Long-term Effects of Neonatal Pain on Adulthood Stress Behavior and Neuroendocrinology

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Abstract:

Each year, thousands of premature babies are treated in neonatal intensive care units and are exposed to invasive noxious procedures, the long term effects of which are unclear. It was once believed that premature babies undergoing invasive treatments did not feel pain because they lacked pain perception circuitry. However, research has shown that early life is a very critical time period for development. Studies utilizing laparotomy, a simple abdominal surgery in rodents to mimic surgeries undergone by humans has shown that when administered to mice early in life, there is a decrease in adulthood pain sensitivity (Sternberg et al. 2005). Furthermore, previous research has consistently found that stress pathways undergo sensitive periods of development during early life. Rat pups separated from their mother for 180 minutes each day for the first two weeks of life showed a significantly increased response in levels of the stress hormone corticosterone in adulthood compared with those in control groups (Plotsky et al. 1993). The “Stress-Induced Analgesia” (SIA) theory has united the pain and stress biological. Endogenous opioid compounds are known to be the compounds responsible for inhibiting pain. They are commonly found along brain and spinal cord regions of the descending pain pathways. Similar to a fight or flight response, the SIA theory postulates that stress prompts an increase in the circulation of stress hormones. In turn, an increase in stimulation of opiate receptors occurs via increased secretion of endogenous opioid compounds. This results in a temporary relief in pain. Given that neonatal pain sensations have been shown to decrease adulthood pain behavior, and furthermore neonatal stress has been shown to increase the amounts of circulating stress hormone, it is hypothesized
that decreases in adulthood pain sensitivity observed after neonatal pain, may be mediated by an increase in a stress response resulting in stress-induced analgesia.

Therefore we conducted an experiment in which mice underwent a neonatal surgical procedure and hormonal stress response and stress behavior was tested in adulthood.

This study utilized a rodent model to explore the effects of neonatal pain on adulthood behavioral and hormonal stress response. Subjects were divided across surgery, sham-surgery and unhandled conditions. On the day of birth, subjects in the surgery group underwent laparotomy, a noxious stimulus consisting of an abdominal incision. Subjects in the sham-surgery group underwent the same stress experiences of the surgery group including cryoanesthesia and maternal separation but did not undergo surgery, and subjects in the unhandled group were returned to mothers. Adulthood behavioral stress testing was conducted using the elevated plus maze at baseline and under induced stress conditions. Adulthood hormonal stress response was studied by measuring levels of corticosterone in blood samples taken at baseline and fifteen, thirty and sixty minutes after exposure to a restraint stressor. Significant behavioral effects were found in that overall subjects spent more time in the closed versus open arms of the EPM. In addition, corticosterone levels increased over time and there was a trend suggestive of an enhanced corticosterone response in the surgery group. Neonatal pain potentially influences adulthood stress behavior and neuroendocrinology which may account for differences in adulthood pain behavior seen after exposure to neonatal pain.
However, studies with a greater sample size and alternate measures of analyzing stress behavioral data need to be conducted in order to draw more conclusive results.

I. Introduction

Approximately 250,000-350,000 newborn infants are admitted to neonatal intensive care units (NICU) each year. Recent advances in medicine have provided means to sustain the life of infants born extremely prematurely. However these means consist of invasive procedures and neonatal intensive care. Preterm neonates are exposed to prolonged hospitalizations consisting of a series of repeated invasive procedures and handling including repeated blood tests, insertion of peripheral lines and surgery, resulting in exposure to acute pain, chronic pain, and prolonged stress (Barker & Rutter, 1996).

It was once believed that neonates were not affected by early life pain and early pain would have no lasting effects on behavior or development. However, research has indicated that the premature neonates possess basic neuronal circuitry to process and behaviorally and physiologically respond to pain exposure (Lidow, 2002) such as the procedures mentioned above. This circuitry is in the developmental phases sensitive to modification from external stimuli. The brain experiences extreme plasticity during the prenatal and neonatal periods and this is a critical period of development that maximizes the influence of the environment on the brain and behavior (Rakic et al., 1986). Plasticity occurs in the form of synaptic activity causing the stabilization of the populations of synapses that are repeatedly and commonly activated while those that are inactive.
undergo apoptosis (Rabinowics et al., 1996). The brain is undergoing major development including the establishment and differentiation of neurons, alignment, orientation and layering of cortical neurons, elaboration of dendrites and axons and the formation of synapses. Thus it is possible that stressful and painful experiences in early life that consist of repetitive exposure to stress or pain during this period of development might have profound and permanent effects on brain by strengthening certain synapses while those atrophied undergo apoptosis and influence the development of stress and pain circuitry. This can have lifelong effects on stress and pain behavior. This hypothesis has been substantiated by clinical and laboratory investigations of humans and rodents.

There are fewer human studies then rodent studies because human studies. Human studies are problematic for multiple reasons, some of which include that researchers cannot manipulate experimental conditions, the difficulty associated with coordinating large sample sizes, as well as to study the long term effects of early life pain would take many years considering the relatively long maturation period for humans.

Researchers have resolved these infeasible issues by making use of animal models. The neonatal rodent has served as an appropriate paradigm for investigating the long-term consequences of neonatal pain in humans. Research comparing the development of newborn pups and corresponding timeframe of development for humans has found that rodent pups are born in the stages of neural development equivalent to a second trimester human fetus (Clancy et al., 2001). This allows researchers to study long-term effects of noxious neonatal procedures on rodents and translate the findings to make predictions about long term effects of exceptionally premature human neonates that undergo procedures of similar nociceptive magnitudes. Furthermore, rodent’s gestation
periods commonly last three weeks and yield relatively large litters. They are weaned
from the dam and separated from parents at approximately twenty days and reach
complete sexual maturation at approximately six weeks (Sternberg & Al-Chaer, 2007).
This allows for a timely completion of an experiment. Jointly, studies of human and
rodent neonates have suggested that neonatal pain has lasting effects in that it contributes
to the altered development of pain and stress behaviors and pathways.

*Pain Circuitry*

Pain circuitry can be divided into two groups: Ascending and descending
pathways. These pathways are mutually inhibiting and the sensation of pain represents a
balance of activation of these pathways.

Ascending pathways are the pathways that transmit the information of noxious
stimuli from the peripheral regions of the nervous system to the brain. Neurons
specialized in pain detection, called nociceptors exist in the peripheral nervous system
and are activated during the experience of tissue damage as a result of high intensity
temperature and mechanical, or chemical stimuli encroaching the body, among another
noxious stimuli. The nociceptors are concentrated in areas including the skin, joints,
connective tissue, skeletal muscles, and blood vessel walls and few are also located in
visceral organs. The cell bodies of the nociceptors extend through long projection fiber
from the periphery to the substantia gelatinosa of the dorsal horn of the spinal cord via
spinal nerves (Iggo et al., 1985). Nociceptive afferents are small in diameter and contain
relatively little amounts of myelination. The dorsal horn of the spinal cord contains the
grey matter of these cells including axon terminals, dendrites and cell bodies of second
order neurons in the somatosensory system that receive input from the nociceptors (Iggo
et al., 1985). The outputs of the tracts in the dorsal horn are sent via white matter through the spinothalamic, spinoreticular and dorsal column pathways to the brain. Once in the brainstem, these pathways synapse on thalamic neurons located in the brainstem as well as the reticular formation arousal areas in the pons and the limbic system which is responsible for emotion. The thalamic neurons direct the information to the corresponding area of the primary somatosensory cortex in the parietal lobe where a somatotopic map of the body exists (Willis et al., 1997). From the somatosensory cortex, action potentials containing information of tissue damage are transmitted to higher order cognitive control areas including the parietal lobe and frontal lobes, as well as areas in the cerebral cortex. Processing in these executive control areas contribute to the quality and levels of intensity that are consciously perceived as characteristics of pain. This interconnected circuitry from the peripheral regions of the body inward to the spinal cord and into brain regions represents the neural connections that allow one to perceive painful stimuli, connect them to emotional sensations and organize behavioral responses to thwart the pain (Sternberg, 2007).

This pathway is activated when nociceptors are stimulated. Studies involving thermal heat stimuli have implicated the activation of nociceptors via two pathways. Heat has found to open a nonselective TRP cation channel in primary sensory neurons. Furthermore studies of another ion channel, vanilloid receptor subtype 1, VR1 gated by capsaicin, the active ingredient of chili peppers works in similar manner to the heat channel (Cesare et al., 1999). The response of the heat channel is potentiated by phosphorylation by protein kinase C, whereas VR1 is potentiated by externally applied protons. Protein kinase C is known to be activated by a variety of inflammatory
mediators, and extracellular acidification is characteristically produced by anoxia and inflammation (Velazquez et al., 2007). Given the nature of stimulation of these pathways, they are likely to be involved in the activation of nociceptors which sparks the transmission of this information through the ascending pain circuitry to the brain.

Descending pain circuitry originates in the hypothalamic regions of the brain and synapses on the spinal cord. While the neural response to pain takes place, the hypothalamus secretes neurohormones which then stimulate the release or inhibition of pituitary hormones in response to painful and stressful stimuli. Axons of the descending pathways project from the hypothalamus down to the periaqueductal grey matter in the midbrain, which then form inhibitory synapses on the dorsal horn of the spinal cord.

The descending pathways are characterized by the inhibition and limiting of upward flow of nociceptive information in the ascending pathways from the spinal cord. They represent the body’s natural system for inhibiting pain. This was first theorized when electric stimulation of the midbrain produced analgesia or pain relief (Mayer et al. 1971). It was later discovered that endogenous opioid compounds were the compounds responsible for inhibiting pain. Opioid compounds achieve analgesic effects by binding to opiate receptors. Opiate receptors are commonly found along brain and spinal cord regions of the descending pathways. Studies exploring the stimulation of the brainstem in rats have found that while this region was stimulated, normative responses to intense pain were abolished resulting in analgesia (Mayer et al., 1971). In addition, when blocking these receptors, analgesic effects are diminished. It has been observed that when rats were administered naloxone, an opiate receptor blocker, analgesic responses were eliminated or weakened (Cannon et al. 1982). The purpose of this type of endogenous
pain inhibition circuitry might be to suppress pains under extreme environmental stressors. When under conditions of extreme stress such as predation, in order to survive an organism must utilize all resources via “fight-or-flight” mechanisms which produce a temporary relief in pain. The descending pathways produce this stress-induced analgesia (SIA) mechanism via the stimulation of opiate receptors via endogenous opioid compounds and represent the interconnected nature of stress and pain systems.

Research has suggested a critical period of sensitivity exists in the development of pain circuitry. One study examined neonatal rat pups and found that neonatal rat pups had lower thresholds and exaggerated behavioral responses to mechanical, thermal (Mclaughlin et al., 1990), and inflammatory (Guy et al., 1992) nociceptive stimuli compared with older pups or adult rats. Furthermore, newborn pups compared to adult pups exhibited lower thresholds to stimulation of the dorsal flexor reflex with Von Frey hairs (Fitzgerald et al., 1998). In addition, newborn pups exhibited lower nociceptive thresholds to thermal stimulation as measured by paw withdrawal latencies (Hu et al., 1997) and tail withdrawal from a heated water bath (Falcon et al., 1996). These findings highlight a critical period during early life of the development of pain response suggesting that the organism vulnerable to effects from environmental stimuli including pain and stress.

Pain Manipulation and Measurement Methods

Multiple techniques exist for applying painful stimuli and measuring pain behavior in rodent studies. Studies referenced in this paper utilize the following techniques. One technique used is repeated noxious insults in which pups receive foot shocks at a controlled frequency. Another technique used is inflammatory insults in
which subjects are administered an inflammation inducing substance such as complete
Freund’s adjuvant in their paws, and inflammation may persist for a number of days. In
addition, visceral stimuli, a technique consisting of inflammatory treatments to the colons
of rodents is another type of inflammation inducing procedure in use. Furthermore,
injection of acetic acid is known to cause abdominal constrictions in a subject. The
greater the amount of abdominal constrictions indicates the greater the pain sensitivity of
the subject. A technique more equivalent to the experiences undergone by premature
human neonates is surgery. A model of noxious surgical stimuli in neonates in use is the
administration of a laparotomy, or incision in the abdominal area, on P0, the day of birth.
This surgery is performed after pups are anesthetized using cryoanesthesia techniques.
This technique was developed because it better reflects the noxious surgeries that many
preterm human neonates undergo in the NICU. This allows for results from rodent studies
using this technique to be more translatable to human studies. These techniques have
shown that early life pain can have lifelong affects on pain behavior as measured by paw-
lick latency or how long a subject takes to lick its paw after a stimulus is applied,
morphine induced analgesia or how much morphine must be administered in order to
achieve pain relief, and paw and tail withdrawal times when in contact with a thermal
stimulus.

Stress Circuitry

The hypothalamic-pituitary-adrenal (HPA) axis is a major part of the
neuroendocrine system that is involved in the stress response. This axis represents a
series of direct connections as well as feedback mechanisms between the hypothalamus
located in the ventral region of the diencephalon, the pituitary gland at the base of the
brain, and the adrenal gland located on top of each kidney. The paraventricular nucleus of the hypothalamus mainly secretes two peptide hormones, vasopressin and corticotropin-releasing hormone (CRH). These two hormones act on the anterior pituitary which in turn releases adrenocorticotropic hormone (ACTH). ACTH then initiates the synthesis and secretion of glucocorticoid hormones from the adrenal cortex (Matthews, 2002). Glucocorticoid hormones are steroid hormones that bind with corticosteroid hormone receptors. It has been well established that cortisol is the chief hormone involved in the response to stress in humans. Furthermore, decades of research have found that in rodents, the glucocorticoid hormone corticosterone (CORT) is released as a result of HPA axis stimulation from stress inducing stimuli and furthermore, the effects of CORT are mediated via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (de Kloet 1991). The interconnected circuitry of the HPA axis mediates the response to stress via the release of glucocorticoid hormones such as corticosterone in rodents.

A critical period of development of the HPA axis also exists. The development of the HPA axis is highly sensitive to the surrounding environment during the fetal and neonatal period. Prenatal and neonatal stimuli have served to manipulate HPA development and function. Glucocorticoids (GCs) have been implicated as programming factors of the fetal HPA axis during prenatal stress. One mode of studying the role of GC’s involves maternal treatment with endogenous synthetic glucocorticoids (sGCs). Studies have shown that in rats, daily treatment with sGC in the final week of gestation resulted in elevated basal plasma corticosterone levels in adult male offspring (Levitt et al., 1996). Another study found that in rats, male offspring of mothers treated with sGC
late in the gestational period, mounted a greater corticosterone response to stress (Muneoka et al., 1997). Furthermore, adult male offspring of mothers treated with sGC in the last week of gestation exhibited reduced GR and MR mRNA in the hippocampus and increased CRH mRNA in the paraventricular nucleus indicating reduced feedback sensitivity from GCs resulting in less regulated, and greater HPA activity (Welberg et al., 2001). Postnatal stimulation has also served to manipulate development and function of the HPA axis. A study using neonatal handling as a stressor found that neonatal handling resulted in elevated hippocampal GR implying increased GC negative feedback and reduction in HPA activity (Meaney et al., 2000).

These studies highlight the sensitivity of the HPA axis and illustrate how the development and function of the HPA axis can be enhanced or diminished by environmental stimuli during fetal and neonatal development. Additional research has shown that alterations in HPA axis development and function can be seen to cause changes in basal hormonal levels and hormonal response to stress in adulthood. These findings suggest that the immediate effects of neonatal stress on the HPA axis may produce long-term effects on stress behavior. Behavioral studies of stress have corroborated this hypothesis.

**Stress Manipulation and Measurement Methods**

Multiple techniques allow for the stressful manipulation and stress response measurement. Techniques discussed in this paper that are used to induce stress in early life consist of maternal separation, and separation from the dam or separating the pup from the mother and the dam for a controlled amount of time, and daily physical handling which consists of being picked up, held in restraint and moved against choice daily.
Furthermore there is considerable stress associated with undergoing procedures such as
cryoanesthesia or being anesthetized by immersion in ice. In addition, to measure the
stress response, studies have utilized biological and behavioral measures. Some
biological measures consist of measuring the amount of glucocorticoid receptors or
hormonal mRNA in a specific area. The greater the number of receptors or mRNA
indicates the greater the amount of stress hormone. Other neuroendocrine measures
consist of measuring the amount of circulating stress hormones such as corticosterone in
rodents and cortisol in humans. In addition, to measure stress behavior in rodents a maze
called the Elevated Plus Maze (EPM) has been established. The EPM is a platform in the
shape of a plus that sits approximately 62.5cm above the ground. One set of opposing
arms are enclosed on the all sides by walls whereas the other set of opposing arms are
open with no enclosures. This apparatus measures behavioral anxiety by monitoring the
time a subject spends in the enclosed arms versus the open arms in that the more anxious
a subject is, the more time it will spend in the closed arms, areas where the height above
the ground is not visible, and the less anxious a subject is, the more time it will spend in
the open arms not stressed by the sight of being above the ground. Handley and Mithani
originally evaluated the elevated plus-maze as a possible animal model of anxiety (1984).
These investigators evaluated the influence of anxiolytic and anxiogenic drugs on the
amount of open arm entries, and found a decrease in open arm entries was correlated with
an increase in anxiety inducing drugs and the number of open arm entries increased with
anxiety relieving drugs, validating the EPM as an accurate measure of behavioral stress.
The EPM has been used as a behavioral measure of anxiety in mice and rats for decades.

*Neonatal Stress and Adulthood Stress*
As would be expected based on findings of altered HPA activity, neonatal stress has been shown to affect adulthood stress behavior. One study focused on human neonatal infants found that stressful conditions at birth were similarly associated with increased salivary cortisol responses to vaccination at 4 and 6 months of age (Ramsay et al., 1995). However, little is known about the long-term effects of stress on adulthood in humans because researchers cannot assign conditions to babies. Thus any study of humans can only be correlation where causality of results cannot be concluded. However animal models have allowed for further investigation in this area. A study involving squirrel monkeys was suggestive of the notion that moderately stressful early experiences strengthened socioemotional and neuroendocrine resistance to subsequent stressors (Parker et al., 2004). This study is illustrative of the notion of stress inoculation. Stress inoculation suggests that exposure to mild neonatal stress reduces stress behavior in adulthood (Sternberg & Al-Chaer, 2007). Contributions have also been made from rodent studies. Adult mice exposed to neonatal stressor paradigm of brief maternal separation during the first two weeks of life exhibited reduced stress-induced behavior compared to unhandled subjects (Sternberg & Ridgeway, 2003). Furthermore, neonatal rat pups exposed to daily physical handling or subcutaneous injection of saline developed an adult phenotype of decreased pituitary-adrenal responses to stress (Ader et al., 1969, Meaney et al., 1989). Supporting this notion of a reduced stress response, when exposed to stressors in adulthood, rats that had been neonatally handled mounted a smaller CORT response with a faster return to baseline compared with those of nonhandled rats. These differences endured throughout the course of their lifetime (Meaney et al., 1988, Meaney et al., 1991).
On the other hand, some studies found however that neonatal stress produces an increased stress response. Rat pups separated from their mother for 180 minutes each day for the first two weeks of life showed a significantly increased CORT response in adulthood compared with those in control groups. Also, newborn rats exposed to prolonged maternal separation had marked increases in CRF mRNA in the hypothalamic paraventricular nucleus (Plotsky et al., 1993).

Some studies observed an increased stress response to stressors while others observed a decrease stress response. One hypothesis that mediates this conflict is the idea that different levels of severity of neonatal stress may produce different effects on stress response. It has been suggested that mild neonatal stress exposure reduces stress responses in adulthood while severe neonatal stress exposure increases stress response in adulthood (Sternberg & Al-Chaer, 2007). Possible hypothalamic mechanisms explaining change in GC response from the HPA axis are focused around receptor density differences in stressed versus non-stressed subjects. Glucocorticoid receptor (GR) density was significantly reduced in the hippocampus and median eminence of adult animals exposed to neonatal stressors including maternal care (Meaney et al., 1994, Liu et al., 1997). On the other hand, GR density was significantly increased in the hippocampus in neonatal handled versus nonhandled animals (Meaney et al., 1989). In the neonatal handling group, increased numbers of GR in the hippocampus increased negative-feedback inhibition of the HPA axis stress responses producing decreases in basal and stress-induced expression of CRF and lower corticosterone responses (Sapolsky et al., 1984, Herman et al., 1989, Jacobson et al., 1991).
Human and animal studies illustrate that neonatal stress can manipulate stress circuitry during the critical period of development and can have long term effects on adulthood stress response. Mild neonatal stress stimuli reduce the adulthood stress response and severe neonatal stress stimuli enhance the stress response. Possible mechanisms focus on effects on the amount of glucocorticoid receptor density in brain regions part of the HPA axis.

*Neonatal Pain and Adulthood Pain*

Just as neonatal stress stimuli have been shown to affect adulthood stress response, neonatal noxious stimuli have been shown to produce lasting effects on adulthood pain behavior in studies employing a variety of manipulations in both human and animal studies. The extent to which this has been able to be studied in humans is based on already occurring painful stimuli. It has been found that preterm neonates who had experienced 4 weeks of NICU therapy including multiple noxious stimuli manifested decreased behavioral responses to the pain of a heel prick as compared to full-term neonates and furthermore differences in these response patterns were strongly correlated with the number of invasive procedures experienced since birth (Johnston & Stevens, 1996). On the other hand it was shown that 27-32 week gestational age infants who underwent repeated heel-stick procedures for blood draws exhibited hyperalgesic (increased pain sensitivity) responses to subsequent heel sticks (Fitzgerald et al., 1989). Furthermore, unanesthetized circumcision in the neonatal period was associated with an increased behavioral responsiveness to the pain of vaccination at 4-5 months of age (Taddio et al., 1997). Given the inability to control which baby receives noxious stimulation, the data is correlational and it is possible that alternate reasons might
contribute to the behavior observed. Animal paradigms have allowed researchers to conduct scientific research using a variation of noxious stimuli and to identify effects that might last well into adulthood. Studies using the repeated noxious insults technique have shown that rat pups that underwent daily footshock twice a day from postnatal day 0 (symbolized by P0 meaning date of birth) to P21, displayed a significant increase in paw-lick latency and enhanced morphine-induced analgesia as compared to control groups indicating decreased pain sensitivity (Shimada et al., 1990). Furthermore studies using the inflammation technique manipulated neonatal rat pups with daily doses of complete Freund’s adjuvant. In adulthood at 56-84 days of age, neonatally CFA-injected hindpaws of these animals were tested in the latencies of paw withdrawal to thermal stimulation and no significant difference was found between this group and the control group. However, when the same paw was re-inflamed by new CFA injections, subjects displayed higher thermal hyperalgesia (increased pain sensitivity) as measured via latency withdrawal to thermal stimulation, compared to the control group (Ruda et al., 2000). Similar findings were found in inflammation studies using carrageenan as an inflammatory agent. However, in comparing neonates injected at P0 versus P14, long term effects on nociception were seen in subjects injected on P0 but not P14, emphasizing the neonatal window of vulnerability and critical period of development that exists in early life (Cleland et al., 1999). Furthermore, studies exploring the use of visceral stimulation have observed results consistent with findings of increased pain sensitivity from previous studies discussed utilizing alternate techniques. It has been found that rats that were exposed to neonatal colon inflammation displayed an increased response to pain in adulthood (Al-Chaer et al., 2000).
A study that used the surgical laparotomy procedure as a neonatal noxious stimuli divided pups into three groups: the unhandled group, the surgery group in which subjects are exposed to maternal separation, cryoanesthesia and laparotomy, and the sham group in which subjects undergo all the same stressors of the surgery group including maternal separation and cryoanesthesia except the laparotomy itself. Thus, we can interpret the specific long term effects stress and pain alteration from just neonatal stress, as well as neonatal stress and pain. It was found that those who were in the neonatal noxious surgery group injected with a control injection had significantly longer tail-withdrawal latencies from a warm water bath, significantly longer latencies to shake and withdraw the paw from a hot-plate, significantly less number of abdominal constrictions, all indications of decreased pain sensitivity (Sternberg et al., 2005).

These studies taken together illustrate consistent findings that early life pain experiences and stimuli can influence adulthood pain behavior as shown in results from research utilizing a variety of techniques. Furthermore, the idea that a window of vulnerability or critical period of development exists in early life is reinforced.

**Neonatal Stress and Adulthood Pain**

Given that in early life both pain and stress circuitry undergo a critical period of development, neonatal stress can affect adulthood stress response, and neonatal pain can affect adulthood pain behavior, it is plausible that neonatal stress stimuli can produce an acute pain response and thus can also affect the development of pain circuitry. A subset of research suggests there is an intertwined relationship between stress and pain pathway development and function. Few human studies have been conducted, some of which are mentioned as follows. One study has shown that neonates that undergo the stress of being
handled exhibit greater increases in pulse rate, facial activity, and time spent crying in response to the heel stick (a painful procedure) than did the nonhandled infants (Porter et al., 1998) illustrating that the stress of handling can affect pain behavior. In rodent studies, it has been found in some techniques that neonatal stress increases pain sensitivity while in other techniques decreases pain sensitivity, possibly explained by the severity hypothesis mentioned earlier. Studies conducted utilizing maternal separation have found that rat pups exposed to 180 minutes of daily maternal separation for the first 2 weeks of life displayed heightened visceral sensitivity to colonic irritation as well as reduced sensitivity to thermal somatic stimulation in the tail-flick test in adulthood at age P60 (Coutinho et al., 2002). It is important to note that these findings are similar to studies of neonatal noxious visceral irritation that have been shown to produce visceral hyperalgesia (Al-Chaer et al., 2000) and neonatal carrageenan-based somatic inflammation to induce somatic hypoalgesia (Lidow et al., 2001) suggesting that neonatal stress might be intertwined with neonatal pain in causing adulthood effects seen from neonatal pain. The following studies have shown decreased pain sensitivity responses to neonatal stress. One study subjecting mice to the daily stress of handling and subcutaneous saline injections found that these mice display less sensitivity to noxious thermal stimuli in hot-plate and tail-flick tests in adulthood at age P45 (Pieretti et al., 1991). In addition, it has been found that in mice exposed to daily handling stress including separation from the dam for 15 minutes for the first two weeks of life had displayed elevated thermal withdrawal latencies in adulthood indicating decreased pain sensitivity (Sternberg & Ridgeway, 2003). Another study utilizing this same stressor
reported reduced pain sensitivity in the tail-flick task as well as in formalin tests in comparison with control animals (D’Amato, et al., 1999).

The findings of altered pain sensitivity are similar to the studies involving the effects of neonatal pain on adulthood pain behavior produced from noxious stimuli including surgery, footshock and inflammation mentioned in the neonatal pain effects on adulthood pain section. If neonatal stress can produce effects on adulthood pain behavior that mirror the effects of neonatal pain on adulthood pain behavior, it is possible that the differences in adulthood pain behavior are secondary responses to differences in stress behavior and that stress mediates effects of pain stimuli on pain responses. Furthermore it is possible that these effects are carried out via the stress-induced-analgesia mechanisms discussed previously in which opiate receptors on the descending pain pathways are activated by opioid release during stress, producing analgesia. To elucidate this hypothesis, it would be plausible to study whether differences in adulthood stress response can be observed after the administration of neonatal pain.

**Neonatal Pain and Adulthood Stress**

Limited studies have been conducted on whether neonatal pain can affect adulthood stress response. In human newborns, studies have found that in infants born less than or equal to 28 weeks of gestational age, a higher number of skin-breaking procedures since birth was associated with lowered cortisol responses to stress as compared to full-term infants (Grunau et al., 2005). In addition, it has been found that infants born extremely preterm showed higher salivary cortisol levels compared to full term controls after the introduction of visual novelty. Furthermore it was found that the cumulative number of skin-breaking procedures was associated with greater response to
novelty cortisol levels at 8 months (Grunau et al., 2004). These studies illustrate that repeated pain in the NICU possibly influences stress hormone responses. In rodent studies, a rat model employing neonatal local inflammatory insult found that in adulthood these rats had different behavioral and hormonal stress responses. Rats displayed reduced anxiety on an EPM, as well as reduced basal and stress-induced plasma levels of CRF and ACTH (Anseloni et al., 2005).

The purpose of our study is to further explore the effects of neonatal pain on adulthood stress using a different technique then those previously researched. This study will explore the effects of neonatal pain on adulthood stress behavior utilizing a simple laparotomy, an established neonatal surgical pain manipulation employed in Sternberg et al. (2005) as a neonatal noxious stimulus. This technique was chosen because it better represents the noxious experiences of preterm human neonates. Furthermore adulthood stress response will be measured behaviorally at baseline and after exposure to a stressor utilizing the EPM, and hormonally by measuring baseline and stress-induced levels of corticosterone, the glucocorticoid utilized by the HPA axis to respond to and influence stress. Given previous research findings that laparotomy results in decreased pain sensitivity (Sternberg et al., 2005), it is plausible that the neonatal laparotomy, a severe stimuli might induce an enhanced stress response as potentially observed through enhanced corticosterone levels, a neuroendocrine stress marker, and an enhanced behavioral stress response observed in time spent in the open arms versus closed arms of the EPM. This enhanced stress response may result in stress-induced analgesia (similar to the numbing of pain that occurs in a stress induced fight-or flight response) and may explain decreased pain sensitivity effects seen from early life painful and stressful...
stimuli. Similar to the paradigm of the Sternberg et al. (2005) study we will also utilize a sham group in addition to the surgery group. The sham group undergoes all the same stressors as the surgery group including maternal separation and time spent anesthetized, but not the surgery itself. This helps elucidate the causes of any effects seen in that if seen in the sham and surgery group, they are attributed to stress alone but if seen in just the surgery group it is possible that the stimulus causes changes above and beyond those of just the stress procedure. Furthermore, our hypothesis is substantiated by studies exploring neonatal pain and stress in infants finding an elevated cortisol response correlated with the number of painful surgeries (Grunau et al., 2004, Grunau et al., 2005), neonatal stress and stress in infants, finding an elevated cortisol response correlated with stressful experiences (Ramsay et al., 1995), increased levels of corticosterone after neonatal stress in rodents (Plotsky et al., 1993) as well as increased anxiety behavior displayed in the EPM who experienced inflammation insult (Anseloni et al., 2005). If this hypothesis were confirmed this may suggest that differences in adulthood pain behavior arising from neonatal noxious stimuli are a secondary response of stress, produced from vigorous activation of the HPA axis in the critical neonatal time period which results in an altered adulthood stress response. Furthermore, if this were not found, it is possible that differences in adulthood pain behavior arising from neonatal manipulations may occur through another route.
II. Methods

Subjects

The subjects in this study were the offspring of timed pregnant CD-1 female mice (Harlan Sprague-Dawley, Indianapolis, IN). Twelve female pregnant CD-1 mice were delivered to Haverford College while in late periods of gestation. Upon birth, pups were randomly assigned to conditions. Housed littermates that had undergone the same manipulation were placed under care of one mother regardless of biological origin. Maternal care as has been previously shown does not differentiate between biologically related pups and non-biologically related pups in this strain. We combined pups from across litters born on the same day in order to insure that all manipulations were consistently administered at the same age and that adulthood stress behavior would be measured at the same ages. Subjects were weaned from the mother at approximately three weeks of age and were housed with same-sex, same condition littermates in groups of up to 8 until adulthood testing at 20-25 weeks of age. There were a total of ninety-one subjects: nine males and fifteen females in the surgery group, eighteen males and fourteen females in the sham group, and fifteen males and twenty females in the unhandled group for a total of forty-two males and forty-nine females, of whom twenty-four total were in the surgery group, thirty-two in the sham group and thirty-five in the unhandled group. Animals were housed in plastic cages in a light (12:12h, lights on at
08:00) and temperature (20°C) controlled environment with ad libitum access to food (Harlan Teklad 8604) and tap water.

**Surgical Procedure**

The neonatal noxious stimulation used was a simple laparotomy (Sternberg et al., 2005). Pups were anesthetized using cryoanesthesia (Phifer & Terry, 1986). Cryoanesthesia consisted of covering the pup in crushed ice for approximately two minutes until the pup was immobile, pale and failed to respond to tail-pinches. After this, the pup was placed on a bed of crushed ice for the duration of the surgery. An abdominal incision was made extending from umbilicus to genitalia followed by insertion and rotation of a curved tip forceps. Sutures were placed using chromic gut. Pups were placed under a heat lamp on a warming bed until regaining abilities to move on their own and a pink color.

**Behavioral Stress Apparatus**

The elevated plus maze was used to measure stress behavior. The elevated plus maze (Hamilton-Kinder, San Diego, CA, USA) consists of two open arm platforms (length: 35 cm, width:5.2 cm) and two closed arms (with the same dimensions as the open arm platforms except enclosed on three sides by black walls 14.5 cm above the platform) emanating from a central platform (5.2 cm × 5.2 cm) to form a plus shape. Time spent in closed arm versus open arm is operationalized for anxiety (refer to introduction). The entire maze was raised 62.5 cm above the floor. Mice were placed in
the center of the maze facing an open arm and their behavior was videotaped for five minutes.

*Neonatal Manipulation Procedure*

Near the expected time of complete gestation, visibly pregnant mice would be monitored once a day to check for the birth of pups. Upon birth, all newborn pups were manipulated within twenty four hours between 08:30 and 14:30 during the light cycle. The entire set of pups combined across all birthed litters for that day were sexed, and randomly assigned to one of three conditions: surgery, sham-surgery, and the unhandled group. The unhandled subjects were immediately returned to a mother and kept in the lab for the duration of the procedure. For the laparotomy and sham surgery subjects, one pup from the surgery group and a same-sex pup from the sham surgery group simultaneously underwent equal duration of all stressors except the laparotomy which only the surgical group endured. Thus for every one subject in surgical condition, there would be a counterpart of the same gender in the sham-surgery condition. For each testing day, equal numbers of same-sex pairs were formed between the sham-surgery and surgery groups. This would result in equal numbers of males and females in the sham-surgery group and the surgery group. Both the sham-surgery pup and surgery pup were concurrently cryoanesthetized and the surgery pup underwent the laparotomy procedure as described above. While the surgery was being performed, the sham-surgery pup was also kept on a surface of a bed of crushed ice for equal amounts of time (ranging from 7 to 15 minutes per pair) as the paired surgery pup. The purpose for this was for the sham subjects to experience all the same stressors as the surgery group including exposure to ice, duration of separation from the dam, except the laparotomy, in order to isolate this manipulation
and be able to draw specific conclusions from the surgical procedure. The length of the entire surgery/sham-surgery procedure ranged from 100 minutes to 125 minutes from separation to recovery. After the surgery was complete, both pups were removed from atop the layer of ice and were warmed on a heating pad under a heat lamp. When affects of anesthesia wore off from both pups by indications of pinkish color and intentional movement, pups were returned to dams grouped by condition.

**Adulthood behavioral stress response measurement**

Stress behavior was measured via the elevated plus maze at approximately 23-25 weeks of age. Testing of subjects was done in random order. Mice were placed individually in the center of the maze facing an open arm. All testing was conducted during the light phase and in a dimly-lit room. The behavior of the animal was videotaped. The maze was thoroughly cleaned after each test using a spray cleaner and paper towels to rid of urine and feces and associated odor in order to prevent individual mice from following the scent of a previously tested animal. The videotaped behavior was scored by an investigator recording whether the subject entered with all four paws positioned in an arm runway, the open arm, the closed arm, the middle neutral area, and furthermore elicited a “head poke” or extension of the head and front paws into one of the arms without complete entry. Each observation was recorded utilizing a time sampling technique in which observations would be made during a span of five seconds for every twenty second period of videotaping. Behavior was videotaped for five minutes in the EPM, yielding fifteen five-second segments of behavior that were scored. EPM testing was conducted twice per subject: first at baseline and then after 15 minutes of exposure to a restraint stressor. A five minute habituation period was allowed for subjects just before
baseline EPM data collection, however not for stress-induced EPM data collection, which was conducted approximately within one to two weeks of baseline testing. Mice were remarked every two days until adulthood stress testing was complete. N= 27M, 27F for the stress EPM experiment, nine subjects per each condition (3) for each gender (2). N=37M, 42F for the baseline EPM experiment, 14 subjects per each condition (3) for each gender (2), except for the male surgical condition which had only 9.

Analysis of EPM data was conducted using total number of closed arm entries and total number of open arm entries per subject per EPM test, because these categories are important for the measurement of anxiety.

*Adulthood hormonal stress response measurement*

The amount of corticosterone levels at baseline and in response to a stressor was measured to identify potential differences in adulthood hormonal stress response across groups. The paradigm to conduct this measurement was structured to allow us to take measures of blood across four time points, at baseline (T1), fifteen minutes after the stressor (T2), thirty minutes after the stressor (T3), and sixty minutes after the stressor (T4). This paradigm allowed us to run eighteen subjects per session. Three sessions had to be conducted in order to draw blood samples from all fifty four subjects. Furthermore, an EPM test for each subject was conducted in between T2 and T3 to collect EPM data after exposure to a stressor.

All baseline EPM data were collected one to two weeks before the EPM stress data. The procedure for exposure to the stressor allowing for the collection of blood as well as EPM stress data was conducted as follows. Before each session, four plastic tubes (for blood storage) per subject would be labeled for each of the blood draws at four time
points. Each tube was filled with 6 μl of EDTA to serve as an anticoagulant that stops blood that is drawn from clotting.

Subjects were placed into a 50ml tube with air holes cut at one end and holes allowing for the tail to protrude at the other end. This tube served as a restraint stressor in that the subjects were tightly confined and unable to move. Immediately after placement in the restraint tube, the distal tip of the tail was snipped using scissors to access the tail vein for drawing blood and draw one was taken at T1. Blood was stored on ice and centrifuged. The serum was pipetted. Subjects remained confined in the restraint tube for fifteen minutes. Thereafter, draw two was taken at T2. Immediately after draw two, subjects were placed in the EPM to collect stress induced behavioral data. EPM behavior was recorded for five minutes and was later analyzed after the completion of the session. After five minutes, subjects were taken out of the EPM and returned to their cages until the time came for draw three. At T3 draw three was taken and then subjects were returned to their cage until draw four, which was taken at T4. After all blood samples were collected, the blood EDTA solution was centrifuged at 5000 RPM for 5-10 minutes. Centrifuging separated the blood into two visual layers, a clear serum layer and a darker layer of red blood cells. Serum, the component of blood that contains hormones and no clotting factors, was isolated by the removal of the red blood cells layer. Vials containing the serum from each session were stored in a freezer at -70°C until corticosterone analysis.

Analysis of corticosterone levels from blood samples

In order to determine the levels of corticosterone in the serum, a double antibody radioimmunoassay method specific for rats and mice was employed using a commercial
kit (MP Biomedicals LLC, Orangeburg, NY, USA). The principle of this assay was to measure the concentration of antigen (in this study, corticosterone) using a radioactive label that quantifies the amount of antigen by determination of the extent to which it combines with its antibody. The amount of antibody-bound corticosterone was separated from unbound antigen and its radioactivity was measured. The greater the amount of bound radioactive corticosterone indicated less corticosterone from the serum sample and vice versa. A set of standards containing known amounts and varied concentrations of corticosterone allowed us to develop a standard curve and a linear aggression formula to view the relationship between the concentrations of bound corticosterone and the radioactivity emitted. We then applied the quantified radioactivity measured from unknown samples against the standard curve formula to determine the unknown concentration of corticosterone in each sample.

This procedure was conducted in the laboratory of Tracy Bale at the University of Pennsylvania, School of Veterinary Medicine, and followed ImmuChem double antibody corticosterone$^{125}$I RIA kit protocol as follows. For accuracy, two tubes were used for each sample or concentration of which the average was taken and used as the gamma reading. Two vials served as non-specific binding tubes containing no corticosterone to represent the minimum amount of activity in the gamma counter (in which radioactivity is measured) which was subtracted from all other gamma readings. Another group of vials in which no corticosterone was added, but 0.1ml of steroid diluent and 0.2ml of antiserum was added, served as the zero concentration standards. Furthermore another group of vials served as corticosterone calibrators for the standard curve in which .1ml of different known concentrations ranging from 25ng/ml -1000ng/ml was added. Another
group of vials consisted of controls in which a high and low concentration of corticosterone had been added to the steroid diluent and were used to test the accuracy of the standard curve. The remaining vials were used for samples from the serum taken from subjects. The mouse serum was diluted. Next, radioactive corticosterone-\textsuperscript{125}I was added to all tubes. Following this of the antiserum anti-corticosterone were added. All vials were vortexed and incubated at room temperature for two hours to allow for competition between the radioactive corticosterone and mice corticosterone to bind to the antibody. After incubation, precipitant solution was added to all tubes and vortexed thoroughly. The precipitation allowed for the bound antigen to separate from the unbound antigen. All assay tubes were centrifuged at 2500rpm for fifteen minutes and the supernatant was aspirated to isolate the pellet of bound radioactive and unknown corticosterone. The radioactivity was counted in a gamma counter, a standard curve was formed from readings from the corticosterone calibrators in the associated vials as mentioned above, and readings from the unknown samples were applied to the formula of the standard curve in order to measure the unknown levels of corticosterone for each sample. The levels of unknown corticosterone (corticosterone from the subject’s serum) could be determined from the standard curve because the amount of radioactivity was inversely proportional to the amount of unknown corticosterone. The average of each of the two readings per sample was taken to come to an accurate value of corticosterone levels in ng/ml per sample.
III. Results

Data Analysis: EPM Behavioral Data

Available EPM data for each subject included numbers of open arm entries, closed arm entries, head-pokes and neutral area entries for baseline EPM and stress induced EPM tests. Closed and open arm entries were used as dependent variables. We expected to see a difference across condition in number of open arm entries compared to the closed arm entries when comparing baseline data to stress induced data, in that the surgical group might display an enhanced stress response and have more entries in the closed arm (suggestive of increased anxiety behavior) then in the open arm after being stressed compared to the other groups. A 2 X 3 X 2 mixed factorial ANOVA with gender (m/f) and neonatal conditions (surgery/sham-surgery/unhandled) as between-subjects variables and the within subjects variable being behavior (open entries/closed entries), was used to identify significant differences between dependent variables of baseline open arm entries and baseline closed arm entries and stress induced open arm entries and stress induced closed arm entries. To compare stress EPM data to baseline EPM data, a 2 X 3 X 2 X 2 mixed- factorial repeated measures ANOVA with between-subjects factors of gender and neonatal conditions was used and within subjects factors included behavior (open arm entries/closed arm entries, as well as time (baseline data/stress data).

Data Analysis: Corticosterone Levels
Corticosterone samples were taken at four time points: T1, T2, T3, and T4. It was predicted that those in the surgery group would display an enhanced stress response in terms of significantly greater amounts of corticosterone after being stressed compared to subjects in other conditions. To analyze this possibility, a 2 X 3 X 4 mixed factorial ANOVA was conducted with gender and neonatal condition as the between-subject variables and levels of corticosterone across the four time points as the within-subjects factors.

**EPM Data Results**

During baseline EPM data, there was a main effect for behavior, $F(1,73) = 7.87$, $p<.05$, (closed arm entries > open arm entries) for all mice (see Figure 1). This suggests that all mice were more anxious and stressed, possibly occurring from a general fear of heights that can be seen from the open arms.

Furthermore there was a main effect for gender $F(1,73) = 5.37$, $p<.05$, in that females had more entries averaged across closed and open arms compared to males indicating greater movement in females as can be seen in Figure 2. This difference suggests that early life pain may influence EPM behavior at baseline differently in females. The data was split by gender and we analyzed females and males separately using a 3 X 2 mixed factorial ANOVA with neonatal conditions being the between groups factor and behavior as the within groups factor. In females and males, a significant behavior effect was found as was expected from the original analysis and showed that more time was spent in closed then open entries. However for females a significant behavior by condition interaction effect was also observed, $F(2,39) = 3.579$, $p<.001$, in that females in the sham group spent significantly greater time in the closed
arms then the open arms, compared to females in the surgery condition closed arm and open arm entries and females in the unhandled condition closed arm and open arm entries, as averaged across individuals per each group (see Figure 3).

The 2 X 3 X 2 X 2 mixed factorial ANOVA comparing baseline to stress EPM data that would help elucidate whether the surgery caused an enhanced behavioral response only showed a significant behavior effect, F(1,45)=15.93, p<.001, in that all mice spent more time in the closed arms then the opened arms overall during baseline and post-stressor EPM testing as seen in Figure 4. However no behavior by condition by time interaction was shown indicating that there was no significant difference between entries of closed arms compared to entries of open arms compared across conditions and across baseline and stress data. This result was not what we expected in that we thought in general all mice would spend more time in the closed arm then in the open arm after being stressed compared to baseline, and those in the surgery group may spend even greater time in the closed arms then the open arms because of a greater stress response as compared to those in the other conditions.

Corticosterone Response Results

We analyzed corticosterone levels across four time points before and after being stressed. We expected those in the surgery group to display an enhanced corticosterone response compared to those in the other groups. Significant main effects were noted for time, F(3,132)=38.26, p<.001, in that the levels of corticosterone increased significantly over the course of draws taken (T4>T3>T2>T1) as can be seen in Figure 5. This indicates that there is a significant increase in corticosterone levels across all groups after exposure to a stressor.
The analysis most relevant for our hypothesis was testing for a significant time by condition effect which would identify if the neuroendocrine response to stress is affected by neonatal pain. The time by condition effect as tested by the 2 X 3 X 4 mixed factorial ANOVA was near significance with $F(6,132)=1.97$, $p=.074$. There was a trend illustrating that those in the surgery group had an enhanced corticosterone response to the stressor as compared to the other groups, which can be seen in Figure 5. Those in the surgery group mounted enhanced levels of corticosterone in response to the stressor. This result supports the hypothesis that neonatal pain enhances the neuroendocrine stress response, but this effect is not produced by exposure to just neonatal stress, as the sham group did not display a similar enhanced response compared to the unhandled group. Instead the sham group displayed a very similar response to the unhandled group as can be seen in Figure 5.
IV. Discussion

The results of this study are support previous observations of long-lasting effects of neonatal laparotomy on adulthood hormonal and behavioral stress response exist. To sum the results, analysis of the EPM data has shown that at both baseline and stress, all subjects had significantly more entries in the closed versus open arms but this ratio did not change from baseline to stress nor was it mediated by condition. However at baseline, females had significantly more entries then males and separate analysis of females yielded a significant behavior by condition interaction. Females in the sham group had significantly more entries in the closed arms versus open arms compared to the other groups, potentially illustrating greater levels of anxiety and that solely non-noxious neonatal stimuli can alter adulthood stress behavior. Analysis of the corticosterone levels across time yielded a significant time effect in that levels of corticosterone increased over time validating corticosterone as an appropriate measure for neuroendocrine stress response. Furthermore, there was a trend suggesting that this response was enhanced in the surgery group suggesting that neonatal pain can enhance adulthood neuroendocrine stress response.

The most promising novel result was the trend toward a greater corticosterone response to stress in the surgery group in that they were almost significantly greater as compared to the other groups. While perusing the data their seemed to be a significant difference in corticosterone levels in males across conditions, but not the females and
when analyzed this trend was significant. These findings directly address the question on whether neonatal noxious stimuli can affect adulthood stress response.

Furthermore, that there was a significant increase in corticosterone across time indicates the effectiveness of the restraint stressor. It is possible that the corticosterone released in early life in response to stressful stimulation might mediate a potential global nervous system phenomenon and effect alternate pain behavior seen after neonatal exposure to pain, specifically painful surgery (Sternberg et al., 2005). To ensure that the surgery was painful, ultrasonic vocalizations (USV) are used to show that the laparotomy is a painful manipulation. Evidence for this comes from previous research in which a significantly greater amount of USVs was emitted in the group undergoing laparotomy as compared to the other groups and that the amount of USVs emitted was reduced after treatment with morphine (Sternberg et al., 2005). We detected USVs from the subjects in the surgery condition to confirm this was a painful manipulation. In addition, the finding that levels of corticosterone increase over time is generally true, however if examined in closer detail, in the sham and unhandled group the levels of corticosterone drop from draw 2 to draw 3, but then significantly increase at draw 4, as can be seen in Figure 5. This highlights issues with the timings of the draws. If only taking four draws, perhaps it would be better to take draw 3 at a greater time interval then fifteen minutes after draw 2. This perhaps is too soon, and that it represents 25% of all corticosterone data in this experiment perhaps more conclusive results could be drawn from future studies if the draw was taken at a later time. This might better help us understand the corticosterone response and better identify the rate of corticosterone increase that occurs between draw 3 and draw 4. There is a drastic increase between draw 3 and draw 4, but we do not know
if that a linear or exponential increase. Draw 3 if placed at a later time could help elucidate this. Furthermore, it would be ideal to follow corticosterone levels for a longer time period, perhaps 1.5 hours or until they return to baseline. Most previous research of corticosterone responses in rodents are taken at one point after decapitation. However in chickens, corticosterone measurements were taken between 1 hour and 1 day after exposure to a stimulus (Shini et al., 2007). If these changes were made, they would contribute to the understanding of how great and how long the enhanced corticosterone response occurs. If draws were more spaced out, taken more frequently or taken for a longer time course this might yield significant results as opposed to near significant, and would help elucidate how great of an effect the neonatal manipulations have on adulthood corticosterone stress responses.

More detailed draws could also help better understand and differentiate the long-term effects of noxious versus non-noxious stressors. Studies in the past have shown that non-noxious stress including maternal separation causes an enhanced corticosterone response in adulthood (Plotsky et al., 1993) while other studies have shown that rats who have been neonatally handled mounted a decreased corticosterone response to stressors in adulthood (Meaney et al., 1991). The sham group in our study exhibited neither, but instead mirrored the response of the unhandled group. Given previous research, it is unlikely that either the stress undergone by the sham group including cryoanesthesia as well as maternal separation to have no effect on corticosterone response. These results suggest that a more thorough collection of corticosterone data is needed to elucidate if and how cryoanesthesia and brief maternal separation have long term effects on the stress response. It might be that those in the sham group might have shown a faster return to
baseline corticosterone levels then other groups, however with our data we are unable to
tell. These issues should be addressed.

Another point of contention is the mismatch between behavioral and hormonal
adulthood stress responses. Behavioral baseline data suggest that the female sham group
has higher levels of anxiety at baseline as compared to the other groups. However
baseline corticosterone levels were not significantly different across any groups nor did a
trend exist. Furthermore, the trend suggesting an enhanced glucocorticoid response in the
surgery group after being stressed was not apparent in post stressor EPM data. This may
be suggestive of multiple issues. It is possible but not likely that levels of corticosterone
circulating in the blood do not influence behavior in the EPM. Another possibility is that
the way either the corticosterone levels or EPM data were measured was not an accurate
representation of a stress response. Given the straightforward theory of the measurement
of corticosterone and the variability of methods in analyzing EPM data, it is likely that
the EPM data was not an accurate representation of stress behavior. This idea is further
supported by the lack of significant differences in number of entries in the closed arm
compared to number of entries between baseline and post-stress time points. It was
expected that after being stressed that subjects in general should spend more time in the
closed arms because a subject is shielded from seeing the distance above the ground, and
that perhaps those in the surgery group might display an enhanced stress behavior.
However, significant differences were not observed and furthermore, results of the
female sham group showing significantly enhanced stress behavior at baseline, were not
apparent post stress exposure. In our study there was a lack of counterbalancing. All
subjects underwent baseline EPM testing prior to stress EPM testing because it was
necessary to draw blood after being exposed to stress, and this semi-invasive procedure might effect baseline stress behavior. It is possible that since all stress EPM data was collected after baseline EPM data, subjects were already habituated to the maze and that this could account for the lack of significant behavior observed between baseline EPM behavior and post-stress EPM behavior. In addition, an apparent trend was seen in the general number of open arm entries in that they decreased from baseline to stress. However our analysis consisted of comparing these entries in ratios to closed arm entries and not to each other, which yielded no significant results. These findings bring into question our method of analyzing EPM behavior.

To analyze EPM data, our method consisted of analyzing a five second portion of video every twenty seconds for five minutes. If the subject stepped inside a closed or open arm, regardless of the duration of time, at any point in during the five second portion, a single entry was marked in each respective category of where the subject traveled. This method of recording EPM behavior is limiting. This system equates if a subject spends one second on the open arm and four seconds on the closed arm and if a subject spends on second on the closed arm and four seconds on the open arm. In each case there would be a single entry for open and closed arm. Although this system was meant to take into account the proportion of time spent in the open versus closed arms, in actuality it does not allow researchers to distinguish factors such as duration of time spent in the arm, or repeated entries within a single time frame of analysis. It is likely that because this system equalizes potentially different behaviors, this might have prevented us from seeing significant results. Specific research has sought to identify the most valid measures of analyzing data from the EPM to identify anxiety behavior. One study
compared a three-factor model to a two-factor model of theory explaining behavior in the EPM and found a two-factor model to be the better and most parsimonious fit of measuring anxiety. This model utilized measures including closed arm time ratios defined as time spent in the closed arm compared to total time on the maze and open arm time ratios defined as time spent in the open arm out of total time spent in the maze (Wall & Messier, 2000). This method of measurement accounts for the differences in the actual amount of time spent in each arm and further forms a ratio between the amounts of time spent in each arm out of the total time. This method better illustrates how a subject spends its time in each arm and instead of comparing open arm entries to closed arm entries, it compares the actual time spent in each respective entry as a fraction of the total time, giving a more accurate perspective. Furthermore, other studies have found that elevated CORT responses correlate with risk assessment in the EPM (primarily, stretched-attend postures) and not open arm entries (Rogers et al., 1999). It is probably that these behavioral measurements and analyses more accurately represent stress behavior. Future studies utilizing the EPM should make use of these more accurate analytical methods.

EPM baseline and stress data highlight another limitation of this study. Significant effects across condition were found in females at baseline but not after being stressed. Females in the sham condition were found to spend significantly greater amounts of time in the closed arm versus open arm at baseline. This is suggestive that perhaps there is something about the non-painful stress of the laparotomy operation excluding the operation itself that enhances the adulthood behavioral stress response, and the laparotomy procedure may undo the effects caused by the non-noxious stressors. A
possible explanation for this is that differences in severity of each procedure produce different effects on stress behaviors.

However, these results were not observed in the stress EPM data. This highlights the question, what changed? Due to limitations of time allocated for the entire procedure baseline EPM utilized a greater N then all other tests. Furthermore, none of the other analyses including stress EPM data, and comparison of stress EPM data to baseline EPM data showed significant effects. There is a strong possibility that lack of significant effects observed in our data might have been attributed to high within group variability. If more subjects were tested, it is probable (as indicative from the baseline behavior by condition effect in females) that the influence of these individual differences would be diminished and more conclusive results could be drawn from the behavioral data. In addition, the possibility exists that given the complexity and the inherent variability of experimental error introduced by the inexperience of researchers, that significant results might be observed when procedures are conducted by researchers with greater expertise. In sum, a greater number of subjects as well as greater expertise in conducting these procedures might yield more conclusive results.

Apart from these limitations, this study lays a foundation for future studies. Experiments of neonatal surgery and stress in humans are lacking the longitudinal design to draw conclusive effects that may characterize behavior in adulthood. In rodents, this method of noxious stimulus, as opposed to inflammation, may be the most similar model to noxious stimuli that human neonates undergo. Laparotomy involves skin wounding, local inflammation and nociception, all things that pre-term neonates experience in NICU’s. The findings from this study suggest that it is possible that neonatal pain might
affect adulthood stress response hormonally but more subjects are needed in future studies. It is possible that this hormonal change may cause a global change in the nervous system which possibly mediates changes in adulthood pain behavior seen from neonatal noxious stimuli (Sternberg et al., 2005). This study highlights the interconnections that exist between the neuroendocrine element of the nervous system and the physiological mechanisms underlying behavior. Results suggest that noxious stimuli at a time of development may have long-lasting effects extending throughout many different but interwoven domains of stress and pain. Although the nature of the findings are speculative, the trends seen add support to the notion that the great amount of plasticity of the nervous system in early life that exists leaves the development of the nervous system vulnerable to early life stimuli which could produce long-lasting effects. There is a misconception that preterm neonates are incapable of experiencing pain due to their underdeveloped pathways and are often not treated for pain despite undergoing numerous painful procedures (Johnston et al., 1997). These findings add to the evidence of the existence of long-term effects of noxious stimuli on the development of the nervous system in humans, and also provide that noxious stimuli can have branching effects across different developmental pathways. In sum, preterm human neonates being treated in NICUs are vulnerable to stressful and painful stimuli, which can have long term effects on their development. Extreme care should be taken to manage the exposure to and treatment of painful stimuli.
V. Figures

Figure 1. Closed vs. Open arm entries at baseline:
All subjects had significantly more entries in the closed arm compared to the open arm at baseline, indicating greater levels of anxiety across all subjects.
Figure 2. Entries averaged across baseline open and closed arms split by gender: Females had significantly greater entries than males during baseline EPM indicating more movement and perhaps a different mechanism influencing EPM behavior in females leading us to analyze data split by gender.
Figure 3. Female closed vs. open arm entries at baseline split by condition:
When we split the data by gender, a significant behavior by condition interaction was found in females at baseline. Those in the sham group had significantly greater entries in the closed arm than in the open arm compared to this ratio in the surgery and unhandled groups indicating a greater level of behavioral stress. It is possible that the non-painful stress associated with procedures underwent in the sham group specifically influences stress behavioral responses in the EPM.
Figure 4. Stress induced open vs. closed arm entries:
All subjects spent significantly more time in the closed versus open arms of the EPM post stress exposure (above) and baseline EPM testing, as averaged across individuals. However no significant effects were found for condition or day indicating that EPM performance was not influenced by neonatal manipulation or from stress exposure using a restraint tube.
Figure 5. Corticosterone levels across time per each condition:
Corticosterone levels at baseline (draw 1) and fifteen minutes (draw 2), thirty minutes (draw 3), and one hour (draw 4) after exposure to stressor as averaged across individuals per each group. There is an increase in response over time and exposure to neonatal surgery produces enhanced corticosterone response compared to the sham and unhandled groups in which subjects display similar responses suggesting that neonatal noxious stimuli alters adulthood neuroendocrine stress response.
VI. References


