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The Impact Of Neonatal Pain And Reduced Maternal Care On Brain And Behavioral Development

Sean Michael Mooney-Leber
Wayne State University, moone077@gmail.com

THE IMPACT OF NEONATAL PAIN AND REDUCED MATERNAL CARE ON BRAIN AND BEHAVIORAL DEVELOPMENT

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SEAN MOONEY-LEBER

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DEDICATION

To all my friends and family, being away from all of you is, has, and always will be the most difficult part of this journey. To my ever-supportive parents, Mary Mooney and John Leber, for teaching me the value of hard work and dedication and encouraging me to pursue my interests in higher education. To my sister Mary Jeanne (Mooney-Leber) Stephens for always being there for me no matter the circumstances, your support has kept me going during difficult times. Lastly to my future wife Katie Holt, words cannot describe how grateful I am for your unwavering support and the amount of sacrifices you have made to afford me this opportunity. I love you more than you could ever know and I can easily say this project would not have been possible without you.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 3

Chapter 4

LIST OF FIGURES

CHAPTER 1: BACKGROUND INFORMATION

Preterm births account for 11.1% of all live births worldwide (Blencowe et al., 2012) and the numbers are still increasing (by 1% from 1990 to 2010) even in developed countries such as the United States. Survival rates on the other hand have improved dramatically so that even extremely early preterm infants (i.e. 23 weeks) now have a 23-33% chance of survival (Rysavy et al., 2015). However, being born very premature often results in cognitive and behavioral deficits and there is a dearth of knowledge about what contributes to the long-term biobehavioral outcomes following preterm birth. One consistent and widely documented finding relates to altered brain maturation in preterm infants which has been observed at multiple developmental stages including infancy (Woodward, Anderson, Austin, Howard, & Inder, 2006), childhood (Peterson et al., 2000), and young adulthood (Nosarti et al., 2014). These alterations in brain maturation may manifest as cognitive impairments (Bhutta et al., 2001; Fraello et al., 2011; Marlow, Hennessy, Bracewell, Wolke, & Group, 2007; Marlow, Wolke, Bracewell, Samara, & Group, 2005; Stewart et al., 1999) and altered internalizing behaviors (anxiety/depression; Spittle et al., 2009) seen in preterm infants later in life. Further, preterm birth has been associated with a higher risk of psychiatric disorders such as non-affective psychosis, depressive disorder, attention deficit hyperactivity disorder, autism spectrum disorder, and alterations to the hypothalamic pituitary adrenal (HPA) axis (Grunau et al., 2007; Johnson et al., 2010; Nosarti et al., 2012).

Although previous research links preterm birth itself to various negative biological and behavioral outcomes (Aylward, 2005), it has been suggested that the impaired brain maturation and altered behavioral outcomes may be –at least partly- a product of environmental stressors such as pain exposure during early life (Anand & Hickey, 1987; Anand & Scalzo, 2000; Grunau, Holsti, & Peters, 2006). Neonates are exposed to a variety of stressors while in the neonatal intensive care

unit (NICU) including neonatal pain, decreased maternal care, altered auditory and light stimulation, mechanical ventilation, nursing procedures, and medical treatments. Further, preterm infants have a high risk for infections and other medical complications (Platt, 2014). It is conceivable that the multitude of stressors these infants are exposed to contributes to their biobehavioral development. In line with this idea, recent research has shown that increasing the number of stressors (light, sound, handling, or pain) present at any given time typically produces an increase in heart rate, respiratory rate, oxygen saturation, and facial grimacing (Peng et al., 2009), all of which are symptoms of neonatal distress. Further, Holsti, Weinberg, Whitfield, and Grunau (2007) demonstrated that behavioral responses to pain are heightened in preterm neonates after clustered routine nursing interventions. More recently, several studies provided evidence for a direct link between the exposure to painful procedures and altered brain and behavioral development (Brummelte et al., 2012; Ranger et al., 2013).

Preterm infants usually experience less maternal care while they are in the NICU or special care nursery than they would at home. Interestingly, previous animal studies have shown a potential modulatory role of maternal care on the stress of neonatal pain (Blass, Shide, Zaw-Mon, & Sorrentino, 1995). Further, both neonatal pain and reduced maternal care promote HPA axis activation (Kuhn, Pauk, & Schanberg, 1990; Victoria, Karom, Eichenbaum, & Murphy, 2014) which may be exacerbated when neonates are exposed to both stressors simultaneously. These findings suggest that neonatal pain and reduced maternal care may share a common biological mechanism responsible to produce their deleterious effect. However, very few studies exist examining the mechanisms of neonatal pain and reduced maternal care, let alone the combination effect of experiencing both stressors simultaneously. Thus, the current dissertation will examine

the role of the HPA axis in the production of cognitive impairment and altered affective behaviors in adulthood, following neonatal exposure to neonatal pain and/or reduced maternal care.

Neonatal pain

Development of pain pathways and pain exposure

The biological system for the perception of pain in mammals is very complex and relies on input from both the peripheral and central nervous system (for review see Millan, 1999). From the spinal cord, perception of pain is carried in an ascending manner to many supraspinal regions including, but not limited to, the various nuclei of the thalamus and hypothalamus, sensory cortex, amygdala, insula, and various sections of the cingulate and prefrontal cortices (Brooks & Tracey, 2005). It is beyond the scope of this dissertation to review the full complexity of the pain system, but it is clear that pain is a multidimensional and multisensory modality and that it relies on many intact systems and components to produce its affective and sensory experience.

It was once widely believed that neonates were unable to perceive pain due to an underdeveloped neural system and as a consequence some surgeries were performed on neonates without analgesia (Rodkey & Pillai Riddell, 2013). Fortunately, this belief has since been discredited through neuroanatomical and behavioral studies providing evidence for early development of pain pathways (Anand & Hickey, 1987). In rodents, the afferent neurons responsible for relaying the detection of noxious stimuli innervate the dorsal horn as early as embryonic day 14 (Jackman & Fitzgerald, 2000). In humans, the afferents from the dorsal horn that project to the spinal cord develop between the 8th and 19th week of gestation (Konstantinidou, Silos-Santiago, Flaris, & Snider, 1995). Even though peripheral systems may be in place to perceive noxious stimuli at a very early stage, it has been argued that pain cannot be fully perceived until afferent projections from the thalamus have reached their destined targets. Upon the departure

of the subplate zone, neurons innervate their target and allow the connections from the thalamus to be complete as early as 28 weeks gestation (Kostovic & Goldman-Rakic, 1983), though painful procedures can elicit activation of the somatosensory cortex as early as 25 weeks gestation (Bartocci, Bergqvist, Lagercrantz, & Anand, 2006; Slater, Boyd, Meek, & Fitzgerald, 2006). Behavioral findings further support the foregoing neuroanatomical evidence of pain perception in neonates, as even very young preterm infants show heightened levels of facial grimacing, crying, and full body reflexive withdrawal in response to a painful stimulus (Abdulkader, Freer, Garry, Fleetwood-Walker, & McIntosh, 2008; Johnston, Stevens, Yang, & Horton, 1995; Stevens, Johnston, & Horton, 1994). In summary, these findings demonstrate that preterm infants have the proper neuroanatomical systems in place to perceive pain as well as the behavioral tools to respond to painful stimuli.

Although it has been clearly demonstrated that preterm infants are capable of perceiving pain, they are still subjected to many painful medical procedures while in the NICU without any pain management. In fact, a recent study in 430 neonates from France revealed that ~80% of painful procedures were performed without specific analgesia (Carbajal et al., 2008). Preterm infants receive a median of 10 painful procedures per day during a typical stay in the NICU with suctioning and heel stick for medically indicated blood sampling being the most prevalent (Carbajal et al., 2008; Simons et al., 2003). Clearly, many of these procedures are necessary to ensure their survival and can thus not be completely avoided but we must start thinking about how to better control or manage the pain experience of these vulnerable infants.

Neurodevelopmental consequences of neonatal pain on the HPA axis

The study of both acute and long-term biobehavioral consequences of preterm pain exposure is relatively new, though it has been suggested long ago that the early pain exposure may

have deleterious effects on the developing brain and thus contribute to the altered outcomes recorded in preterm infants (Anand & Scalzo, 2000; Grunau et al., 2006). One well-documented consequence of early pain exposure is alterations in pain sensitivity later in life which leads to a hyposensitivity to acute pain but a hypersensitivity to prolonged pain later in life (for an excellent review on this topic see Schwaller & Fitzgerald, 2014). A similar bi-directional effect has also been suggested for the HPA axis response (Victoria, Karom, & Murphy, 2015), however less is known about the long-term effects of pain exposure on the long-term regulation of the HPA axis.

Premature infant's cortisol response to neonatal pain has yielded inconsistent results. For instance, an increase in cortisol levels as well as no change in cortisol levels have both been observed following a painful procedure (Cignacco, Denhaerynck, Nelle, Buhrer, & Engberg, 2009; Cong, Ludington-Hoe, & Walsh, 2011; Herrington, Olomu, & Geller, 2004; Magnano, Gardner, & Karmel, 1992). The differences in cortisol response may be due to their previous exposure to pain (Grunau et al., 2005) and it is conceivable that the multitude of painful procedures in the NICU may contribute to an altered programming of the HPA axis. In fact, a study conducted by Grunau et al. (2005) investigated preterm infant's HPA axis response to the painful heel stick procedure in the NICU and found that younger preterm infants (≤ 28 weeks gestation) who had experienced a higher number of skin-breaking procedures in the NICU in the past displayed a decreased cortisol response to a painful stimuli at 32 weeks corrected gestational age (CGA) compared to older preterm infants (29-32 weeks gestation) who had experienced less painful procedures. At school age and early adolescence, baseline saliva cortisol levels and cortisol response to a cognitive task or stressor did not differ significantly between preterm and full-term children (Brummelte et al., 2015; Buske-Kirschbaum et al., 2007) though Grunau et al. (2013) found lower cortisol levels in hair samples, a more chronic measure of HPA axis activity, of

preterm children at school age compared to full-term children. Importantly, in two of these studies (Brummelte et al., 2015; Grunau et al., 2013), higher numbers of skin-breaking procedures were associated with lower cortisol levels in preterm infants. In particular, infants with the highest number of skin-breaking procedures had lower saliva cortisol during the lab visit for cognitive testing and in the early morning of their diurnal pattern compared to preterm children that received fewer painful procedures (Brummelte et al., 2015). The number of painful procedures that neonates are exposed to during their time in the NICU varies dramatically (i.e. one study reported a range of 4-613 procedures per infant; Carbajal et al., 2008) and this may contribute to the complexity of reported results. Taken together these findings suggest that the simple comparisons of preterm and full-term populations may not be very meaningful and that one needs to take previous pain exposure of preterm infants into consideration when analyzing their HPA axis function. It is possible, that only the cumulative exposure to painful procedures may result in reprogramming of the HPA axis and thus the observed down-regulation in response to subsequent stressors and painful procedures. Clearly, more research is needed to elucidate the relationship between neonatal pain and the HPA axis as there seems to be a multifaceted relationship between severity, gender, and age of pain exposure and HPA axis response.

Intuitively, one might think that more pain exposure should lead to higher cortisol levels, however, as described above, higher number of skin-breaking procedures were generally associated with down-regulation of HPA axis activity. This chronic down-regulation after constant or intense activation is in line with what is typically seen in people suffering from post-traumatic stress disorder (PTSD), i.e. lower baseline levels of cortisol in the absence of stress (Wahbeh & Oken, 2013). In fact, it has even been suggested that early life pain experiences may increase the vulnerability to develop PTSD later in life (Gold, Kant, & Kim, 2008). Further, animal and human

studies have repeatedly shown that early life stress can result in altered HPA axis programming (Essex et al., 2011; Maccari, Krugers, Morley-Fletcher, Szyf, & Brunton, 2014) and, depending on the type and time of the stressor, result in lower adult glucocorticoid levels. Thus, it is not surprising that the exposure to early life pain should have a down-regulating effect on the HPA axis. However, one has to keep in mind that the maintenance of homeostasis requires activation of a complex range of responses involving the endocrine, nervous, and immune systems (Smith & Vale, 2006) and thus adaptions may go beyond the down-regulation of the HPA axis.

Further evidence for a pain-induced change in HPA axis programming comes from animal models that have been constructed to mimic neonatal painful procedures experienced by premature infants in the NICU. These models include repetitive paw needle sticks (Anand, Coskun, Thrivikraman, Nemeroff, & Plotsky, 1999), injection of inflammatory agents (formalin, carrageenan, and complete Freund's adjuvant; Anand et al., 2007; Laprairie & Murphy, 2009), hind paw incision (Young, Baumbauer, Hillyer, & Joynes, 2007), and nerve injury (Perez et al., 2013). Neonatal rat pups display a significant increase in corticosterone release when compared to handled controls (Victoria et al., 2014) 24 hours and 7 days after a carrageenan injection. Interestingly, injured animals displayed decreased corticosterone levels at 48 hours post pain exposure. The increase in corticosterone 24 hours after pain exposure and the subsequent decrease in corticosterone 48 hours later suggests that the injection of the inflammatory agent may have triggered a down-regulation of HPA axis output in response to the pain.

Animal models further allow us to investigate potential underlying mechanisms of altered HPA axis function after early pain exposure. Victoria, Inoue, Young, and Murphy (2013a) found that adult rats that were previously exposed to neonatal pain had decreased binding levels of corticotrophin releasing factor receptors (CRFR) 1 in the basolateral amygdala and ventrolateral

periqueductal gray and increased binding levels of CRFR2 in the lateral septum and cortical amygdala. Further, these animals displayed decreased levels of the glucocorticoid receptor (GR) in the dorsal CA1 and ventral CA1 areas of the hippocampus and increased levels of GR in the paraventricular nucleus (PVN) of the hypothalamus. Further, adult rats that were previously exposed to a painful stimulus shortly after birth displayed decreased basal and stressed induced levels of corticotrophin releasing factor (CRF), vasopressin, and adrencorticotropic hormone (ACTH) when compared to controls (Anseloni et al., 2005). These changes may be a consequence of the early pain exposure and explain the altered HPA output as CRF, ACTH, and GRs are all directly involved in HPA regulations.

Neurodevelopmental consequences of neonatal pain on the brain and behavior

In addition to the above-mentioned changes in receptor levels in the hippocampus and hypothalamus, neonatal pain in rodent models also increased levels of cell death in the frontal cortex, temporal cortex, parietal cortex, amygdala, hippocampus, and hypothalamus (Anand et al., 2007; Duhrsen et al., 2013; Rovnaghi, Garg, Hall, Bhutta, & Anand, 2008). Further, neonatal painful procedures have been linked to an increase in total glial fibrillary acidic protein (marker of glial activation) and c-Fos activation in multiple brain regions (Anand et al., 2007). In line with these findings, neonatal formalin injections produced a decrease in total caspase-3 protein but in no change in BAX or Bcl-2 proteins in female rats, which suggests that the increased cell death in pain-exposed pups could be facilitated by non-apoptotic pathways (Rovnaghi et al., 2008). More recently, human studies could add evidence to the hypothesis of pain-induced brain alterations. Brummelte et al. (2012) found that a higher number of skin-breaking procedures were associated with reduced white and gray matter maturation in preterm infants at term equivalent age. In line with this, Smith et al. (2011) found that exposure to a greater number of stressors, including painful

procedures, was associated with decreased frontal and parietal brain width, altered diffusion measures and functional connectivity in the temporal lobes, and abnormalities in motor behavior on neurobehavioral examination in newborn premature infants. Moreover, neonatal pain has been associated with impaired development of the corticospinal tract of preterm infants (Zwicker et al., 2013) and decreased head circumference (Vinall et al., 2012). In school-aged preterm infants, higher number of painful procedures predicted decreased cortical thickness in the frontal and parietal lobes, and reduced cerebellar volumes (Ranger et al., 2013; Ranger et al., 2015). Further, in school-aged children who were extremely low gestational age (≤ 28 weeks) preterm infants, neonatal procedural pain was associated with altered background cortical rhythmicity (Doesburg et al., 2013). Collectively, these recent studies suggest a link between exposure to neonatal pain and impaired brain development in preterm infants, however the exact mechanism behind this remains to be investigated.

Previously, white matter injury in preterm infants has been attributed primarily to impaired myelination due to diminished differentiation of precursor oligodendrocytes (Volpe, Kinney, Jensen, & Rosenberg, 2011). However, recent diffusion tensor imaging findings point to a pain-associated impairment in axonal development as the water diffusion measure that is believed to reflect the integrity of the axon and its internal components (Song et al., 2002), was most affected (Brummelte et al., 2012). Further, these axonal changes are believed to be secondary to impaired development of subcortical neurons, i.e. fewer or impaired neurons would explain the white matter changes, as damaged neurons would have no or less axonal connections. The above mentioned studies provide the first human evidence for the hypothesis that neonatal pain experience may lead to physical damage or even death of young neurons in the brain (Grunau et al., 2006). A potential mechanism explaining the reduced brain maturation in pain-exposed infants may be that high

stimulation of physiological immature neurons may lead to overstimulation and excitotoxic damage (Anand & Scalzo, 2000). In an electroencephalogram (EEG) study, Slater et al. (2010) showed that preterm infants had significantly larger neuronal activity in response to a noxious procedure compared to age-matched term infants. This may be an indicator of increased activation of vulnerable and immature neurons that are susceptible to overstimulation and excitotoxic damage or it may present a sensitization of the preterm infants' pain-response system. Either way, pain-induced overstimulation may thus lead to neuronal damage, possibly due to excessive glutamate or calcium release and subsequent excitotoxicity. In line with this idea of neuronal impairment, a study in asphyxiated term infants showed that tissue-damaging procedures in the first days of life were associated with altered brain metabolites on magnetic resonance spectroscopic imagining (MRSI) on day 4 (Angeles et al., 2007). In particular, the study found a significant correlation between painful procedures and glutamate/glutamine and creatine ratios in certain brain areas that are suggestive of brain injury and may reflect the vulnerability of the neonatal brain to excessive neuronal stimulation (Angeles et al., 2007).

The altered HPA axis activity in preterm infants as discussed above may contribute to these physiological alterations and the impaired brain development, as evidence from animal studies suggests an interaction between glucocorticoids and the glutamatergic system that could contribute to the pain-induced overstimulation and subsequent neuronal damage. Glucocorticoid receptor activation increases surface expression of the glutamatergic NMDA receptor 2A (NR2A) and 2B (NR2B) and NMDA mediated excitatory postsynaptic currents (Yuen et al., 2009; Yuen et al., 2011). Further, previous results have demonstrated that glucocorticoids inhibit the uptake of glutamate in hippocampal astrocytes (Virgin et al., 1991) and increase the readily releasable pool of glutamate vesicles into synaptic terminals (Treccani et al., 2014). Thus, pain-evoked increases

in glucocorticoids can result in increased glutamate levels which in turn can be neurotoxic in neonatal neuronal tissue. For example, in neonatal cultured hippocampal neurons, administration of glutamate produced a significant increase in cell death when compared to controls (Hilton, Nunez, Bambrick, Thompson, & McCarthy, 2006). Moreover, when looking at the direct interaction between the two systems, blockade of glutamate receptors using MK-801 (NMDA receptor antagonist) in conjunction with dexamethasone administration (glucocorticoid receptor agonist) results in decreased cell death when compared to administration of dexamethasone alone (Lu, Goula, Sousa, & Almeida, 2003; Zhu, Wang, & Bissette, 2006) suggesting a direct interaction between the two systems in regard to cell death. Taken together, these results indicate that acute activation of the HPA axis by neonatal pain may result in an increase in circulating glucocorticoids which in turn modulates and enhances the release of glutamate resulting in neurotoxicity. This hypothesis of glutamate mediated cell death following neonatal pain is supported by the results from Anand et al. (2007) in which neonatal pups were exposed to both neonatal pain (formalin injection) and the glutamate NMDA receptor antagonist ketamine. Pups that received ketamine and formalin had significantly lower levels of cell death in the cortex, amygdala, and hypothalamus when compared to pups that received formalin only. Further, ketamine prevented some of the formalin-induced learning impairments (Anand et al., 2007). Taken together, animal and human studies suggest that the brain may be particularly vulnerable to pain-induced excitation and overstimulation that may result in neuronal damage during a time when neural networks are highly immature and thalamo-cortical connections are waiting in the subplate zone (Brummelte et al., 2012; Kostovic & Judas, 2010).

Alterations to neurodevelopment following early life pain exposure may account for adverse behavioral outcomes later in life. A study conducted by Grunau et al. (2009) investigated

the impact of neonatal pain on cognitive and motor outcomes in preterm infants. At 8 and 18 months CGA, the number of skin-breaking procedures was a significant predictor of cognitive and motor development with a higher number of skin breaks predicting poorer cognitive and motor development. In school-aged children, poorer cognitive outcomes and lower IQ scores were associated with higher number of skin-breaking procedures (Ranger et al., 2015; Vinall et al., 2014). Although there is a lack of longitudinal human studies investigating the cognitive impact of neonatal pain exposure later in life, rodent models suggest that these cognitive impairments persist well into adulthood. Indeed, adult animals that are exposed to early life inflammatory pain exhibit cognitive deficits in the radial arm maze and Morris water maze (Anand et al., 2007; Henderson, Victoria, Inoue, Murphy, & Parent, 2015; Rovnaghi et al., 2008).

Studies also found an association between exposure to neonatal pain and affective behaviors. For instance, Vinall, Miller, Synnes, and Grunau (2013) examined neonatal pain exposure on internalizing behaviors and found that at 18 months CGA, the greater number of skin-breaking procedures was a significant predictor of poorer (higher) internalizing behavioral scores. Consistent with previous findings at 18 months CGA, former preterm school-aged children exhibited issues with internalizing behaviors that were predicted by higher levels of neonatal pain (Ranger, Synnes, Vinall, & Grunau, 2014). Interestingly, animals exposed to early life pain displayed reduced anxiety-like behavior (Anseloni et al., 2005; Victoria, Inoue, Young, & Murphy, 2013b; Victoria et al., 2015) and reduced depressive-like behavior (Anseloni et al., 2005) compared to controls. While these outcomes may seem positive, they could also be viewed as maladaptive behaviors. Rodents that spend more time in the open areas of the open field test or elevated plus maze test (indicative of 'reduced anxiety-like behavior'), spend more time in the

'risky' areas of the arenas. These behaviors, in the wild, could lead to an early death through a predator.

It is important to note that children that were born preterm often present with lower cognitive skills such as language acquisition or mathematical skills (Sansavini et al., 2010; Simms et al., 2013) and display higher internalizing behaviors (Spittle et al., 2009) compared to their full-term peers. However, the link between a higher number of painful procedures and brain development and thus cognitive and behavioral outcomes was only established recently. This association suggests that we may be able to improve the cognitive and emotional outcomes of children born prematurely by improving their early environmental conditions. If lower cognitive skills or behavioral problems in children born preterm are not due to the preterm birth per se, but rather a consequence of the early adverse environment, we may be able to improve their mental health and biobehavioral outcome by improving their NICU conditions and reducing painful procedures. Though more research is needed to establish which factors are essential for impacting long-term development, it is an exciting opportunity to try to help these vulnerable children thrive.

Maternal Care

Reduced maternal care in the NICU

Depending on the gestational age and medical condition, many preterm infants spend several weeks or even months in the hospital after being born (Kowlessar et al., 2013). During this time they are usually kept in neonatal incubators that try to optimize many of the environmental factors, such as temperature and humidity (Antonucci, Porcella, & Fanos, 2009) to promote survival and typical development (Buetow & Klein, 1964). The first case of using an incubator for the care of a premature infant was documented in 1857 (Cone, 1981) and though they have significantly improved since then, they still cannot mimic the conditions in the womb perfectly

and they cannot make up for the lack of maternal touch during this time. Further, there is still a dearth of knowledge of what comprises an 'optimal' environment for a preterm infant. For instance, it has been suggested that the noisy and unnatural conditions of the NICU with recurrent high-frequency sounds and beeps constitutes a trauma to the auditory system though the developmental implications of this remain largely unstudied (Lahav & Skoe, 2014). In line with this, not much is known about the effects of parental touch and skin-to-skin care on the long-term developmental outcome of preterm infants. Though a fetus *in utero* would obviously not be exposed to maternal touch, the uterine environment does provide a soft and warm habitat with boundaries that protects the fetus from noxious stimuli. Thus, it is not surprising that it is believed that the maternal presence and touch is beneficial to the development of preterm infants (Conde-Agudelo, Diaz-Rossello, & Belizan, 2003; Flacking et al., 2012). In fact, a Cochrane study found that skin-to-skin care reduced the behavioral and physiological indicators of pain in neonates in most studies, but it concluded that more research is needed on the optimal duration of the maternal touch as well as on the effects in different gestational age groups and the long-term effects (Johnston et al., 2014).

Obviously, preterm infants usually experience reduced maternal care and feedings compared to full-term infants due to the circumstances of their NICU stay and the fact that mothers usually cannot stay in the hospital beyond a few days after giving birth. One issue with implementing maternal skin-to-skin care in the standard NICU setting is that parents display decreased time visiting the NICU as the infant's duration within the hospital increases (Reynolds et al., 2013). Additional barriers to frequent visitations are associated with lower gestational age, if the infant has siblings, and distance from the hospital (Latva, Lehtonen, Salmelin, & Tamminen, 2007). Moreover, many medical procedures and apparatuses make it difficult for the parents to

hold their newborn infant even while they are visiting (e.g. if the infant needs treatment for jaundice using the bilirubin blanket).

Another obstacle in enhancing maternal care is the fact that preterm infants can be less responsive to maternal cues due to their underdeveloped and immature systems compared to full-term infants (Macey, Harmon, & Easterbrooks, 1987). This lack of response from the infant and the perceived vulnerability can be challenging or stressful for the parent and can affect the mother-infant interactions (Horwitz et al., 2015). Impairments in mother-infant interactions are associated with adverse child outcomes. For instance, difficulties in noticing infant signs of interest and supporting their engagement with the environment is associated with poor cognitive development (Brummelte & Galea, 2016). Thus, preterm infants who suffer from reduced maternal care in their early days may further be exposed to impaired interactions later in life that may be a consequence of interruptions in early bonding. Taken together, there are multiple circumstances that are responsible for the experience of reduced opportunities for maternal care by preterm infants. Considering the reported positive outcomes concerning survival rate, pain response, brain maturation, HPA axis activity, and cognitive functioning following enhanced maternal care (Cong et al., 2011; Corbo et al., 2000; Feldman, Rosenthal, & Eidelman, 2014; Golianu, Krane, Seybold, Almgren, & Anand, 2007; Gray, Watt, & Blass, 2000; Jain, Kumar, & McMillan, 2006; Johnston et al., 2014; Johnston et al., 2008; Johnston et al., 2003; Scher et al., 2009; Schneider, Charpak, Ruiz-Pelaez, & Tessier, 2012), it may be worth trying to facilitate longer visitations in the NICU.

Maternal care and HPA axis

Infants are sensitive to the maternal presence and can react with increased stress hormone levels in response to prolonged separation (Kuhn et al., 1990; McCormick, Kehoe, & Kovacs, 1998; Nishi, Horii-Hayashi, & Sasagawa, 2014). For instance, Larson, Gunnar, and Hertsgaard

(1991) found that when 9-month-old infants were separated from their mothers for 30 minutes, they expressed elevated levels of cortisol compared to when they had 30 minutes of playtime with their mothers. Similarly, infant squirrel and rhesus monkeys express elevated cortisol levels and norepinephrine metabolites when separated from their mothers for 30 minutes (Mendoza, Smotherman, Miner, Kaplan, & Levine, 1978). In rodents, one can investigate the specific effects of reduced maternal care as researchers can manipulate maternal care levels by inducing maternal deprivation for a certain period of time (ranging from 30 mins to 24hr/day for several days) or by using the natural variance in animal nursing and licking behavior (Kuhn et al., 1990; Liu et al., 1997). For instance, maternal separation in rodents leads to increases in circulating corticosterone, ACTH, epinephrine, and norepinephrine (Eghbal-Ahmadi, Hatalski, Avishai-Eliner, & Baram, 1997; Hennessy, Tamborski, Schiml, & Lucot, 1989; Huot, Plotsky, Lenox, & McNamara, 2002; Kuhn et al., 1990; McCormick et al., 1998; Viau, Sharma, & Meaney, 1996), but decreased CRF in the median eminence and circulating corticosteroid-binding globulin (Pihoker, Owens, Kuhn, Schanberg, & Nemeroff, 1993; Viau et al., 1996). These changes in HPA axis related hormones coincide with alterations in receptor subtypes within the HPA axis: 24 hours of maternal separation results in a decrease in the glucocorticoid receptor mRNA within the hippocampus, PVN, and frontal cortex as well as a decrease in CRFR2 in the hypothalamic ventromedial nucleus (Avishai-Eliner, Hatalski, Tabachnik, Eghbal-Ahmadi, & Baram, 1999; Eghbal-Ahmadi et al., 1997). Maternal separation may also prime the HPA axis to respond differently to further stressors as 9 and 12 day old rat pups display a significantly greater ACTH and corticosterone response to a saline injection when compared to non-deprived animals (Suchecki, Nelson, Van Oers, & Levine, 1995). A similar response is still seen in adult rodents that were separated from their dams as pups, i.e. an increase in ACTH and corticosterone in response to an acute stressor (Huot et al., 2002).

Further, adult maternally-separated rats displayed higher levels of CRFR1 in the hypothalamus compared to non-separated rats under basal conditions and higher levels of CRFR1 or CRFR2 in the prefrontal cortex, hippocampus, and amygdala after an acute stressor (O'Malley, Dinan, & Cryan, 2011). In adult maternally-separated rats, CRF binding was significantly decreased in the anterior pituitary but significantly increased in the raphe nuclei, parabrachial nucleus, and median eminence compared to controls (Ladd, Owens, & Nemeroff, 1996). These results emphasize the idea that the experience of reduced maternal care early in life may permanently re-program the HPA axis function. It is feasible that these consequences are due to repetitive or chronic elevations in corticosterone exposure in response to the maternal separation, as studies of chronic early stress exposure result in similar HPA axis modifications (e.g. Isgor, Kabbaj, Akil, & Watson, 2004; Romeo et al., 2006). Further, these results suggest that maternal separation, much like neonatal pain, may re-program the HPA axis through changes to the glucocorticoid, CRF, and ACTH secretion.

Aside from the stress induced by maternal separation, it appears that the quality of the care, i.e. the relationship or interaction between the infant and parent, also moderates the stress response. For instance, infants who have a higher quality of maternal care (as measured by the Aninsworth's Maternal Sensitivity Scale) display lower cortisol response to free play, and a faster cortisol recovery when faced with a stressor (Albers, Riksen-Walraven, Sweep, & de Weerth, 2008; Spangler, Schieche, Ilg, Maier, & Ackermann, 1994). In rodents, offspring of dams who express low levels of licking and grooming (considered lower quality of maternal care) have elevated levels of basal corticosterone and CRF in adulthood when compared to offspring of high-licking and grooming mothers (Champagne et al., 2008; Liu et al., 1997) and display immediate and prolonged increases of both corticosterone and ACTH in response to an acute stressor (Liu et al., 1997).

However, under stressful conditions, low-licking offspring showed better learning and enhanced long-term potentiation (LTP) compared to high-licking offspring (Champagne et al., 2008), suggesting that the quality of maternal care 'transmitted' epigenetic modifications for adapting to their environment. In other words, the offspring may gather information on the quality of the environmental surroundings based on the amount or quality of maternal care they receive. For instance, during challenging conditions such as reduced food availability, a dam would spend more time gathering food than nursing and licking her offspring. This reduced maternal care could lead to HPA axis programming in the offspring that will make them better prepared/adapted for survival under challenging conditions. In line with the HPA axis reprogramming hypothesis, adult rats of low-licking and grooming dams revealed a decrease of glucocorticoid receptor protein in the hippocampus when compared to adult rats of high-licking and grooming dams (Champagne et al., 2008; Liu et al., 1997). These results show that not just the amount but also the quality of maternal care may play a modulatory role in the development of the HPA axis.

In humans, McGowan et al. (2009) found that individuals who had a history of child abuse displayed significantly lower levels of glucocorticoid receptor mRNA in the hippocampus compared to individuals with no history of child abuse. Though child abuse may not be equivalent to reduced maternal touch and involvement in care, this is the first study of its kind showing a connection between early life stress and epigenetic changes in the human brain, in particular in the promoter region of the glucocorticoid receptor gene, suggesting an experience-based element in the alterations observed in the mRNA expression. Further, a study by Oberlander et al. (2008) showed that exposure to prenatal maternal depression, which is associated with impaired mother-infant interactions (Brummelte & Galea, 2016), increased the methylation of the glucocorticoid receptor gene NR3C1. This in turn was associated with increased cortisol stress responses in 3-

month-old infants (Oberlander et al., 2008), further highlighting a potential link between maternal care, epigenetic changes, and HPA axis responses in humans. Taken together, these results suggest that maternal care plays a vital role in the development of a typical HPA axis and reduced maternal care may lead to altered HPA axis development.

Neurodevelopmental consequences of reduced maternal care

The early life stress of child neglect or maternal separation are believed to produce long-term structural changes in the brain and alter the behavioral and cognitive outcome of a child (De Bellis, 2005). However, in humans it is difficult to define or quantify 'reduced maternal care'. One option is to look at children that experienced institutional deprivation as a form of reduced parental care. These children display decreased total grey and white matter volumes but increased amygdala volumes (Mehta et al., 2009; Tottenham et al., 2010). Additionally, institutional deprivation has been associated with accelerations in the maturation of the amygdala-mPFC connection, which was mediated by salivary cortisol levels (Gee et al., 2013). Further, the volumes of the CA3 and dentate gyrus subfields of the hippocampus are reduced in adults who reported higher levels of adverse childhood experiences (Teicher, Anderson, & Polcari, 2012). In line with these neuroanatomical findings, children who experienced maltreatment and had been placed in foster care had lower full-scale IQ scores when compared to a matched control group (Viezel, Freer, & Lowell, 2015). Moreover, childhood maltreatment has been associated with PTSD, oppositional disorder, and major depressive disorder (Famularo, Kinscherff, & Fenton, 1992; Kaufman, 1991). Although the subjects from these studies may have experienced more drastic events than simply reduced maternal care, they indicate that parental care may influence neurodevelopment and cognitive outcome in humans.

In rodents, studies using 24-hour maternal separation found that neonatal rat pups display a decrease in hippocampal volume, a decrease in prefrontal cortex and motor cortex thickness, and an increase in apoptotic cells in various cortices, hippocampus, and cerebellar cortex (Aksic et al., 2013; Zhang et al., 2002). Further, adult rats that experienced 3 hour daily maternal separation from postnatal days 2-14 had decreased mossy fiber density in the hippocampus but no changes in overall hippocampal volume (Huot et al., 2002), suggesting that the impact on the hippocampus may depend on the duration or degree of reduced maternal care. It is conceivable that these neuroanatomical findings are likely caused by the increased glucocorticoid release during periods of maternal separation. As previously mentioned, glucocorticoids stimulate the release of glutamate, as well as increase glutamate receptor subtypes and post synaptic firing (Moghaddam, Bolinao, Stein-Behrens, & Sapolsky, 1994; Yuen et al., 2009; Yuen et al., 2011). Interestingly, maternal separation and reduced maternal care (i.e. low-licking and grooming) have produced different effects on the interaction between glutamate and stress. For instance, adult rats that experienced maternal deprivation display an increase in hippocampal glutamate NR1, NR2A, and GluR4 receptor subtypes (Martisova et al., 2012). Further, application of corticosterone in hippocampal slices results in elevated glutamate release in maternally deprived animals when compared to non-deprived animals (Martisova et al., 2012). Conversely, the down-regulation of glutamate receptors is observed on postnatal day 8 and in adulthood of offspring from low-licking and grooming dams (Liu, Diorio, Day, Francis, & Meaney, 2000). These findings suggest that the glutamatergic system is very sensitive to changes in maternal care, however, further research is needed to clarify the exact relationship between reduced maternal care and the glutamatergic system development.

Similar to neonatal pain, reduced maternal care appears to have a negative impact on cognitive outcomes. For instance, Liu et al. (2000) found that animals of low-licking and grooming mothers displayed cognitive impairments during the Morris water maze probe trials by spending less time in the learned quadrant. Moreover, cognitive impairments in the novel object recognition task have been observed in low-licking and grooming and maternally-separated offspring (Aisa, Tordera, Lasheras, Del Rio, & Ramirez, 2007; Bredy, Humpartzoomian, Cain, & Meaney, 2003). Maternally-separated rat pups further display increased anxiety-like and depressive-like behaviors as indicated by lower amounts of time spent in the open arms in the elevated plus maze, increased fear of novel situations, and increased immobility in the forced swim test, respectively (Aisa et al., 2007; Daniels, Pietersen, Carstens, & Stein, 2004; Lee et al., 2007). Taken together, these findings suggest that early deprivation or a low level of maternal care has a profound impact on the neuroanatomical and behavioral profile of the offspring ranging from altered affective states to cognitive impairments.

Future directions for appropriate models

Although it is apparent that exposure to neonatal pain and reduced maternal care are experienced by preterm infants in the NICU and lead to many similar adverse outcomes, the direct interaction between the two stressors on biobehavioral development has yet to be investigated. This dearth of research is most likely due to the lack of animal models that appropriately mimic NICU settings. For instance, many neonatal pain models are centered on a single injection of an inflammatory agent (formalin and carrageenan) into the paw of neonatal rat pups (Anseloni et al., 2005; Bhutta et al., 2001) with the goal of producing chronic inflammatory pain. Though these models are effective in producing results, they lack the repetitive painful procedures observed in the NICU where preterm infants are exposed to a median of 10 painful procedures a day (Carbajal

et al., 2008), but not necessarily to inflammatory agents. When taking into consideration the large number of painful procedures preterm infants experience, it could be argued that animal models need to adopt an approach that utilizes a high number of painful procedures to better mimic NICU settings. In addition to neonatal pain models, current animal models of reduced maternal care do not appropriately mimic NICU settings which is most likely due to the fact that they were not created with this purpose in mind. Also, animal models of reduced maternal care differ dramatically in the duration of separation or the age of the offspring, and have thus produced inconsistent results (Lehmann & Feldon, 2000). To develop an appropriate model to investigate the interaction between neonatal pain and reduced maternal care, researchers must take the interaction of the two stressors into consideration. For example, in the first study of its kind, Walker, Kudreikis, Sherrard, and Johnston (2003) used thermal heat as a noxious stimulus and 15-minute acute maternal separation. Surprisingly, no deficits in behavior were observed, but the dams expressed significantly higher levels of maternal care when they were reunited with their pups. Thus, this study may be an indication that protective effects of maternal care can be observed in rodents and thus that they can be tested under appropriate conditions.

The biobehavioral impact of NICU stressors results in many adverse outcomes for the infant and it is often difficult to disentangle the impact of individual stressors as preterm infants are exposed to a set of adverse experiences. Though animal models may not be able to test all of the NICU stressors such as pain, infections, mechanical ventilation, lower maternal contact etc. simultaneously, it is important to keep in mind that these stressors may impact each other and result in worse outcomes than if they are experienced alone. Some of the observed outcomes of preterm infants such as impaired brain maturation, altered pain thresholds, altered stress response, and impaired cognitive functioning may be at least partly due to the experience of these early life

stressors rather than the preterm birth per se. Thus, it is important to continue to develop animal models that will help us to better understand the consequences of these stressors- individually and in combination- on the above-mentioned outcomes.

Activation of the HPA axis and subsequent release of glucocorticoids following neonatal pain exposure or reduced maternal care may be one mechanism explaining some of the negative outcomes, however more research is needed to determine the exact underlying molecular pathways. The beneficial outcomes observed after Kangaroo care, increased skin-to-skin contact between the neonate and mother during painful procedures, indicates a modulatory role of maternal care on neonatal pain, which may be produced by dampening the stress response to pain exposure. The goal of this dissertation is to develop a model that mimics neonatal pain and reduced maternal care experienced in today's NICU. To do so, we will adopt the repetitive needle poke model of pain, as it closely resembles one of the most prevalent painful procedure in the NICU the heel stick. For reduced maternal care, we will create a novel model utilizing a tea-ball infuser, which will prevent skin-to-skin contact/comforting from the dam to the pup after pain exposure but allow for the dam and pup to be in the same environment. Using these models, we will examine the role of the HPA axis and various brain metabolites, specifically amino acids, as a potential mechanism for the adverse outcomes of reduced maternal care and neonatal pain. Moreover, to further characterize our model of NICU pain and reduced maternal care, we will investigate the long-term consequences of both stressors in adult cognitive functioning, affective behaviors, and HPA axis reactivity.

CHAPTER 2: RESEARCH QUESTIONS/HYPOTHESES

Based off the literature investigating neonatal pain and reduced maternal care, it is apparent that both lead to acute and long-term developmental consequences. Currently, the mechanism by which these stressors produce developmental deficits remains unknown. In an attempt to uncover the biological mechanisms and long-term behavioral consequences following neonatal pain and reduced maternal care the following research questions and hypotheses were generated:

1) Are tea-ball infusers a valid model of reduced maternal care that closely mimics maternal isolation seen in today's NICU?

Hypothesis: Pups placed in tea-ball infusers will experience reduced maternal care and maternal behaviors expressed to non-tea-ball pups will not be altered due to the presence of tea-balls in home-cages.

2) Do repetitive needle pokes and maternal isolation produce a stress response in neonatal rat pups?

Hypothesis: Both pain exposure and maternal isolation will result in an increase serum corticosterone during the stress hypo-responsive period. Moreover, the combination of both stressors will produce an exacerbated increase in corticosterone.

3) Are there acute changes in frontal cortex and hippocampus metabolites, specifically amino acids, due to pain exposure or maternal isolation?

Hypothesis: In the frontal cortex and hippocampus, pain and maternal isolation will alter glutamate, glutamine, and GABA. Further, both stressors will produce a decrease in N-acetylaspartate in the same regions. Additionally, alterations in glutamate, glutamine, and GABA and decreases in N-acetylaspartate will be more drastic in pups that experience both stressors simultaneously.

4) Do repetitive needle pokes and reduced maternal care alter cell proliferation in the frontal cortex, medial prefrontal cortex, or dentate gyrus of the hippocampus?

Hypothesis: Both stressors will result in decreased cell proliferation in the frontal cortex, medial prefrontal cortex and dentate gyrus of the hippocampus. Pups that experience both neonatal pain and maternal isolation will have exacerbated reductions in cell proliferation.

5) How are long-term affective behaviors and stress-reactivity impacted by neonatal pain and maternal isolation?

Hypothesis: For anxiety-like behaviors and HPA axis reactivity, it is believed that pain and maternal isolation will result in opposing phenotypes. Pain exposure will result in a decreased in anxiety-like behaviors and blunted stress response. Maternal isolation will produce an increase in anxiety-like behaviors and an increased stress response. Due to the expected opposing outcomes for both pain and maternal isolation, the hypothesized outcome for animals experiencing both is unknown.

6) What are the long-term cognitive consequences of neonatal pain and maternal isolation?

Hypothesis: Pain and maternal isolation will produce cognitive deficits in spatial learning and long-term memory, reference memory, and cognitive flexibility. Deficits in cognitive functioning will be exacerbated in pups experiencing both stressors.

CHAPTER 3: ACUTE BRAIN AND ENDOCRINE RESPONSES TO NEONATAL PAIN AND MATERNAL ISOLATION (HYPOTHESES: 1, 2, 3, & 4)

Introduction

Premature births account for 11.1% of births worldwide (Blencowe et al., 2012). Unfortunately, children born preterm experience heightened levels of biobehavioral problems including cognitive delays and impaired development (Anderson, Doyle, & Victorian Infant Collaborative Study, 2003; Bhutta, Cleves, Casey, Cradock, & Anand, 2002; Peterson et al., 2000). Until recently, it was believed that these adverse outcomes were solely attributable to being born premature, however, new research suggests that some of these adverse outcomes may be, at least in part, due to environmental conditions that preterm infants are exposed to during the perinatal period (Brummelte et al., 2012; Cong et al., 2017; Grunau et al., 2009; Ranger et al., 2013; Vinall et al., 2014). In the neonatal intensive care unit (NICU), premature neonates experience a multitude of stressors including pain, reduced maternal contact, auditory stimuli, and excessive lighting (Cong et al., 2017; Konig, Stock, & Jarvis, 2013; Newnham, Inder, & Milgrom, 2009). Previous research has established a link between exposure to NICU stressors and changes in heart rate, oxygen saturation, and facial grimacing (Peng et al., 2009), all of which are signs of neonatal distress. Further, Smith et al. (2011) found an association between NICU stressors (including painful procedures) and brain development, further suggesting a role for the environment in the negative outcomes following premature birth.

While in the NICU, preterm infants experience an average of 16 invasive procedures a day (Carbajal et al., 2008), with setting an IV and heel lances being the most prevalent (Stevens et al., 2003). Near infrared spectroscopy recordings of cortical activity in preterm infants following noxious stimuli exposure indicate a functional pain system in neonates as early as 25 weeks gestation (Slater et al., 2006). Thus, it is not surprising that repetitive pain exposure during a critical

developmental period results in adverse neuronal outcomes. For example, in a study conducted by Brummelte et al. (2012), preterm infants with heightened levels of skin-breaking procedures exhibited lower levels of the neuronal biomarker N-acetylaspartate/choline ratio in subcortical gray matter when compared to preterm infants that had experienced lower levels of skin-breaking procedures, suggesting impaired brain maturation following increased early-life pain exposure. Further, in former preterm school-aged children, the number of skin-breaking procedures was a significant predictor of cortical thinning of the frontal and parietal lobes, which the authors suggest may be indicative of delayed neuronal growth or increased cell death (Ranger et al., 2013). A study conducted by Grunau et al. (2005) found that preterm infants (≤28 weeks gestation) who were exposed to high levels of painful procedures had a blunted cortisol response to a noxious stimulus at 32 weeks corrected gestational age compared to preterm infants exposed to low levels of painful procedures. This down-regulation of the hypothalamic-pituitary-adrenal (HPA) axis is also seen in former preterm school-aged children (Brummelte et al., 2015; Grunau et al., 2013) and suggests a physiological reorganization within the neuroendocrine system as a consequence of pain exposure.

Animal models of early-life pain have found increased cell death in the frontal cortex, temporal cortex, parietal cortex, amygdala, hippocampus, and hypothalamus in animals exposed to pain compared to controls (Anand et al., 2007; Duhrsen et al., 2013; Rovnaghi et al., 2008). Further, animals exposed to neonatal inflammatory pain exhibit cognitive deficits in the radial arm maze (Rovnaghi et al., 2008) and Morris water maze (Henderson et al., 2015). Although it is clear that neonatal pain produces profound impacts on neonatal development, the biological mechanisms responsible for producing the observed deleterious effects are currently unknown. It has been suggested that neonatal pain is the result of overstimulation via excitatory amino acids

(Anand & Scalzo, 2000), a hypothesis that has gained support by observations of a protective effect produced by ketamine, a NMDA receptor antagonist, in conjunction with pain exposure (Anand et al., 2007). However, neurochemical changes within the glutamatergic system have yet to be documented following neonatal pain exposure. The HPA axis influences glutamatergic release in the brain with increased levels of glucocorticoids resulting in increased glutamate release in the hippocampus and frontal cortex (Abraham, Juhasz, Kekesi, & Kovacs, 1998; Moghaddam et al., 1994). Based off this interaction one may speculate that repetitive pain exposure in the NICU leads to elevated cortisol release, which in turn leads to excitotoxic levels of glutamate release in the immature neonatal brain. Although increased cortisol has been documented following neonatal pain (Victoria et al., 2014), the link between the HPA axis and glutamatergic signaling has not been established.

Preterm infants are also exposed to reduced maternal contact while in the NICU compared to infants that are cared for at home. Moreover, the longer the infant is required to stay in the NICU the more difficult it is for the parents to visit regularly (Latva et al., 2007). Maternal contact has a profound influence on the biobehavioral development of the neonate and skin-to skin contact between the infant and parent is encouraged as often as feasible while the infant remains in the NICU. Positive outcomes following Kangaoo care or skin-to-skin contact between the mother and the premature infant during painful procedures suggest an interaction between neonatal pain and maternal care (Conde-Agudelo et al., 2003; Hall & Kirsten, 2008; Johnston et al., 2008; Lawn, Mwansa-Kambafwile, Horta, Barros, & Cousens, 2010). Currently, the analgesic effect of maternal contact is poorly understood, however, recent research suggests that Kangaroo care suppresses the HPA axis response in the neonatal infant when challenged with a noxious stimulus (Cong et al., 2011).

Various animals have been utilized to elucidate the modulatory role of maternal care on development. In the rat pup, maternal care influences cognitive and emotional development (Bredy et al., 2003; Liu et al., 2000). For example, intermittent or prolonged periods of maternal separation during the neonatal period produces impaired brain development in the form of cell death (Zhang et al., 2002) and cognitive deficits seen in the Morris water maze and novel object recognition task (Aisa et al., 2007). The impact of maternal care on offspring development can be viewed as a selective evolutionary pressure on the mother, with higher environmental demands on the dam to be off the nest, resulting in specific developmental changes in the offspring to thrive in a rougher environment. It is believed that a portion of the developmental changes produced by reduced maternal care are driven by reprogramming of the HPA axis (for review see Caldji, Diorio, & Meaney, 2000). This hypothesis is supported by the observed increases in corticosterone and ACTH in rodent pups following maternal separation during the neonatal period (Hennessy et al., 1989; Kuhn et al., 1990; McCormick et al., 1998). Further, in adulthood, maternal separation as a neonate leads to increased basal and stress induced corticosterone levels when compared to normally reared animals (Aisa et al., 2007).

Interestingly, neonatal pups do not show a common increase in corticosterone in response to a foot shock pain during their stress-hyporesponsive period when their dam is present (Moriceau & Sullivan, 2006). This highlights the importance of maternal care and the maternal presence in regulating physiological responses to pain and stress in early life. Currently, preterm infants experience large amounts of painful procedures without maternal presence in the NICU. It is feasible that continuous exposure to pain in the absence of maternal contact accounts for impaired brain development in preterm populations, however, this relationship remains widely understudied in preclinical settings.

Thus, the present study sought to elucidate alterations within the HPA axis and glutamatergic signaling following two common NICU stressors: repetitive neonatal pain and reduced maternal care in a neonatal rat population. We will analyze serum corticosterone levels as well as certain amino acids and cell proliferation in the frontal cortex and hippocampus in response to neonatal pain and reduced maternal contact in our rodent model. Due to the modulatory role of maternal care on the HPA axis, we hypothesize that both neonatal pain and reduced maternal care will lead to increased circulating levels of corticosterone, and therefore elevated levels of glutamate and decreased cell proliferation. It is further hypothesized that increases in corticosterone and glutamate and reductions in cell proliferation will be exacerbated following simultaneous exposure to neonatal pain and reduced maternal care.

Methods

Animals

Seventeen Sprague-Dawley females and four Sprague-Dawley males were purchased from Charles River Laboratory (Portage MI, USA) and were mated to produce seventeen litters. On postnatal day (PD) one, litters were culled to 5 males and 5 females and were assigned to one of the following groups each: touch control, isolation, pain, and isolation and pain (described below). All animals were housed in 12:12-hour light:dark cycle controlled vivarium, with food and water available *ad libitum*.

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals revised in 1996, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University and can be provided upon request. All efforts were made to reduce the number of animals used and their suffering.

Groups and Procedures

Based on group assignment, pups in each litter were exposed to different early-life conditions. Maternal isolation pups were exposed to 30 minutes of maternal isolation via encapsulation in a tea-ball infuser (see Figure 1) four times a day from PD1 to PD4. During tea-ball encapsulation, pups were placed back into the nest allowing for olfactory cues, however dams were unable to provide direct maternal contact. Pups in the pain group were exposed to repetitive painful procedures four times day from PD1 to PD4. Painful procedures used in this study were modified from (Anand et al., 1999) and consisted of a 26-gauge needle rapidly inserted into the paws and were spread out over four sessions a day. To mimic NICU settings, painful procedures dissipated over the course of the first four days with 8 pokes on PD1 (2 per session), 6 pokes on PD2 (1-2 per session), 4 pokes on PD3 (1 per session), and 4 pokes on PD4. Isolation and pain pups experienced tea-ball encapsulation immediately following pain exposure. Touch control pups experienced tactile stimulation via a cotton swab on the paws. These procedures were carried out at 8:00, 9:30, 11:00, and 13:30, which allowed time for maternally isolated pups to nurse in between each session. As no more than one male and female per group was used from each litter, we had 17 males and 17 females per group with pups from n= 9 litters being sacrificed on PD4 and pups from n=8 litters being sacrificed on PD 8.

Experimental timeline

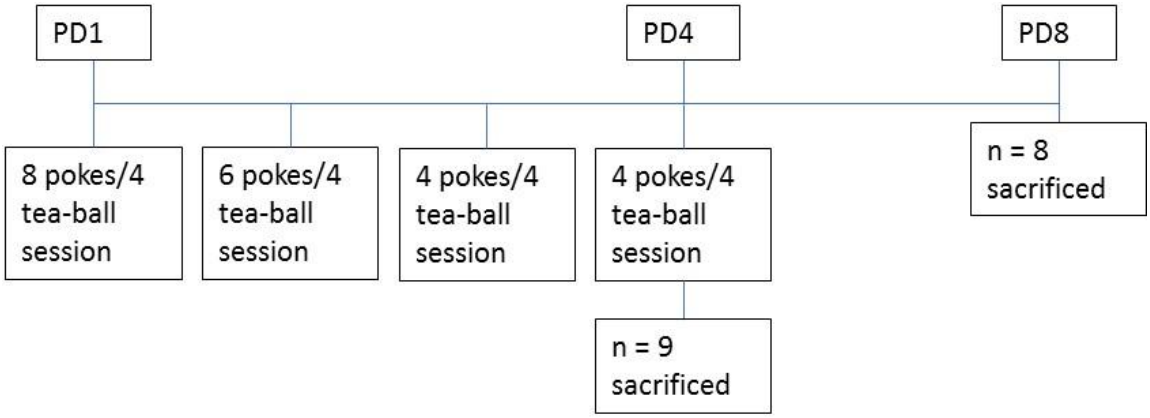

Figure 1. Postnatal day 1 rat pup placed in an open (A) and closed (B) tea ball infuser and dam nest with pups placed in tea ball infusers (C).

Maternal care observation

During the first two sessions of every day, the amount of time spent on and off the nest and the amount of time dams spent licking and grooming their pups were collected every 5 seconds during the 30 minutes of pup tea-ball encapsulation and 20 minutes immediately after the pups were released.

Body weight and tissue collection

Body weights were collected on PD1, PD4, and PD8 (2nd group only). Pups in the first group were sacrificed on PD4 immediately following the final stress exposure (i.e. 30 minutes after pain exposure and immediately after removal from tea-ball encapsulation) via rapid

decapitation and brains were collected and flash frozen on dry ice. Trunk blood was collected for corticosterone measurements at the time of sacrifice. Pups in the second group underwent a cardiac perfusion with 4% paraformaldehyde on PD8. Brains were extracted and kept in paraformaldehyde for 24 hours before transfer to a 30% sucrose solution in which they remained until processing.

Magnetic resonance spectroscopy (MRS) imagining

Brain tissue collected from PD4 pups was analyzed via magnetic resonance spectroscopy imagining. To obtain specific regions, brains were sectioned using a brain matrix. For frontal cortex, a 3mm in diameter and 2mm thick punch was collected 2mm in from the most anterior portion of the brain and for the hippocampus, a same size punch was collected 6mm in from the most anterior portion of the brain. Tissue was weighted and then placed into a 12 µL Bruker zirconium rotor with chilled buffer (pH 7.4, 100 mM K2HPO4/KH2PO4, 200 mM HCOONa, 1 g/l NaN3 diluted 50% with D2O containing 3 mM trimethylsilylpropinoate), with the weight of the sample and volume of buffer adding to equal 12 µL. A vertical 8.9-cm bore Bunker 11.7-T magnet, controlled via an Avance DRX-500 console was utilized for sample analysis, which was carried out by placing the rotor (containing both sample and buffer) into a multinuclear Bruker magic angle spinning probe. All analyses were conducted with temperature maintained at 4℃ and rotors spun at 4.2 ± 0.002kHz at 54.7° comparative to the static magnetic field B_0. For water suppression, a 1-D Carr-Purcell-Meiboom-Gil pulse sequence with a pre-saturation pulse was used. Further, a semi-automated shimming procedure, to adjust for field inhomogeneities, was used to obtain data using Bruker-XWINNMR. A custom LCModel analyzed raw spectral data with a custom basis set of individual neurochemical spectra and non-specific lipid signals created to fit the brain tissue spectrum and analyzed concentrations of neurochemicals with peaks between 1.0 and 4.2 ppm (Provencher, 1993). Neurochemical recordings were adjusted for tissue weight and

were reported in nmol of neurochemical/mg tissue and normalized to the creatine signal to account for variance within neonatal tissue. Glutamate, gamma-aminobutyric acid (GABA), glutamine, glutamate & glutamine (GLX), GABA/glutamate, and n-acetylaspartate were analyzed.

Serum corticosterone

Trunk blood samples were collected from the first group during sacrifice. Samples were kept at -4℃ and allowed to clot overnight. The next day, samples were spun down at 8000*g* for 10 minutes, serum was removed and stored at -20℃ until further processing. Serum was analyzed for corticosterone using a standard corticosterone EIA kit from Arbor Assays (catalog # K014-H1; Ann Arbor, MI USA) per the manufacturer's instructions. Samples were run in duplicates and the inter- and intra- assay coefficients were less than 10%.

Ki-67 immunohistochemistry

Brains collected from PD8 group were sectioned at 40 micrometers with a sliding microtome (Thermo Fischer) and stored at -20℃ in antifreeze. Slices containing frontal cortex, medial prefrontal cortex, and hippocampus were stained with Ki-67 to detect newly proliferated cells as previously described (Kee et al., 2002). Briefly, free floating tissue was rinsed overnight on a rotator to remove any residual antifreeze. Tissue was then rinsed 3 times for 10 minutes in phosphate-buffered saline (PBS). Next, slices were submerged in 3% H202 for 30 minutes and then rinsed again 3 times in PBS. Tissue was then incubated overnight in 0.5% triton, 1% normal goat serum, and rabbit-anti Ki-67 (1:1000, Vector Labs (frontal cortex) & 1:1000, Abcam (hippocampus) on a rotator. On the following day, tissue was rinsed 3 times in PBS and then exposed to a goat anti-rabbit secondary antibody (1:200 Vector labs) for 1 hour and 30 minutes. Tissue was again rinsed 3 times in PBS and then placed in an avidin-biotin complex (ABC, Vector labs) for 1 hour. Next, slices were rinsed 3 more times in PBS before being submerged in DAB

solution (Vector labs) for 4 minutes. Immediately after DAB exposure, tissue was rapid rinsed in PBS 3 times and then mounted and coverslipped. Due to discontinued production of our 1st Ki67 antibody, two separate antibodies were used for frontal cortex (Vector Labs, Burlingame, CA) and hippocampal tissue (Abcam, Cambridge, MA).

Cell counting

Cell proliferation in the frontal cortex, medial prefrontal cortex, and hippocampus were analyzed using an Olympus BX51 microscope and Olympus cellSens Dimension 1.14 with count and measure 1.14 (Olympus, Tokyo, Japan) according to the areas described in Paxinos & Watson (2004). For frontal cortex, medial prefrontal cortex, and dentate gyrus of the hippocampus, a designed-based stereology method was employed, with six tissue pieces that contain the regions of interest used for counting. For the frontal cortex one counting frame (186,459.26 μ^2; 10x magnification) was used to quantify the number of Ki-67 positive cells. Similarly, for the medial prefrontal cortex one counting frame (124,285.01 μ^2; 10x magnification) was used. For slices containing the hippocampus, cell counting occurred in five counting frames (3593.22 μ^2 each; 10x magnification) that were placed along the dentate gyrus. Researchers responsible for cell counting were blind to the animal's conditions.

Statistics

Maternal behaviors during the 30 minutes in which pups were in tea-balls and the 20 minutes following the removal of pups from the tea-balls were compared using a paired samples t-test. A repeated measures ANOVA was conducted with sex (male, female), tea-ball encapsulation (no isolation, isolation), and pain (no pain, pain) as between subject's and days (PD1, PD4, PD8) as repeated measures to assess body weight during PD1, PD4, and PD8. All other analyses were carried out with factorial ANOVAs with sex, tea-ball encapsulation, and pain

as between-subject factors. All significant interactions were followed up with a Fishers LSD post-hoc analysis. All statistical analyses were conducted using IBM SPSS 24. If present, extreme outliers were removed. Results were considered significant at $p<0.05$.

Results

Maternal care

No statistical differences were found in percent of time spent on nest when pups were enclosed in tea-balls compared to when pups were out of tea-balls ($p > .05$; see Figure 2a). A paired samples t-test revealed a significant difference in the percent of time dams spent licking and grooming their pups during tea-ball exposure compared to when pups were out of tea-balls ((T, 16) = 2.354, p = .032), with dams licking and grooming their pups slightly more when all pups were removed from tea-balls (see Figure 2b).

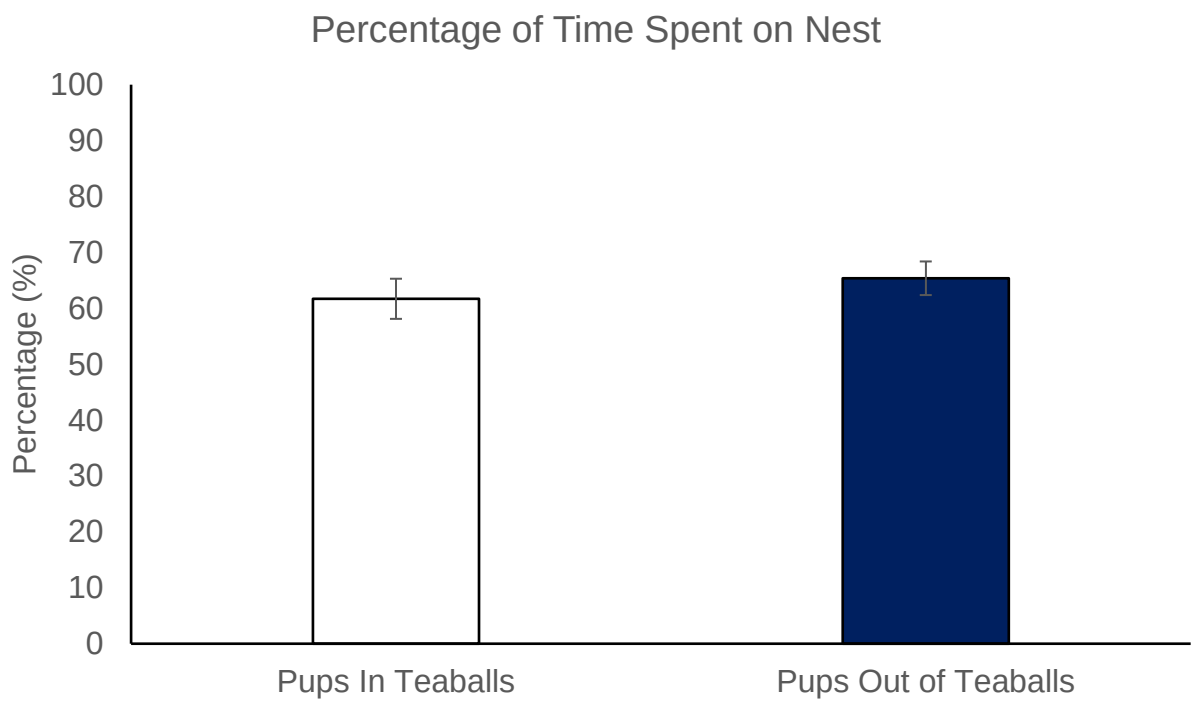

Figure 2a.

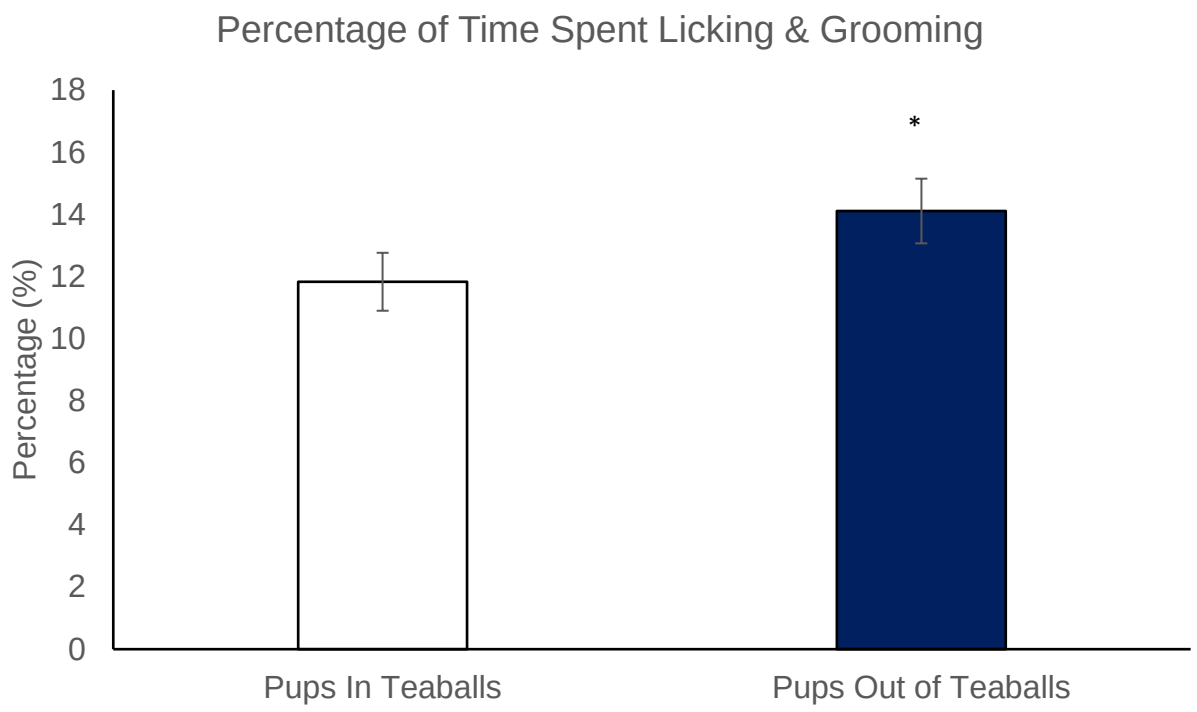

Figure 2b.

Figure 2. A) Displays the percentage of time spent on nest during the 30 minutes pups were in tea-balls compared to the 20 minutes immediately after pups were removed from tea-balls. B) Shows the percentage of time licking and grooming during the 30 minutes pups were in tea-balls compared to the 20 minutes immediately after pups were removed from tea-balls. Data are represented as mean ± SEM. * $p<0.05$

Body weight

A repeated measures ANOVA analyzing PD1, PD4, and PD8 body weight found a main effect of days ($F(1, 56) = 4230.19$, $p = .0001$) and a non-significant interaction between days and maternal isolation ($F(1.203, 67.363) = 2.554$, $p = .092$), but no effect of maternal isolation, pain exposure, or sex (p's >.05; see Table 1).

Table 1. Body weight on PD1, 4, & 8 (g)

	Touch	Isolation	Pain	Isolation & Pain
PD1	6.81 ± 0.21	7.02 ± 0.18	6.83 ± 0.20	6.93 ± 0.21
PD4	11.03 ± 0.31	10.71 ± 0.31	10.78 ± 0.31	10.44 ± 0.33
PD8	18.18 ± 0.44	17.58 ± 0.40	17.58 ± .43	17.33 ± 0.42

Data are represented as mean ± SEM.

Serum corticosterone

A factorial ANOVA found a main effect of isolation ($F(1, 63) = 11.357$, $p = .001$) and a trend for pain ($F(1, 63) = 3.145$), $p = .081$) to increase corticosterone on PD4, but no effect of sex and no interaction effects ($p > .05$; see Figure 3).

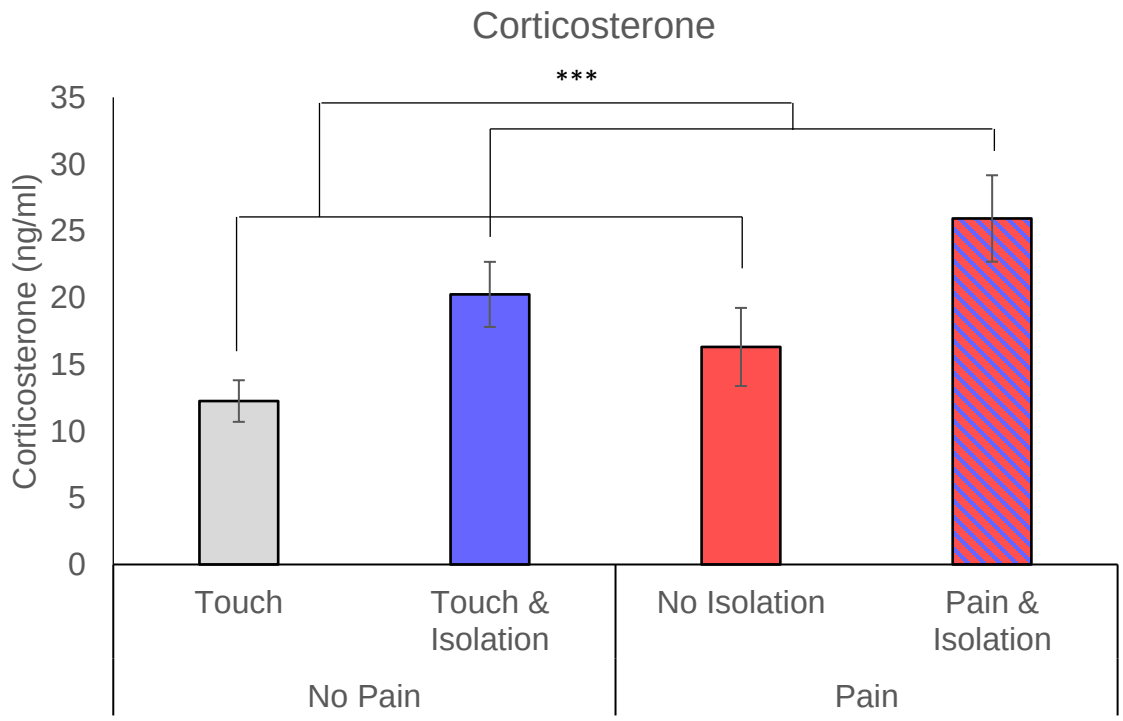

Figure 3. Serum corticosterone levels on PD4 measured immediately after the last tea-ball encapsulation (isolation). Isolated pups had higher corticosterone levels compared to pups that were not enclosed in a tea-ball (*** p=0.001). Data are represented as mean ± SEM.

Magnetic resonance Spectroscopy Imaging

Frontal cortex

A factorial ANVOA analyzing frontal cortex glutamate/creatine ratio found significantly reduced levels in pain exposed pups compared to non-pain pups ($F(1, 53) = 4.270$, $p = .044$; see Figure 4a) but no effect of isolation or sex (p's > 05). Glutamate & glutamine (GLX)/creatine was not impacted by pain or sex (p's > .05) but was reduced in isolated pups compared to non-isolated pups, albeit in a non-significant fashion ($F(1, 51) = 3.732$, $p = .059$; see Figure 4c). GABA/glutamate ratio showed a significant increase in isolation pups ($F(1, 52) = 15.834$, $p = .001$; see Figure 4d) compared to non-isolated pups and in males compared to females ($F(1, 52) = 4.435$, $p = .040$; see Figure 4d) but no effect of pain ($p > .05$). No main effects were observed for GABA/creatine (see Figure 4e), glutamine/creatine (see Figure 4b), or NAA/creatine ratios (see Figure 4f).

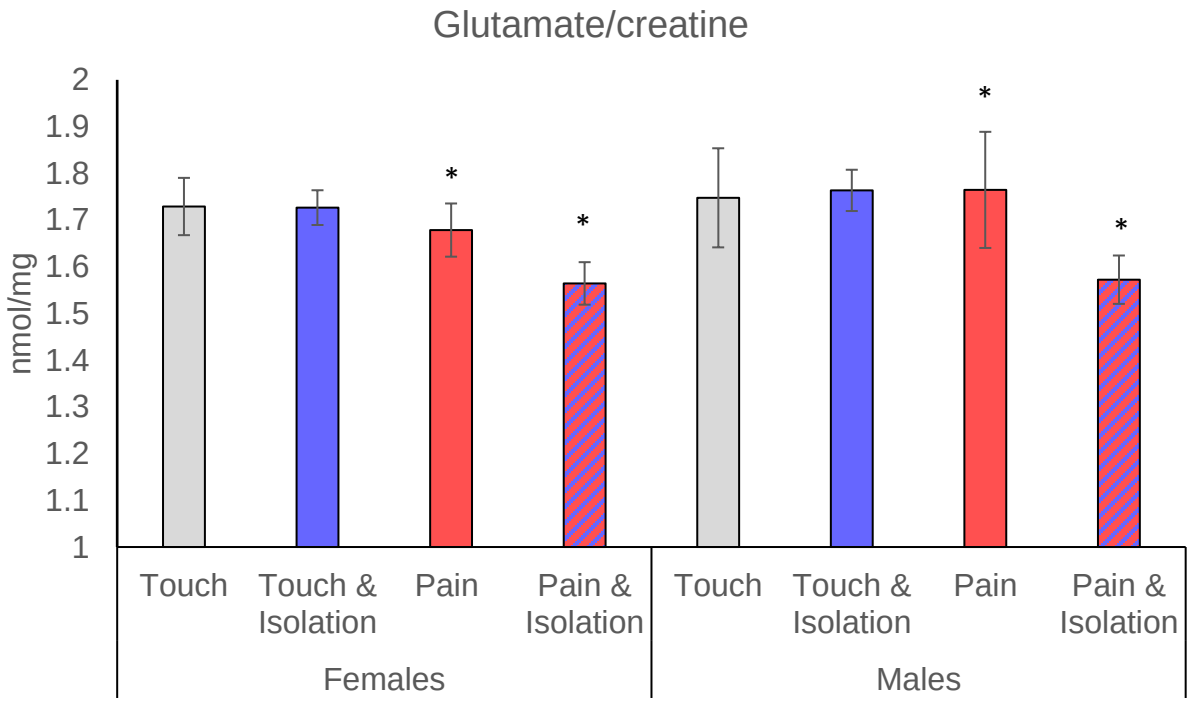

Figure 4a.

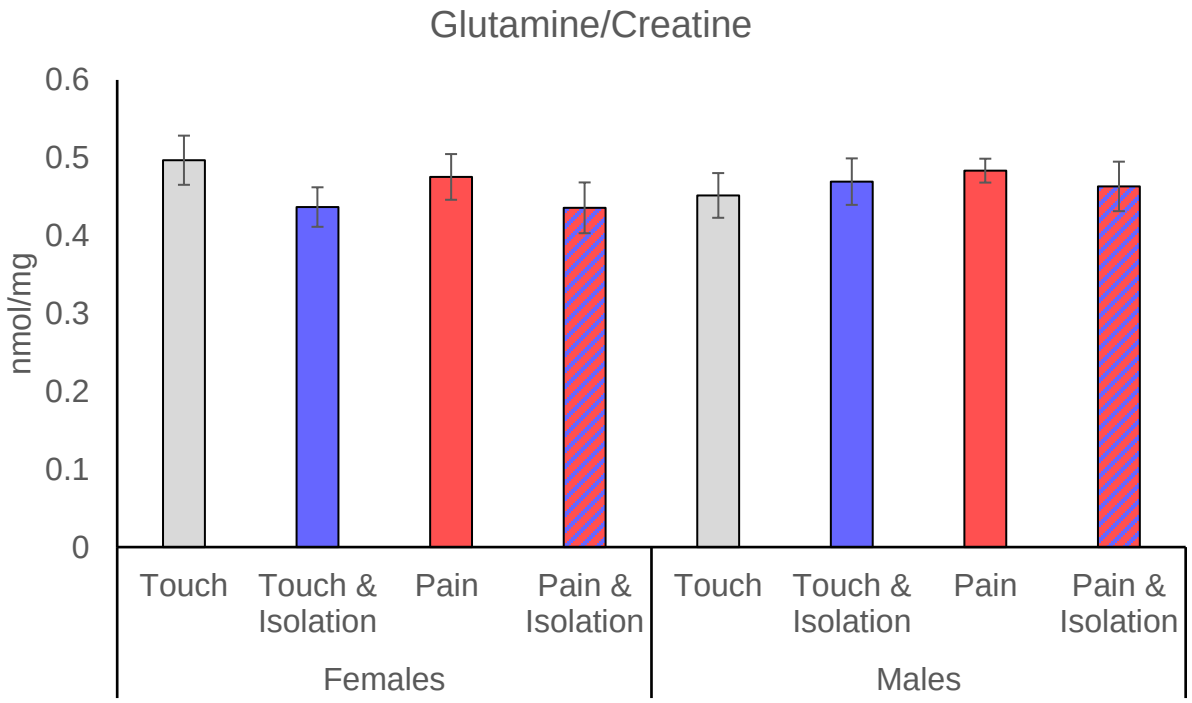

Figure 4b.

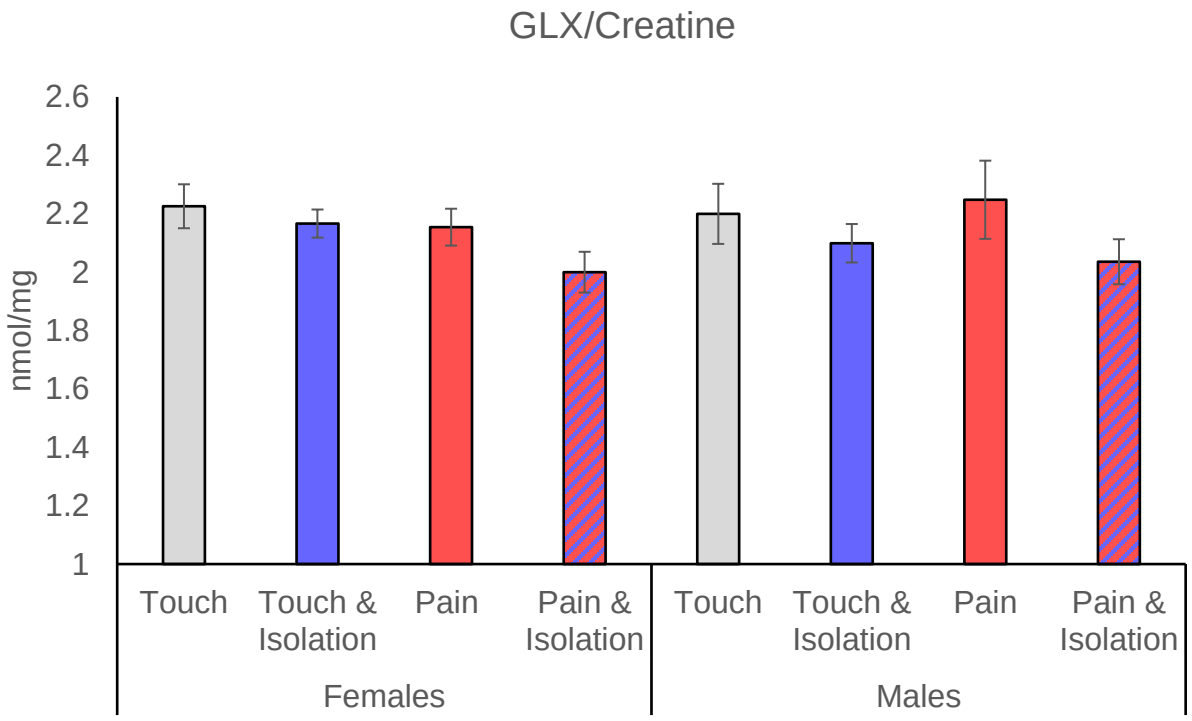

Figure 4c.

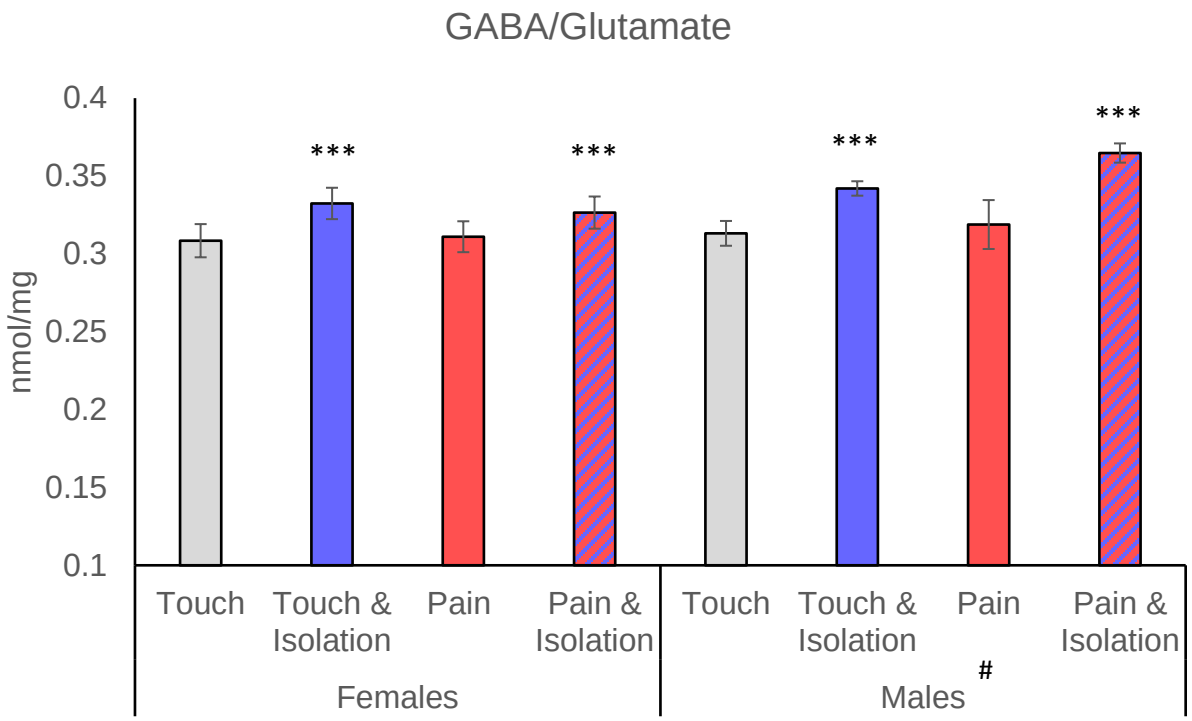

Figure 4d.

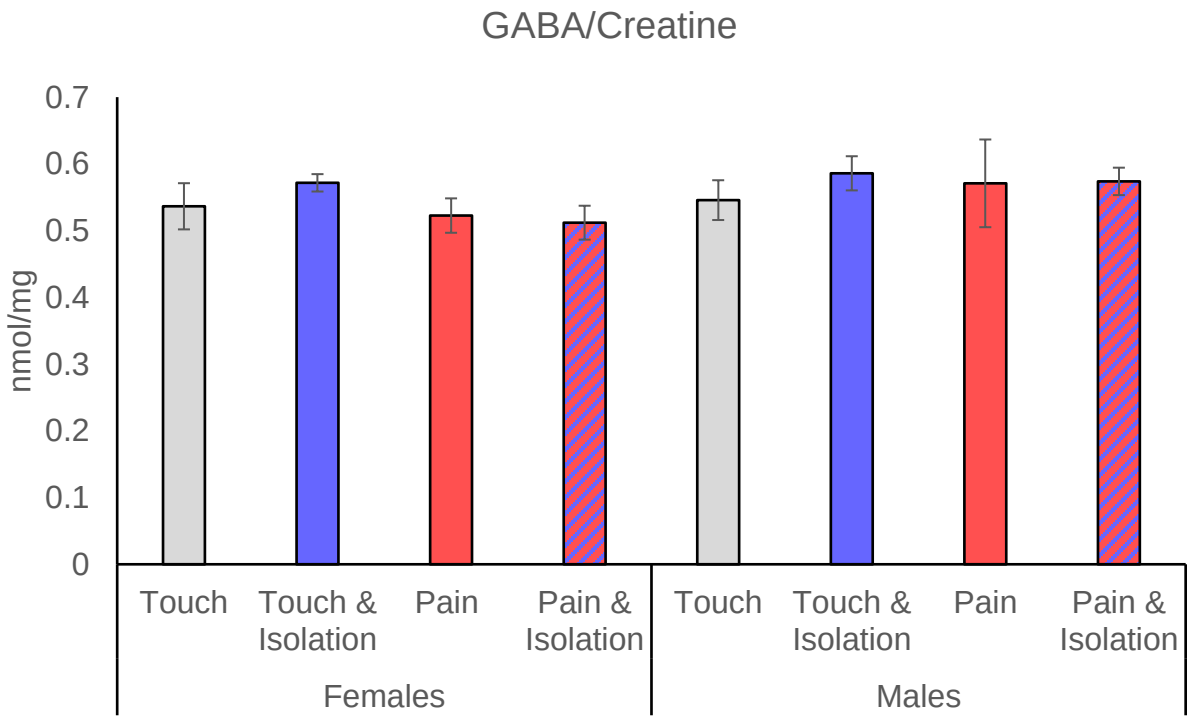

Figure 4e.

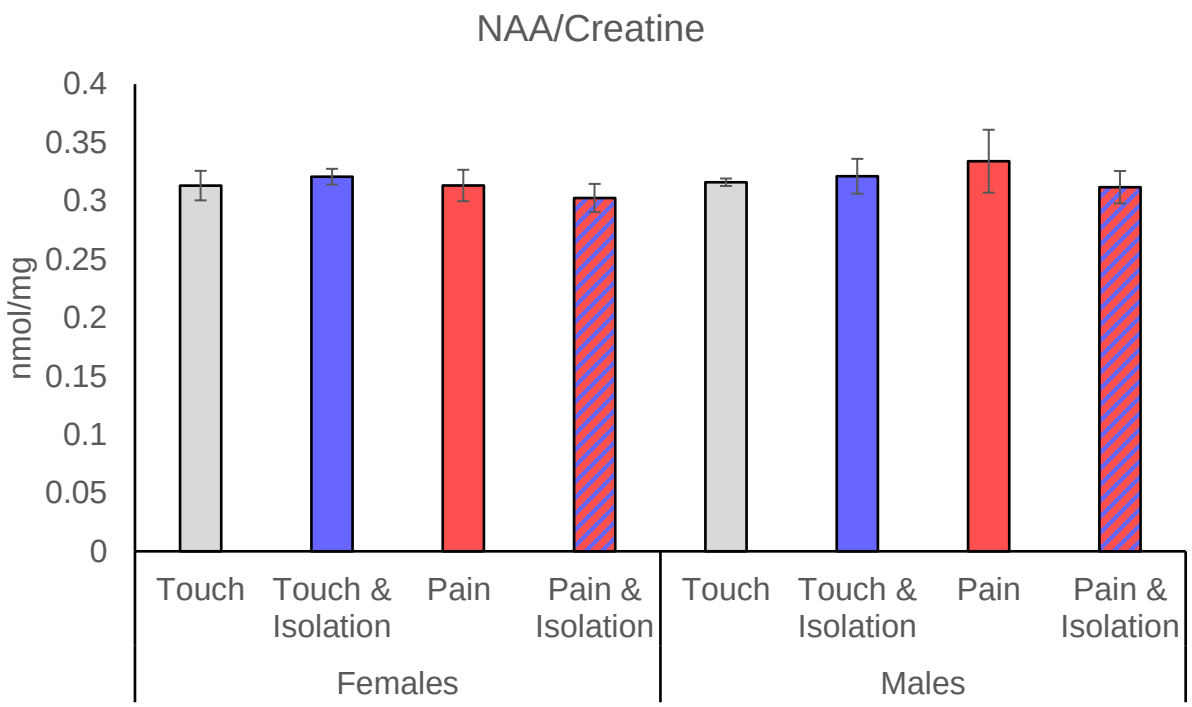

Figure 4f.

Figure 4. Frontal cortex brain metabolites analyzed on PD4 immediately after last tea-ball exposure. A) Pain exposed pups had increased glutamate compared to non-exposed pups (*p = .044). B) Neither sex, pain, nor isolation impacted glutamine levels. C) Frontal cortex GLX levels were not altered by sex, pain, or maternal isolation. D) GABA/glutamate levels were increase in isolated pups compared to non-isolated pups (*p = .001) and in males compared to females (#p = .040). E) Sex, pain, or isolation did not impact GABA levels. F) Neither sex, pain, or maternal isolation NAA in the frontal cortex. Data are represented as mean ± SEM.

Hippocampus

A factorial ANOVA analyzing hippocampal glutamate/creatine ratio found a significant reduction in isolated pups compared to non-isolated pups ($F(1, 52) = 4.193$, $p = .049$; see Figure 5a) but no effect of pain or sex (p's > .05). No main effects for GABA/creatine were found (p's >.05) however a sex by pain interaction was observed ($F(1, 51) = 4.502$, $p = .039$; see Figure 5b). Post-hoc analysis revealed a significant increase in GABA/creatine in male pain exposed pups compared to female pain exposed pups ($p = .038$). Glutamine/creatine showed a non-significant decrease in pain exposed animals compared to non-pain exposed ($F(1, 49) = 3.977$, $p = .052$) but

no effect of isolation or sex (p's > .05). No group differences were observed for GLX/creatine (see Figure 5e), GABA/glutamate (Figure 5c), or n-acetylaspartate/creatine (see Figure 5f).

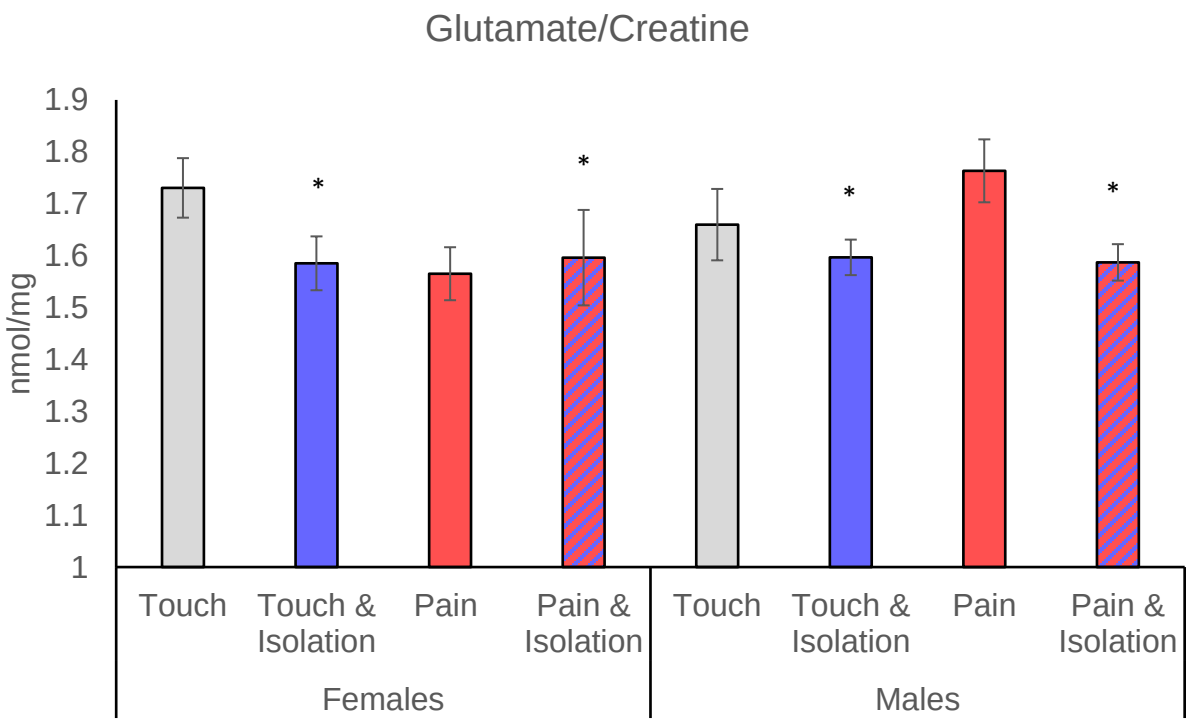

Figure 5a.

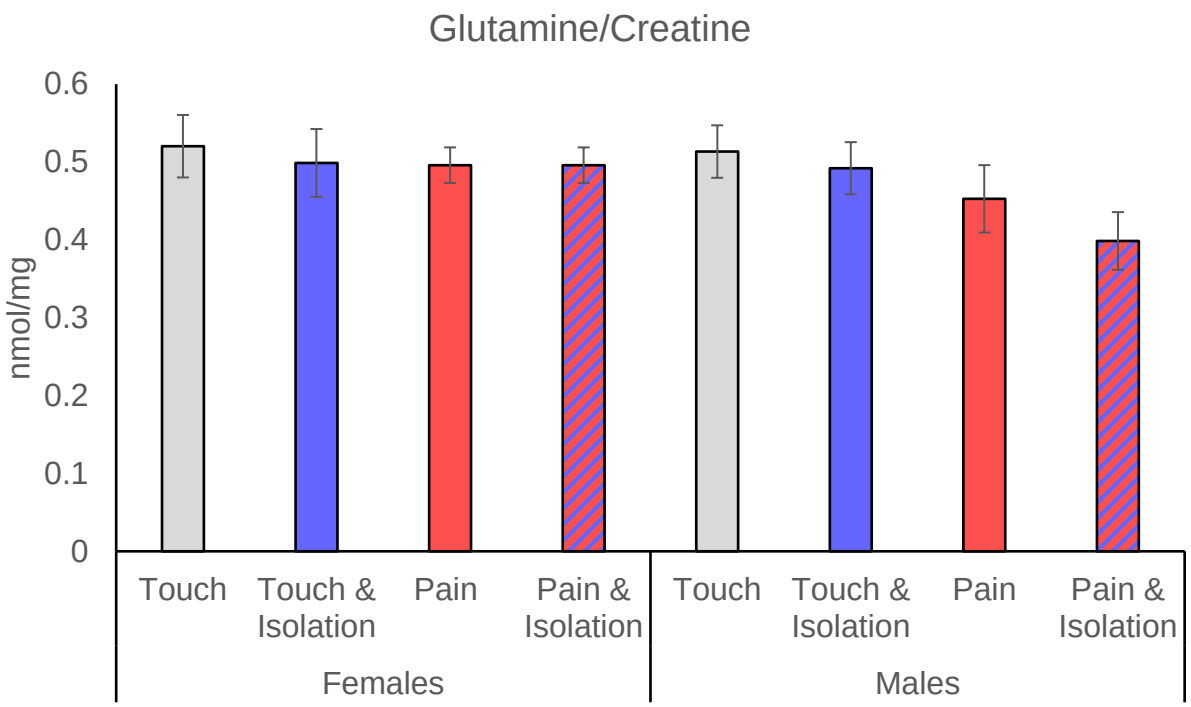

Figure 5b.

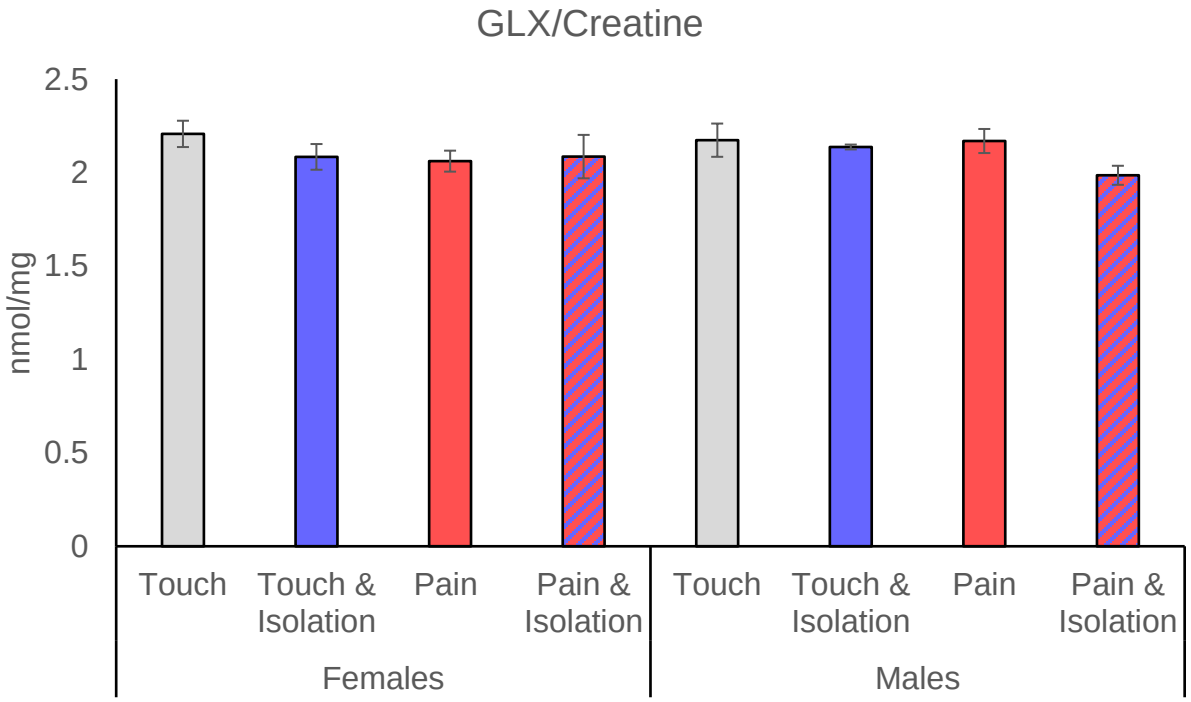

Figure 5c.

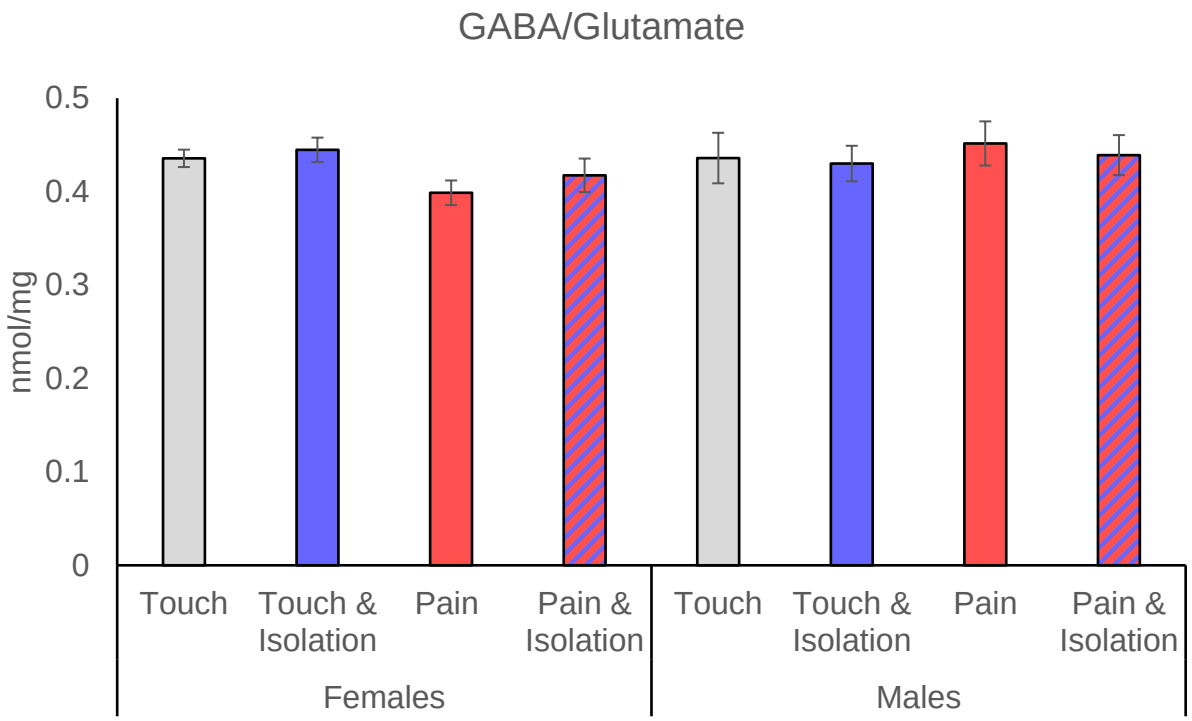

Figure 5d.

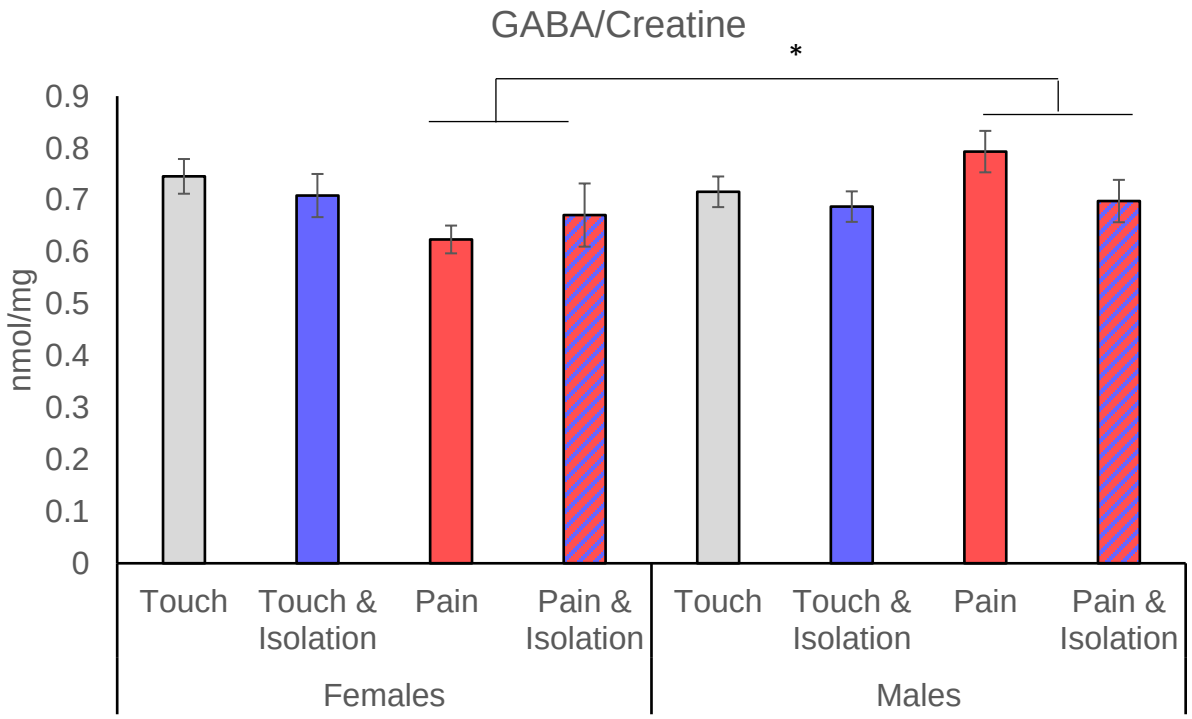

Figure 5e.

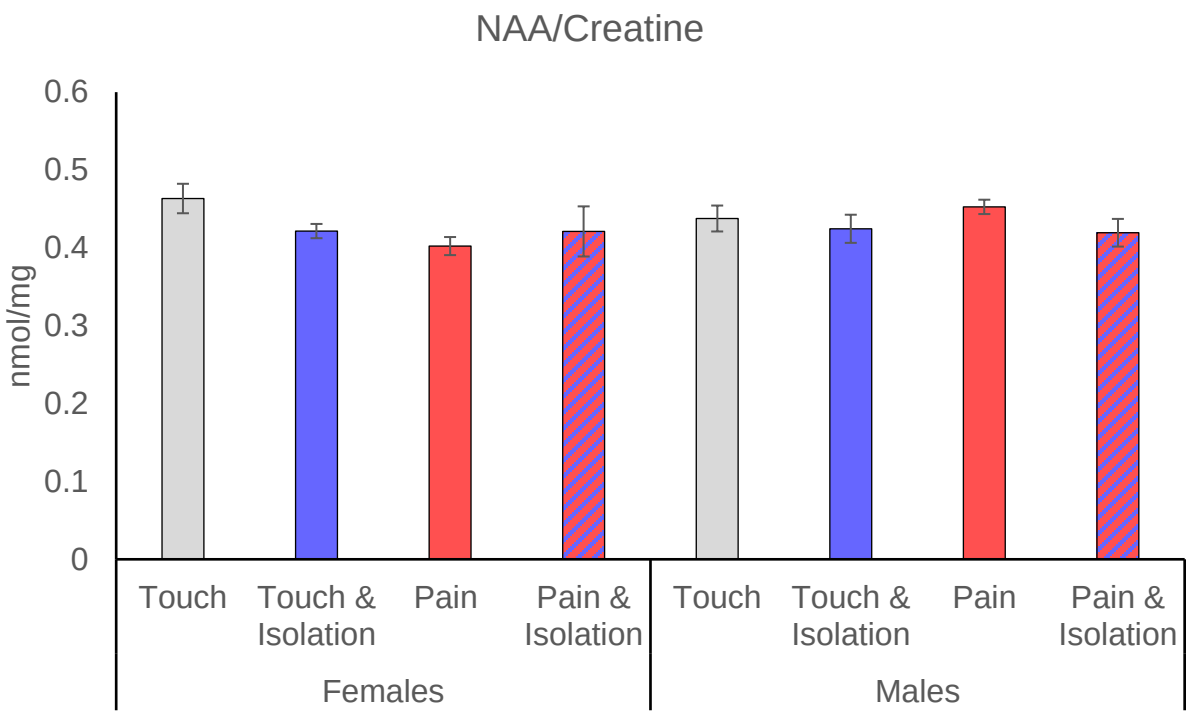

Figure 5f.

Figure 5. Hippocampal brain metabolites analyzed on PD4 immediately after last tea-ball exposure. A) Maternal isolation significantly reduced glutamate/creatine levels compared to non-isolate pups (*p = .049). B) Sex, pain, or maternal isolation did not impact glutamine levels. C) Hippocampal GLX levels were not altered by sex, pain, or maternal isolation. D) GABA/glutamate levels were significantly increased in male pain exposed animals compared to female pain exposed animals (*p = .038). E) Neither sex, pain, nor isolation altered hippocampal GABA levels. F). No group differences were observed in NAA in the hippocampus. Data are represented as mean ± SEM.

Ki-67 staining

Neither isolation nor pain impacted the number of Ki-67 positive cells in the frontal cortex or medial prefrontal cortex (p's > .05; see Figure 6a & b). Cell proliferation in the dentate gyrus showed a slight but non-significant decrease in the pain group compared to non-pain groups ($F(1, 51) = 2.835$, $p = .098$; see Figure 6c) and no differences for isolation or sex (p's > .05).

Table 2. Cell proliferation count in the frontal cortex, mPFC, and dentate gyrus

	Touch	Isolation	Pain	Pain & Isolation
Frontal cortex	1446.18 ± 71.27	1537.50 ± 112.35	1480.03 ± 64.31	1563.73 ± 93.36
Medial prefrontal cortex	438.95 ± 21.56	454.04 ± 32.79	445.87 ± 28.89	446.76 ± 34.10
Dentate Gyrus	59.79 ± 9.26	63.12 ± 5.21	52.00 ± 4.34	49.29 ± 3.81

Data are represented as means ± SEM.

Discussion

Results from the present study indicate that tea-ball encapsulation ("isolation") is a novel model to reduce maternal care provided to rat pups. Further, both neonatal isolation and pain produced a stress response on PD4 and we found stressor- and sex-specific alterations in neurochemistry within the frontal cortex and hippocampus, with no change to the number of newly proliferated cells.

Tea-ball encapsulation as a novel model of reduced maternal care

We used a novel approach to restrict maternal contact in our experiments. Pups were individually enclosed in tea-balls and we observed maternal behavior during the 30 minutes that half of the pups were enclosed in tea-balls and then compared that to the 20 minutes immediately after the pups were removed from the tea-balls. Results showed that dams spend the same percentage of time on the nest with or without the tea-balls present, indicating that isolated pups received less maternal care while they were in the tea-ball, as they were missing out on maternal licking and grooming behaviors during those 30 minutes. The fact that dams did not increase time on nest behaviors after pups were released from the tea-ball encapsulation (i.e. dams did not

increase their maternal contact substantially when pups were released), indicates that tea-ball isolation is indeed a valid model to restrict maternal care to specific pups within a litter while not disrupting the amount of maternal care provided to the other pups. This form of reduced maternal care closely mimics NICU settings as pups are still in the same environment as the care-giver but direct contact is restricted. It should be noted however, that the percentage of time spent licking and grooming was slightly, but significantly enhanced upon tea-ball removal (11.8% during tea-ball presence and 14.1% after tea-ball removal). This increase is in line with previous research, which shows an increase in licking and grooming behaviors in dams whose pups were returned following prolonged isolation (Kosten & Kehoe, 2010; Zimmerberg, Rosenthal, & Stark, 2003). Variations in licking and grooming behavior are critical for typical brain and behavioral development (Bredy et al., 2003; Liu et al., 2000) and it is feasible that increased maternal care after stressful procedures may help to buffer against the negative consequences of stress. In fact, Walker et al. (2003) investigated pain exposure in rat pups but found no differences in neuroendocrine responses to stress later in life which they suggested might be due to increased maternal care expressed by dams after they were reunited with their pups. Moreover, de Medeiros, Fleming, Johnston, and Walker (2009) found that maternal contact significantly reduced swelling from daily formalin injections when compared to artificial maternal contact (multiple anogenital and whole-body stimulation with a paintbrush). Importantly, in our study the slight increase in licking and grooming behavior upon release of the pups was observed for the whole litter. Unfortunately, with our study design, it was not possible to determine which individual pups were licked as this would have required more extreme daily marking and handling of all pups that we were trying to avoid (Ragan, Loken, Stifter, & Cavigelli, 2012). Thus, we cannot be sure, if the

dam indeed licked the isolated pups more or if she just spent more time licking and grooming all pups in general once the foreign objects were removed from the nest.

Neither maternal isolation nor pain exposure altered body weight gain on PD1, PD4, or PD8. These results suggest that the daily 4 x 30 minutes of maternal separation experienced by the tea-ball enclosed pups does not hinder gross body development. This intended null finding is most likely due to allowing for adequate nursing time in between sessions. Further, rat dams display bouts of time off nest throughout the day to maintain proper thermal control over their litters (Woodside & Leon, 1980), indicating that periods of maternal separation are typical.

Maternal isolation and pain increase serum corticosterone on PD4

During typical early-life development, corticosterone peaks immediately after birth and subsides to low basal levels between postnatal days 2-14 (Sapolsky & Meaney, 1986). This marked period of reduced circulating glucocorticoids is known as the stress hypo-responsive period (SHRP) and is believed to protect the brain from excessive amounts of glucocorticoids which have been shown to cause devastating effects to an immature brain (Sapolsky & Meaney, 1986). Previous studies using early-life pain models utilizing inflammatory agents have found increases in corticosterone 30 minutes, 24 hours, and 7 days after pain exposure despite pups being in the SHRP (Butkevich, Mikhailenko, Makukhina, Bagaeva, & Stolyarova, 2013; Victoria et al., 2014). Moreover, Moriceau and Sullivan (2006) have demonstrated that a foot shock increases corticosterone levels in rat pups only if the dam is not present during the painful experience. This suggests that pain is a powerful stressor that can activate the HPA axis even during a time of low corticosterone levels (i.e. during SHRP). Here, we demonstrate that our repetitive needle prick model, that does not rely on an inflammatory agent to produce a prolonged pain signal is also capable of causing an (albeit not significant) increase in corticosterone. Importantly, our sample

was collected on PD4 at the end of the last 30 minute isolation session, i.e. 30 minutes after the last pain exposure and thus the corticosterone levels may reflect the recovery stage for the pain groups. In humans, Grunau et al. (2005) found that at 32 weeks corrected gestational age, younger preterm infants (≤ 28 weeks gestational age) that experienced high amounts of painful procedures had a blunted cortisol response to a noxious stimulus compared to older preterm infants (29-32 weeks gestation age) that had experienced less painful procedures, suggesting a pain mediated down-regulation of HPA axis responding. Further, Brummelte et al. (2015) found that cortisol response as well as diurnal levels in preterm children at school-age are predicted by neonatal pain-related stress in the NICU and Grunau et al. (2013) confirmed that lower hair cortisol levels in school-aged preterm children were also associated with greater neonatal pain exposure. From these results, the slightly elevated levels of corticosterone observed in the current study may indicate a down-regulatory shift of the HPA axis. In other words, the chronic exposure to pain over 4 days may have already induced a re-programming of the HPA axis with neonatal rats showing less of a response to the pain on PD4. Though results from studies measuring *acute* cortisol responses following pain exposure are inconsistent in humans with some studies showing elevated levels and others showing no change in cortisol in response to a painful stimulus (Cignacco et al., 2009; Cong et al., 2011; Herrington et al., 2004; Magnano et al., 1992), it seems like repeated early pain exposure may result in reprogramming of the HPA axis in preterm children.

The increase of corticosterone following maternal isolation in preclinical models is well established (Kuhn et al., 1990; Levine, Huchton, Wiener, & Rosenfeld, 1991; McCormick et al., 1998). Tea-ball encapsulation produced a significant increase in serum corticosterone levels in our pups, demonstrating the effectiveness of our stressor. However, the increase in both groups (isolation and pain) suggests, that both stressors are indeed effective in activating the HPA axis

stress response. Interestingly, although corticosterone levels were noticeably the highest in pups that experienced maternal isolation and pain, there was no significant interaction effect, indicating that the experience of both stressors did not significantly exacerbate HPA axis activation.

Pain and maternal isolation specific alterations in brain neurochemistry

During the neonatal period, glutamate levels in the frontal cortex increase over the course of development (Burri et al., 1990; Tkac, Rao, Georgieff, & Gruetter, 2003). Alterations in glutamate signaling during the neonatal period lead to impaired brain and behavioral development (Pancaro et al., 2016; Soriano et al., 2010), indicating the need of homeostatic glutamate levels for proper development. For example, Hilton et al. (2006) found that application of glutamate in cultured hippocampal neurons produced a robust increase in the amount of cell death. It has been suggested before that the negative consequences of early pain exposure may be due to increased glutamatergic signaling and excitotoxicity in response to overstimulation of immature neurons (Anand & Scalzo, 2000; Brummelte et al., 2012). Support of this idea is observed in both animal and human literature. For instance, blockade of the glutamate NMDA receptor, in conjunction with neonatal inflammatory pain, mitigates the typical increase in cell death in rodents, suggesting a role of the glutamate receptor in pain produced apoptosis (Anand et al., 2007; Rovnaghi et al., 2008). Moreover, in human neonates pain is associated with increased GLX (Angeles et al., 2007), indicating that neonatal glutamate is elevated in response to pain. In our study, pain and maternal isolation reduced frontal cortex and hippocampal glutamate respectively. Interestingly, these outcomes are opposite of our original hypothesis that exposure to a stressor (pain or isolation) would result in increased levels of glutamate, a relationship that is observed 30 minutes after restraint stress in adult rodents (Moghaddam et al., 1994). It is important to note that the neurochemical findings in the current study are not in response to a single pain or maternal

isolation exposure but rather after four days of continuous stress exposure. Thus, it is feasible that the initial pain and maternal care exposure produced increases in glutamate but over multiple exposures the neonatal pups down-regulates their glutamate system as a protective measure. Similar to corticosterone outcomes, we did not see a statistical exacerbation in the pain in combination with maternal isolation group, however from Figure 4a, it is noticeable that the pain and maternal isolation group displays the largest decrease regardless of sex.

In addition to changes glutamate, maternal isolation and pain altered GABA/glutamate and GABA levels respectively. Unlike in the adult brain, where GABA is the primary inhibitory neurotransmitter, there is much debate about the role of GABA during the neonatal period. Until recently most studies indicated that GABA served an excitatory role during development (Cancedda, Fiumelli, Chen, & Poo, 2007; Cherubini, Gaiarsa, & Ben-Ari, 1991), however, current findings suggest that GABA is an inhibitory neurotransmitter during postnatal days 3-4 (Kirmse et al., 2015). Due to conflicting findings of outcomes surrounding the role of early-life GABA, it is hard to make an interpretation on alterations within the GABAergic system. Currently, very little is known about the relationship between neonatal pain exposure and GABA, however, down-regulation of the GABAergic system in the adult spinal cord has been documented following neonatal visceral pain (Sengupta et al., 2013). Further, Knott, Quairiaux, Genoud, and Welker (2002) demonstrated that even a continuous non-noxious tactile stimulation can lead to quick adaptations in inhibitory systems to monitor future excitation. In the current study, we found that pain increased hippocampal GABA levels in males compared to females. Due to the conflicting reports regarding neonatal GABA, it is unclear how this change in GABA modulates neuronal development. However, in human preterm infant populations, female neonates have more favorable neurological outcomes compared to male neonates in the NICU (Peacock, Marston,

Marlow, Calvert, & Greenough, 2012), which may be related to the observed changes in hippocampal GABA. Changes within the GABAergic system following maternal isolation have been documented, however, many of the findings have been inconsistent with some studies finding down- and others finding up- regulation of various components within GABAergic systems (Caldji, Diorio, et al., 2000; Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Feng et al., 2014; Leussis, Freund, Brenhouse, Thompson, & Andersen, 2012). In the present study, we found a significant increase in GABA/glutamate ratio, suggesting a potential developmental trajectory of enhanced GABAergic signaling. However, it should be noted that this effect is most likely driven by the reduction in glutamate rather than the increase in GABA as there are no group differences in frontal cortex GABA levels (figure 4e) but a significant reduction in glutamate (figure 4a) levels in the pain and isolation group.

Surprisingly, we did not observe any changes in the neuronal biomarker NAA following maternal isolation and/or pain exposure. Previous studies have demonstrated that both maternal isolation and neonatal pain result in decreased NAA (Angeles et al., 2007; Brummelte et al., 2012; Llorente, Villa, Marco, & Viveros, 2012). Obvious methodological differences arise when comparing between clinical and preclinical findings but it is feasible that the models utilized in the current study were not severe enough to induce changes in NAA.

Pain and maternal isolation do not impact cell proliferation

Exposure to early-life pain or reduced maternal care results in increased apoptosis and loss of neurons (Aksic et al., 2013; Anand et al., 2007; Duhrsen et al., 2013; Rovnaghi et al., 2008; Zhang et al., 2002) and an increase in cortical thinning of the frontal and parietal lobes (Aksic et al., 2013; Ranger et al., 2013), indicating impaired brain development. Conversely, in the current study pain or maternal isolation failed to impact cell proliferation in the frontal cortex, medial

prefrontal cortex, or hippocampus. This is somewhat surprising as both stressors increased corticosterone which has been shown to decrease neurogenesis (Brummelte & Galea, 2010). Moreover, early-life exposure to dexamethasone decreases cell proliferation in the hippocampus (Claessens et al., 2012), however this effect was gone 7 days after treatment. From these findings, it is feasible that the effect of both stressors on cell proliferation had already passed between the last stress exposure (PD4) and the time of perfusion (PD8).

Conclusion

The goal of this study was to investigate the impact of neonatal pain and maternal isolation on neonatal endocrine, neurochemical, and cellular outcomes. Briefly, neonatal pain and maternal isolation increased corticosterone on PD4 and altered glutamate levels in the frontal cortex and hippocampus respectively. These findings are the first of their kind to demonstrate that repetitive pain exposure in the neonatal rat pup is capable of influencing neurochemistry. Further, we produced a translational novel model that reduces maternal contact/comfort during painful procedures, similar to what is observed in today's NICU. These findings add to the growing data on the mechanism responsible for impaired development following neonatal pain and reduced maternal care. Despite these outcomes, it should be noted that limitations exist within the current study. First, we observed an increase in maternal care after pups were removed from tea-balls, which may have been an attempt to buffer stress experienced by tea-ball pups. Second, the amount of pain experienced in the current study is still well below the average 16 invasive procedures experienced by NICU preterm infants. Nevertheless, it is important to note, that despite these limiting factors, pain and maternal isolation exposure still produced impairments in endocrine and neurochemical outcomes, indicating the power of these early-life stressors. Collectively, our data suggest that impairments observed following neonatal pain and maternal isolation may be

facilitated by increases in corticosterone and alterations within the glutamatergic system. More research utilizing the current novel model will help elucidate the role of the observed endocrine and neurochemical changes in the long-term biobehavioral consequences associated with neonatal pain and maternal isolation.

CHAPTER 4: LONG-TERM BIOBEHAVIORAL CONSEQUENCES OF NEONATAL PAIN AND MATERNAL ISOLATION (HYPOTHESES 1, 5, & 6)

Introduction

Early-life stress has a profound impact on cognitive and affective behavioral development (Aisa et al., 2007; Aisa, Tordera, Lasheras, Del Rio, & Ramirez, 2008; Hedges & Woon, 2011). Preterm infants experience heightened levels of stressors while in the neonatal intensive care unit (NICU) (Newnham et al., 2009; Peng et al., 2009). These stressors include but are not limited to exposure to painful procedures, reduced maternal contact, auditory stimuli, and excessive lighting (Cong et al., 2017; Konig et al., 2013; Newnham et al., 2009). One of the more prominent stressors in the NICU is exposure to painful procedures, with preterm infants experiencing a median of 10, medically relevant, painful procedures a day (Carbajal et al., 2008). In rodent models of early-life pain exposure, the hypothalamic-pituitary-adrenal axis does indeed respond to the painful stimuli with elevated levels of corticosterone 24 hours later in pups that experience pain compared to controls (Victoria et al., 2014). In our laboratory, utilizing a repetitive needle poke, similar to the heel stick NICU procedure, we observed an increase in corticosterone 30 minutes after pain exposure in 4-day old pups compared to controls. Recent human studies indicate that early-life pain exposure in preterm infants negatively impacts cognitive and affective behavioral development in infancy (Grunau et al., 2009) and school-aged children (Ranger et al., 2015; Vinall et al., 2014) but the impact of early-life pain exposure on cognition and behavior in adulthood is unknown.

Early-life pain exposure produces a down-regulation of the hypothalamic-pituitary-adrenal (HPA) axis response in very premature infants who have experienced a large number of painful procedures and in school-aged former preterm infants (Brummelte et al., 2015; Grunau et al., 2013; Grunau et al., 2005). With the brain being vulnerable to elevated levels of

glucocorticoids, it is not surprising to see changes in cognitive functioning and affective behavior following early-life pain exposure. For example, Grunau et al. (2009), found that the number of skin-breaking procedures was associated with poor cognitive and motor function at 8 and 18 months corrected gestational age and Vinall et al. (2014) found that the number of painful procedures was a significant predictor of lower IQ in former preterm school-aged children. Although there is a lack of adult longitudinal human studies, findings from animal models indicate that early-life pain exposure results in long-term cognitive deficits as well. For example, Anand et al. (2007) found that animals exposed to repetitive inflammatory pain via paw formalin injections required more time to consume food in baited arms in the radial arm maze when compared to controls, suggesting impaired spatial memory. In line with these findings, a single exposure of neonatal chronic-inflammatory pain produced deficits in memory in the Morris water maze in middle-aged rats (Henderson et al., 2015). In addition to cognitive deficits, elevated levels of early-life pain have been linked to poorer internalizing behavioral outcomes in former preterm 18-month-old infants (Vinall et al., 2013) and in school-aged children (Ranger et al., 2014). Surprisingly, rat pups exposed to early-life inflammatory pain exhibit decreased anxiety-like behavior in the elevated plus maze and decreased depressive-like behavior in the forced swim test (Anseloni et al., 2005) when compared to controls. However, less is known whether simple skin-breaking procedures, similar to the heel stick in the NICU, will have long-lasting consequences that last into adulthood. In the current study we are going to use a model of early life pain exposure that closely mimics the experiences of preterm infants in the NICU by using repetitive needle pokes throughout the first days of life, a model which we have previously shown to increase corticosterone and reduce glutamate on PD4. Due to these changes, we will investigate the

consequences of early-life pain on learning and memory performance, anxiety-like behaviors, and stress response in adult male and female rats.

In addition to experiencing many painful procedures, NICU preterm infants also experience early-life stress in the form of reduced maternal contact. In the NICU, an infant's environment is maintained by a neonatal incubator, which is in place to promote the survival of the infant, however, these incubators inadvertently reduce maternal contact. Due to the vast amounts of confounding factors, the exact stress response and consequences of reduced maternal contact produced in the NICU is not known. However, adopted children that experienced initialized care, a much more extreme form of reduced maternal care, displayed increased ADHD symptomology and lower IQ scores 2.5-5 years later when compared to non-adopted children (Doom, Georgieff, & Gunnar, 2015). In animal models, exposure to reduced maternal care triggers the stress response and leads to increased corticosterone (Kuhn et al., 1990; McCormick et al., 1998). Further, from our previous work utilizing a tea-ball infuser, a novel model of reduced maternal care, we found that 30 minutes of maternal isolation through tea-ball encapsulation produced a significant increase in corticosterone. Behaviorally, early-life maternal isolation is associated with many adverse outcomes. For example, Aisa et al. (2007) found that 3 hour daily maternally separation during the first 3 weeks of life produced impaired spatial reference memory testing during the Morris water maze and recognition memory during the novel object recognition test when compared to controls. Although these findings suggest cognitive deficits following maternal deprivation it should be noted that not all previous findings are consistent, with some finding cognitive deficits (Baudin et al., 2012; Garner, Wood, Pantelis, & van den Buuse, 2007) and others finding cognitive enhancement (Zhang et al., 2014). Based off these results, further

research is needed to understand the behavioral outcomes, especially in cognitive functioning, following early-life changes in maternal care.

Interestingly, one stressor that maternal contact appears to modulate is neonatal pain. For example, increasing skin-to-skin contact or Kangaroo care during a painful procedure reduces the behavioral responses associated with neonatal pain (Johnston et al., 2008). Moreover, in rodents the presence of the mother reduces the corticosterone response in pups to a painful foot shock (Moriceau & Sullivan, 2006), indicating that maternal contact negates the stress response produced by neonatal pain. Despite this influential role of maternal care on neonatal pain, very few preclinical studies control for maternal care when investigating neonatal pain, indicating that previous deficits produced by neonatal pain may be more drastic than previously thought.

It is apparent that pain and maternal care modulate adult cognitive and affective behaviors and HPA axis functionality. However, the exact mechanism by which these stressors influence adult behaviors remains unknown. Using a repetitive needle poke model and reduced maternal care via the tea-ball infuser model, we have previously reported that pain increases serum corticosterone but reduces frontal cortex and hippocampal glutamate respectively on PD4. Based on these findings, the current study investigates the adult behavioral consequences of early-life pain and/or reduced maternal care in rats by utilizing the neonatal pain model described above and the novel tea-ball infuser model of reduced maternal care. To assess cognitive functioning, animals exposed to pain and/or reduced maternal care performed the Morris water maze and novel object recognition test. Further, the open field test and restraint stress testing were employed to investigate changes in affective behaviors and HPA axis functionality. We hypothesize that early-life pain exposure and reduced maternal care will result in cognitive deficits and altered stress responding. Further, due to the modulatory role of maternal care on pain, we hypothesize that the

consequences following each stressor alone will be exacerbated in animals that experience both neonatal pain and reduced maternal care simultaneously.

Methods

Animals

Eight Sprague-Dawley females and four Sprague-Dawley males were purchased from Charles River Laboratory (Portage MI, USA) and were mated to produce eight litters. On postnatal day (PD) one, litters were culled to 10 pups. Pups remained with their mother until weaning on PD21. Post-weaning, animals were grouped housed with same sex litter mates until PD51, in which they were pair housed with same sex litter mates. Animals were housed in 12:12-hour light:dark cycle controlled vivarium, with food and water available ad libitum. Three days prior to behavioral testing, animals were briefly handled to attenuate handling stress.

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approval for all procedures was granted by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University and can be provided upon request. All efforts were made to reduce the number of animals used and their suffering.

Groups and Procedures

Male and female pups in each liter were exposed to five different early life conditions. Maternal isolation pups experienced 30 minutes of maternal isolation via encapsulation in a tea-ball infuser four times a day from PD1 to PD4. Pups enclosed in tea-balls were placed back into the nest allowing for olfactory cues, however, dams were not able to provide direct maternal contact. Pain group pups were exposed to repetitive painful procedures from PD1 to PD4. Painful procedures utilized in this study were modified from Anand et al. (1999), which consisted of a 26-

gauge needle rapidly inserted into the paws and were dispersed over four sessions a day. To replicate NICU settings, the number of painful procedures dissipated over the course of the first four days with 8 pokes on PD1 (2 per session), 6 pokes on PD2 (1-2 per session), 4 pokes on PD3 (1 per session), and 4 pokes on PD4. Pups in the isolation and pain group experienced tea-ball encapsulation instantly after pain exposure. Touch control pups experienced tactile stimulation on the paws via a cotton swab and unhandled controls pups were left undisturbed. If litters had less than 5 males or females (n = 4 litters), dams kept 10 pups with an uneven distribution of the sexes. This resulted in some groups having more animals assigned than others. For instance, one litter had only three males, so instead of a touch control male this litter provided two female pain pups. No litter had more than two pups in any given group. This resulted in the following number of animals per group: pain group (male n = 9, female n = 9), maternal isolation group (male n = 8, female n = 8), pain and isolation group (male n = 8, female n = 9), handled control group (male n = 7, female n = 8), and unhandled (male n = 6, female n = 7). Procedures were conducted at 8:00, 9:30, 11:00, and 13:30 to allow for maternally isolated pups time to nurse. Body weights were collected before the start of the first session or at 9:00 on PD1, PD4, PD8, PD21, and PD99.

Maternal care observation

Time spent on and off nest and the time dams spent licking and grooming pups were obtained in 5 second intervals during the 30 minutes of pup tea-ball encapsulation and 20 minutes after pups were released for the first two sessions of every day (i.e. PD1-4).

Open field test (OFT)

Adult animals (PD 79-95) were placed into an 80 x 80 x 36 cm open arena and allowed to freely explore for 10 minutes. Each session was video recorded using a Basler ace area scan camera (acA1300, Basler, Hamburg Germany). While in the arena, total distance traveled, time spent in

the center (inner 50%) of the arena, latency to enter the center of the arena, and number of crosses into the center arena were recorded and analyzed using EthoVision XT 10 (Noldus, Wageningen, Netherlands).

Novel object recognition (NOR)

One day after the OFT, animals were subjected to the novel object recognition test to assess recognition memory. Briefly, animals were placed in an arena with two identical objects that were placed equidistance from one another and the arena walls. Animals were allowed to explore both objects freely for 10 minutes. Animals were then removed from the arena and placed back into their home-cages for 1 hour. Following the delay, animals were placed back into the arena with one of the previously used objects replaced with a novel/unfamiliar object. Animals were allowed to freely explore the objects for 5 minutes. Time spent investigating both objects was video recorded (acaA1300, Basler, Wageningen, Netherlands) and hand scored by an experimenter, who was blind to the animals' group assignment. Discrimination index was calculated as ((time exploring the novel object – time spent exploring the old object)/ (time exploring the novel object + time spent exploring the old object))* 100. To adjust for habituation, novel object recognition testing occurred in the same arena used for open field testing.

Morris water maze

One week after the NOR, animals performed a modified version of the Morris water maze spatial learning task that consisted of 5 training days with 3 sessions per day (Callan et al., 2017). Briefly, animals were exposed to a large circular pool (160-cm diameter, 50-cm depth) filled with water mixed with black tempera paint. Within the pool, a square platform was submerged 2 cm below the water surface. During training phases, animals were placed in the pool facing the pool wall and were then allowed to swim until they had reached the platform location. Upon reaching

the platform the animals stayed on the platform for 10 seconds. If the animal failed to reach the platform during the 90 second trail, they were placed on the platform for 10 seconds. Each animal experience three training trials a day, with an inter-trial interval of 30 minutes, for five consecutive days. Starting points during training trials were counter-balanced between groups. After the last training trial on the fifth day, animals were subjected to a 60-second probe trial to assess acute spatial reference memory, in which the platform was removed from the pool. Seven days later another probe trial was conducted, identical to the first probe trial, to examine long-term spatial reference memory. Immediately after the second probe trial, animals underwent a reversal learning protocol in which the platform location was moved to the opposite quadrant from its original location. Three training trials were conducted, identical to the training phases prior, with the new platform location. During training sessions, total distance swam was videotaped via Basler ace area scan camera (acA1300, Basler, Hamburg Germany) and recorded and analyzed using EthoVision XT 10 (Noldus, Wageningen, Netherlands). For probe trials, time spent in learned quadrant was analyzed.

Restraint stress

To assess HPA axis functioning animals were exposed to an acute restraint stressor one week after the final trial in the Morris water maze test. Before the induction of stress, a baseline blood sample was collected via a saphenous vein blood draw (t0) within 3 minutes of touching the animal's cage. After the initial sample, animals were immediately placed and enclosed in a plastic restraint stress tube (Plas labs, model 553-BSSR & 554- BSSR) for 30 minutes. When the animals were removed, another blood sample was collected (t30) and they were placed back into their home-cages and returned to the vivarium. One hour after stress exposure a final blood sample was collected to assess the physiological recovery from the acute stressor (t90).

Serum corticosterone

Blood samples were kept at -4℃ and allowed to clot overnight. On the following day, samples were spun down at 8000g for 10 minutes and serum was extracted and maintained at -20℃ until further processing. Serum was analyzed for corticosterone using a standard CORT EIA kit (catalog # K014-H1; Arbor assays, Ann Arbor MI) per manufacturers recommendations. Samples were run in duplicates and inter- and intra- assay coefficients were less than 10%.

Vaginal Lavage

On behavioral testing days, female animals were lavaged to determine their estrous cycle phase. Briefly, three to four drops of water were administered into the vagina and then immediately reabsorbed via an eyedropper. From the eyedropper, samples were placed on a glass slide and stained with Cresyl Violet. Once dry, samples were analyzed under a microscope and the estrous cycle phase was determined from the types of cells observed as previously described (McLean et al., 2012).

Statistics

Maternal behaviors during the 30 minutes of tea-ball escalation and the 20 minutes after pups were removed from tea-balls were compared using a paired samples t-test. Repeated measures ANOVAs with sex (male, female), tea-ball encapsulation (no isolation, isolation), and pain (no pain, pain) as between-subject factors were utilized to assess group differences in spatial learning over the 5 training days and the reversal learning. To avoid list wise deletions, extreme outliers in the repeated measures models were replaced with the group average for the sex of that animal. Due to natural adult differences in weight, separate ANOVAs for male and females were used to assess weight on PD1, PD4, PD8, PD21, and PD99 with tea-ball encapsulation and pain as between subject's factors. All other analyses were carried out with a factorial ANVOA with

sex, tea-ball encapsulation, and pain as between-subject factors. All significant interactions were followed up with a Fishers LSD post-hoc analysis. To examine the role of the estrous cycle on behavioral outcomes, a separate factorial ANOVA investigating isolation and pain with estrous phases as a covariate was conducted for all behavioral outcomes. Unless stated otherwise below, estrous cycle did not influence behavioral results. All statistical analyses were conducted using IBM SPSS 24 and results were considered significant at $p < 0.05$.

Results

Maternal care

No statistical differences were observed when comparing percentage of time on nest or licking and grooming during the 30 minutes when pups were in tea-balls to the 20 minutes following removal of the pup from the tea-balls (p's > .05; see figure 6 a & b).

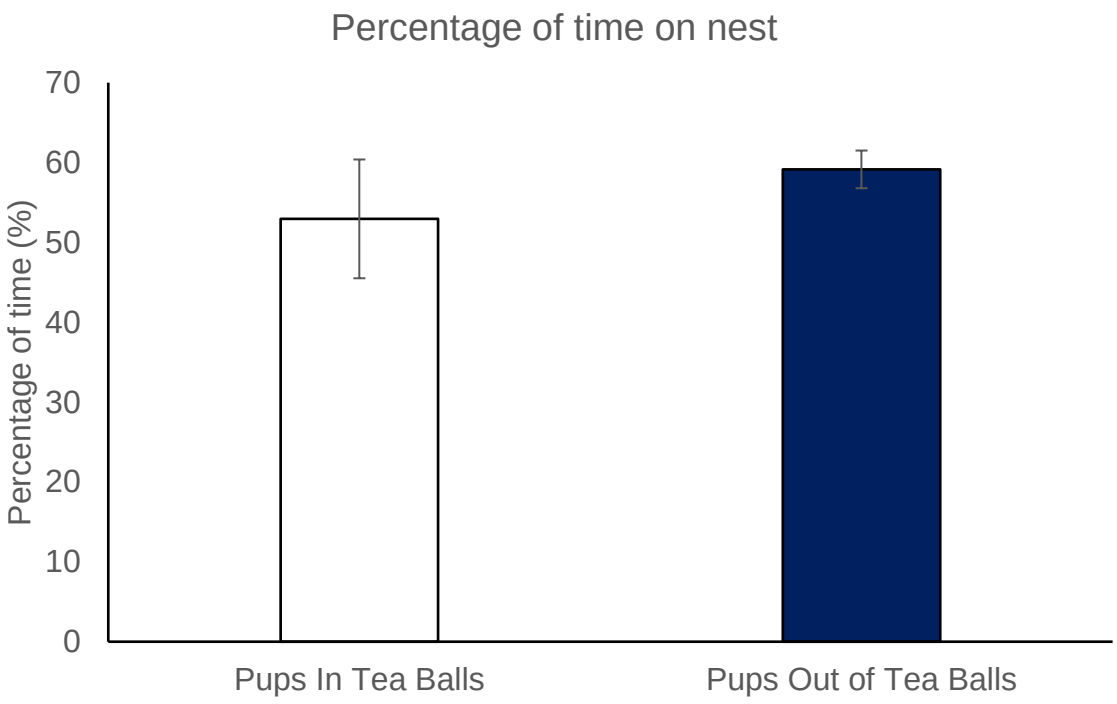

Figure 6a.

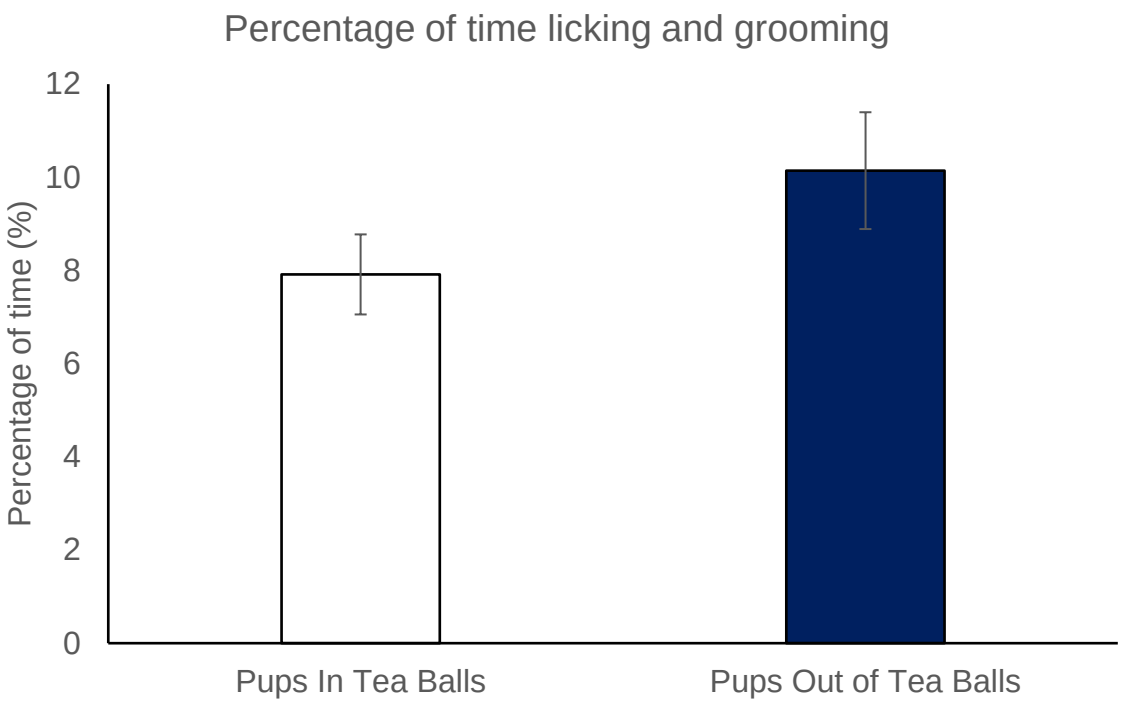

Figure 6b.

Figure 6. A) Displays the percentage of time on nest during the 30 minutes when pups were in tea-balls compared to the 20 minutes immediately after pups were removed from tea-balls. B) Displays the percentage of time spent licking and grooming during the 30 minutes when pups were in tea-balls compared to the 20 minutes immediately after pups were removed from tea-balls.

Body weight

A repeated ANOVA analyzing male body weight from PD1, PD4, PD8, PD21, and PD99 revealed no effect of pain or tea-ball encapsulation (p's > .05) but a significant main effect of days ($F(1.036, 29.003) = 14391.79$, $p = .001$; see Table 3) with weight increases over the course of development. In females a similar outcome was observed with no effect of pain or tea-ball encapsulation (p's > .05) but a significant main effect of days ($F(1.047, 31.402) = 6716.058$, $p = .001$; see Table 4). Handling did not impact weight over the course of development (p >.05).

Table 3. Male body weights (g)

	Touch	Isolation	Pain	Isolation & Pain
PD1	7.04 ± 0.30	7.51 ± 0.44	6.87 ± 0.07	7.38 ± 0.30
PD4	10.97 ± 0.39	10.95 ± 0.45	10.97 ± 0.30	10.14 ± 0.47
PD8	18.68 ± 0.57	17.99 ± 0.66	17.83 ± 0.27	17.22 ± 0.58
PD21	53.63 ± 1.40	51.66 ± 1.88	52.19 ± 1.59	49.94 ± 1.67
PD99	513.67 ± 8.77	525.14 ± 6.33	524.4. ± 10.78	508.86 ± 12.41

Data are represented as means ± SEM.

Table 4. Female body weights (g)

	Touch	Isolation	Pain	Isolation & Pain
PD1	7.30 ± 0.23	7.02 ± 0.32	6.99 ± 0.17	7.27 ± 0.26
PD4	10.93 ± 0.27	10.51 ± 0.34	10.57 ± .022	9.95 ± 0.30
PD8	18.45 ± 0.39	17.53 ± 0.43	17.85 ± 0.35	17.56 ± 0.42
PD21	52.33 ± 1.67	49.54 ± 0.56	51.94 ± 1.40	51.29 ± 0.57
PD99	294.29 ± 5.98	290.57 ± 7.42	291.25 ± 5.93	304.38 ± 7.08

Data are represented as means ± SEM.

Open field test (OFT)

When comparing distance traveled across groups, a factorial ANOVA found no effects of isolation or pain (p's > .05; see table 5 & 6) but a main effect of sex ($F(1, 58) = 31.701$, $p = .0001$) with females traveling more distance compared to males. No differences in the percentage of time spent in the center arena were found (p's > .05; see table 5 & 6). Latency to enter the center arena was not impacted by sex, pain, or isolation (p's > .05; see table 5 & 6). No differences in the

number of center arena entries were found between groups (p's > .05; see table 5 & 6). No open field tests outcomes were affected by handling (p >.05).

Table 5. Male open field test outcomes

	Touch	Isolation	Pain	Isolation & Pain
Distance Traveled (cm)	4637.39 ± 304.69	4850.47 ± 164.84	4582.25 ± 142.31	4802.40 ± 202.26
Percent time in center arena (%)	8.63 ± 0.76	8.25 ± 1.27	6.89 ± 1.06	8.40 ± 1.21
Number of center entries (#)	13.46 ± 4.72	28.39 ± 6.44	29.36 ± 6.4	18.43 ± 8.80

Data are represented as means ± SEM.

Table 6. Female open field test outcomes

	Touch	Isolation	Pain	Isolation & Pain
Distance Traveled (cm)	5531.56 ± 316.86	5683.10 ± 292.44	5613.43 ± 239.81	5900.60 ± 239.93
Percent time in center arena (%)	9.97 ± 2.02	6.90 ± 1.41	8.58 ± 0.99	7.37 ± 1.11
Number of center entries (#)	27.89 ± 6.92	44.22 ± 15.37	18.53 ± 9.39	21.27 ± 6.02

Data are represented as means ± SEM.

Novel object recognition

A factorial ANOVA analyzing percentage of time exploring the novel object found a main effect of sex ($F(1, 55) = 5.751$, $p = .020$) ,with females performing better than males, but no effect

of isolation or pain (p's > .05; see table 7). Discrimination index was not impacted by handling (p >.05).

Table 7. Male and female discrimination index during novel object recognition test

	Touch	Isolation	Pain	Pain & Isolation
Males	0.00 ± 0.24	-0.11 ± 0.17	0.17 ± 0.06	0.04 ± 0.12
Females	0.19 ± 0.11	0.27 ± 0.05	0.14 ± 0.08	0.27 ± 0.05

Discrimination index value of zero represented chance exploration (50%). Data are represented as means ± SEM.

Morris water maze

A repeated measures ANOVA found no main effects of isolation, pain, or sex (p's > .05) but a main effect of training days ($F(2.99, 173.95) = 100.044$, $p = .0001$; see figure 7a) with animals swimming less distance over the course of training and a significant interaction between training days and pain ($F(2.99, 173.95) = 3.181$, $p = .025$; see figure 7a). A follow up post-hoc test found that pain animals swam less distance on day 2 of training than non-pain exposed animals ($p = .008$). No main effects were found for time spent in the learned quadrant during the second probe trial (p's > .05; see figure 7c) but a significant pain by isolation interaction was observed ($F(1, 58) = 4.279$, $p = .043$; see figure 7c). A follow up post-hoc test found that pain animals performed worse than handled control animals ($p = .056$) and isolation animals performed worse than handled control animals ($p = .025$). No main effects of pain and isolation were found for reversal learning (p's > .05; see figure 7d) but a significant between subjects main effect of sex ($F(1, 58) = 8.928$, $p = .004$) and a significant between subjects sex by isolation by pain interaction was found ($F(1, 58) = 4.807$, $p = .032$; see figure 7c). A follow up post-hoc test found that control males swam more than isolated males ($p = .041$), isolated females swam more than isolated males ($p = .027$), and a

non-significant increase in isolated females compared to pain and isolated females (p = .071). Time spent in the learned quadrant in the first probe trial was not impacted by pain or isolation (p's >. 05), but by sex (F(1, 57) = 4.469, p = .039; see figure 7b), with males performing better than females but no other main effects (see figure 7b). No behavioral outcomes were altered by handling (p's >.05).

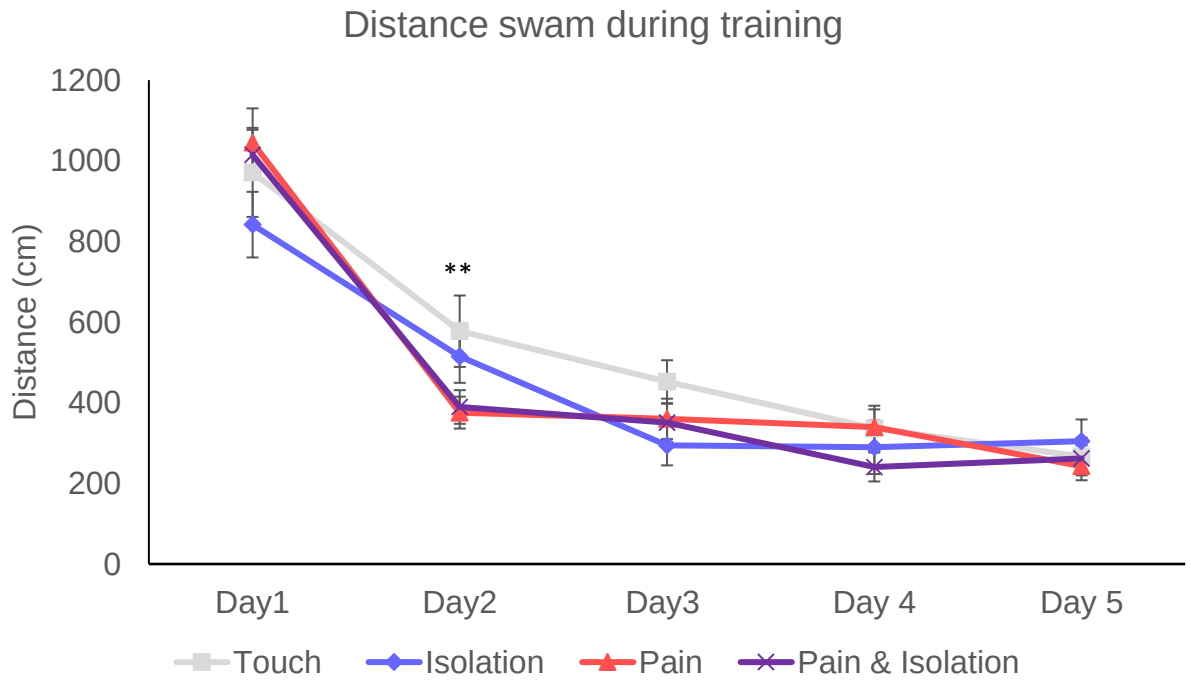

Figure 7a

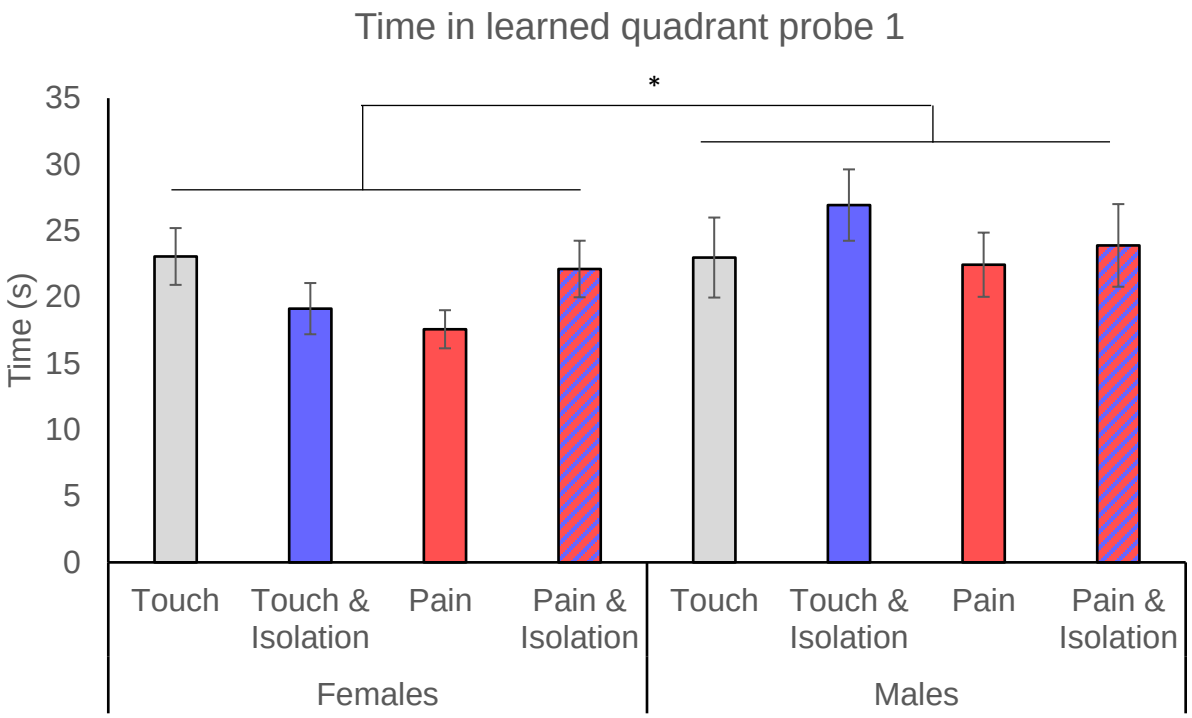

Figure 7b.

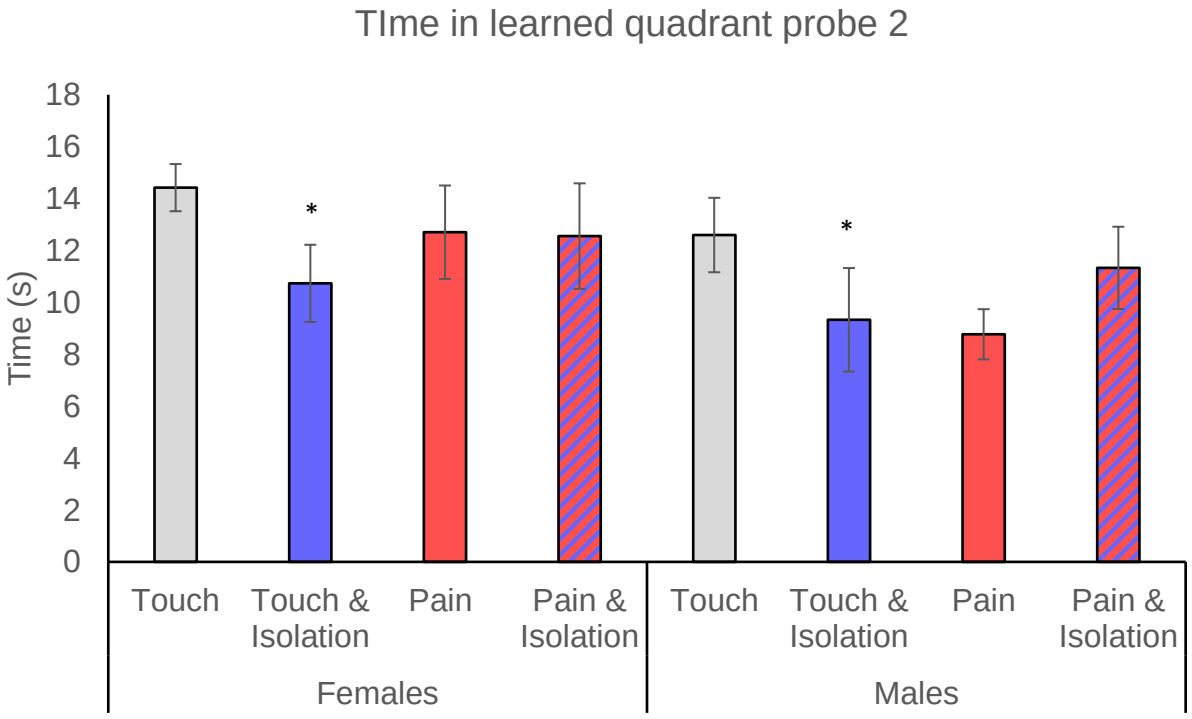

Figure 7c.

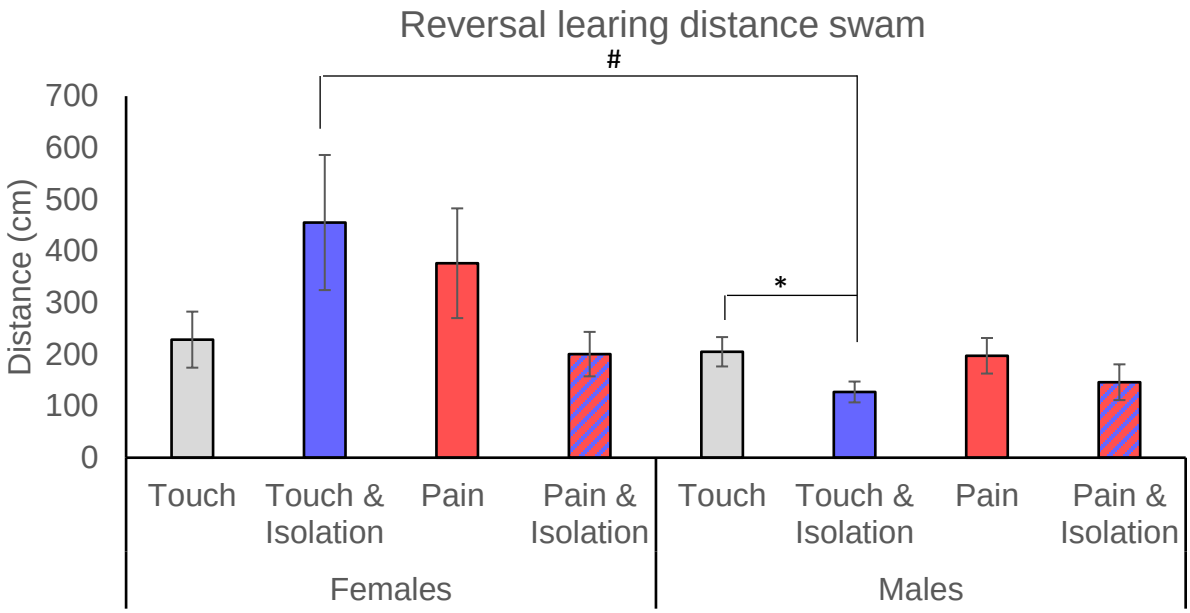

Figure 7d.

Figure 7. Various cognitive outcomes during Morris water maze testing (PD87-102). A) Pain exposed animals swam significant less distance on day 2 of training compared to non-pain exposed animals (*p = .025). B) During the first probe trial, immediately after the last training session, male animals spent more time in the learned quadrant than females (*p = .039). C) Isolated animals spent significantly less time in the learned quadrant than non-isolated animals during the second probe trial (7 days after the last day of training; *p = .025). D) During reversal leaning, male isolated animals swam significant less than control males (*p = .041) and isolated females (# p = .027)

Restraint stress testing

Due to natural sex differences in corticosterone levels, males and females were analyzed separately. Separate factorial ANOVAs analyzing female and male baseline corticosterone found no effects of isolation or pain exposure (p's > .05; see figures 8a & b). Further, there were no significant effects for corticosterone levels immediately after an acute stressor (p's > .05; see figure 8c & d). Female corticosterone levels one hour after the cessation of the restraint stress were significantly elevated in pain exposed animals compared to non-pain animals ($F(1, 28) = 4.422$, $p = .045$), with no effect of maternal isolation (p > . 05; see figure 8e). Further, when controlling for estrous cycle, female pain exposed animals had significantly elevated levels of corticosterone one

hour after stress exposure compared to non-pain exposed animals ($F(1, 26) = 6.081$, $p = .021$; see figure 6e) but no effect of maternal isolation ($p > .05$; see figure 8e). No main effects were found when comparing male corticosterone levels one hour after stress exposure (p's $> .05$; see figure 8f).

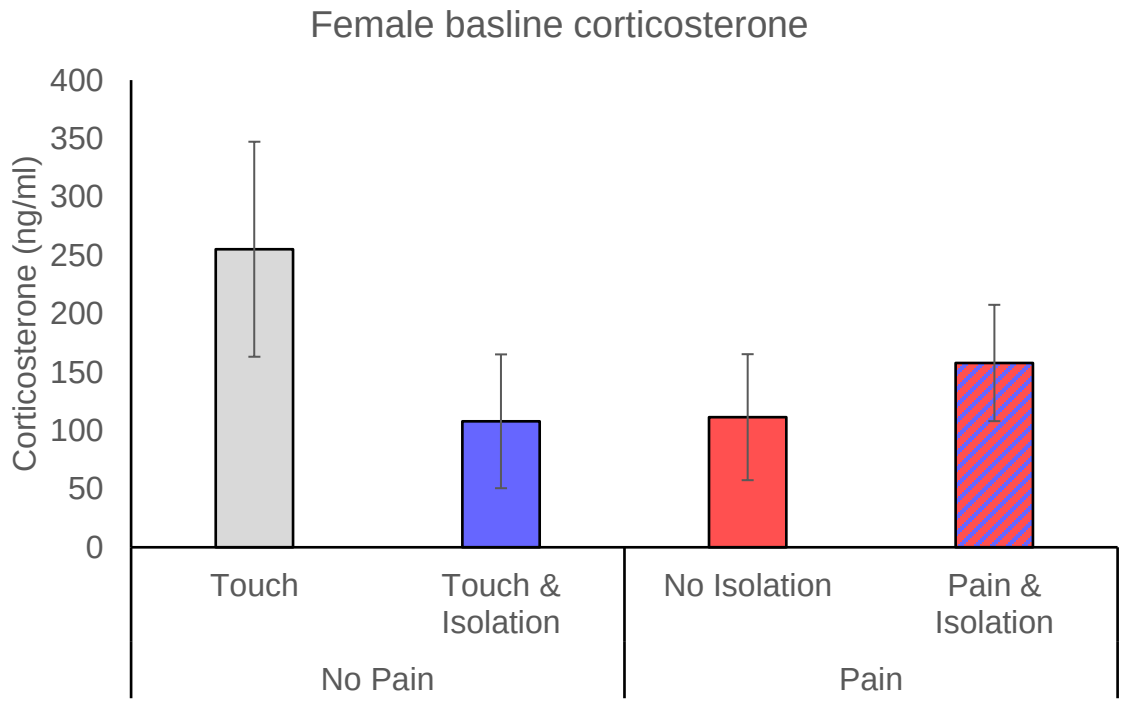

Figure 8a.

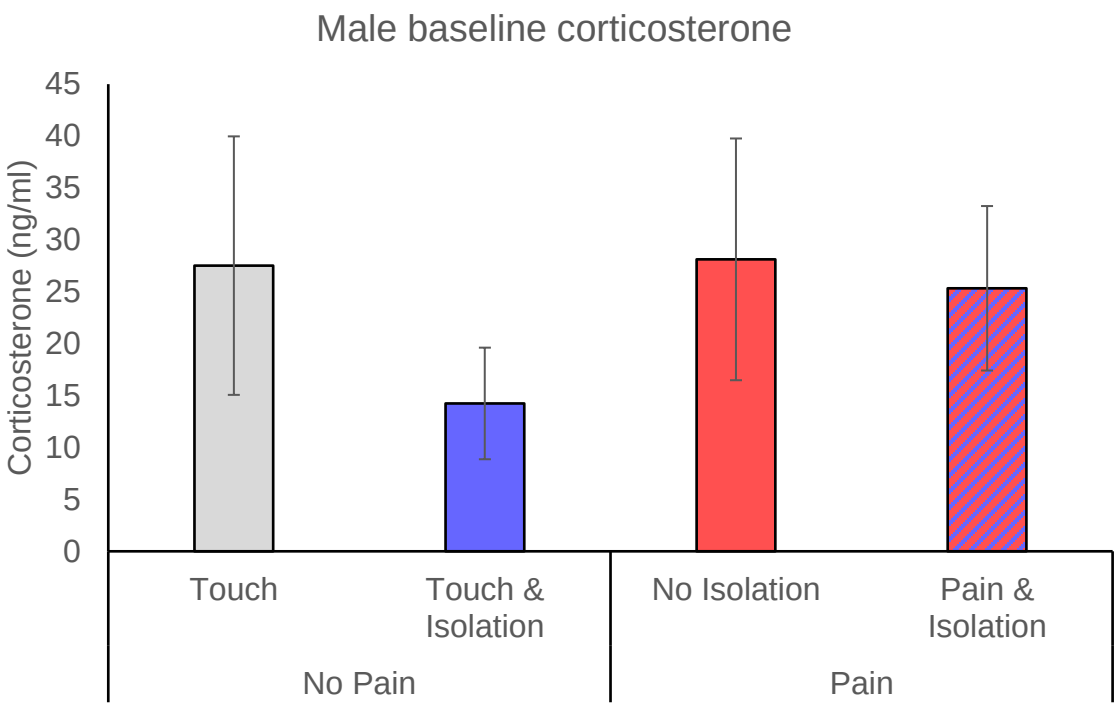

Figure 8b.

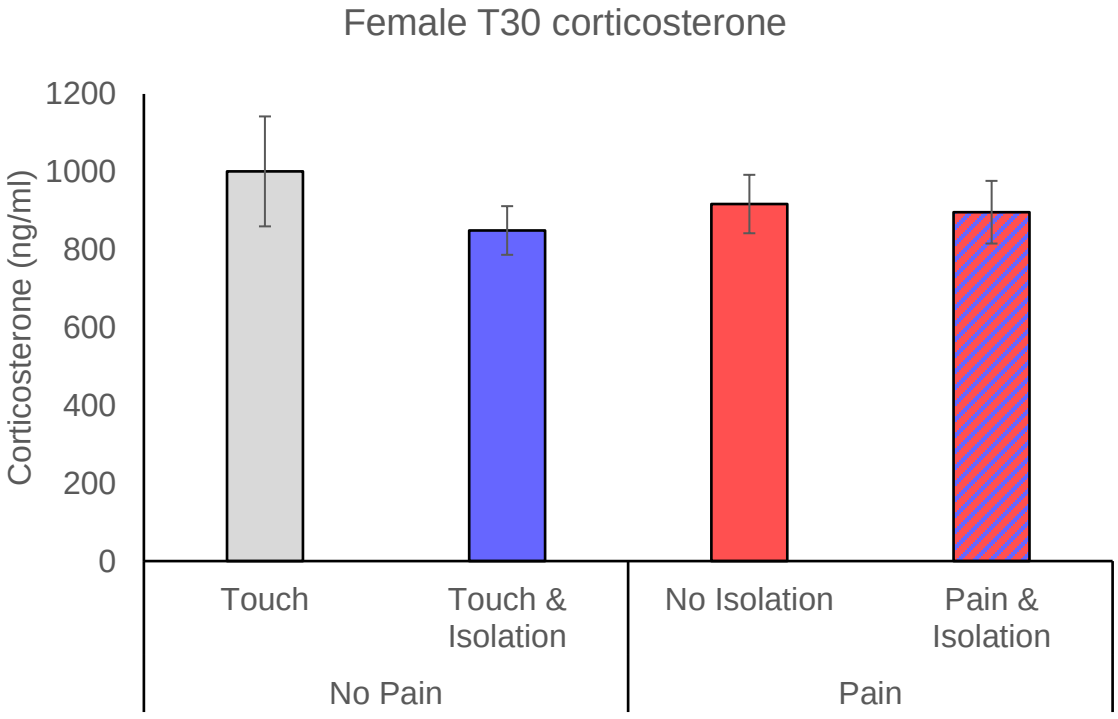

Figure 8c.

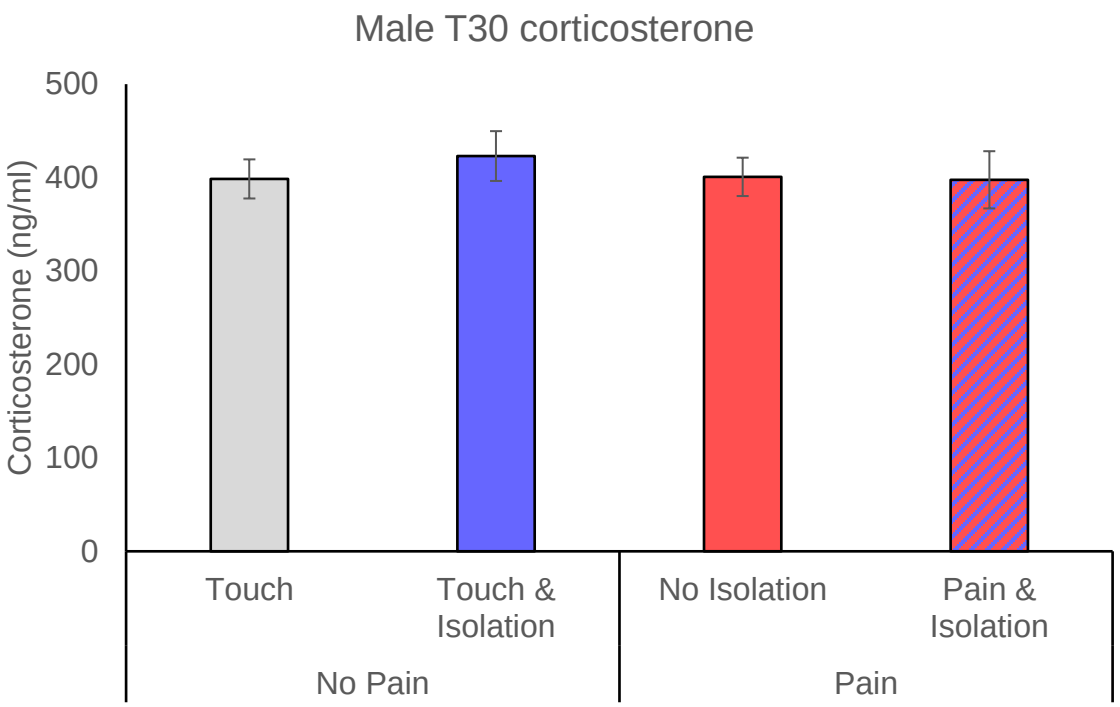

Figure 8d.

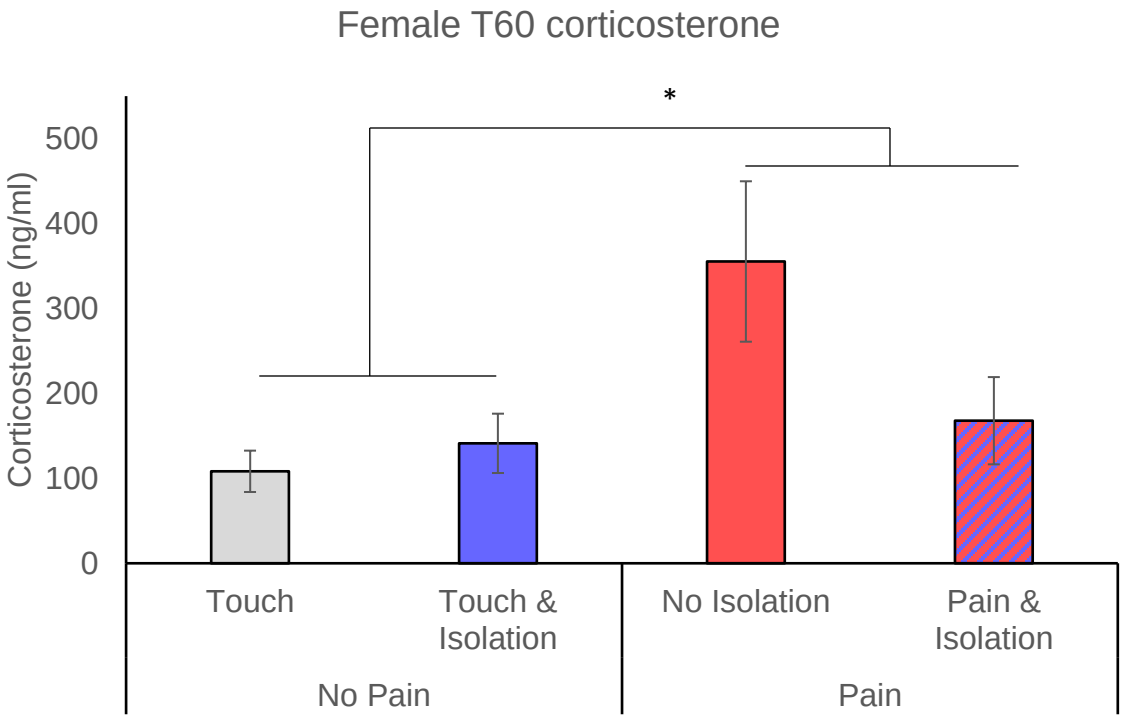

Figure 8e.

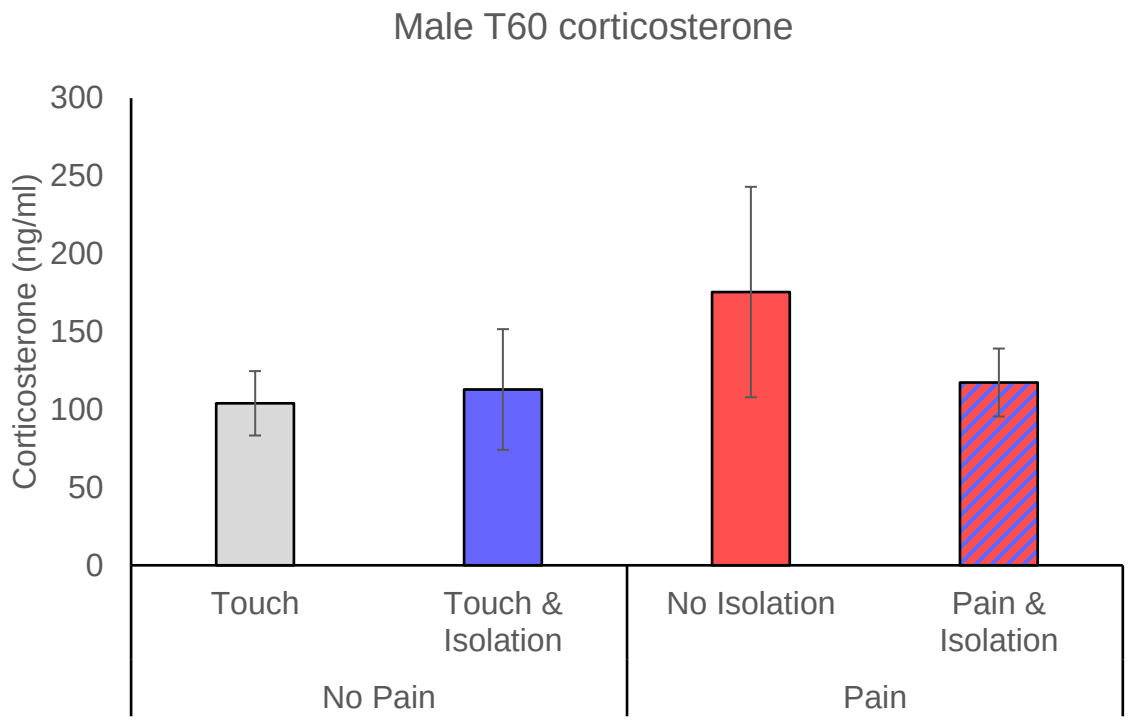

Figure 8f.

Figure 8) Serum corticosterone levels at baseline (A,B), 30-minutes (C,D), and 60-minutes after (E,F) restraint stress exposure. Serum corticosterone levels did not differ between groups before or immediately after acute stress exposure in females and males respectively (A,B,C,D). E) Female pain exposed animals had significantly higher serum corticosterone 1 hour after stress exposure compared to non-pain exposed females (*p = .021). F) Serum corticosterone levels in males were not different between groups 1 hour after stress exposure.

Discussion

In line with previous findings, tea-ball encapsulation reduced maternal care for encapsulated pups did not influence maternal care behaviors after pups were removed from the tea-balls. Behavioral results indicate that tea-ball encapsulation (isolation) and pain exposure enhance unique aspects of learning but impaired long-term memory. Further, pain impaired HPA axis recovery from an acute stressor in a sex dependent manner.

Tea-ball encapsulation reduces maternal contact

Similar to previous findings, the percentage of time dams spent on the nest did not differ during the 30 minutes pups were placed in tea-balls compared to the 20 minutes after pups were

removed. These results indicate that pups placed in the tea-ball infusers received less maternal care than their litter mates that were not enclosed in tea-balls and that this reduction is not being "made up" for pups in the tea-balls once they are removed. A similar effect was found when comparing the percentage of time dams spent licking and grooming their pups.

Neither pain nor tea-ball encapsulation impact body weight during development

Weight gain was monitored during exposure to neonatal pain and maternal isolation over the course of development. In line with previous results from our lab, we did not notice group differences in the amount of body weight gain during the early postnatal development period (PD1,4, & 8). Body weight is typically reduced following maternal isolation (McIntosh, Anisman, & Merali, 1999) and unchanged following neonatal pain exposure (Anand et al., 1999; LaPrairie & Murphy, 2007; Walker et al., 2003). The lack of weight reduction in the maternally isolated animals in the current study is most likely due to the duration of maternal isolation (30-minutes) and the spacing (1-hour) in between sessions to allow for nursing. Moreover, the null finding in early-life weight gain for both maternal isolation and neonatal pain suggests that pups are not experiencing malnourishment.

Maternal isolation does not impact affective behaviors in the open field test

One of the most widely documented consequences of early-life stress is alterations in affective behavior later in life (Pechtel & Pizzagalli, 2011). Interestingly, maternal isolation and neonatal pain typically produce different affective phenotypes with increased anxiety-like behavior in isolated animals (Aisa et al., 2007; Daniels et al., 2004; Kalinichev, Easterling, Plotsky, & Holtzman, 2002) and decreased anxiety in neonatal pain exposed animals (Anseloni et al., 2005; Victoria et al., 2013b; Victoria et al., 2015). In the current study, we failed to observe any changes in affective or locomotor behavior during the open field test following maternal isolation or

neonatal pain. In line with our findings, Zhang et al. (2012) and Lajud, Roque, Cajero, Gutierrez-Ospina, and Torner (2012) both found no change in adult anxiety-like behavior in pups that were exposed to 3 hour daily maternal separation for two weeks. Moreover, Negrigo, Medeiros, Guinsburg, and Covolan (2011) found no difference in the amount of time spent in the open arms of the elevated plus maze in both males and females exposed to PD1 formalin injections compared to control animals. When taken collectively, it is apparent that the development of affective behaviors may be sensitive to the types of early-life stress the organism is exposed to and that differences between previous studies and the current study are most likely due to methodological differences in neonatal stress exposure.

Maternal isolation and pain impact various components of spatial learning and memory

It has been well established that spatial learning and long-term memory formation are dependent on the hippocampus (Logue, Paylor, & Wehner, 1997; Morris, Garrud, Rawlins, & O'Keefe, 1982), a brain region which is sensitive to maternal isolation and inflammatory neonatal pain (Aksic et al., 2013; Anand et al., 2007; Duhrsen et al., 2013; Huot et al., 2002; Rovnaghi et al., 2008; Zhang et al., 2002). Thus, it is not surprising that previous studies have found adult alterations in learning and memory in the Morris water maze following maternal isolation and inflammatory neonatal pain (Aisa et al., 2009; Henderson et al., 2015; Solas et al., 2010; Suri et al., 2013). Interestingly, in the current study using a translational repetitive needle poke model, we found that pain exposure accelerated learning during acquisition of the platform location and maternal isolation enhanced reversal learning when the platform was placed in the opposite quadrant in a sex dependent fashion. Moreover, both stressors impaired long-term memory during the second probe trial. Although enhanced cognitive outcomes were opposite of what was hypothesized, these findings are not the first to demonstrate improved cognitive functioning after

early-life stress (Pryce, Bettschen, Nanz-Bahr, & Feldon, 2003; Zhang et al., 2014). For instance, Champagne et al. (2008) found that pups from low-licking and grooming dams (reduced maternal care) displayed enhanced cognitive functioning in a stressful fear conditioning paradigm when compared to pups from high-licking and grooming dams. Moreover, hippocampal slices from low-licking and grooming pups exhibited enhanced LTP when incubated in corticosterone but not under standard conditions; a reverse relationship existed for hippocampal slices from high-licking and grooming pups. Given the fact that the Morris water maze is a potent stressor to rodents (Harrison, Hosseini, & McDonald, 2009), it is feasible that exposure to neonatal pain or maternal isolation during critical neurodevelopmental periods rewires the brain to enhance cognitive functioning when challenged with a stressful event. Further, early-life stress may prioritize learning functionality over long-term memory capabilities as learning may be more beneficial for survival in stressful environments. Taken together these findings suggest that early-life stress may produce an adaptation in an organism to enhance cognitive functioning in challenging or stressful environments. It should be noted, however, that the current findings are incongruent with human outcomes which demonstrate cognitive impairments in infants and school-aged children that experienced increased levels of neonatal pain (Grunau et al., 2009; Ranger et al., 2015; Vinall et al., 2014). Nevertheless, human preterm infants experience more stressors than the two modeled in the current study, many of which may contribute to the impaired documented cognitive deficits in humans.

Alterations in stress reactivity are sex and stressor dependent

It has been well documented in both human and rodent findings that early-life noxious insults impact HPA axis functioning and development (Grunau et al., 2005; Victoria et al., 2014). For example, Anseloni et al. (2005) found that inflammatory pain exposed animals had decreased

basal and stress induced corticotrophin release factor and ACTH when compared to control animals. Further, Victoria et al. (2013a) found that male rats exposed to early-life inflammatory pain had decreased serum corticosterone during the recovery from an acute stressor. In line with these findings Grunau et al. (2013) and Brummelte et al. (2015) both found reduced cortisol levels in preterm school-aged boys that experienced increased number of skin-breaking procedures. Collectively, these results suggest that neonatal pain down-regulates bassline HPA axis activity and reactivity to an acute stressor. It is possible that the incongruent findings in the current study stem from the type of pain experienced. Inflammatory pain models produce a chronic pain state lasting upwards of 72 hours (Victoria et al., 2015) compared to the acute rapid needle pokes utilized in the current study. Further, human preterm infants often undergo hundreds of painful procedures and experience many other confounding factors such as infection, mechanical ventilation, or opioid exposures that could contribute to their later outcomes. It is feasible that chronic pain alters the HPA axis differently than repetitive acute pain exposure, especially in females. Interestingly, this is not the first study to suggest that females may be more vulnerable to the long-term consequences of early-life pain exposure. For instance, early-life noxious stimuli produced heightened hypoalgesia and enhanced inflammatory induced hyperalgesia in female rodents when compared to male rodents, suggesting that early-life sex differences, such as estradiol levels, may be protective for males in response to neonatal pain (LaPrairie & Murphy, 2007). However, it is worth mentioning that male corticosterone levels during recovery follow a similar pattern (see Figure 3f) but failed to reach significance, suggesting that repetitive pain may also influence male HPA axis development.

The lack of HPA axis changes within the maternally separated animals is somewhat surprising, as there is an excess of evidence suggesting that maternal care alters HPA axis

development (Llorente-Berzal et al., 2012; Slotten, Kalinichev, Hagan, Marsden, & Fone, 2006). One clear explanation for the lack of changes to HPA axis reactivity observed in the current study stems from methodological differences. For instance, traditional models of maternal isolation remove the pup from the dam and the home-cage for multiple hours over the course of weeks (Aisa et al., 2007), compared to the current model in which the pup remains in the home-cage and are only separated from the dam for 30 minutes four times a day for the first four days of life. It could be argued that the current model is not severe enough to induce long-term HPA axis changes, which may be true, however our model was designed to prevent direct contact between the dam and pup in response to painful procedures but still have both the dam and pup in the same environment, to mimic today's NICU environment.

Conclusion

The aim of the current study was to examine the long-term cognitive and affective consequences of neonatal pain and maternal isolation. We found that neonatal pain exposure accelerated spatial learning and maternal isolation enhanced reversal learning in males only. Moreover, both neonatal pain and maternal isolation impaired long-term spatial memory, suggesting that both stressors lead to adaptations in cognitive functioning that favor learning but impairments in long-term memory. These findings add to the inconsistent literature surrounding early-life stress exposure and cognitive functioning, however, based on the models selected they may provide the best depiction of neonatal pain in combination with reduced maternal care outcomes for preterm NICU infants.

CHAPTER 5: GENERAL DISCUSSION/CONCLUSION

This project was designed to characterize the endocrine, neurochemical, and behavioral outcomes following maternal isolation and neonatal pain. In the process, we created and validated a novel model of reduced maternal care similar to what is seen in today's NICUs. The collection of studies described above demonstrate that maternal isolation through tea-ball encapsulation and simple skin-breaking needle pokes can alter acute and long-term biobehavioral outcomes. The current dissertation advances the field of early-life stress and development and generates new research questions and hypotheses.

Tea-ball infusers and repetitive needle pokes as model NICU stress

To produce a novel model of reduced maternal care, the current studies utilized a tea-ball infuser (see Figure 1) which encloses a rat pup in a wire mesh ball and prevents the dams from providing skin-to-skin contact and licking and grooming. This novel model contrasts with traditional reduced maternal care models which rely on removing the pup from the nest/home-cage for extended periods of time lasting for multiple hours and spanning over multiple days or weeks (Aisa et al., 2007). Although these models are sufficient to produce acute and long-term adverse outcomes in the pups they do not mimic the reduced maternal care observed in today's NICU care (i.e. reducing maternal contact during a painful procedure), which is not surprising since they were not with this goal in mind. From our results, it is apparent that the tea-ball infuser model is sufficient to reduce maternal care (Figure 2a, Figure 6a). That is, dams still provided maternal care for non-tea-ball enclosed pups while the tea-balls were present in the cage. Moreover, there was no increase in time on nest behaviors (blanket nursing, arch back nursing, and nursing and grooming) once pups were removed from tea-balls, indicating that on nest behaviors were consistent regardless of the tea-ball being present in the home-cage. This was not the case,

however, for time spent licking and grooming. From the two sets of analyzed maternal behavior (study 1 vs study 2), we observed conflicting outcomes with dams from the first studies increasing licking and grooming when pups were removed from tea-balls compared to when pups were in tea-balls and dams from the second study displaying no difference in licking and grooming behaviors when pups were in tea-balls or out of tea-balls. It should be noted, that the number of dams in the first study was drastically higher than the second study (n = 17 for study 1 and n = 8 for study 2), suggesting the effect observed in the first study is the typical behavioral response. Moreover, dams from study two did show a similar increase in licking and grooming once pups were removed from tea-balls but this effect failed to reach significance (see Figure 6b). It is possible that the low sample size (n = 8) in the second study produced a false negative and that licking and grooming was increased similar to study 1. In line with the study, increased licking and grooming has been observed after a dam and a recently separated pup are reunited (Kosten & Kehoe, 2010; Zimmerberg et al., 2003). Further, Walker et al. (2003) found that dams acutely increased licking and grooming if pups were exposed to pain compared to pups not exposed to pain, indicating that stress exposure (in the form of pain) in pups can augment maternal behaviors. Increased licking and grooming behaviors have been linked with various developmental outcomes with pups from higher-licking and grooming dams having more favorable outcomes in brain development (Liu et al., 2000) and cognitive functioning (Bredy et al., 2003; Liu et al., 2000). From these results, the increased licking and grooming in our studies must be taken into consideration when interpreting biobehavioral outcomes, however, based on the maternal observations collected, it is unclear if a certain group (i.e. pain or maternal isolation) received the increased licking and grooming or if it was just a global increase. Moreover, it should be mentioned that the increase in licking and grooming is quite small for study 1 (11% vs 14%) and study 2 (8%

vs 10%), suggesting that the impact of the increased licking and grooming may not be as severe. As mentioned previously, utilizing methods of single pup identification may help illuminate the role of maternal care on biobehavioral outcomes following neonatal pain and maternal isolation. Interestingly, previous results using the single pup maternal care observations have found that males experience increased levels of maternal care between PD5-8 (Ragan et al., 2012) and pups that received more maternal care than their littermates displayed worse behavioral outcomes, with high-licked pups displaying increased latency to explore novel objects compared to low-licked pups. From this relationship, the authors suggest that the amount of maternal behavior a pup receives depends on need. That is, a dam will express higher levels of licking and grooming for pups who may need more support to ensure the survival of her offspring (Ragan et al., 2012). In the current study, it is feasible that the observed increase in licking and grooming is directed at pups in need, intuitively this would be the pups exposed to pain and maternal isolation, which in turn would influence our biobehavioral results. Clearly more research is needed, but if pain and maternal isolation pups are indeed receiving more maternal care, that may explain the lack of observed exacerbated behavioral effects.

Repetitive pain was administered via rapid needle insertions in alternating paws, to mimic the heel stick, which is one of the most prevalent painful procedure in the NICU (Stevens et al., 2003). It is important to note, that this model is drastically different than the common inflammatory neonatal pain model, which relies on a single or repetitive injection of an inflammatory agent (Anand et al., 2007; Victoria et al., 2013a). The inflammatory model creates a chronic pain state that lasts upwards of 72 hours (Victoria et al., 2015), which is a stark difference to the single acute needle poke used in the current dissertation. These differences in pain type and duration must be kept in mind when comparing biobehavioral outcomes discussed below.

Neonatal HPA and glutamatergic response to pain and reduced maternal care

As hypothesized, we found that neonatal pain increased serum corticosterone during the SHRP (see Figure 3). These findings are in line with Victoria et al. (2014), who found that pups exposed to a single plantar injection of 1% carrageenan (inflammatory agent) had increased corticosterone 24 hours later compared to control pups. Interestingly, they found that this relationship was reversed 48 hours later with pain exposed pups having reduced corticosterone compared to non-pain exposed pups and then reversed again 7 days after pain exposure. These results indicate that pain plays a modulatory role on neonatal endocrine levels even after the experience of pain has subsided. In the current study, corticosterone levels were only recorded at one time point, which was 30 minutes after the last pain exposure. Collection of multiple time points similar to Victoria et al. (2014) may provide a deeper understanding of the stress response to a more ecologically valid needle poke model compared inflammatory pain. Similar to pain, our novel model of reduced maternal care was sufficient to induce a stress response on PD4 (see Figure 3 study). These findings are in support of previous reports of increased corticosterone following reduced maternal care (Kuhn et al., 1990; Levine et al., 1991). It should be noted however, that based on our multiple day exposure to brief (30 minute) episodes of maternal isolation, we may be observing a sensitization effect of corticosterone to maternal isolation. In support of this hypothesis, McCormick et al. (1998), found that pups exposed to 30 minutes of maternal isolation on PD2 had increased levels of corticosterone compared to control pups, however, if a pup experienced daily 30-minute episodes of maternal isolation from PD2-8 they then had an exacerbated corticosterone release response to a 30-minute maternal isolation episode on PD9 compared to control pups that also experienced 30-minutes of maternal isolation. These findings

suggest, that the observed increase in serum corticosterone in the current studies may reflect a potentiated response produced by the previous 15 maternal isolation episodes.

In adult rodents, exposure to a stressor produces a robust release of glutamate in the frontal cortex and hippocampus that lasts upwards of an hour (Moghaddam et al., 1994). Once a stress response has been triggered, activation of glucocorticoid and mineralocorticoid receptors is vital for the enhanced glutamate response in the hippocampus and frontal cortex (Popoli, Yan, McEwen, & Sanacora, 2011). However, less is known about this connection between stress and the excitatory neurotransmitter glutamate in neonates. During the SHRP, most stressors do not elicit a typical stress response, though previous research indicates that key physiological components of the stress system are functional in neonates, however, the system seems less responsive (Sapolsky & Meaney, 1986) and a stressor must be severe to elicit a neonatal stress response.

Glucocorticoid and mineralocorticoid receptors are present in fetal brain tissue (Diaz, Brown, & Seckl, 1998), thus the circuitry to elicit a stress-induced increase in glutamate in the brain should be in place. However, we found that neonatal pain and maternal isolation produced regionally distinct decreases in glutamate. One explanation for the reduction observed in the current studies may be that pups were exposed to multiple stressors over an extended period of time. In adult rodents, exposure to chronic restraint stress has a similar reduction on basal prefrontal frontal cortex glutamate (Luczynski, Moquin, & Gratton, 2015). It is still feasible that the stress that accompanies pain and maternal isolation increases frontal cortex and hippocampal glutamate acutely, but over multiple exposures, produces a down-regulation of the glutamate system. Further examination of glutamate levels in response to a single episode of maternal isolation or needle pokes would narrow down the neurophysiological response to these neonatal stressors. Despite this potential explanation for down-regulation of glutamate, the regional

specificity of pain and maternal isolation on glutamate remains perplexing. On PD4, glucocorticoid receptors are more abundant in various parts of the hippocampus compared to frontal cortex (Yi, Masters, & Baram, 1994), which would suggest that the hippocampus is more sensitive to increased levels of glucocorticoids from a recent stress response and that a more robust stress response would be needed to impact the frontal cortex. From this, it would be expected that pups that had altered frontal cortex glutamate would also have the highest amount of corticosterone, which is somewhat true. It must be pointed out that the pain specific effect on frontal cortex glutamate is most likely driven by the combination group (pain and maternal isolation simultaneously exposed) which can be seen on figure 4a. Moreover, it is also apparent that the combination group had the highest levels of corticosterone (Figure 3), despite the lack of an interaction effect. Given both observations, the regional differences can be explained by glucocorticoid receptor distribution differences in the frontal cortex and hippocampus. From this, the stress experienced by the maternal isolation group would be sufficient to induce glutamatergic changes in the hippocampus, but the more severe stress experienced by the combination group would alter both the frontal cortex and hippocampus. This hypothesized outcome could be investigated further but increasing the amount of stress (more pokes or longer maternal isolation periods) to see if regional differences become more robust.

Neonatal pain and reduced maternal care alter cognitive functioning

Homeostatic maintenance of glutamate in the neonatal period is vital for proper neuronal and cognitive development. For example, blockade of the NMDA receptor via MK801(NMDA antagonist) in 7-day old rat pups leads to robust increases cell death in the hippocampus, thalamus, and various cortices 24 hours later (Ikonomidou et al., 1999). Moreover,

Kawabe, Iwasaki, and Ichitani (2007) found that exposure to MK-801 from PD7-20 produced adult impairments in the radial arm maze, indicating the cognitive consequences of early-life changes in the glutamatergic system. When taken together, one would intuitively think that the reduction in glutamate due to pain and maternal isolation would lead to adult cognitive impairments, which is somewhat true. In the second experiment, we found that exposure to pain accelerated spatial learning during acquisition of the Morris water maze and maternal isolation enhanced reversal learning in males but both stressors impaired long-term memory during the second probe trial. Although these findings indicate altered cognitive functioning because of neonatal pain and maternal isolation, they do not exactly match our neonatal findings. For example, from our neonatal neurochemistry findings it might be expected that the group with altered hippocampal glutamate would have altered spatial learning during the Morris water maze, as this is a hippocampal dependent task (Logue et al., 1997; Morris et al., 1982). However, we found reduced hippocampal glutamate in the isolation group, which displayed the same pace of learning as controls during spatial learning acquisition. Moreover, we found a pain specific reduction in frontal cortex glutamate, but no effect of pain on frontal cortex dependent reversal learning (Clark, Cools, & Robbins, 2004). Based on these inconsistencies, it is possible that the observed changes in early-life glutamate are transient and are not responsible for the observed changes in adult cognitive functioning or that the time point at which glutamate was sampled does not appropriately depict how each stressor alters glutamate.

Since our data points to an alternative mechanism, it is important to discuss other biological systems that may be responsible for deficits produced by neonatal pain and maternal isolation. One system that was altered in the current study and may explain the observed behavioral outcomes is the HPA axis. As discussed prior, both neonatal pain and maternal isolation produce alterations in

glucocorticoid receptor development in adulthood (Liu et al., 1997; Victoria et al., 2013a). Previous research has shown that the glucocorticoid receptor modulates spatial learning in the Morris water maze (Oitzl, de Kloet, Joels, Schmid, & Cole, 1997) and that continuous blockade of the glucocorticoid receptor via intracranial injections of glucocorticoid receptor antagonist leads to enhanced cognitive performance during spatial acquisition of the water maze (Oitzl, Fluttert, & de Kloet, 1998). Moreover, prolonged blockade of the glucocorticoid receptor leads to down-regulation of the receptor in the hippocampus (Oitzl et al., 1998) which is also seen in adult rats exposed to neonatal pain (Victoria et al., 2013a) and reduced maternal care (Liu et al., 1997). Based off these results, it is feasible that the cognitive outcomes obtained in the current study stem from stressor specific changes in glucocorticoid receptor distribution due to early-life stress exposure. In addition to the HPA axis, previous studies have demonstrated that maternal isolation and neonatal pain up-regulate various inflammatory markers, such as tumor necrosis factor alpha (TNF α) and interleukin 1 beta (Lee et al., 2016; Pinheiro et al., 2015). Interestingly, both TNF α and interleukin 1 beta have been shown to increase cell death in neonatal brain tissue (Cai, Lin, Pang, & Rhodes, 2004) and when they are increased with chronic lipopolysaccharide exposure in adults, they lead to altered cognitive functioning (Belarbi et al., 2012). Although the link between cytokines and neonatal pain and reduced maternal care is relatively new, these results suggest that the inflammatory system is worth investigating as a potential mechanism.

Regardless of a mechanistic explanation, it is important to note cross study differences in an attempt to explain the paradoxical enhanced learning following pain exposure and maternal isolation. For instance, Henderson et al. (2015) found age-specific impairments in the Morris water maze following a single inflammatory injection, with adult male and female rats (PD144-158) but not middle-aged rats (PD439-442), displaying increased latency to platform during training trials

that were spaced either 2 hours or 24 hours apart, indicating an impairment in memory more so than learning. Moreover, during a probe trial conducted 48 hours after the last training trial middle-aged rats, but not adult rats, exhibited reduced time in the learned quadrant. Collectively, these findings suggest that inflammatory pain impairs memory capacity but not necessarily learning, in an age depend manner. In our study, we found accelerated learning but impaired long-term memory. Besides the obvious methodological difference in type of pain administered, the animals used in the current study were considerably younger (PD87-102), which suggests that the impact of neonatal pain on learning and memory may be age dependent. Moreover, Suri et al. (2013) found that rat pups subjected to maternal separation from PD2-14 displayed enhanced learning during the Morris water maze when tested 2 months later but no differences in learning and impaired long-term memory when tested 15 months later. Based off these results it is feasible that the contrast in learning between our findings and Henderson et al. (2015) is a function of age. Further examination of learning at different ages (younger and older) utilizing the repetitive needle poke and tea-ball infuser model may uncover a sensitive age threshold for altered learning and memory following neonatal pain.

We observed a three-way interaction for reversal learning with the post-hoc test revealing that isolated females and control males performed worse than isolated males. Interestingly, reversal learning has been shown to be enhanced following an acute stressor or damage to the ventral medial prefrontal cortex (Graybeal et al., 2011). Based on these findings, it could be argued that enhanced reversal learning is reflecting an impairment in the HPA axis or frontal cortex development specific to maternally isolated males. Since we did not see any changes in baseline or stress induced corticosterone in maternally isolated males, it is not likely that the stress response played a role in reversal learning. Clearly more research is needed to disentangle this complex

interaction. Examination of neurophysiological markers of learning, such as NMDA receptors and long-term potentiation thresholds within the prefrontal cortex (region associated with reversal learning), may help shed light on the mechanism responsible for the observed sex differences.

Although these previous findings point to methodological and age-related explanations for our observed findings, they fail to posit how early-life stress improves learning in adulthood. One current explanation is centered on the biological basis of early-life programming. As explained in Daskalakis, Bagot, Parker, Vinkers, and de Kloet (2013), early-life biological programming is an interplay between an organism's genetic variation and non-genetic factors such as the environment. From this interplay, environmental pressures influence the organism's genetic expression, which alters the development of brain and behavior especially during critical developmental periods. In the current dissertation, early-life rat pups were exposed to environments with no manipulation or stress in the form of neonatal pain or maternal isolation. In response to these different environments, rat pup physiology may drastically change to prime the pup to thrive in an environment that matches their early-life environment. From this, it is conceivable that the pups exposed to early-life stressors (pain or maternal isolation) were best suited for stressful environments. Support for this hypothesis comes from Champagne et al. (2008), who found that adult rats born from low-licking and grooming dams (poorer environment) performed better in a hippocampal based fear conditioning paradigm than adult rats born from high-licking and grooming dams, indicating that exposure to a stressful environment during the neonatal period breed rats that thrive under stressful situations. Further evidence comes from their physiological findings, which report that hippocampal slices of adult low-licking and grooming pups had enhanced LTP when incubated in corticosterone and impaired LTP under standard conditions, conversely, hippocampal slices from high-licking and grooming pups exhibit the

opposite outcome. Collectively, these results indicate that early-life stress exposure can permanently manipulate an organism's biobehavioral development. In the case of neonatal pain and maternal isolation exposure, it is possible that these pups were primed to thrive in stressful environments, like the Morris water maze (Harrison et al., 2009). Clearly, it is not this simple as different types of learning were altered in a stress and sex specific manner, but through this logic, components of maternal isolation and neonatal pain differ, such as duration of stress and amount of stress created, which would lead to different behavioral phenotypes.

In line with our original hypothesis, both neonatal pain and maternal isolation impaired long-term memory. These findings are supported by previous literature of both neonatal pain and maternal isolation (Aisa et al., 2007; Henderson et al., 2015). It is worth mentioning that in the current dissertation, we measured long-term memory via a probe trial 7 days after the first probe trial (last day of training). This time point differs quite drastically from Aisa et al. (2007) who found long-memory deficits in maternally separated adults rats in a probe trial 24-hours after the last training trial and Henderson et al. (2015) who found long-term memory impairments in adult rats exposed to neonatal pain during the 24-hour period in-between training sessions. Based on previously used methods, it is feasible that our model of pain and maternal isolation may produce more robust deficits in memory that would be apparent in a shorter delay of long-term memory testing. Follow up studies investigating the threshold of when long-term memory impairments surface using the current models will provide further characterization of the consequences of neonatal pain and maternal isolation. Moreover, biological markers responsible for long-term memory must be examined such as brain derived neurotrophic factor (BDNF), which not surprisingly is down-regulated in the hippocampus of adult animals either exposed to neonatal pain or maternal isolation (Liu et al., 2000; Nuseir et al., 2017).

Impact of neonatal pain and reduced maternal care on HPA-axis and affective behaviors

Lastly, we examined adult affective behaviors and HPA axis functionality. In the current study we failed to find any differences in anxiety-like behaviors in pain exposed animals, which contrasts with previous findings using inflammatory pain (Anseloni et al., 2005; Victoria et al., 2015). As discussed above, drastic methodological differences arise between inflammatory models of neonatal pain and the repetitive needle poke model used in the current dissertation. For instance, a single injection of an inflammatory agent produces continuous pain that lasts upwards of 72 hours (Victoria et al., 2015). Thus, it is likely that this extended duration plays a role in the formation of anxiety-like behaviors. When measuring HPA axis functionality we found no differences in male or female baseline and peak corticosterone response to an acute stressor. However, during recovery (1 hour after stress exposure) we found that females exposed to pain failed to return to baseline and had significantly higher corticosterone when compared to non-pain exposed animals, a similar pattern was observed in males but did not reach significance. To the best of our knowledge, these are the first findings to demonstrate adult HPA axis reactivity alterations in response to a simple needle poke model. Previous research has established that inflammatory pain exposure during the neonatal period modulates adult HPA axis development (Anseloni et al., 2005), however, the results obtained in the current dissertation point in the opposite direction. For instance, Victoria et al. (2013b) found that adult animals exposed to a single injection of an inflammatory agent as pups had an overall reduction in an area under the curve analysis for corticosterone during a 15-minute restraint or 5-minute forced swim stressor. Moreover, males exhibited a faster recovery from either stressor 75 minutes later. It is possible that the differences in the duration of stress exposure (30-minute vs 15-minute & 5-minute) account for the differences between the two studies. It is also possible that the observed differences

are once again a product of the type of pain administered. Methodological differences aside, it is hard to draw comparisons due to the differences in "recovery time" (60 minutes vs 75 minutes). Interestingly, the significantly higher levels of CORT during recovery in females observed in the current dissertation may also be due to the stress experienced during the Morris water maze, as Victoria et al. (2015) demonstrated that pain exposed animals as adults appear to have a hypo/hyper switch in HPA axis reactivity when challenged with mild chronic stress. Specifically, they found that when pain exposed animals were subjected to a series of mild chronic stressors (over 7 days) in adulthood and then were challenged with an acute swim stressor they had increased values of corticosterone 75-minutes later when compared to non-pain exposed animals. These results indicate that the HPA axis reactivity tested in the current dissertation may not actually be the baseline response in pain exposed animals but rather the flip to a hyperactive HPA axis due to multiple episodes of stress exposure produced by the Morris water maze. It should be noted, that restraint stress testing was conducted one week after the last Morris water maze trial to avoid an interaction effect between the two behavioral tests.

Maternal isolation failed to impact affective behaviors or HPA axis reactivity. These findings are surprising given the fact that maternal isolation produced such a robust increase in corticosterone on PD4 (see Figure 3). Further, these findings are in contrast to previous findings of altered adult HPA axis reactivity following prolonged maternal isolation (3hr daily for the first two weeks of life; Kalinichev et al., 2002; Slotten et al., 2006). Although we failed to achieve significant changes in affective and HPA axis responding in our maternal isolation model, it is worth pointing out that our model is not that drastically different from traditional models with our animals being exposed to 2 hour daily maternal separation (spaced out over 4 x 30 minutes sessions), which is 1 to 2 hours less than most other models, our animals stay in the home-cage

during “isolation” compared to being completely removed from the environment, and we only administered isolation for 4 days compared to two weeks. These differences indicate that slight changes in maternal care lead to severely different affective behavioral and HPA development. Clearly there are significant differences in our model and previous models, especially the 4 days vs 2 week duration, but these differences provide future directions for the tea-ball model. That is, maybe the duration chosen in the current studies are too weak and that continuing the tea-ball exposure to a full-term equivalency (PD8-10) may produce more robust results. Testing these limits with the goal of producing a more translation model would go a great distance in characterizing a preclinical model of NICU reduced maternal care.

Although hypothesized, we failed to find biobehavioral outcomes that point to an additive effect of simultaneous exposure to neonatal pain and maternal isolation. In fact, when statistical interactions between the two stressors were observed (long-term memory and reversal learning), animals exposed to both stressors for the most part performed better than animals exposed to either stressor alone. It is possible that maternal care may be blocking the hypothesized additive effect. For instance, as discussed in Ragan et al. (2012) maternal licking and grooming behaviors may be targeted towards specific pups that are in need and that dams will provide more licking and grooming towards these pups to ensure their survival. It is possible that pups exposed to pain in combination with isolation were deemed to be in more need by the dam and thus received more licking and grooming, which in turn prevented the hypothesized exacerbated deficits. There are some instances within the data in which simultaneous exposure led to exacerbated effects (serum corticosterone and frontal cortex glutamate) but it is important to note that these measurements were taken immediately after a maternal isolation session, so the acute influence of licking and grooming would not be observed. Clearly, individual pup observations would be vital in

understanding how maternal care plays a role in the pain and tea-ball interaction when pups are experiencing the stressors but also after the stressors had subsided. That is, it could be possible that the maternal behaviors towards pain and tea-ball pups were augmented form PD5-21, which reduced the hypothesized exacerbated effects, however, based on the current study, this remains unknown.

Limitations and future directions

The studies in this dissertation serve as a stepping stone in the process of producing a novel model and characterizing the acute and long-term biobehavioral outcomes of neonatal pain and reduced maternal care. Like many studies before, certain limitations exist within the experimental design that hinder interpretability but simultaneously point to future directions. First, we observed a significant increase in licking and grooming behaviors in the first two studies, which as previously mentioned, may influence biobehavioral development. Nevertheless, the fact that we still observed acute and long-term alterations from both neonatal pain and maternal isolation indicates that both are potent stressors, however, it is feasible that these alterations may be more severe if licking and grooming was not augmented. Future studies utilizing a more intense single pup observation method discussed above would help solve and control for this limitation. Second, the single time point for serum corticosterone and brain metabolites on PD4 does not provide information about the exact acute response to neonatal pain and maternal isolation. That is, the alterations in serum and corticosterone reflect 4 days of multiple stress exposure, which may account for the observed reduction in glutamate, but does not allow us to make claims about the initial experience of pain or maternal isolation. Future studies utilizing *in vivo* microdialysis would provide not only the best temporal picture of metabolite change but also the extracellular changes in contrast to total levels. Lastly, using the same animals for multiple behavioral tests may produce

changes in behaviors in the latter tests. Future studies utilizing single cohorts for behavioral tests would provide the best indication of altered behavioral profiles due to neonatal pain or maternal isolation exposure.

In conclusion, this dissertation provides preclinical evidence that a simple skin-breaking model of neonatal pain and a tea-ball infuser novel model of reduced maternal care are enough to produce robust acute and long-term biobehavioral alterations. Clearly, the next logical step is to investigate the adult neurophysiological changes that are responsible for the changes in learning and memory observed in the current study. Further, the needle poke and tea-ball infuser model of NICU stress should be applied to other behavioral domains, such as cognitive testing in a non-stressful environment. Collectively, our model of NICU pain and reduced maternal care suggest that preterm infants experience continuous stress during a critical neurodevelopmental period. Mitigating this stress response through pharmacological or non-pharmacological interventions may help prevent some of the deficits associated with neonatal pain and reduced maternal care, however, optimal treatment for these preterm infants remains an unknown. Further examination of the impact of neonatal pain and reduced maternal care on the systems that comprise the HPA axis may help in the production of an appropriate treatment for preterm NICU infants.

REFERENCES

Abdulkader, H. M., Freer, Y., Garry, E. M., Fleetwood-Walker, S. M., & McIntosh, N. (2008). Prematurity and neonatal noxious events exert lasting effects on infant pain behaviour. *Early Hum Dev, 84*(6), 351-355. doi: 10.1016/j.earlhumdev.2007.09.018

Abraham, I., Juhasz, G., Kekesi, K. A., & Kovacs, K. J. (1998). Corticosterone peak is responsible for stress-induced elevation of glutamate in the hippocampus. *Stress, 2*(3), 171-181.

Aisa, B., Elizalde, N., Tordera, R., Lasheras, B., Del Rio, J., & Ramirez, M. J. (2009). Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. *Hippocampus, 19*(12), 1222-1231. doi: 10.1002/hipo.20586

Aisa, B., Tordera, R., Lasheras, B., Del Rio, J., & Ramirez, M. J. (2007). Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology, 32*(3), 256-266. doi: 10.1016/j.psyneuen.2006.12.013

Aisa, B., Tordera, R., Lasheras, B., Del Rio, J., & Ramirez, M. J. (2008). Effects of maternal separation on hypothalamic-pituitary-adrenal responses, cognition and vulnerability to stress in adult female rats. *Neuroscience, 154*(4), 1218-1226. doi: 10.1016/j.neuroscience.2008.05.011

Aksic, M., Radonjic, N. V., Aleksic, D., Jevtic, G., Markovic, B., Petronijevic, N., . . . Filipovic, B. (2013). Long-term effects of the maternal deprivation on the volume and number of neurons in the rat neocortex and hippocampus. *Acta Neurobiol Exp (Wars), 73*(3), 394-403.

Albers, E. M., Riksen-Walraven, J. M., Sweep, F. C., & de Weerth, C. (2008). Maternal behavior predicts infant cortisol recovery from a mild everyday stressor. *J Child Psychol Psychiatry, 49*(1), 97-103. doi: 10.1111/j.1469-7610.2007.01818.x

Anand, K. J., Coskun, V., Thrivikraman, K. V., Nemeroff, C. B., & Plotsky, P. M. (1999). Long-term behavioral effects of repetitive pain in neonatal rat pups. *Physiol Behav, 66*(4), 627-637.

Anand, K. J., Garg, S., Rovnaghi, C. R., Narsinghani, U., Bhutta, A. T., & Hall, R. W. (2007). Ketamine reduces the cell death following inflammatory pain in newborn rat brain. *Pediatr Res, 62*(3), 283-290. doi: 10.1203/PDR.0b013e3180986d2f

Anand, K. J., & Hickey, P. R. (1987). Pain and its effects in the human neonate and fetus. *N Engl J Med, 317*(21), 1321-1329. doi: 10.1056/NEJM198711193172105

Anand, K. J., & Scalzo, F. M. (2000). Can adverse neonatal experiences alter brain development and subsequent behavior? *Biol Neonate, 77*(2), 69-82. doi: 14197

Anderson, P., Doyle, L. W., & Victorian Infant Collaborative Study, G. (2003). Neurobehavioral outcomes of school-age children born extremely low birth weight or very preterm in the 1990s. *JAMA, 289*(24), 3264-3272. doi: 10.1001/jama.289.24.3264

Angeles, D. M., Ashwal, S., Wycliffe, N. D., Ebner, C., Fayard, E., Sowers, L., & Holshouser, B. A. (2007). Relationship between opioid therapy, tissue-damaging procedures, and brain metabolites as measured by proton MRS in asphyxiated term neonates. *Pediatr Res, 61*(5 Pt 1), 614-621. doi: 10.1203/pdr.0b013e318045bde9

Anseloni, V. C., He, F., Novikova, S. I., Turnbach Robbins, M., Lidow, I. A., Ennis, M., & Lidow, M. S. (2005). Alterations in stress-associated behaviors and neurochemical

markers in adult rats after neonatal short-lasting local inflammatory insult. *Neuroscience, 131*(3), 635-645. doi: 10.1016/j.neuroscience.2004.11.039

Antonucci, R., Porcella, A., & Fanos, V. (2009). The infant incubator in the neonatal intensive care unit: unresolved issues and future developments. *J Perinat Med, 37*(6), 587-598. doi: 10.1515/JPM.2009.109

Avishai-Eliner, S., Hatalski, C. G., Tabachnik, E., Eghbal-Ahmadi, M., & Baram, T. Z. (1999). Differential regulation of glucocorticoid receptor messenger RNA (GR-mRNA) by maternal deprivation in immature rat hypothalamus and limbic regions. *Brain Res Dev Brain Res, 114*(2), 265-268.

Aylward, G. P. (2005). Neurodevelopmental outcomes of infants born prematurely. *J Dev Behav Pediatr, 26*(6), 427-440.

Bartocci, M., Bergqvist, L. L., Lagercrantz, H., & Anand, K. J. (2006). Pain activates cortical areas in the preterm newborn brain. *Pain, 122*(1-2), 109-117. doi: 10.1016/j.pain.2006.01.015

Baudin, A., Blot, K., Verney, C., Estevez, L., Santamaria, J., Gressens, P., . . . Naudon, L. (2012). Maternal deprivation induces deficits in temporal memory and cognitive flexibility and exaggerates synaptic plasticity in the rat medial prefrontal cortex. *Neurobiol Learn Mem, 98*(3), 207-214. doi: 10.1016/j.nlm.2012.08.004

Belarbi, K., Jopson, T., Tweedie, D., Arellano, C., Luo, W., Greig, N. H., & Rosi, S. (2012). TNF-alpha protein synthesis inhibitor restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. *J Neuroinflammation, 9*, 23. doi: 10.1186/1742-2094-9-23

Bhutta, A. T., Cleves, M. A., Casey, P. H., Cradock, M. M., & Anand, K. J. (2002). Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. *JAMA, 288*(6), 728-737.

Bhutta, A. T., Rovnaghi, C., Simpson, P. M., Gossett, J. M., Scalzo, F. M., & Anand, K. J. (2001). Interactions of inflammatory pain and morphine in infant rats: long-term behavioral effects. *Physiol Behav, 73*(1-2), 51-58.

Blass, E. M., Shide, D. J., Zaw-Mon, C., & Sorrentino, J. (1995). Mother as shield: differential effects of contact and nursing on pain responsivity in infant rats--evidence for nonopioid mediation. *Behav Neurosci, 109*(2), 342-353.

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., . . . Lawn, J. E. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet, 379*(9832), 2162-2172. doi: 10.1016/S0140-6736(12)60820-4

Bredy, T. W., Humpartzoomian, R. A., Cain, D. P., & Meaney, M. J. (2003). Partial reversal of the effect of maternal care on cognitive function through environmental enrichment. *Neuroscience, 118*(2), 571-576.

Brooks, J., & Tracey, I. (2005). From nociception to pain perception: imaging the spinal and supraspinal pathways. *J Anat, 207*(1), 19-33. doi: 10.1111/j.1469-7580.2005.00428.x

Brummelte, S., Chau, C. M., Cepeda, I. L., Degenhardt, A., Weinberg, J., Synnes, A. R., & Grunau, R. E. (2015). Cortisol levels in former preterm children at school age are predicted by neonatal procedural pain-related stress. *Psychoneuroendocrinology, 51*, 151-163. doi: 10.1016/j.psyneuen.2014.09.018

Brummelte, S., & Galea, L. A. (2010). Chronic high corticosterone reduces neurogenesis in the dentate gyrus of adult male and female rats. *Neuroscience, 168*(3), 680-690. doi: 10.1016/j.neuroscience.2010.04.023

Brummelte, S., & Galea, L. A. (2016). Postpartum depression: Etiology, treatment and consequences for maternal care. *Horm Behav, 77*, 153-166. doi: 10.1016/j.yhbeh.2015.08.008

Brummelte, S., Grunau, R. E., Chau, V., Poskitt, K. J., Brant, R., Vinall, J., . . . Miller, S. P. (2012). Procedural pain and brain development in premature newborns. *Ann Neurol, 71*(3), 385-396. doi: 10.1002/ana.22267

Buetow, K. C., & Klein, S. W. (1964). Effect of Maintenance of "Normal" Skin Temperature on Survival of Infants of Low Birth Weight. *Pediatrics, 34*, 163-170.

Burri, R., Bigler, P., Straehl, P., Posse, S., Colombo, J. P., & Herschkowitz, N. (1990). Brain development: 1H magnetic resonance spectroscopy of rat brain extracts compared with chromatographic methods. *Neurochem Res, 15*(10), 1009-1016.

Buske-Kirschbaum, A., Krieger, S., Wilkes, C., Rauh, W., Weiss, S., & Hellhammer, D. H. (2007). Hypothalamic-pituitary-adrenal axis function and the cellular immune response in former preterm children. *J Clin Endocrinol Metab, 92*(9), 3429-3435. doi: 10.1210/jc.2006-2223

Butkevich, I. P., Mikhailenko, V. A., Makukhina, G. V., Bagaeva, T. R., & Stolyarova, Y. A. (2013). Tonic pain response during inflammation and stress-induced corticosterone variations in prenatally stressed infant rats: effects of maternal buspirone injected during pregnancy. *Bull Exp Biol Med, 155*(2), 194-196.

Cai, Z., Lin, S., Pang, Y., & Rhodes, P. G. (2004). Brain injury induced by intracerebral injection of interleukin-1beta and tumor necrosis factor-alpha in the neonatal rat. *Pediatr Res, 56*(3), 377-384. doi: 10.1203/01.PDR.0000134249.92944.14

Caldji, C., Diorio, J., & Meaney, M. J. (2000). Variations in maternal care in infancy regulate the development of stress reactivity. *Biol Psychiatry, 48*(12), 1164-1174.

Caldji, C., Francis, D., Sharma, S., Plotsky, P. M., & Meaney, M. J. (2000). The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology, 22*(3), 219-229. doi: 10.1016/S0893-133X(99)00110-4

Cancedda, L., Fiumelli, H., Chen, K., & Poo, M. M. (2007). Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo. *J Neurosci, 27*(19), 5224-5235. doi: 10.1523/JNEUROSCI.5169-06.2007

Carbajal, R., Rousset, A., Danan, C., Coquery, S., Nolent, P., Ducrocq, S., . . . Breart, G. (2008). Epidemiology and treatment of painful procedures in neonates in intensive care units. *JAMA, 300*(1), 60-70. doi: 10.1001/jama.300.1.60

Champagne, D. L., Bagot, R. C., van Hasselt, F., Ramakers, G., Meaney, M. J., de Kloet, E. R., . . . Krugers, H. (2008). Maternal care and hippocampal plasticity: evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. *J Neurosci, 28*(23), 6037-6045. doi: 10.1523/JNEUROSCI.0526-08.2008

Cherubini, E., Gaiarsa, J. L., & Ben-Ari, Y. (1991). GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci, 14*(12), 515-519.

Cignacco, E., Denhaerynck, K., Nelle, M., Buhrer, C., & Engberg, S. (2009). Variability in pain response to a non-pharmacological intervention across repeated routine pain exposure in preterm infants: a feasibility study. *Acta Paediatr, 98*(5), 842-846. doi: 10.1111/j.1651-2227.2008.01203.x

Claessens, S. E., Belanoff, J. K., Kanatsou, S., Lucassen, P. J., Champagne, D. L., & de Kloet, E. R. (2012). Acute effects of neonatal dexamethasone treatment on proliferation and astrocyte immunoreactivity in hippocampus and corpus callosum: towards a rescue strategy. *Brain Res, 1482*, 1-12. doi: 10.1016/j.brainres.2012.08.017

Clark, L., Cools, R., & Robbins, T. W. (2004). The neuropsychology of ventral prefrontal cortex: decision-making and reversal learning. *Brain Cogn, 55*(1), 41-53. doi: 10.1016/S0278-2626(03)00284-7

Conde-Agudelo, A., Diaz-Rossello, J. L., & Belizan, J. M. (2003). Kangaroo mother care to reduce morbidity and mortality in low birthweight infants. *Cochrane Database Syst Rev*(2), CD002771. doi: 10.1002/14651858.CD002771

Cone, T. E., Jr. (1981). The first published report of an incubator for use in the care of the premature infant (1857). *Am J Dis Child, 135*(7), 658-660.

Cong, X., Ludington-Hoe, S. M., & Walsh, S. (2011). Randomized crossover trial of kangaroo care to reduce biobehavioral pain responses in preterm infants: a pilot study. *Biol Res Nurs, 13*(2), 204-216. doi: 10.1177/1099800410385839

Cong, X., Wu, J., Vittner, D., Xu, W., Hussain, N., Galvin, S., . . . Henderson, W. A. (2017). The impact of cumulative pain/stress on neurobehavioral development of preterm infants in the NICU. *Early Hum Dev, 108*, 9-16. doi: 10.1016/j.earlhumdev.2017.03.003

Corbo, M. G., Mansi, G., Stagni, A., Romano, A., van den Heuvel, J., Capasso, L., . . . Paludetto, R. (2000). Nonnutritive sucking during heelstick procedures decreases behavioral distress in the newborn infant. *Biol Neonate, 77*(3), 162-167. doi: 14211

Daniels, W. M., Pietersen, C. Y., Carstens, M. E., & Stein, D. J. (2004). Maternal separation in rats leads to anxiety-like behavior and a blunted ACTH response and altered neurotransmitter levels in response to a subsequent stressor. *Metab Brain Dis, 19*(1-2), 3-14.

Daskalakis, N. P., Bagot, R. C., Parker, K. J., Vinkers, C. H., & de Kloet, E. R. (2013). The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology, 38*(9), 1858-1873. doi: 10.1016/j.psyneuen.2013.06.008

De Bellis, M. D. (2005). The psychobiology of neglect. *Child Maltreat, 10*(2), 150-172. doi: 10.1177/1077559505275116

de Medeiros, C. B., Fleming, A. S., Johnston, C. C., & Walker, C. D. (2009). Artificial rearing of rat pups reveals the beneficial effects of mother care on neonatal inflammation and adult sensitivity to pain. *Pediatr Res, 66*(3), 272-277. doi: 10.1203/PDR.0b013e3181b1be06

Diaz, R., Brown, R. W., & Seckl, J. R. (1998). Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. *J Neurosci, 18*(7), 2570-2580.

Doesburg, S. M., Chau, C. M., Cheung, T. P., Moiseev, A., Ribary, U., Herdman, A. T., . . . Grunau, R. E. (2013). Neonatal pain-related stress, functional cortical activity and visual-

perceptual abilities in school-age children born at extremely low gestational age. *Pain, 154*(10), 1946-1952. doi: 10.1016/j.pain.2013.04.009

Doom, J. R., Georgieff, M. K., & Gunnar, M. R. (2015). Institutional care and iron deficiency increase ADHD symptomology and lower IQ 2.5-5 years post-adoption. *Dev Sci, 18*(3), 484-494. doi: 10.1111/desc.12223

Duhrsen, L., Simons, S. H., Dzietko, M., Genz, K., Bendix, I., Boos, V., . . . Felderhoff-Mueser, U. (2013). Effects of repetitive exposure to pain and morphine treatment on the neonatal rat brain. *Neonatology, 103*(1), 35-43. doi: 10.1159/000341769

Eghbal-Ahmadi, M., Hatalski, C. G., Avishai-Eliner, S., & Baram, T. Z. (1997). Corticotropin releasing factor receptor type II (CRF2) messenger ribonucleic acid levels in the hypothalamic ventromedial nucleus of the infant rat are reduced by maternal deprivation. *Endocrinology, 138*(11), 5048-5051. doi: 10.1210/endo.138.11.5647

Essex, M. J., Shirtcliff, E. A., Burk, L. R., Ruttle, P. L., Klein, M. H., Slattery, M. J., . . . Armstrong, J. M. (2011). Influence of early life stress on later hypothalamic-pituitary-adrenal axis functioning and its covariation with mental health symptoms: a study of the allostatic process from childhood into adolescence. *Dev Psychopathol, 23*(4), 1039-1058. doi: 10.1017/S0954579411000484

Famularo, R., Kinscherff, R., & Fenton, T. (1992). Psychiatric diagnoses of maltreated children: preliminary findings. *J Am Acad Child Adolesc Psychiatry, 31*(5), 863-867. doi: 10.1097/00004583-199209000-00013

Feldman, R., Rosenthal, Z., & Eidelman, A. I. (2014). Maternal-preterm skin-to-skin contact enhances child physiologic organization and cognitive control across the first 10 years of life. *Biol Psychiatry, 75*(1), 56-64. doi: 10.1016/j.biopsych.2013.08.012

Feng, M., Sheng, G., Li, Z., Wang, J., Ren, K., Jin, X., & Jiang, K. (2014). Postnatal maternal separation enhances tonic GABA current of cortical layer 5 pyramidal neurons in juvenile rats and promotes genesis of GABAergic neurons in neocortical molecular layer and subventricular zone in adult rats. *Behav Brain Res, 260*, 74-82. doi: 10.1016/j.bbr.2013.11.040

Flacking, R., Lehtonen, L., Thomson, G., Axelin, A., Ahlqvist, S., Moran, V. H., . . . Closeness Experiences in the Neonatal Environment, g. (2012). Closeness and separation in neonatal intensive care. *Acta Paediatr, 101*(10), 1032-1037. doi: 10.1111/j.1651-2227.2012.02787.x

Fraello, D., Maller-Kesselman, J., Vohr, B., Katz, K. H., Kesler, S., Schneider, K., . . . Spann, M. N. (2011). Consequence of preterm birth in early adolescence: the role of language on auditory short-term memory. *J Child Neurol, 26*(6), 738-742. doi: 10.1177/0883073810391904

Garner, B., Wood, S. J., Pantelis, C., & van den Buuse, M. (2007). Early maternal deprivation reduces prepulse inhibition and impairs spatial learning ability in adulthood: no further effect of post-pubertal chronic corticosterone treatment. *Behav Brain Res, 176*(2), 323-332. doi: 10.1016/j.bbr.2006.10.020

Gee, D. G., Gabard-Durnam, L. J., Flannery, J., Goff, B., Humphreys, K. L., Telzer, E. H., . . . Tottenham, N. (2013). Early developmental emergence of human amygdala-prefrontal connectivity after maternal deprivation. *Proc Natl Acad Sci U S A, 110*(39), 15638-15643. doi: 10.1073/pnas.1307893110

Gold, J. I., Kant, A. J., & Kim, S. H. (2008). The impact of unintentional pediatric trauma: a review of pain, acute stress, and posttraumatic stress. *J Pediatr Nurs, 23*(2), 81-91. doi: 10.1016/j.pedn.2007.08.005

Golianu, B., Krane, E., Seybold, J., Almgren, C., & Anand, K. J. (2007). Non-pharmacological techniques for pain management in neonates. *Semin Perinatol, 31*(5), 318-322. doi: 10.1053/j.semperi.2007.07.007

Gray, L., Watt, L., & Blass, E. M. (2000). Skin-to-skin contact is analgesic in healthy newborns. *Pediatrics, 105*(1), e14.

Graybeal, C., Feyder, M., Schulman, E., Saksida, L. M., Bussey, T. J., Brigman, J. L., & Holmes, A. (2011). Paradoxical reversal learning enhancement by stress or prefrontal cortical damage: rescue with BDNF. *Nat Neurosci, 14*(12), 1507-1509. doi: 10.1038/nn.2954

Grunau, R. E., Cepeda, I. L., Chau, C. M., Brummelte, S., Weinberg, J., Lavoie, P. M., . . . Turvey, S. E. (2013). Neonatal pain-related stress and NFKBIA genotype are associated with altered cortisol levels in preterm boys at school age. *PLoS One, 8*(9), e73926. doi: 10.1371/journal.pone.0073926

Grunau, R. E., Haley, D. W., Whitfield, M. F., Weinberg, J., Yu, W., & Thiessen, P. (2007). Altered basal cortisol levels at 3, 6, 8 and 18 months in infants born at extremely low gestational age. *J Pediatr, 150*(2), 151-156. doi: 10.1016/j.jpeds.2006.10.053

Grunau, R. E., Holsti, L., Haley, D. W., Oberlander, T., Weinberg, J., Solimano, A., . . . Yu, W. (2005). Neonatal procedural pain exposure predicts lower cortisol and behavioral reactivity in preterm infants in the NICU. *Pain, 113*(3), 293-300. doi: 10.1016/j.pain.2004.10.020

Grunau, R. E., Holsti, L., & Peters, J. W. (2006). Long-term consequences of pain in human neonates. *Semin Fetal Neonatal Med, 11*(4), 268-275. doi: 10.1016/j.siny.2006.02.007

Grunau, R. E., Whitfield, M. F., Petrie-Thomas, J., Synnes, A. R., Cepeda, I. L., Keidar, A., . . . Johannesen, D. (2009). Neonatal pain, parenting stress and interaction, in relation to cognitive and motor development at 8 and 18 months in preterm infants. *Pain, 143*(1-2), 138-146. doi: 10.1016/j.pain.2009.02.014

Hall, D., & Kirsten, G. (2008). Kangaroo Mother Care--a review. *Transfus Med, 18*(2), 77-82. doi: 10.1111/j.1365-3148.2007.00812.x

Harrison, F. E., Hosseini, A. H., & McDonald, M. P. (2009). Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. *Behav Brain Res, 198*(1), 247-251. doi: 10.1016/j.bbr.2008.10.015

Hedges, D. W., & Woon, F. L. (2011). Early-life stress and cognitive outcome. *Psychopharmacology (Berl), 214*(1), 121-130. doi: 10.1007/s00213-010-2090-6

Henderson, Y. O., Victoria, N. C., Inoue, K., Murphy, A. Z., & Parent, M. B. (2015). Early life inflammatory pain induces long-lasting deficits in hippocampal-dependent spatial memory in male and female rats. *Neurobiol Learn Mem, 118*, 30-41. doi: 10.1016/j.nlm.2014.10.010

Hennessy, M. B., Tamborski, A., Schiml, P., & Lucot, J. (1989). The influence of maternal separation on plasma concentrations of ACTH, epinephrine, and norepinephrine in guinea pig pups. *Physiol Behav, 45*(6), 1147-1152.

Herrington, C. J., Olomu, I. N., & Geller, S. M. (2004). Salivary cortisol as indicators of pain in preterm infants: a pilot study. *Clin Nurs Res, 13*(1), 53-68. doi: 10.1177/1054773803259665

Hilton, G. D., Nunez, J. L., Bambrick, L., Thompson, S. M., & McCarthy, M. M. (2006). Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca++ from intracellular stores and is prevented by estradiol. *Eur J Neurosci, 24*(11), 3008-3016. doi: 10.1111/j.1460-9568.2006.05189.x

Holsti, L., Weinberg, J., Whitfield, M. F., & Grunau, R. E. (2007). Relationships between adrenocorticotropic hormone and cortisol are altered during clustered nursing care in preterm infants born at extremely low gestational age. *Early Hum Dev, 83*(5), 341-348. doi: 10.1016/j.earlhumdev.2006.08.005

Horwitz, S. M., Storfer-Isser, A., Kerker, B. D., Lilo, E., Leibovitz, A., St John, N., & Shaw, R. J. (2015). A model for the development of mothers' perceived vulnerability of preterm infants. *J Dev Behav Pediatr, 36*(5), 371-380. doi: 10.1097/DBP.0000000000000173

Huot, R. L., Plotsky, P. M., Lenox, R. H., & McNamara, R. K. (2002). Neonatal maternal separation reduces hippocampal mossy fiber density in adult Long Evans rats. *Brain Res, 950*(1-2), 52-63.

Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., . . . Olney, J. W. (1999). Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science, 283*(5398), 70-74.

Isgor, C., Kabbaj, M., Akil, H., & Watson, S. J. (2004). Delayed effects of chronic variable stress during peripubertal-juvenile period on hippocampal morphology and on cognitive and stress axis functions in rats. *Hippocampus, 14*(5), 636-648. doi: 10.1002/hipo.10207

Jackman, A., & Fitzgerald, M. (2000). Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. *J Comp Neurol, 418*(3), 281-298.

Jain, S., Kumar, P., & McMillan, D. D. (2006). Prior leg massage decreases pain responses to heel stick in preterm babies. *J Paediatr Child Health, 42*(9), 505-508. doi: 10.1111/j.1440-1754.2006.00912.x

Johnson, S., Hollis, C., Kochhar, P., Hennessy, E., Wolke, D., & Marlow, N. (2010). Psychiatric disorders in extremely preterm children: longitudinal finding at age 11 years in the EPICure study. *J Am Acad Child Adolesc Psychiatry, 49*(5), 453-463 e451.

Johnston, C., Campbell-Yeo, M., Fernandes, A., Inglis, D., Streiner, D., & Zee, R. (2014). Skin-to-skin care for procedural pain in neonates. *Cochrane Database Syst Rev, 1*, CD008435. doi: 10.1002/14651858.CD008435.pub2

Johnston, C. C., Filion, F., Campbell-Yeo, M., Goulet, C., Bell, L., McNaughton, K., . . . Walker, C. D. (2008). Kangaroo mother care diminishes pain from heel lance in very preterm neonates: a crossover trial. *BMC Pediatr, 8*, 13. doi: 10.1186/1471-2431-8-13

Johnston, C. C., Stevens, B., Pinelli, J., Gibbins, S., Filion, F., Jack, A., . . . Veilleux, A. (2003). Kangaroo care is effective in diminishing pain response in preterm neonates. *Arch Pediatr Adolesc Med, 157*(11), 1084-1088. doi: 10.1001/archpedi.157.11.1084

Johnston, C. C., Stevens, B. J., Yang, F., & Horton, L. (1995). Differential response to pain by very premature neonates. *Pain, 61*(3), 471-479.

Kalinichev, M., Easterling, K. W., Plotsky, P. M., & Holtzman, S. G. (2002). Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. *Pharmacol Biochem Behav, 73*(1), 131-140.

Kaufman, J. (1991). Depressive disorders in maltreated children. *J Am Acad Child Adolesc Psychiatry, 30*(2), 257-265. doi: 10.1097/00004583-199103000-00014

Kawabe, K., Iwasaki, T., & Ichitani, Y. (2007). Repeated treatment with N-methyl-d-aspartate antagonists in neonatal, but not adult, rats causes long-term deficits of radial-arm maze learning. *Brain Res, 1169*, 77-86. doi: 10.1016/j.brainres.2007.06.062

Kirmse, K., Kummer, M., Kovalchuk, Y., Witte, O. W., Garaschuk, O., & Holthoff, K. (2015). GABA depolarizes immature neurons and inhibits network activity in the neonatal neocortex in vivo. *Nat Commun, 6*, 7750. doi: 10.1038/ncomms8750

Knott, G. W., Quairiaux, C., Genoud, C., & Welker, E. (2002). Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. *Neuron, 34*(2), 265-273.

Konig, K., Stock, E. L., & Jarvis, M. (2013). Noise levels of neonatal high-flow nasal cannula devices--an in-vitro study. *Neonatology, 103*(4), 264-267. doi: 10.1159/000346764

Konstantinidou, A. D., Silos-Santiago, I., Flaris, N., & Snider, W. D. (1995). Development of the primary afferent projection in human spinal cord. *J Comp Neurol, 354*(1), 11-12.

Kosten, T. A., & Kehoe, P. (2010). Immediate and enduring effects of neonatal isolation on maternal behavior in rats. *Int J Dev Neurosci, 28*(1), 53-61. doi: 10.1016/j.ijdevneu.2009.09.005

Kostovic, I., & Goldman-Rakic, P. S. (1983). Transient cholinesterase staining in the mediodorsal nucleus of the thalamus and its connections in the developing human and monkey brain. *J Comp Neurol, 219*(4), 431-447. doi: 10.1002/cne.902190405

Kostovic, I., & Judas, M. (2010). The development of the subplate and thalamocortical connections in the human foetal brain. *Acta Paediatr, 99*(8), 1119-1127. doi: 10.1111/j.1651-2227.2010.01811.x

Kuhn, C. M., Pauk, J., & Schanberg, S. M. (1990). Endocrine responses to mother-infant separation in developing rats. *Dev Psychobiol, 23*(5), 395-410. doi: 10.1002/dev.420230503

Ladd, C. O., Owens, M. J., & Nemeroff, C. B. (1996). Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. *Endocrinology, 137*(4), 1212-1218. doi: 10.1210/endo.137.4.8625891

Lahav, A., & Skoe, E. (2014). An acoustic gap between the NICU and womb: a potential risk for compromised neuroplasticity of the auditory system in preterm infants. *Front Neurosci, 8*, 381. doi: 10.3389/fnins.2014.00381

Lajud, N., Roque, A., Cajero, M., Gutierrez-Ospina, G., & Torner, L. (2012). Periodic maternal separation decreases hippocampal neurogenesis without affecting basal corticosterone during the stress hyporesponsive period, but alters HPA axis and coping behavior in adulthood. *Psychoneuroendocrinology, 37*(3), 410-420. doi: 10.1016/j.psyneuen.2011.07.011

LaPrairie, J. L., & Murphy, A. Z. (2007). Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury. *Pain, 132 Suppl 1*, S124-133. doi: 10.1016/j.pain.2007.08.010

Laprairie, J. L., & Murphy, A. Z. (2009). Neonatal injury alters adult pain sensitivity by increasing opioid tone in the periaqueductal gray. *Front Behav Neurosci, 3*, 31. doi: 10.3389/neuro.08.031.2009

Larson, M. C., Gunnar, M. R., & Hertsgaard, L. (1991). The effects of morning naps, car trips, and maternal separation on adrenocortical activity in human infants. *Child Dev, 62*(2), 362-372.

Latva, R., Lehtonen, L., Salmelin, R. K., & Tamminen, T. (2007). Visits by the family to the neonatal intensive care unit. *Acta Paediatr, 96*(2), 215-220.

Lawn, J. E., Mwansa-Kambafwile, J., Horta, B. L., Barros, F. C., & Cousens, S. (2010). 'Kangaroo mother care' to prevent neonatal deaths due to preterm birth complications. *Int J Epidemiol, 39 Suppl 1*, i144-154. doi: 10.1093/ije/dyq031

Lee, J. H., Espinera, A. R., Chen, D., Choi, K. E., Caslin, A. Y., Won, S., . . . Yu, S. P. (2016). Neonatal inflammatory pain and systemic inflammatory responses as possible environmental factors in the development of autism spectrum disorder of juvenile rats. *J Neuroinflammation, 13*(1), 109. doi: 10.1186/s12974-016-0575-x

Lee, J. H., Kim, H. J., Kim, J. G., Ryu, V., Kim, B. T., Kang, D. W., & Jahng, J. W. (2007). Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. *Neurosci Res, 58*(1), 32-39. doi: 10.1016/j.neures.2007.01.008

Lehmann, J., & Feldon, J. (2000). Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? *Rev Neurosci, 11*(4), 383-408.

Leussis, M. P., Freund, N., Brenhouse, H. C., Thompson, B. S., & Andersen, S. L. (2012). Depressive-like behavior in adolescents after maternal separation: sex differences, controllability, and GABA. *Dev Neurosci, 34*(2-3), 210-217. doi: 10.1159/000339162

Levine, S., Huchton, D. M., Wiener, S. G., & Rosenfeld, P. (1991). Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. *Dev Psychobiol, 24*(8), 547-558. doi: 10.1002/dev.420240803

Liu, D., Diorio, J., Day, J. C., Francis, D. D., & Meaney, M. J. (2000). Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci, 3*(8), 799-806. doi: 10.1038/77702

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., . . . Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science, 277*(5332), 1659-1662.

Llorente-Berzal, A., Mela, V., Borcel, E., Valero, M., Lopez-Gallardo, M., Viveros, M. P., & Marco, E. M. (2012). Neurobehavioral and metabolic long-term consequences of neonatal maternal deprivation stress and adolescent olanzapine treatment in male and female rats. *Neuropharmacology, 62*(3), 1332-1341. doi: 10.1016/j.neuropharm.2011.07.031

Llorente, R., Villa, P., Marco, E. M., & Viveros, M. P. (2012). Analyzing the effects of a single episode of neonatal maternal deprivation on metabolite profiles in rat brain: a proton nuclear magnetic resonance spectroscopy study. *Neuroscience, 201*, 12-19. doi: 10.1016/j.neuroscience.2011.11.033

Logue, S. F., Paylor, R., & Wehner, J. M. (1997). Hippocampal lesions cause learning deficits in inbred mice in the Morris water maze and conditioned-fear task. *Behav Neurosci, 111*(1), 104-113.

Lu, J., Goula, D., Sousa, N., & Almeida, O. F. (2003). Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic N-methyl-D-aspartate receptors. *Neuroscience, 121*(1), 123-131.

Luczynski, P., Moquin, L., & Gratton, A. (2015). Chronic stress alters the dendritic morphology of callosal neurons and the acute glutamate stress response in the rat medial prefrontal cortex. *Stress, 18*(6), 654-667. doi: 10.3109/10253890.2015.1073256

Maccari, S., Krugers, H. J., Morley-Fletcher, S., Szyf, M., & Brunton, P. J. (2014). The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. *J Neuroendocrinol, 26*(10), 707-723. doi: 10.1111/jne.12175

Macey, T. J., Harmon, R. J., & Easterbrooks, M. A. (1987). Impact of premature birth on the development of the infant in the family. *J Consult Clin Psychol, 55*(6), 846-852.

Magnano, C. L., Gardner, J. M., & Karmel, B. Z. (1992). Differences in salivary cortisol levels in cocaine-exposed and noncocaine-exposed NICU infants. *Dev Psychobiol, 25*(2), 93-103. doi: 10.1002/dev.420250203

Marlow, N., Hennessy, E. M., Bracewell, M. A., Wolke, D., & Group, E. P. S. (2007). Motor and executive function at 6 years of age after extremely preterm birth. *Pediatrics, 120*(4), 793-804. doi: 10.1542/peds.2007-0440

Marlow, N., Wolke, D., Bracewell, M. A., Samara, M., & Group, E. P. S. (2005). Neurologic and developmental disability at six years of age after extremely preterm birth. *N Engl J Med, 352*(1), 9-19. doi: 10.1056/NEJMoa041367

Martisova, E., Solas, M., Horrillo, I., Ortega, J. E., Meana, J. J., Tordera, R. M., & Ramirez, M. J. (2012). Long lasting effects of early-life stress on glutamatergic/GABAergic circuitry in the rat hippocampus. *Neuropharmacology, 62*(5-6), 1944-1953. doi: 10.1016/j.neuropharm.2011.12.019

McCormick, C. M., Kehoe, P., & Kovacs, S. (1998). Corticosterone release in response to repeated, short episodes of neonatal isolation: evidence of sensitization. *Int J Dev Neurosci, 16*(3-4), 175-185.

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonte, B., Szyf, M., . . . Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci, 12*(3), 342-348. doi: 10.1038/nn.2270

McIntosh, J., Anisman, H., & Merali, Z. (1999). Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. *Brain Res Dev Brain Res, 113*(1-2), 97-106.

Mehta, M. A., Golembo, N. I., Nosarti, C., Colvert, E., Mota, A., Williams, S. C., . . . Sonuga-Barke, E. J. (2009). Amygdala, hippocampal and corpus callosum size following severe early institutional deprivation: the English and Romanian Adoptees study pilot. *J Child Psychol Psychiatry, 50*(8), 943-951. doi: 10.1111/j.1469-7610.2009.02084.x

Mendoza, S. P., Smotherman, W. P., Miner, M. T., Kaplan, J., & Levine, S. (1978). Pituitary-adrenal response to separation in mother and infant squirrel monkeys. *Dev Psychobiol, 11*(2), 169-175. doi: 10.1002/dev.420110209

Millan, M. J. (1999). The induction of pain: an integrative review. *Prog Neurobiol, 57*(1), 1-164.

Moghaddam, B., Bolinao, M. L., Stein-Behrens, B., & Sapolsky, R. (1994). Glucocorticoids mediate the stress-induced extracellular accumulation of glutamate. *Brain Res, 655*(1-2), 251-254.

Moriceau, S., & Sullivan, R. M. (2006). Maternal presence serves as a switch between learning fear and attraction in infancy. *Nat Neurosci, 9*(8), 1004-1006. doi: 10.1038/nn1733

Morris, R. G., Garrud, P., Rawlins, J. N., & O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature, 297*(5868), 681-683.

Negrigo, A., Medeiros, M., Guinsburg, R., & Covolan, L. (2011). Long-term gender behavioral vulnerability after nociceptive neonatal formalin stimulation in rats. *Neurosci Lett, 490*(3), 196-199. doi: 10.1016/j.neulet.2010.12.050

Newnham, C. A., Inder, T. E., & Milgrom, J. (2009). Measuring preterm cumulative stressors within the NICU: the Neonatal Infant Stressor Scale. *Early Hum Dev, 85*(9), 549-555. doi: 10.1016/j.earlhumdev.2009.05.002

Nishi, M., Horii-Hayashi, N., & Sasagawa, T. (2014). Effects of early life adverse experiences on the brain: implications from maternal separation models in rodents. *Front Neurosci, 8*, 166. doi: 10.3389/fnins.2014.00166

Nosarti, C., Nam, K. W., Walshe, M., Murray, R. M., Cuddy, M., Rifkin, L., & Allin, M. P. (2014). Preterm birth and structural brain alterations in early adulthood. *Neuroimage Clin, 6*, 180-191. doi: 10.1016/j.nicl.2014.08.005

Nosarti, C., Reichenberg, A., Murray, R. M., Cnattingius, S., Lambe, M. P., Yin, L., . . . Hultman, C. M. (2012). Preterm birth and psychiatric disorders in young adult life. *Arch Gen Psychiatry, 69*(6), E1-8. doi: 10.1001/archgenpsychiatry.2011.1374

Nuseir, K. Q., Alzoubi, K. H., Alhusban, A., Bawaane, A., Al-Azzani, M., & Khabour, O. F. (2017). Sucrose and naltrexone prevent increased pain sensitivity and impaired long-term memory induced by repetitive neonatal noxious stimulation: Role of BDNF and beta-endorphin. *Physiol Behav, 179*, 213-219. doi: 10.1016/j.physbeh.2017.06.015

O'Malley, D., Dinan, T. G., & Cryan, J. F. (2011). Neonatal maternal separation in the rat impacts on the stress responsivity of central corticotropin-releasing factor receptors in

adulthood. *Psychopharmacology (Berl), 214*(1), 221-229. doi: 10.1007/s00213-010-1885-9

Oberlander, T. F., Weinberg, J., Papsdorf, M., Grunau, R., Misri, S., & Devlin, A. M. (2008). Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics, 3*(2), 97-106.

Oitzl, M. S., de Kloet, E. R., Joels, M., Schmid, W., & Cole, T. J. (1997). Spatial learning deficits in mice with a targeted glucocorticoid receptor gene disruption. *Eur J Neurosci, 9*(11), 2284-2296.

Oitzl, M. S., Fluttert, M., & de Kloet, E. R. (1998). Acute blockade of hippocampal glucocorticoid receptors facilitates spatial learning in rats. *Brain Res, 797*(1), 159-162.

Pancaro, C., Segal, B. S., Sikes, R. W., Almeer, Z., Schumann, R., Azocar, R. J., & Marchand, J. E. (2016). Dexmedetomidine and ketamine show distinct patterns of cell degeneration and apoptosis in the developing rat neonatal brain. *J Matern Fetal Neonatal Med, 29*(23), 3827-3833. doi: 10.3109/14767058.2016.1148132

Peacock, J. L., Marston, L., Marlow, N., Calvert, S. A., & Greenough, A. (2012). Neonatal and infant outcome in boys and girls born very prematurely. *Pediatr Res, 71*(3), 305-310. doi: 10.1038/pr.2011.50

Pechtel, P., & Pizzagalli, D. A. (2011). Effects of early life stress on cognitive and affective function: an integrated review of human literature. *Psychopharmacology (Berl), 214*(1), 55-70. doi: 10.1007/s00213-010-2009-2

Peng, N. H., Bachman, J., Jenkins, R., Chen, C. H., Chang, Y. C., Chang, Y. S., & Wang, T. M. (2009). Relationships between environmental stressors and stress biobehavioral responses

of preterm infants in NICU. *J Perinat Neonatal Nurs, 23*(4), 363-371. doi: 10.1097/JPN.0b013e3181bdd3fd

Perez, M., Benitez, S. U., Cartarozzi, L. P., Del Bel, E., Guimaraes, F. S., & Oliveira, A. L. (2013). Neuroprotection and reduction of glial reaction by cannabidiol treatment after sciatic nerve transection in neonatal rats. *Eur J Neurosci, 38*(10), 3424-3434. doi: 10.1111/ejn.12341

Peterson, B. S., Vohr, B., Staib, L. H., Cannistraci, C. J., Dolberg, A., Schneider, K. C., . . . Ment, L. R. (2000). Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. *JAMA, 284*(15), 1939-1947.

Pihoker, C., Owens, M. J., Kuhn, C. M., Schanberg, S. M., & Nemeroff, C. B. (1993). Maternal separation in neonatal rats elicits activation of the hypothalamic-pituitary-adrenocortical axis: a putative role for corticotropin-releasing factor. *Psychoneuroendocrinology, 18*(7), 485-493.

Pinheiro, R. M., de Lima, M. N., Portal, B. C., Busato, S. B., Falavigna, L., Ferreira, R. D., . . . Schroder, N. (2015). Long-lasting recognition memory impairment and alterations in brain levels of cytokines and BDNF induced by maternal deprivation: effects of valproic acid and topiramate. *J Neural Transm (Vienna), 122*(5), 709-719. doi: 10.1007/s00702-014-1303-2

Platt, M. J. (2014). Outcomes in preterm infants. *Public Health, 128*(5), 399-403. doi: 10.1016/j.puhe.2014.03.010

Popoli, M., Yan, Z., McEwen, B. S., & Sanacora, G. (2011). The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci, 13*(1), 22-37. doi: 10.1038/nrn3138

Pryce, C. R., Bettschen, D., Nanz-Bahr, N. I., & Feldon, J. (2003). Comparison of the effects of early handling and early deprivation on conditioned stimulus, context, and spatial learning and memory in adult rats. *Behav Neurosci, 117*(5), 883-893. doi: 10.1037/0735-7044.117.5.883

Ragan, C. M., Loken, E., Stifter, C. A., & Cavigelli, S. A. (2012). Within-litter variance in early rat pup-mother interactions and adult offspring responses to novelty. *Dev Psychobiol, 54*(2), 199-206. doi: 10.1002/dev.20581

Ranger, M., Chau, C. M., Garg, A., Woodward, T. S., Beg, M. F., Bjornson, B., . . . Grunau, R. E. (2013). Neonatal pain-related stress predicts cortical thickness at age 7 years in children born very preterm. *PLoS One, 8*(10), e76702. doi: 10.1371/journal.pone.0076702

Ranger, M., Synnes, A. R., Vinall, J., & Grunau, R. E. (2014). Internalizing behaviours in school-age children born very preterm are predicted by neonatal pain and morphine exposure. *Eur J Pain, 18*(6), 844-852. doi: 10.1002/j.1532-2149.2013.00431.x

Ranger, M., Zwicker, J. G., Chau, C. M., Park, M. T., Chakravarthy, M. M., Poskitt, K., . . . Grunau, R. E. (2015). Neonatal Pain and Infection Relate to Smaller Cerebellum in Very Preterm Children at School Age. *J Pediatr, 167*(2), 292-298 e291. doi: 10.1016/j.jpeds.2015.04.055

Reynolds, L. C., Duncan, M. M., Smith, G. C., Mathur, A., Neil, J., Inder, T., & Pineda, R. G. (2013). Parental presence and holding in the neonatal intensive care unit and associations with early neurobehavior. *J Perinatol, 33*(8), 636-641. doi: 10.1038/jp.2013.4

Rodkey, E. N., & Pillai Riddell, R. (2013). The infancy of infant pain research: the experimental origins of infant pain denial. *J Pain, 14*(4), 338-350. doi: 10.1016/j.jpain.2012.12.017

Romeo, R. D., Bellani, R., Karatsoreos, I. N., Chhua, N., Vernov, M., Conrad, C. D., & McEwen, B. S. (2006). Stress history and pubertal development interact to shape hypothalamic-pituitary-adrenal axis plasticity. *Endocrinology, 147*(4), 1664-1674. doi: 10.1210/en.2005-1432

Rovnaghi, C. R., Garg, S., Hall, R. W., Bhutta, A. T., & Anand, K. J. (2008). Ketamine analgesia for inflammatory pain in neonatal rats: a factorial randomized trial examining long-term effects. *Behav Brain Funct, 4*, 35. doi: 10.1186/1744-9081-4-35

Rysavy, M. A., Li, L., Bell, E. F., Das, A., Hintz, S. R., Stoll, B. J., . . . Human Development Neonatal Research, N. (2015). Between-hospital variation in treatment and outcomes in extremely preterm infants. *N Engl J Med, 372*(19), 1801-1811. doi: 10.1056/NEJMoa1410689

Sansavini, A., Guarini, A., Justice, L. M., Savini, S., Broccoli, S., Alessandroni, R., & Faldella, G. (2010). Does preterm birth increase a child's risk for language impairment? *Early Hum Dev, 86*(12), 765-772. doi: 10.1016/j.earlhumdev.2010.08.014

Sapolsky, R. M., & Meaney, M. J. (1986). Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Res, 396*(1), 64-76.

Scher, M. S., Ludington-Hoe, S., Kaffashi, F., Johnson, M. W., Holditch-Davis, D., & Loparo, K. A. (2009). Neurophysiologic assessment of brain maturation after an 8-week trial of skin-to-skin contact on preterm infants. *Clin Neurophysiol, 120*(10), 1812-1818. doi: 10.1016/j.clinph.2009.08.004

Schneider, C., Charpak, N., Ruiz-Pelaez, J. G., & Tessier, R. (2012). Cerebral motor function in very premature-at-birth adolescents: a brain stimulation exploration of kangaroo mother care effects. *Acta Paediatr, 101*(10), 1045-1053. doi: 10.1111/j.1651-2227.2012.02770.x

Schwaller, F., & Fitzgerald, M. (2014). The consequences of pain in early life: injury-induced plasticity in developing pain pathways. *Eur J Neurosci, 39*(3), 344-352. doi: 10.1111/ejn.12414

Sengupta, J. N., Pochiraju, S., Kannampalli, P., Bruckert, M., Addya, S., Yadav, P., . . . Banerjee, B. (2013). MicroRNA-mediated GABA Aalpha-1 receptor subunit down-regulation in adult spinal cord following neonatal cystitis-induced chronic visceral pain in rats. *Pain, 154*(1), 59-70. doi: 10.1016/j.pain.2012.09.002

Simms, V., Gilmore, C., Cragg, L., Marlow, N., Wolke, D., & Johnson, S. (2013). Mathematics difficulties in extremely preterm children: evidence of a specific deficit in basic mathematics processing. *Pediatr Res, 73*(2), 236-244. doi: 10.1038/pr.2012.157

Simons, S. H., van Dijk, M., Anand, K. S., Roofthooft, D., van Lingen, R. A., & Tibboel, D. (2003). Do we still hurt newborn babies? A prospective study of procedural pain and analgesia in neonates. *Arch Pediatr Adolesc Med, 157*(11), 1058-1064. doi: 10.1001/archpedi.157.11.1058

Slater, R., Boyd, S., Meek, J., & Fitzgerald, M. (2006). Cortical pain responses in the infant brain. *Pain, 123*(3), 332; author reply 332-334. doi: 10.1016/j.pain.2006.05.009

Slater, R., Fabrizi, L., Worley, A., Meek, J., Boyd, S., & Fitzgerald, M. (2010). Premature infants display increased noxious-evoked neuronal activity in the brain compared to healthy age-matched term-born infants. *Neuroimage, 52*(2), 583-589. doi: 10.1016/j.neuroimage.2010.04.253

Slotten, H. A., Kalinichev, M., Hagan, J. J., Marsden, C. A., & Fone, K. C. (2006). Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. *Brain Res, 1097*(1), 123-132. doi: 10.1016/j.brainres.2006.04.066

Smith, G. C., Gutovich, J., Smyser, C., Pineda, R., Newnham, C., Tjoeng, T. H., . . . Inder, T. (2011). Neonatal intensive care unit stress is associated with brain development in preterm infants. *Ann Neurol, 70*(4), 541-549. doi: 10.1002/ana.22545

Smith, S. M., & Vale, W. W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci, 8*(4), 383-395.

Solas, M., Aisa, B., Mugueta, M. C., Del Rio, J., Tordera, R. M., & Ramirez, M. J. (2010). Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. *Neuropsychopharmacology, 35*(8), 1664-1673. doi: 10.1038/npp.2010.13

Song, S. K., Sun, S. W., Ramsbottom, M. J., Chang, C., Russell, J., & Cross, A. H. (2002). Dysmyelination revealed through MRI as increased radial (but unchanged axial) diffusion of water. *Neuroimage, 17*(3), 1429-1436.

Soriano, S. G., Liu, Q., Li, J., Liu, J. R., Han, X. H., Kanter, J. L., . . . Ibla, J. C. (2010). Ketamine activates cell cycle signaling and apoptosis in the neonatal rat brain. *Anesthesiology, 112*(5), 1155-1163. doi: 10.1097/ALN.0b013e3181d3e0c2

Spangler, G., Schieche, M., Ilg, U., Maier, U., & Ackermann, C. (1994). Maternal sensitivity as an external organizer for biobehavioral regulation in infancy. *Dev Psychobiol, 27*(7), 425-437. doi: 10.1002/dev.420270702

Spittle, A. J., Treyvaud, K., Doyle, L. W., Roberts, G., Lee, K. J., Inder, T. E., . . . Anderson, P. J. (2009). Early emergence of behavior and social-emotional problems in very preterm

infants. *J Am Acad Child Adolesc Psychiatry, 48*(9), 909-918. doi: 10.1097/CHI.0b013e3181af8235

Stevens, B., McGrath, P., Gibbins, S., Beyene, J., Breau, L., Camfield, C., . . . Yamada, J. (2003). Procedural pain in newborns at risk for neurologic impairment. *Pain, 105*(1-2), 27-35.

Stevens, B. J., Johnston, C. C., & Horton, L. (1994). Factors that influence the behavioral pain responses of premature infants. *Pain, 59*(1), 101-109.

Stewart, A. L., Rifkin, L., Amess, P. N., Kirkbride, V., Townsend, J. P., Miller, D. H., . . . Murray, R. M. (1999). Brain structure and neurocognitive and behavioural function in adolescents who were born very preterm. *Lancet, 353*(9165), 1653-1657.

Suchecki, D., Nelson, D. Y., Van Oers, H., & Levine, S. (1995). Activation and inhibition of the hypothalamic-pituitary-adrenal axis of the neonatal rat: effects of maternal deprivation. *Psychoneuroendocrinology, 20*(2), 169-182.

Suri, D., Veenit, V., Sarkar, A., Thiagarajan, D., Kumar, A., Nestler, E. J., . . . Vaidya, V. A. (2013). Early stress evokes age-dependent biphasic changes in hippocampal neurogenesis, BDNF expression, and cognition. *Biol Psychiatry, 73*(7), 658-666. doi: 10.1016/j.biopsych.2012.10.023

Teicher, M. H., Anderson, C. M., & Polcari, A. (2012). Childhood maltreatment is associated with reduced volume in the hippocampal subfields CA3, dentate gyrus, and subiculum. *Proc Natl Acad Sci U S A, 109*(9), E563-572. doi: 10.1073/pnas.1115396109

Tkac, I., Rao, R., Georgieff, M. K., & Gruetter, R. (2003). Developmental and regional changes in the neurochemical profile of the rat brain determined by in vivo 1H NMR spectroscopy. *Magn Reson Med, 50*(1), 24-32. doi: 10.1002/mrm.10497

Tottenham, N., Hare, T. A., Quinn, B. T., McCarry, T. W., Nurse, M., Gilhooly, T., . . . Casey, B. J. (2010). Prolonged institutional rearing is associated with atypically large amygdala volume and difficulties in emotion regulation. *Dev Sci, 13*(1), 46-61. doi: 10.1111/j.1467-7687.2009.00852.x

Treccani, G., Musazzi, L., Perego, C., Milanese, M., Nava, N., Bonifacino, T., . . . Popoli, M. (2014). Stress and corticosterone increase the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex. *Mol Psychiatry, 19*(4), 433-443. doi: 10.1038/mp.2014.5

Viau, V., Sharma, S., & Meaney, M. J. (1996). Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. *J Neuroendocrinol, 8*(1), 1-8.

Victoria, N. C., Inoue, K., Young, L. J., & Murphy, A. Z. (2013a). Long-term dysregulation of brain corticotrophin and glucocorticoid receptors and stress reactivity by single early-life pain experience in male and female rats. *Psychoneuroendocrinology, 38*(12), 3015-3028. doi: 10.1016/j.psyneuen.2013.08.013

Victoria, N. C., Inoue, K., Young, L. J., & Murphy, A. Z. (2013b). A single neonatal injury induces life-long deficits in response to stress. *Dev Neurosci, 35*(4), 326-337. doi: 10.1159/000351121

Victoria, N. C., Karom, M. C., Eichenbaum, H., & Murphy, A. Z. (2014). Neonatal injury rapidly alters markers of pain and stress in rat pups. *Dev Neurobiol, 74*(1), 42-51. doi: 10.1002/dneu.22129

Victoria, N. C., Karom, M. C., & Murphy, A. Z. (2015). Analgesia for early-life pain prevents deficits in adult anxiety and stress in rats. *Dev Neurosci, 37*(1), 1-13. doi: 10.1159/000366273

Viezel, K. D., Freer, B. D., & Lowell, A. (2015). Cognitive Abilities of Maltreated Children. *Psychology in the School, 52*(1), 92-106.

Vinall, J., Miller, S. P., Bjornson, B. H., Fitzpatrick, K. P., Poskitt, K. J., Brant, R., . . . Grunau, R. E. (2014). Invasive procedures in preterm children: brain and cognitive development at school age. *Pediatrics, 133*(3), 412-421. doi: 10.1542/peds.2013-1863

Vinall, J., Miller, S. P., Chau, V., Brummelte, S., Synnes, A. R., & Grunau, R. E. (2012). Neonatal pain in relation to postnatal growth in infants born very preterm. *Pain, 153*(7), 1374-1381. doi: 10.1016/j.pain.2012.02.007

Vinall, J., Miller, S. P., Synnes, A. R., & Grunau, R. E. (2013). Parent behaviors moderate the relationship between neonatal pain and internalizing behaviors at 18 months corrected age in children born very prematurely. *Pain, 154*(9), 1831-1839. doi: 10.1016/j.pain.2013.05.050

Virgin, C. E., Jr., Ha, T. P., Packan, D. R., Tombaugh, G. C., Yang, S. H., Horner, H. C., & Sapolsky, R. M. (1991). Glucocorticoids inhibit glucose transport and glutamate uptake in hippocampal astrocytes: implications for glucocorticoid neurotoxicity. *J Neurochem, 57*(4), 1422-1428.

Volpe, J. J., Kinney, H. C., Jensen, F. E., & Rosenberg, P. A. (2011). The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int J Dev Neurosci, 29*(4), 423-440. doi: 10.1016/j.ijdevneu.2011.02.012

Wahbeh, H., & Oken, B. S. (2013). Salivary cortisol lower in posttraumatic stress disorder. *J Trauma Stress, 26*(2), 241-248. doi: 10.1002/jts.21798

Walker, C. D., Kudreikis, K., Sherrard, A., & Johnston, C. C. (2003). Repeated neonatal pain influences maternal behavior, but not stress responsiveness in rat offspring. *Brain Res Dev Brain Res, 140*(2), 253-261.

Woodside, B., & Leon, M. (1980). Thermoendocrine influences on maternal nesting behavior in rats. *J Comp Physiol Psychol, 94*(1), 41-60.

Woodward, L. J., Anderson, P. J., Austin, N. C., Howard, K., & Inder, T. E. (2006). Neonatal MRI to predict neurodevelopmental outcomes in preterm infants. *N Engl J Med, 355*(7), 685-694. doi: 10.1056/NEJMoa053792

Yi, S. J., Masters, J. N., & Baram, T. Z. (1994). Glucocorticoid receptor mRNA ontogeny in the fetal and postnatal rat forebrain. *Mol Cell Neurosci, 5*(5), 385-393. doi: 10.1006/mcne.1994.1048

Young, E. E., Baumbauer, K. M., Hillyer, J., & Joynes, R. L. (2007). Local anesthetic treatment significantly attenuates acute pain responding but does not prevent the neonatal injury-induced reduction in adult spinal behavioral plasticity. *Behav Neurosci, 121*(5), 1073-1081. doi: 10.1037/0735-7044.121.5.1073

Yuen, E. Y., Liu, W., Karatsoreos, I. N., Feng, J., McEwen, B. S., & Yan, Z. (2009). Acute stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory. *Proc Natl Acad Sci U S A, 106*(33), 14075-14079. doi: 10.1073/pnas.0906791106

Yuen, E. Y., Liu, W., Karatsoreos, I. N., Ren, Y., Feng, J., McEwen, B. S., & Yan, Z. (2011). Mechanisms for acute stress-induced enhancement of glutamatergic transmission and working memory. *Mol Psychiatry, 16*(2), 156-170. doi: 10.1038/mp.2010.50

Zhang, L., Hernandez, V. S., Liu, B., Medina, M. P., Nava-Kopp, A. T., Irles, C., & Morales, M. (2012). Hypothalamic vasopressin system regulation by maternal separation: its impact on anxiety in rats. *Neuroscience, 215*, 135-148. doi: 10.1016/j.neuroscience.2012.03.046

Zhang, L. X., Levine, S., Dent, G., Zhan, Y., Xing, G., Okimoto, D., . . . Smith, M. A. (2002). Maternal deprivation increases cell death in the infant rat brain. *Brain Res Dev Brain Res, 133*(1), 1-11.

Zhang, X., Wang, B., Jin, J., An, S., Zeng, Q., Duan, Y., . . . Cao, X. (2014). Early deprivation reduced anxiety and enhanced memory in adult male rats. *Brain Res Bull, 108*, 44-50. doi: 10.1016/j.brainresbull.2014.08.005

Zhu, M. Y., Wang, W. P., & Bissette, G. (2006). Neuroprotective effects of agmatine against cell damage caused by glucocorticoids in cultured rat hippocampal neurons. *Neuroscience, 141*(4), 2019-2027. doi: 10.1016/j.neuroscience.2006.05.011

Zimmerberg, B., Rosenthal, A. J., & Stark, A. C. (2003). Neonatal social isolation alters both maternal and pup behaviors in rats. *Dev Psychobiol, 42*(1), 52-63. doi: 10.1002/dev.10086

Zwicker, J. G., Grunau, R. E., Adams, E., Chau, V., Brant, R., Poskitt, K. J., . . . Miller, S. P. (2013). Score for neonatal acute physiology-II and neonatal pain predict corticospinal tract development in premature newborns. *Pediatr Neurol, 48*(2), 123-129 e121. doi: 10.1016/j.pediatrneurol.2012.10.016

ABSTRACT

THE IMPACT OF NEONATAL PAIN AND REDUCED MATENRAL CARE ON BRAIN AND BEHAVIORAL DEVELOPMENT

by

SEAN M. MOONEY-LEBER

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Advisor: Dr. Susanne Brummelte

Major: Psychology

Degree: Doctor of Philosophy

In the neonatal intensive care unit (NICU) preterm infants are exposed to a multitude of stressors, which include both neonatal pain and reduced maternal care. Clinical and preclinical research has demonstrated that exposure to neonatal pain and reduced maternal care has a profound negative impact on brain and behavioral development. Currently, the biological mechanism by which both of these stressors impacts brain and behavioral outcomes remains widely unknown. To uncover a potential biological mechanism, the current dissertation project utilized a preclinical model of repetitive needle pokes and developed a novel model of reduced maternal care through tea-ball encapsulation. Briefly, rat pups were separated into one of five groups: touch control, isolation and touch, pain, pain and isolation, and unhandled controls. Pups in the isolation conditions were enclosed in tea-ball infusers from postnatal day (PD) 1-4, four times a day for 30 minutes. Pups in pain conditions experienced repetitive needle pokes into alternating paws starting on PD1 and ending on PD4. Unhandled control pups were left undisturbed throughout each experiment. For experiment 1, pups were sacrificed immediately after the last tea-ball exposure on PD4 and serum corticosterone and various brain metabolites were analyzed. We observed a significant increase in serum corticosterone on PD4 in maternally isolated animals and a non-

significant increase in pain exposed animals. Further, glutamate/creatine ratios were reduced in the frontal cortex and hippocampus in pain and maternally isolated animals respectively. For experiment 2, pups matured into adulthood and affective and cognitive behaviors were assessed through the open field test, novel object recognition test, Morris water maze, and restraint stress testing. During the Morris water maze, pain exposed animals displayed accelerated learning but both stressors impaired long-term memory. Moreover, reversal learning was enhanced in male isolated animals compared to touch males and isolated females. Finally, female pain exposed animals displayed impaired HPA-axis recovery following an acute stressor. Collectively, these studies demonstrate that both neonatal pain and reduced maternal care are potent neonatal stressors and can influence neonatal neurochemistry and adult cognitive and HPA-axis functioning. These findings highlight the need of interventions mitigating neonatal stress in the NICU.

AUTOBIOGRAPHICAL STATEMENT

During my undergraduate years at the University of Minnesota Morris, I was fortunate to work under the direction of Dr. Leslie Meek investigating the effects of paternal alcohol consumption during conception on aggressive behaviors in offspring mice. This wonderful experience provided me my first experience into the field of behavioral neuroscience and more importantly the two subfields of behavioral pharmacology and developmental psychobiology. After obtaining my Bachelors degree in psychology, I was accepted into the experimental psychology Masters program at Northern Michigan University, where I worked for Dr. Adam Prus examining the impact of various antidepressants on the schedule-induced polydipsia animal model. Upon graduating from Northern Michigan University, I began my Ph.D. studies at Wayne State University in the behavioral neuroscience program under the direction of Dr. Susanne Brummelte. While at Wayne State University, I worked on multiple projects which include the effects of *in utero* antidepressants on biobehavioral outcomes of offspring rats and the potential therapeutic effect of probiotics in a rat model of depression. From these projects, I gained valuable experience in various wet lab techniques as well as behavioral assays, which were critical for the success of the current project.