARY RESEARCH PUBLICATIONS

1. **LaPrairie J.L**., Murphy A.Z. Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury. Pain. 2007 Nov; 132 Suppl 1:S124-33.
2. **LaPrairie J.L**., Johns M., Murphy A.Z. Pre-emptive morphine analgesia attenuates the long-term consequences of neonatal inflammation in male and female rats. Pediatric Research (*accepted Pediatric Research*).
3. **LaPrairie J.L**., Famojure E.O., Murphy A.Z. Neonatal injury-induced hypoalgesia is mediated by increased endogenous opioid tone in the periaqueductal gray. (*submitted; Science*).
4. **LaPrairie J.L**., Murphy A.Z. The impact of neonatal noxious stimulation on developing nociceptive circuits. *(submitted; Frontiers in Neuroscience).*

ABSTRACT/POSTER PRESENTATIONS

1. Bales K.L., Smith L.G., **LaPrairie J.L.**, Plotsky P.M., Sanchez M.M. Mapping of Urocortin II and III in rhesus macaque brain. American Society of Primatologists Conference, Calgary, Canada, 2003.
2. Bales K.L., Smith L.G., **LaPrairie J.L.**, Plotsky P.M., Sanchez M.M. Mapping of Urocortin II and III in rhesus macaque brain. Annual Meeting of the Society for Neuroscience, New Orleans, LA, 2003.

3. McCormack K., Grand A., **LaPrairie J.**, Fulks R., Graff A., Maestripieri D., Plotsky P., Sanchez M. Behavioral and neuroendocrine outcomes of infant maltreatment in rhesus monkeys: the first four months. Annual Meeting of the Society for Neuroscience, New Orleans, LA, 2003.
4. Martin I**., LaPrairie J.L.**, Lonstein J.S. Effects of Dopamine D1 and D2 receptor antagonists in the preoptic area in maternal behavior in lactating rats. Annual Meeting of the Society for Neuroscience, San Diego, CA, 2004.
5. **LaPrairie J.L**., Murphy A.Z. Neonatal injury differentially affects males and females and response to re-injury in adulthood. Annual Meeting of the Society for Neuroscience, Washington DC, 2005.
8. **LaPrairie J.L**., Murphy A.Z. Pharmacological pretreatment attenuates the long-term behavioral consequences of neonatal inflammatory injury. Annual Meeting of the Society for Neuroscience, Atlanta, GA, 2006.
9. **LaPrairie J.L**., Murphy A.Z. Neonatal inflammatory injury-induced hypoalgesia is mediated by increased central endogenous opioid tone. Annual Meeting of the Society for Neuroscience, San Diego, CA, 2007.

SEMINAR PRESENTATIONS

1. **LaPrairie J.L.** "The Long-Term Effects of Neonatal Pain", 8th Annual W.M. Keck Center for Behavioral Biology Symposium. North Carolina State University, Durham, NC, 2007.

2. **LaPrairie J.L.** "The Long-Term Impact of Neonatal Injury on Adult Pain Sensitivity", Brains and Behavior Program Annual Retreat. Georgia State University, Atlanta, GA, 2008.