EXAMINING THE EFFECTS OF EARLY LIFE STRESS FROM MATERNAL SEPARATION ON MEASURES OF PAIN AND ANXIETY

by

MEGAN LYNNE UHELSKI

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ABSTRACT

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Megan L. Uhelski, M. S.

The University of Texas at Arlington, 2009

Supervising Professor: Perry N. Fuchs

Animal models of stress-induced conditions have provided important insight into the physiological mechanisms of many chronic disorders. Models of early life stress involve procedures designed to induce prenatal or postnatal stress upon pups, which produces adult rats with enhanced stress responses and behavioral similarities to animal models of schizophrenia, anxiety, and depression. Since little research has focused on the effect of maternal separation on adult pain processing, the purpose of the current study is to examine both sensory and affective pain measures in adult rats following repeated maternal separation in infancy, a common model of early life stress. Eighty-six male pups were utilized following either early maternal separation (EMS) or early handling (EH). Although sensory thresholds remained unaltered for adult EMS rats, their emotional response to nociceptive stimuli intensified under certain conditions. In addition, EMS rats demonstrated more hyperactivity and anxiety-like behavior in the elevated plus maze. This indicates that early life stress leads to exaggerated emotional responses to novel or nociceptive stimuli in adulthood. Further research could determine whether or not this pattern holds true for different pain models, or if post-weaning enrichment could reverse the effects of maternal separation on pain processing.

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CHAPTER 1

INTRODUCTION

The physiological response to stress is a subject of significant research interest in a broad range of scientific disciplines. The first evidence of connections between stress and chronic disease forced the medical community to begin evaluating patients' mental health as well as their physical well-being when considering diagnosis and treatment options. The types of illness caused by long term stress and other psychological factors range from high blood pressure and arteriosclerosis to stomach cancer and a weakened immune system. The so-called 'Type A' personality disorder, characterized by impatience, hostility, and time urgency, is a strong precursor to the development of coronary heart disease (Friedman & Booth-Kewley, 1987 & 1988; Matthews, 1982 & 1988). Stress exposure has been shown to decrease the natural killer (NK) cell response and lymphocyte production following antigen exposure (Stein, et al., 1985). Perceived uncontrollable stress has even led to cancer development in laboratory animals, with further studies indicating that lack of control over the stressful events was the crucial detrimental element (Laudenslager, et al., 1983). Thus, stress management and the maintenance of perceived control over one's situation are imperative in the treatment of individuals who are at risk for chronic disease or have already been diagnosed (Taylor, 1990). Understanding the complexities of the stress response cycle is critical for psychologists and medical personnel to develop effective prevention programs for chronic illnesses that result from stress or are exacerbated by its presence. By examining the effects of early life stress induced by maternal separation, we can investigate the factors involved in childhood trauma leading to adult neurological dysfunction.

1.1 The Stress-Response Cycle

The human stress-response cycle is a complex neuroendocrine response triggered by perceived or real threats from the environment or internal stimuli. The sympathetic nervous system and the hypothalamus-pituitary-adrenal (HPA) axis are primarily responsible for the physiological changes associated with a stressful state (Selye, 1937). The initial catecholamine response triggered from the sympathetic nervous system involves a rapid release of epinephrine and norepinephrine into the bloodstream and throughout the body, inducing the changes characteristic of the 'fight or flight' response—increased heart rate and respiration, pupil dilation, and more blood flow to the skeletal muscles, vigilance, and so forth (Cannon, 1929; but see also Bracha et al., 2004).These changes are rapid but readily reversed when the initial danger has passed. A slower, more profound effect is also produced by the activity of the HPA axis. Corticotrophin-releasing hormone or factor (CRH) is synthesized in the hypothalamus and released into the hypothalamic-pituitary portal system, where it flows to the pituitary. In turn, the pituitary gland releases adrenocorticotropic hormone (ACTH) into the bloodstream and past the blood-brain barrier. Once this arrives at the adrenal gland, the cortex releases glucocorticoids into the bloodstream, which have longer lasting effects than the catecholamines released from the sympathetic nervous system and adrenal medulla. In humans, the main glucocorticoid is cortisol, a hormone with wide-ranging effects in the body that is derived from cholesterol in the zona fasciculata of the adrenal cortex. Upon its release, cortisol's objective is to return the organism to homeostasis following a stressful event. This adaptive mechanism evolved to promote survival in early warm-blooded creatures by minimizing loss of bodily resources and recuperating from damage due to injury or disease (Selye, 1937; Haller, et al., 1998). Cortisol helps maintain a state of elevated blood pressure by sensitizing catecholamine receptors in the blood vessels. Blood sugar is increased by reducing glucogenesis and increasing the breakdown of glycogen, protein, and lipids in the liver. The immune system is disrupted and suppressed in a variety of ways. Cytokine signaling is also disrupted by the suppression of nuclear factor κβ

signaling pathways, which can block the normal inflammatory response and prevent T-cell proliferation (Ashwell et al., 2000; Raison & Miller, 2003). Histamine production is also suppressed, and immune cells are redistributed back to the bone marrow (Wiegers & Reul, 1998). Although cortisol is normally released in a pattern corresponding to the circadian rhythm (lowest around midnight and highest early morning) and is present in the bloodstream at all times, excessive stimulation of the HPA axis can be detrimental to the organism. The cortisol response itself has a self-limiting component: the negative feedback loop formed when cortisol is released inhibits the release of CRH from the hypothalamus.

Animal models of stress-induced diseases and conditions have provided important insight into the physiological mechanisms. The physiological response to stress—a flood of catecholamines followed more slowly by the release of glucocorticoids—is found in all mammals, birds, and some reptiles, with a glucocorticoid-like receptor present in amphibians that is believed to effect GABA transmission (Haller, et al., 1998). Rat models of the stress response focus on the release of corticosterone (CORT), the rodent equivalent of the human glucocorticoid cortisol. Investigating the complex relationship between stress and pain processing is an important aspect of research with significant clinical implications. The stress response involves nearly every aspect of an organism's physiology, and its impact upon sensory and affective nervous system processes clearly includes the processing of nociceptive stimuli.

1.2 Stress and Early Life Experience

Models of early life stress involve procedures designed to induce prenatal or postnatal stress upon pups, which has a lifelong impact on neuroendocrine functioning (Meaney et al. 1990 & 1991) and has been shown to alter development robustly and permanently. Clinically, it has been suggested that the stress of certain traumatic events in early infancy and childhood can be a risk factor in adult mental illness (Friedman et al., 2002; Fumagalli et al, 2007), which has focused attention on investigating the exact mechanisms that take place in individuals who experience stressful or traumatic events in early life. Ethical concerns preclude the examination

of casual relationships between early life stress and its consequences in adulthood, and so the development of animal models is essential for the progress of research in this area. A well-established model of early life stress in rats is maternal separation (EMS). In this procedure, newborn pups are isolated from the dams for an extended period designed to exceed the normal 20-25 minute separations normally induced by the dam between feedings (the inter-nest bout interval, Leon et al., 1978). It can be carried out acutely or repeatedly anywhere from postnatal day 1 until about two weeks after birth. Around the 15^{th} postnatal day is the end of what is called the Stress Hyporesponsive Period (SHRP, Schapiro, 1968), a period during early postnatal development that protects infant pups from the effects of excessive hormone release in the presence of stress for the first few weeks of life. During the late fetal period, rats have a functioning HPA axis and are able to secrete CORT prior to birth. Shortly after birth (PND 2), the HPA axis becomes virtually non-responsive to a variety of stressors, including loud noise, injection with needles, surgical procedures, and so forth (Levine, 1957; Schapiro, 1968). This period allows for normal central nervous system development in the presence of common stressors, since glucocorticoids are catabolic and inhibit cell division and protein synthesis (Sapolsky & Meaney, 1986). CORT is a trophic factor in the determination of the final programming of neonatal superior cervical ganglion cells, which can either be cholinergic or adrenergic but will become primarily adrenergic in the presence of high CORT levels (Sze, 1980). Low CORT levels also facilitate the creation of myelination by stimulating a glial specific enzyme, glycerol phosphate dehydrogenase (Sapolsky & Meaney, 1986). Artificially restoring CORT to normal adult levels during the SHRP leads to reduced brain weights and lower levels of DNA content in areas of extensive mitosis (such as the cerebellum and dentate gyrus) as well as impaired social behavior and cognitive capabilities. The only procedure found to induce a CORT response is maternal separation (Pihoker et al., 1993).

Various protocols have been carried out that have manipulated the length and number of days of EMS in order to evaluate different aspects of development or adult behavior that are

altered by early life stress. Pups exposed to EMS as compared to early handling (brief [~15 minute]) period of handling while separated from the dam) demonstrate significant differences in reaching certain neurodevelopmental milestones, such as earlier eye opening and delayed development of righting reflexes (Mesquita et al., 2007). Adult rats demonstrate higher levels of fearfulness and anxiety in open field and elevated plus mazes, and appear to mimic animal models of schizophrenia in PPI (Caldji et al., 1998 & 2000; Ellenbroek et al., 1998) and those of depression in the forced swim test (Saenz et al., 2006; see Fumagalli et al., 2007, for review). In any conditioning paradigm where fear or aversive stimuli is involved, learning is drastically altered for EMS adult rats (Fumagalli et al 2007). Adult EMS rats also hyper-secrete CORT in response to stressors, and this response is abnormally extended due to an impaired negative feedback loop caused by reduced glucocorticoid receptor binding in the hippocampus and enhanced expression of CRF mRNA (Meaney et al., 1985).

Tests of nociceptive functioning in EMS rats are less frequent, and mainly focus on assessment of sensory thresholds. Chung et al (2007) found that EMS rats were more sensitive to noxious visceral stimulation and had drastically increased *cFos* expression in the cingulate cortex. Another study failed to detect any differences in baseline thresholds for tail-flick and hotplate measures, but in response to morphine EMS rats showed a higher level of tolerance, more withdrawal symptoms, and less sensitivity to the antinociceptive effects of morphine (Kalinichev et al, 2001a & b). Weaver et al. (2007) further confirmed that EMS rats are less sensitive to the effects of morphine. In addition, autoradiography of mu-opioid receptors in this same group of rats showed that EMS animals had significantly fewer binding sites than handled or non-handled controls. However, differences in morphine sensitivity only reached significance at one (5mg/kg) of four doses given (1, 2, 5, & 10 mg/kg), and the dams in the study were shipped during pregnancy, which may have confounded any effects due to prenatal stress.

Anatomically, early life stress has been correlated with a decrease in the size of the anterior cingulate cortex in humans (Cohen et al 2006), an area of the brain intimately involved

in affective pain processing (LaGraize et al 2001). Whether this anatomical alteration translates to rats exposed to early life stress has not been established.

1.3 Purpose and Hypotheses

Although not fully examined, it appears that maternal separation may induce permanent changes in the processing of nociceptive stimuli. Because little research has focused on the effect of maternal separation on adult pain processing, the purpose of the current study was to examine both sensory and affective pain measures in adult rats following repeated maternal separation in infancy. The first hypothesis was that the maternal separation procedure would alter neurodevelopment in the pups, as shown by earlier eye opening for the EMS pups. Average pup weights were not expected to differ between the treatment groups. The second hypothesis was that maternal separation would lead to increased anxiety in adulthood, as shown by decreased exploratory behavior in the open arms of the elevated plus maze. These tests would confirm that the maternal separation protocol has sufficiently altered HPA axis activity compared to rats that are only briefly handled. The third hypothesis was that although maternal separation rats were expected to demonstrate normal sensory pain thresholds as measured by hot plate latencies and baseline mechanical paw withdrawal thresholds, in the presence of an inflammatory pain condition they were expected to show significantly lower thresholds that may or may not differ from the handled control group. The final hypothesis was that maternal separation would increase the emotional response to nociceptive stimuli and therefore increase affective pain behavior in both the formalin tests and the place escape avoidance paradigm.

CHAPTER 2

METHOD

2.1 Subjects

Sixteen female Sprague-Dawley rat dams were utilized for this study, with an average litter size of 10.69+/-3.88 pups (range 3-19). Eighty-six male pups were obtained from the litters. After weaning, male SD pups were housed in groups of 3-4 littermates and maintained on a 12 hour light/dark cycle (lights on 7:00 a.m.) prior to experimental testing. Approval for the maternal separation protocol and testing procedures was obtained from the University of Texas at Arlington Institutional Animal Care and Use Committee, and all animals were treated in accordance with the guidelines set forth by the International Association for the Study of Pain (Zimmerman, 1983).

2.2 Materials & Procedure

2.2.1 Experimental Procedure

Starting two days after birth (Post-Natal Day 2), early life stress procedures were initiated. Pups were counted, weighed as a group, and ear-notched for identification. Pups from randomly selected mothers began daily maternal separation, where they were isolated from their mother for six hours during the light cycle (starting at 9:00am and ending at 3:00pm) each day until PND 15. Both the dam and the pups were placed in clean cages for the separation period. Litters assigned to the early handling (EH) control group were similarly separated from the dams in clean cages for only 15 minutes at 9:00 am and 3:00 pm each day, to control for any effects of daily handling and movement of the EMS pups. Pups were placed together on clean bedding, with heat lamps and cage thermometers to monitor temperature conditions. Thermometers were routinely checked throughout the day to adjust heat lamps. The ideal body

temperature for pups is around 38 degrees C (100.4 degrees F), and so the cage temperature was maintained between 33 degrees C and 38 degrees C (90-100 F) and pups were kept in groups to conserve heat (Alberts, 1978). Starting on Post-Natal Day 12, all pups were checked daily (9:00 am, following weighing) for eye-opening. Pups with at least one partially open eye were marked as 'slit', while those at least one fully open eye were labeled as 'open'. Slit eyes tended to precede fully opened eyes by about 24 hours in most cases. On Post-Natal Day 21, all pups were weaned, sexed, and separated from the dams. Males were then housed in groups of 3-4 per cage and allowed ad libitum access to food and water, with normal animal husbandry. Experimental testing did not begin until PND 60 and was scheduled so that all procedures would occur between PND 60 and 65.

Animals were randomly assigned to one of three testing groups following assessment of anxiety in the elevated plus maze. For the elevated plus maze, all animals from both groups (EMS, n=44 and EH, n=42) were tested in the elevated plus maze for five minutes. In the second set of experiments, sensory processing was evaluated by measuring sensory thresholds in both normal animals and those with experimentally induced inflammatory conditions. A group of 35 animals (17 EMS and 18 EH) was tested to determine hot plate latencies (a measure of thermal threshold). A separate group of animals was used to evaluate mechanical thresholds in response to inflammatory pain (EMS, n=12; EH, n=13). After baseline measures were completed, the animals were injected with a 1% carrageenan solution in the left plantar hind paw, which induced an acute inflammatory state. Following period during which the inflammation develops and reaches its peak, testing for mechanical hypersensitivity (MPWT) was carried out at 3 hours and 45 minutes post-carrageenan injection. After an animal had completed MPWT testing, they were then evaluated for the processing of pain affect using the place escape/avoidance paradigm. A separate set of animals (EMS, n=10; EH, n=9) was used for the formalin test, based on ethical guidelines and to avoid potential confounding effects of the carrageenan procedure. An overview of the procedures is presented in Figure A.2.

2.2.2 Assessment of Anxiety

The elevated plus maze consisted of a raised plus-shaped platform (50 cm platform, arms 111 cm long by 10 cm wide), with two opposite arms enclosed by solid walls (52 cm tall, 102 cm total height) and the other set of arms with unrestricted edges (see figure A.1). Rats were placed in the center of the maze and their exploratory behavior observed and recorded for five minutes using video equipment and Ethovision tracking software. The amount of time spent in each area of the maze was recorded, as well as the overall mobility of the animal. This was rated by the Ethovision software and categorized as 'non-mobile', 'mobile', or 'strongly mobile', the last of which can be interpreted as hyperactive behavior. This maze was designed to test the level of anxiety experienced by the animal, which is reflected in the amount of time spent out in the open arms. Rats with higher levels of anxiety in this novel environment spent more time in the closed arms, where they instinctively seek the perceived protection of the walls and the darkness, as compared to the exposed areas of the maze. This test was designed to evaluate whether the maternal separation protocol successfully altered the stress responsiveness of the rats, since previous research has reported more anxiety and fearfulness in maternally separated rats as compared to handled controls (Caldji et al., 1998 & 2000).

2.2.3 Assessment of Sensory Thresholds

Two procedures were used to assess sensory pain thresholds, the hot plate test and the assessment of mean paw withdrawal threshold using von Frey monofilament mechanical stimulation. The hot plate test measured the level of sensitivity to a noxious thermal stimulus. The hot plate (Ugo Basile) consisted of a round metal plate surrounded by a cylindrical Plexiglas enclosure. The surface of the hot plate is heated to 54 degrees C (+/-0.5). The animal was placed in the center of the plate, with a timer started when the hind paws made contact with the surface. The animal was observed until it began to respond to the nociceptive stimulation by rapidly flinching and licking the hind paws or jumping towards the top of the enclosure. The

amount of time it took for them to respond was measured in seconds, with a 20 second cutoff value.

Assessment of sensory thresholds during an acute inflammatory condition was carried out at on a separate set of animals. Baseline Mechanical Paw Withdrawal Threshold (MPWT) scores were assessed using eight von Frey monofilaments (3.85, 5.68, 9.74, 18.39, 39.42, 77.3, 135.3, 251.34 mN) in the up-down method (Dixon, 1980) prior to further manipulations to ensure normal functioning in all subjects. This test was conducted by stimulating the center of the rat's hind paws for one second with the lowest testing von Frey monofilament, applying pressure until it bent slightly. This procedure was repeated until a response was made by the rat, in the form of rapid withdrawal of paw, which may have been followed by shaking or licking of the paw. After this first response, the next lowest force monofilament was applied, and if a response occured the next lowest was be tested (and so forth), until the animal failed to respond, at which point the next highest force will be used. This pattern continued until four additional stimuli had been applied following the first response (in other words, until five total responses had been recorded) or the animal failed to respond at the highest force stimulation. Withdrawal thresholds were calculated using the following formula: $\log(X^{th}) = \log(vFr) + kx$, where *vFr* is the force of the last von Frey used, *k* = 0.2593 which is the average interval (in log units) between the von Frey monofilaments, and *x* is the value that depends upon the pattern of withdrawal responses. Three MPWT trials were conducted, and the scores were averaged across trials to determine mean left and right paw values for each animal. After baseline MPWT were been evaluated, subjects were injected with .05 ml 1% carrageenan in normal saline in the left plantar hind paw. Carrageenan is an inflammatory agent that induces swelling, redness, and pain which develops over several hours and peaks between 4 and 5 hours. At 3 hours 45 minutes post-carrageenan injection, a second MPWT measure was taken. This round of testing was followed immediately by place escape avoidance testing, which is described in the next section. Maternally separated rats were expected to show a small decrease in sensory thresholds, if any, compared to

handled controls. There is some evidence that the mechanisms of sensory pain are altered by the EMS procedure (Chung et al., 2007), although some studies report no differences in baseline thresholds before manipulations (Kalinichev et al., 2001 a & b; Weaver et al., 2007).

2.2.4 Assessment of Pain Affect

The Place Escape Avoidance Paradigm (PEAP, LaBuda & Fuchs, 2000) chamber was 60 x 30 x 30 cm Plexiglas with a light and dark side (painted white and black, respectively) placed on an elevated platform with a mesh screen to access the paws during testing. For this paradigm, following post-carrageenan MPWT, the animal was placed in the chamber and stimulated with the highest von Frey monofilament on the plantar surface of its paws every fifteen seconds throughout the 30-minute duration of the test. If the subject was located on the dark side of the chamber, the left paw was stimulated, and if the animal was on the light side of the chamber, the right paw is stimulated. The animal's location in the chamber and the number of crosses were recorded for each fifteen second interval during the course of the thirty-minute testing period and collapsed to form six five-minute time points for analyses. This test was designed to examine pain affect by quantifying escape/avoidance behavior. The animals naturally prefer the dark half of the chamber, due to the perception of safety in the darkness. When presented with this novel environment, normal animals remained almost exclusively on the dark side of the chamber. On the other hand, animals with an experimentally induced pain condition crossed over to the light half more often (as a means of escaping the painful stimulus), eventually remaining on this side for the majority of the time to avoid the aversive stimulation. Testing for mean paw withdrawal thresholds established the level of sensory pain (i.e., the nociceptive stimulus clearly evokes sensory nociceptive responses) and the place escape/avoidance paradigm assessed how bothersome the pain of the stimulation is by observing whether the animal was willing to actively avoid the associated area. Drugs and manipulations that decreased the amount of pain affect (with or without lowering sensory pain) led to a reduction in escape/avoidance behavior. Lesions of the anterior cingulate cortex (ACC),

a brain region critical in processing of the affective component of pain, nearly abolished escape/avoidance behavior without altering sensory thresholds (LaGraize et al., 2001). Morphine administration also decreases the amount of escape/avoidance behavior compared to saline controls (LaBuda & Fuchs, 2000). In the proposed experiment, maternal separation was expected to increase escape/avoidance behavior. Maternally separated animals have demonstrated higher levels of anxiety and fearfulness, as well as stronger responses to novel or aversive stimuli (Caldji et al. 1998 & 2000). Thus, repeated mechanical stimulation of the inflamed paw should provoke a greater response in terms of pain affect as shown by the amount of escape/avoidance behavior.

The second procedure used to measure pain affect was the formalin test. In this procedure, the animal was injected in the left plantar hind paw with 1% formalin (dilute formaldehyde), an inflammatory agent that induces an acute state of inflammatory pain lasting approximately an hour, and placed in the test chamber, consisting of a square opaque Plexiglas enclosure (31 cm by 31 cm by 31 cm) elevated on a Plexiglas platform with mirrors below to observe the animal's hind paws and behavior in the chamber. The state of inflammation induced several behavioral responses to the afflicted paw, including biting, licking, and guarding. This period of inflammatory pain occurred in two phases, the first five to ten minutes (acute phase) and the last forty or so minutes (tonic phase), with a five to ten minute interphase between the two, during which little to no formalin induced behaviors were observed. This initial response phase was primarily due to the activity of Aβ, Aδ, and high threshold C fibers, while the extended tonic phase was due to activation of C fibers and mechanically insensitive afferents (Puig & Sorkin, 1996; Porro et al., 1999). In the formalin test, the number of seconds the animal spent licking, elevating, and resting their left paw on the floor surface was recorded on a computer program as the behaviors were observed. A weighted formalin pain score was calculated based on the following formula: [0(time spent paw down) + 1(time spent elevating paw) + 2(time spent licking paw)]/300 (seconds). Scores were calculated for every 5 minutes,

and then averaged together to determine an overall pain score. In addition to pain scores, the raw scores for each behavioral category (paw down, paw up, paw licking) were also summed for each 5 minute period. Maternally separated animals were expected to show increased formalin pain behaviors, especially during the second phase of the test.

2.3 Statistical Analysis

Elevated plus maze data were evaluated using independent group *t*-tests for the amount of time spent in the open areas and again for the closed areas. Mobility ratings assessed during the elevated plus maze were evaluated using MANOVA with frequency and duration of rating as the dependent variables. Univariate tests were examined for results indicating significant multivariate tests overall. For the hot plate latencies, an independent groups *t*-test was used to evaluate group differences in paw withdrawal latencies. For the MPWT, post-injection scores were compared to baseline measures using repeated measures ANOVA (mixed design, group as between factor and time as within factor). For the PEAP, the percent of time spent in the light and the number of crosses throughout the duration of the test was compared between groups using two repeated measures ANOVA (mixed design, group as between factor and time as within factor). For the formalin test, four measures were evaluated (paw down, paw up, paw licking, and pain score) using repeated measures ANOVA, with the early (acute and interphase) and late (tonic) phases assessed separately. Post-hoc tests (Bonferroni) were used to determine any significant differences for any overall significance found during analysis of variance

CHAPTER 3

RESULTS

3.1 Confirmation of Maternal Separation Efficacy

Average litter weights were assessed to ensure that repeated lengthy separations from the dam did not induce malnutrition in the pups. Two of the assessments were designed to behaviorally evaluate whether or not the maternal separation procedure was an effective stressor for inducing the neurophysiological changes associated with early life stress: eye opening and elevated plus maze measurements.

3.1.1 Average Litter Weights

Pups were weighed daily as a group from postnatal day 2 through 15. Litter weights were divided by the number of pups in the litter to obtain an average weight per pup. These values were utilized for analyses. A repeated measures ANOVA was performed, with early life stress group as the between subjects factor and time (14 time points) as the within subjects factor. There was a significant main effect for time, $F(1,14) = 469.88$, $p<.001$, indicating that the weights of the pups increased steadily over the course of the two-week manipulation period. There was not a significant main effect for group or an interaction (F's < 1, ns), indicating that the maternal separation treatment did not negatively affect the weight of the pups (see figure A.3).

3.1.2 Neurodevelopment: Eye Opening

The number of pups in each litter with fully opened eyes was recorded daily from postnatal day 12 through 17. For each day, the number of additional pups with open eyes was compared between the two treatment groups. Chi-square analyses were performed for each day (except PND12, as both cell values were zero). The number of pups with eyes opening was significantly different between the two groups on PND 13 ($X^2[1] = 4.99$, $p<.05$), PND 14 ($X^2[1] =$

8.74, $p<.005$), and PND 16 ($X^2[1] = 4.26$, $p<.05$), but not PND 15 ($X^2[1] = .28$, *ns*), or PND 17 ($X^2[1] = 1.15$, *ns*). Maternal separation pups opened their eyes sooner than early handling pups by about two days, indicating that the treatment had an effect on developmental milestones for these pups (see Figure A.4).

3.1.3 Assessment of Anxiety

Elevated plus maze trials were recorded and analyzed using Ethovision software. The total duration in the open areas of the maze and the closed areas of the maze (see Figure A.5) were analyzed using multivariate ANOVA. The overall multivariate test was significant, mult. $F(2, 82) = 35.69$, $p<.001$. Univariate tests were examined for the two dependent variables. There was a significant difference between the groups for duration in open areas, $F(1,83) = 19.20$, $p<.001$, while the duration in closed areas did not differ between the two, $F(1,83) = 2.45$, *ns*. Maternal separation rats tended to spend less time in the open areas compared to early handling rats. Measures of mobility within the elevated plus maze were also analyzed. The software rated the behavior of the animal as 'immobile', 'mobile', or 'strongly mobile' throughout the duration of the test. The frequency and duration of these variables were analyzed separately for each rating. Multivariate ANOVA revealed no significant differences between the groups for immobility, mult. $F(2,82) = .60$, *ns*, and mobility, mult. $F(2,82) = .07$, *ns*, but there was a significant overall test for strong mobility, $F(2,82) = 4.17$, $p<.05$. Univariate tests revealed that there were significant differences between the groups in both the duration, $F(1,83) = 6.95$, $p=.01$, and frequency, $F(1,83) = 8.11$, $p<.01$, of strong mobility. Maternal separation rats tended to be rated as strongly mobile more often and for a longer duration than early handling rats (see Figure A.6).

3.2 Assessment of Sensory Thresholds

3.2.1 Thermal Stimulus: Hot Plate Test

An independent samples t-test was performed to determine if the early life manipulations altered sensory thresholds in adulthood as shown by thermal stimulation in the

hot plate test. Latency to lick the rear paw was the dependent variable examined. The results showed that there was no significant differences between early handling (n=18) and maternal separation (n=17) rats in thermal thresholds, $t(33) = -.69$, *ns*. See figure A.7.

3.2.2 Mechanical Stimulus: Mean Paw Withdrawal Thresholds

A repeated measures ANOVA was performed to evaluate whether any differences in mechanical thresholds existed between the two groups before and after injection of the inflammatory agent, carrageenan. There were two time points, baseline and 4 hours post-carrageenan injection, and the between subjects factor was the type of early life experience manipulation, maternal separation (n=12) or early handling (n=14). There was a significant effect of time, $F(1,24) = 2767.78$, $p<.001$, and a significant interaction, $F(1,24) =6.18$, $p<.05$, but not a significant effect for group, $F(1,24) = 1.09$, *ns*. Analyses revealed that while there was a significant decrease in thresholds from baseline to 4 hours post-injection, the thresholds did not significantly differ between the treatment groups at either time point. See Figure A.8.

3.3 Assessment of Pain Affect

3.3.1 Place Escape Avoidance Paradigm

3.3.1.1 Percentage of Time Spent in the Light Side of the Chamber

A repeated measures ANOVA was performed to evaluate whether there were any differences between the two groups in the proportion of time spent in the light half of the chamber during the test. There were no significant main effects (group: $F(1,24) = 1.21$, *ns*; time: $F(5,120) = .30$, *ns*) and no significant interaction, $F(5,120) = .41$, *ns*. Although the maternal separation animals spent more time on average in the light side than the early handling rats, this was a non-significant trend. See Figure A.9.

3.3.1.2 Number of Midline Crosses

A repeated measures ANOVA was performed to evaluate whether the number of midline crosses differed between the two groups or changed over time. There was a significant effect of time, $F(5,120) = 57.36$, $p<.001$, but not a significant main effect for group, $F(1,24) =$

.12, *ns*, or a significant interaction, $F(5,120) = 1.15$, *ns*. The number of crosses significantly decreased during the time course of the test, but did not differ between the treatment groups. The number of crosses at time point one was significantly higher than all other time points, and the second time point was significantly higher than all except the first time point. The last time point was significantly lower than the first four time points. No other differences existed. See Figure A.10.

3.3.2 Formalin Test

Three behavioral activities were recorded for the duration of the 60-minute formalin test (paw down, paw up, and paw licking), and a composite pain score was calculated from these as well. For the analyses, each variable was collapsed into 5-minute time bins, and the acute (0-20min) and tonic (25-45min) phases were analyzed separately, with early life manipulation as the between subjects factor (maternal separation, n=10, and early handling, n=9).

3.3.2.1 Composite Pain Score

Repeated measures ANOVA for the acute phase was performed with four time points (5, 10, 15 and 20 minutes). There was a significant effect for time, $F(3,51) = 57.31$, $p<.001$, but not a significant main effect for group, $F(1,17) = .28$, *ns*, or interaction, $F(3,51) = 1.81$, *ns*. Early life stress did not influence the pain score for the acute phase of the formalin test. For the tonic phase, 5 time points were used (25, 30, 35, 40, and 45 min). There was a significant main effect for group, $F(1,17) = 4.43$, $p=.05$, but not a significant interaction, $F(4,68) = .26$, *ns*, or time effect, $F(4,68) = .75$, *ns*. Maternal separation rats had significantly higher pain scores overall during the tonic phase of the formalin test compared to the early handling rats. See Figure A.11.

3.3.2.2 Time Spent Licking Paw

For the acute phase (5, 10, 15, and 20 min), there was a significant effect of time, $F(3,51) = 35.00$, $p<.001$, but not a significant interaction, $F(3,51) = 1.05$, ns, or main effect for group, $F(1,17) = .15$, *ns*. For the tonic phase (25, 30, 35, 40, and 45 min), there was a significant main effect for group, $F(1,17) = 6.52$, $p<.05$, but not a significant interaction, $F(4,68)$

= 1.93, *ns*, or time effect, *F*(4,68) = .25, *ns*. Maternal separation rats spent significantly more time licking their formalin-injected paw during the tonic phase of the formalin test than early handling rats. See Figure A.12.

3.3.2.3 Time Spent with Paw Up

For the acute phase, there was a significant effect of time, *F*(3,51) = 23.73, *p*<.001, but not a significant interaction, *F*(3,51) = .88, *ns*, or main effect for group, *F*(1,17) = .17, *ns*. For the tonic phase, there were no significant main effects for time, *F*(4,68) = 1.53, *ns*, group, *F*(1,17) = 1.92, *ns*, or the interaction, *F*(4,68) = 3.88, *p*=.07. Early life manipulations did not significantly alter paw elevation in the formalin test. See Figure A.13.

3.3.2.4 Time Spent with Paw Down

For the acute phase, there was a significant effect of time, *F*(3,51) = 52.99, *p*<.001, but not a significant interaction, *F*(3,51) = 1.72, *ns*, or main effect for group, *F*(1,17) = .25, *ns*. For the tonic phase, there were no significant main effects for time, *F*(4,68) = 2.33, *ns*, group, *F*(1,17) = .96, *ns* or the interaction, *F*(4,68) = 1.31, *ns*. See Figure A.14.

CHAPTER 4

CONCLUSIONS

4.1 Confirmation of Maternal Separation Efficacy

4.1.1 Average Litter Weight and Eye Opening

Average litter weights did not significantly differ between the two treatment groups, indicating that the six hours of daily deprivation did not lead to malnutrition or disrupt growth in the maternal separation pups. In contrast, these pups did demonstrate significantly earlier eye opening compared to the handled controls, demonstrating that while the pups were not in danger of starvation, the absence of the dam was stressful enough to alter the normal course of neurological development. These results replicate one of many shown by Mesquita et al., 2007, in an examination of how maternal separation alters the development of developmental milestones, such as eye and ear opening and righting reflexes. These results confirmed that the maternal separation procedure was stressful not due to the loss of nutrients but because of the absence of the tactile stimuli of the dam. Another important factor is maintaining a suitably heated environment in the mother's absence. Not only are pups more vulnerable to hypothermia and death during the neonatal period, but the mother actively monitors and maintains the pups' temperature while in the nest (see Leon et al., 1978). Removal of this thermal regulation without providing adequate cage heating adds another element of stress to the procedure, which also risks significant pup loss.

4.1.2 Assessment of Anxiety

It was predicted that maternal separation would increase overall anxiety levels in adulthood, and this hypothesis was tested utilizing the elevated plus maze. Analysis of the elevated plus maze data revealed that EMS animals spent less time in the open areas of the maze compared to EH animals, indicating a higher level of anxiety in these rats. The time spent

in the closed areas did not differ, however, and this was likely related to the fact that the EMS animals were rated as strongly mobile more frequently and for a longer duration than the EH animals. Any entry into the open areas by the EMS animals was likely quick, darting movements from one closed area to the next, and therefore only brief encounters were made in the open areas. The increased motor activity could also be indicative of higher anxiety levels; while normal rats would explore and eventually habituate to the environment, the EMS rats continued to move in an agitated manner. Rats raised in social isolation demonstrate a similar pattern of anxious and hyperactive behavior in the elevated plus maze (Wright et al., 1991). Our hypothesis was supported by these results, indicating that EMS rats demonstrate higher anxiety levels than EH rats.

4.2 Assessment of Sensory Thresholds

The results revealed no discernable differences between the two groups in the quantified response to thermal and mechanical stimuli at baseline. Thus, our second hypothesis concerning sensory thresholds was supported, although no difference was present for the inflammatory condition as predicted. This is also consistent with published data regarding sensory thresholds (Kalinichev et al., 2001a & b). The only evidence of altered sensory thresholds following maternal separation involves the enhancement of noxious visceral stimulation (Chung et al., 2007). Rats with corticosterone implants in the central amygdaloid nucleus also demonstrate enhanced responses to noxious visceral stimulation, as well as decrease mechanical thresholds under normal conditions (Myers, et al., 2007). This may explain the lack of sensory threshold differences shown in adult rats following EMS. The animals may need to be stressed prior to sensory measures in order to demonstrate altered thresholds. One of the various restraint or social isolation procedures may have elicited the appropriate neurophysiological condition for significant differences to appear—EMS animals possess an enhanced endocrine response to acute stress, releasing copious amounts of

glucocorticoids coupled with a diminished negative feedback system in the hippocampus (Meaney et al., 1985).

A second possibility is that these behavioral responses are merely spinal reflexes in response to noxious stimulation, and that maternal separation only enhances the emotional response to noxious stimuli. It has been argued that the withdrawal reflex is merely a passive behavioral response that does not evoke emotional pathways, but evidence shows that the responses to different modalities of sensory stimulation are differentially under descending inhibitory or facilitatory control—severing the spinal cord enhanced the thermal withdrawal response while substantially reducing mechanical and cold-induced withdrawal reflexes (Kauppila et al., 1998).

4.3 Assessment of Pain Affect

Findings regarding the final hypothesis were mixed, with supportive evidence from the formalin test but not the place escape avoidance paradigm. In the formalin test, EMS animals exhibited more paw licking and had higher pain scores during the tonic phase of the test than the EH animals. These measures both indicate a higher level of affective pain in response to this inescapable chemical stimulus. In the place escape avoidance paradigm, EMS animals showed a non-significant tendency to spend more time on the light side of the chamber.

Alterations in the behavioral response to the tonic phase of the formalin test are associated with the affective or emotional component of pain. Manipulations within the limbic system have a dramatic effect upon formalin-evoked behaviors. Lesions to the anterior cingulate cortex, an area vital to the processing of the emotional quality of noxious stimuli, significantly decrease the behavioral response to formalin (Donahue et al., 2001), and to the noxious stimuli in the place escape avoidance paradigm (LaGraize et al., 2004). These lesions, like the maternal separation procedures, alter pain affect without interfering with sensory processing, leaving thresholds intact while reducing the unpleasantness of the stimulus, which is the opposite of what was demonstrated in this experiment. Although the effect of EMS is not

as robust as direct lesions or chemical blocks of the ACC, it is clear that early life stress influences the limbic system of subjects. Maternal separation led to increased *cFos* expression in the cingulate cortex in adult rats (Chung et al., 2007), and anatomical evidence from MRI research in humans has shown that individuals with a history of more traumatic events in early life have smaller anterior cingulate cortices and caudate nuclei (Cohen et al., 2006).

4.4 General Discussion

The influence of early life stress on the development of the mammalian brain is not entirely clear, especially in humans. Although its impact upon the endocrine system, most notably the HPA axis, is well established, there are still many issues that need to be addressed in the brain. Alterations in growth or neurochemical pathways during infancy and early childhood could theoretically be reversed throughout the remainder of childhood through environmental enrichment, adequate nurturing, personality type, and other factors. Environmental enrichment reversed the negative impact of EMS on HPA axis reactivity in rats (Francis et al., 2002). The length of the separation, quality of maternal care, environmental conditions (such as temperature and social versus isolated separation), and even the day of the separation can all result in vastly different outcomes in adulthood (Holmes et al., 2005; Matsumoto et al., 2006; Coutellier et al., 2008; Macri et al., 2008; Leon et al., 1978). Sex differences emerge in both the behavioral and physiological responses to stress in adult EMS rats, and although some sources report more behavioral reactivity in females (Korsten et al., 2006; Renard et al., 2007), others report the opposite (Kalinichev et al., 2001 a & b).

Such an array of factors that can be altered can create uncertainty in the interpretation of results from a single study. A comprehensive design that systematically varies the length of the separation (15 min through 24 hours), number of days (a single 24 period through 21 days or more), and the type of maternal behavior (attentive versus detached) may be necessary to elucidate the exact impact of early life stress on pain processing. The protocol chosen for the current study was based on the repeated maternal separation procedure as opposed to a single

24-hour separation. The former is more frequently employed and has produced positive results in related fields, including pain research (Kalinichev et al., 2001 a & b; Chung et al., 2007), fear conditioning and anxiety (Korsten et al., 2006; Pryce et al., 2001), and development (Mesquita et al., 2007). Six hours per day of separation was the maximum employed from these studies, and thus the protocol used was modeled after this method (Mesquita et al., 2007). This length of time was sufficient to produce significant alterations in the development of infant rats, indicating that maternal separation had a substantial neurological effect. Additional pain assays may be necessary to determine precisely what mechanisms are responsible for the changes in pain-related behavior present in adult rats. Further, human studies should examine whether early life trauma corresponds with elevated basal cortisol levels as well as altered pain processing in adulthood, which could be clinically relevant for the future treatment of chronic pain conditions.

APPENDIX A

ILLUSTRATIONS

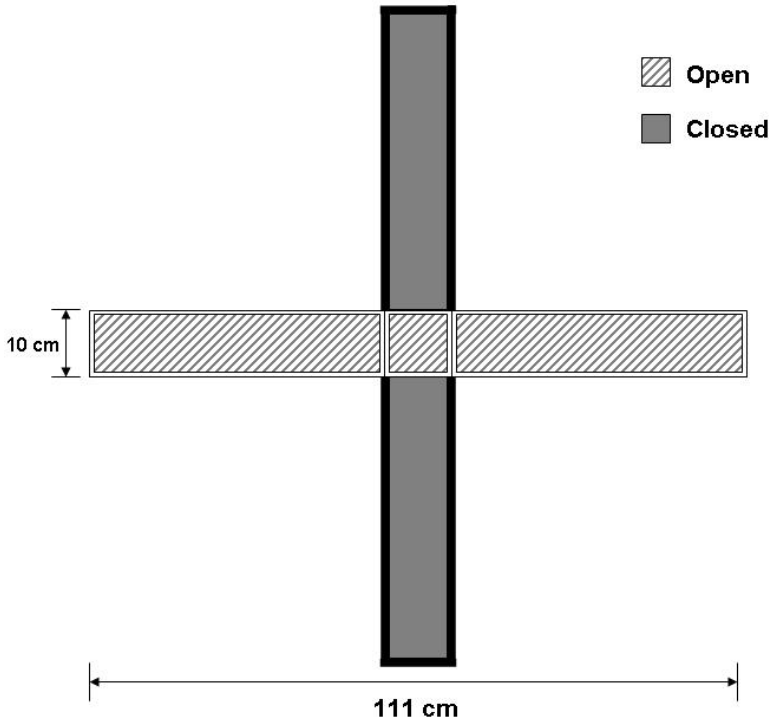

Figure A.1 Elevated Plus Maze Bird's Eye View Diagram

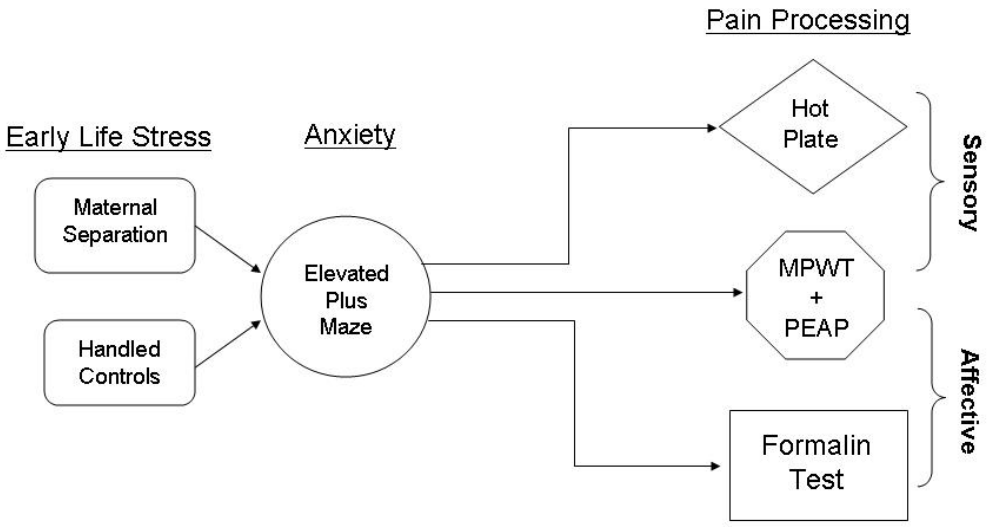

Figure A.2. Overview of Procedures

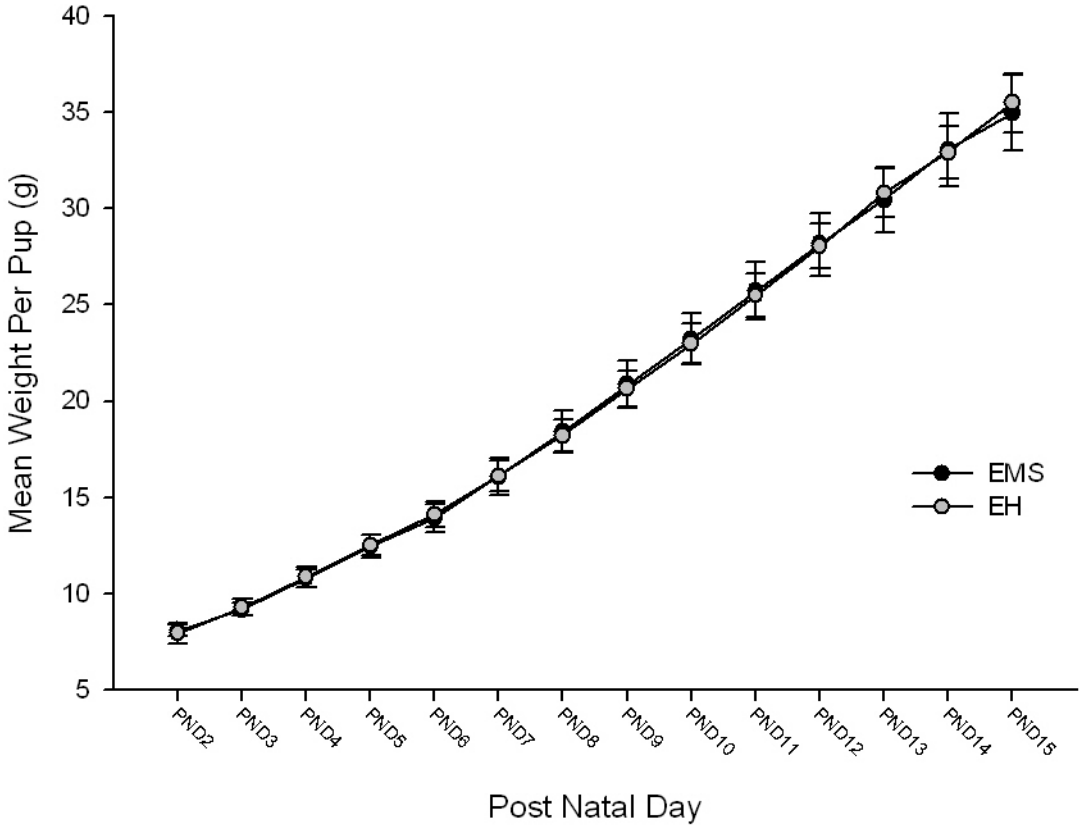

Figure A.3 Average Litter Weight by Post-Natal Day

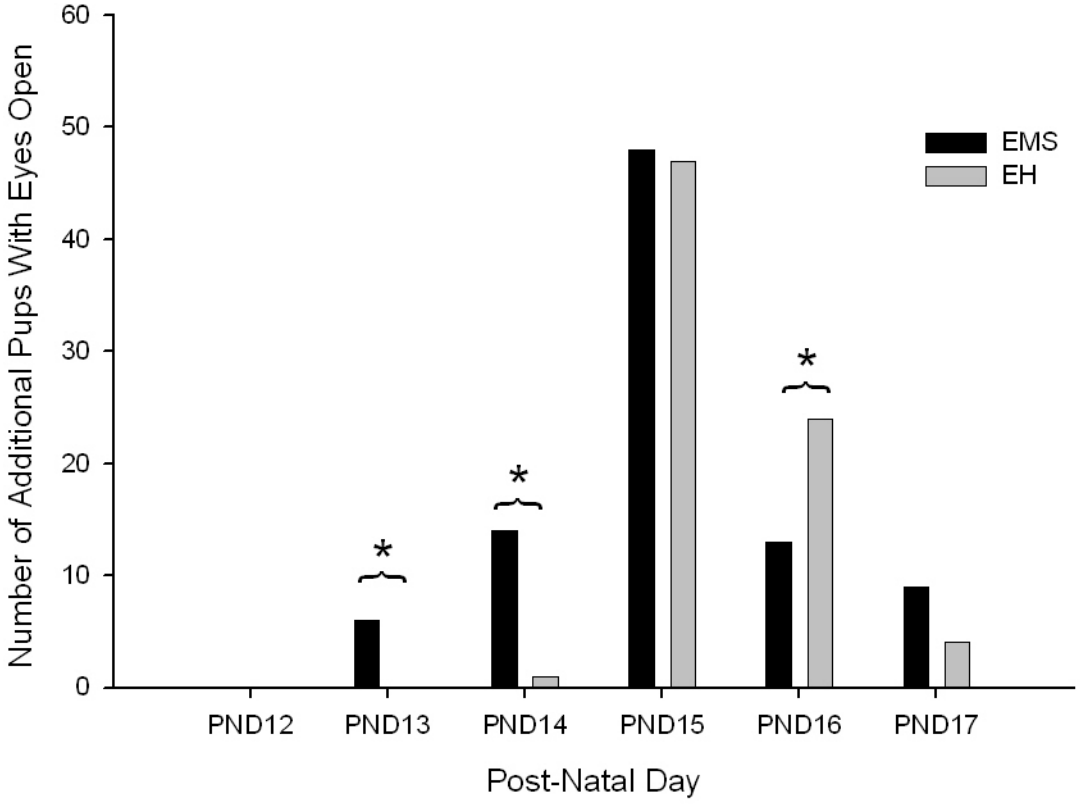

Figure A.4 Number of Additional Pups with Eyes Open by Post-Natal Day. Maternal separation animals had significantly more pups open their eyes on PND 13 and 14, and significantly fewer pups open their eyes on PND16. Overall, EMS pups tended to open their eyes two days earlier than the EH pups.

***** *p<.05*

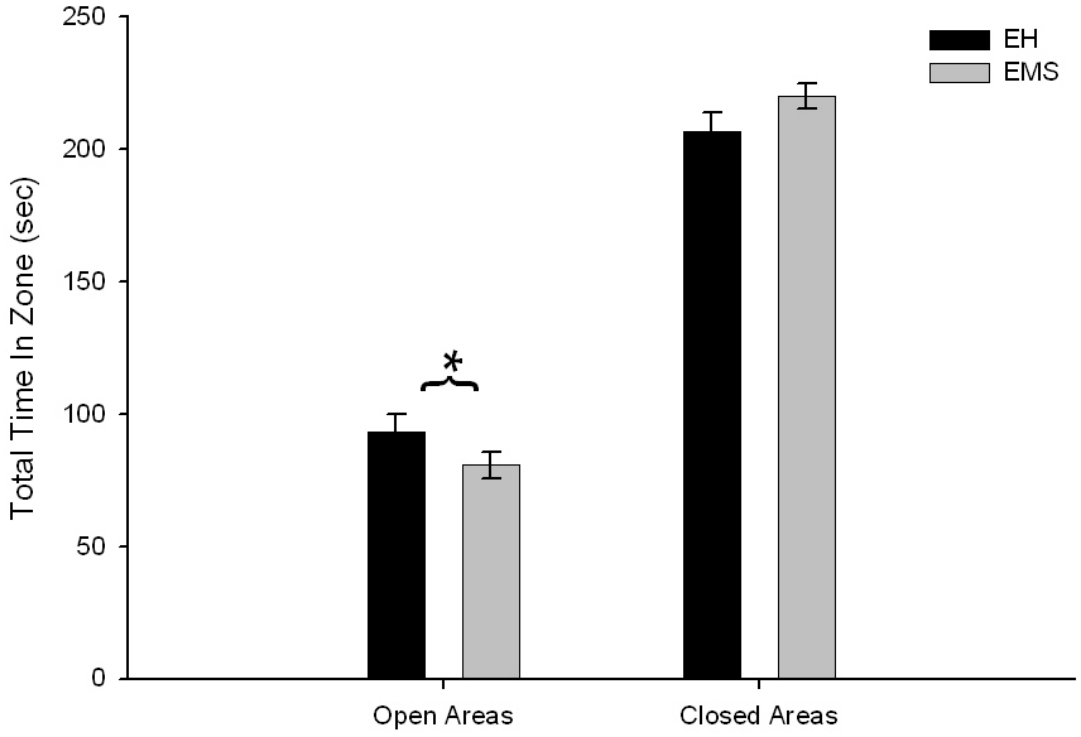

Figure A.5 Total Duration in Open vs. Closed Areas of Elevated Plus Maze. Maternal separation rats spent significantly less time in the open areas of the maze than the early handled rats, but the two groups did not differ in time spent in the closed areas of the maze.

***** *p<.001*

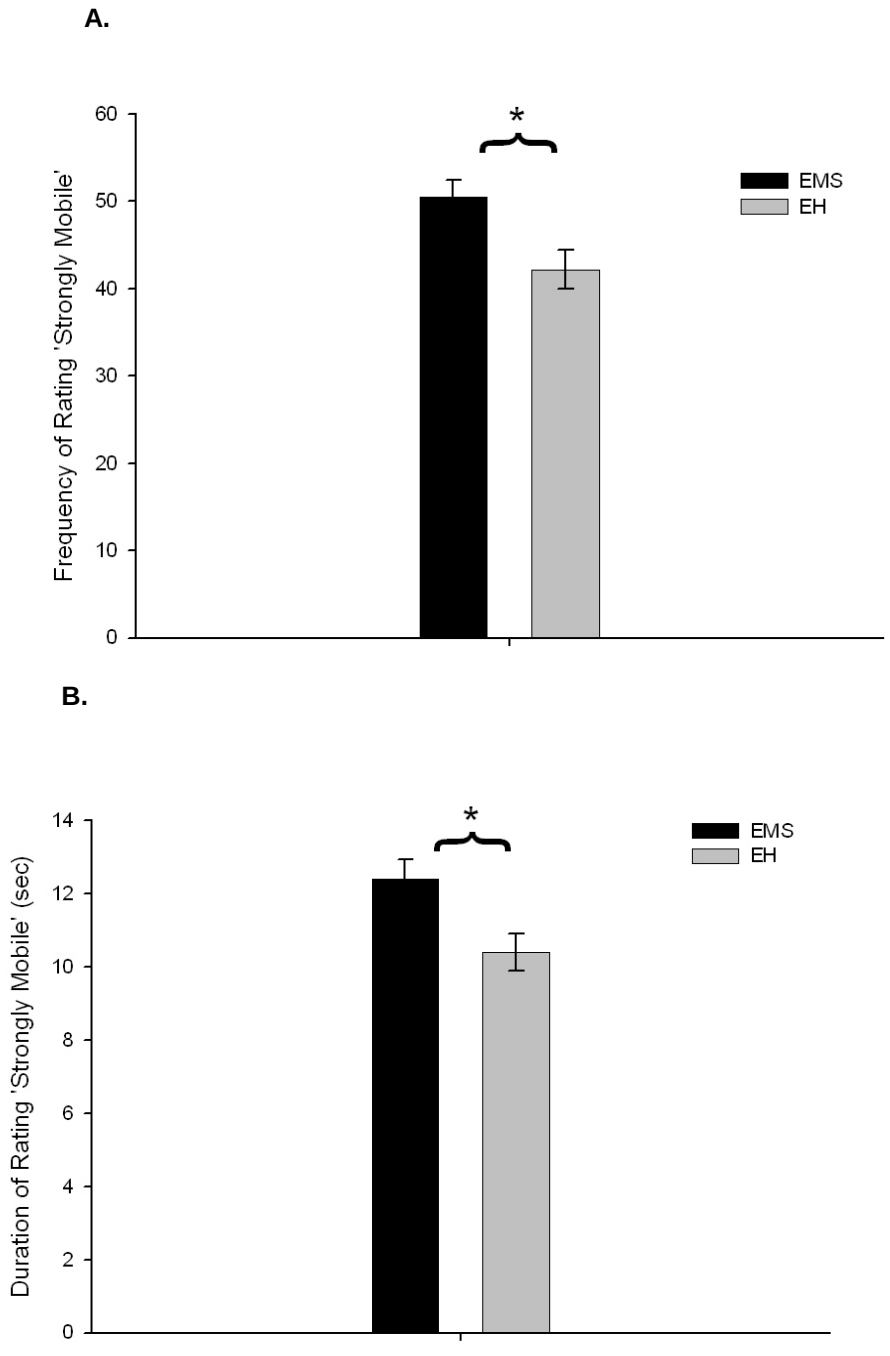

Figure A.6 Frequency and Duration of 'Strongly Mobile' Rating in Elevated Plus Maze (±SEM). **A.**) Maternal separation animals tended to be rated 'Strongly Mobile' more frequently than early handled animals, *$p<.01$, and **B.**) they were rated 'Strongly Mobile' for longer duration, *$p=.01$.

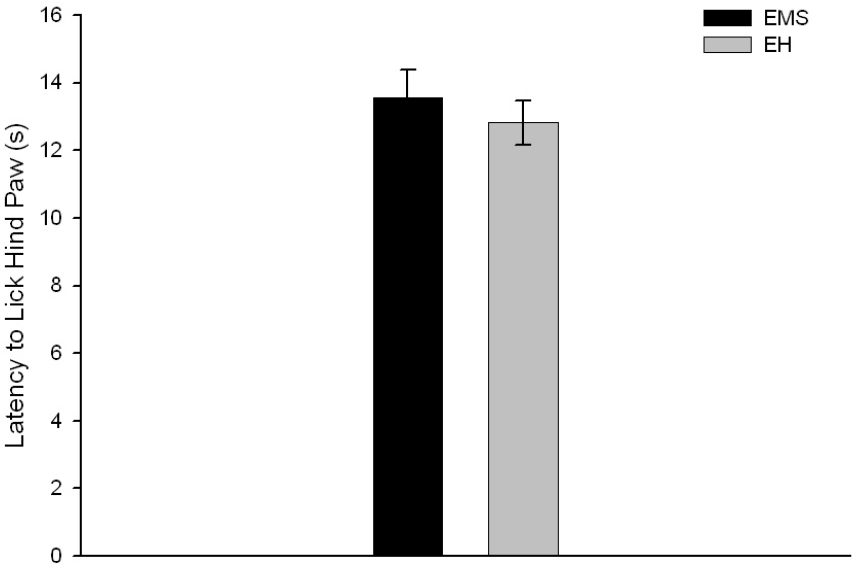

Figure A.7 Latency to Lick Hind Paw in Hot Plate Test (±SEM).

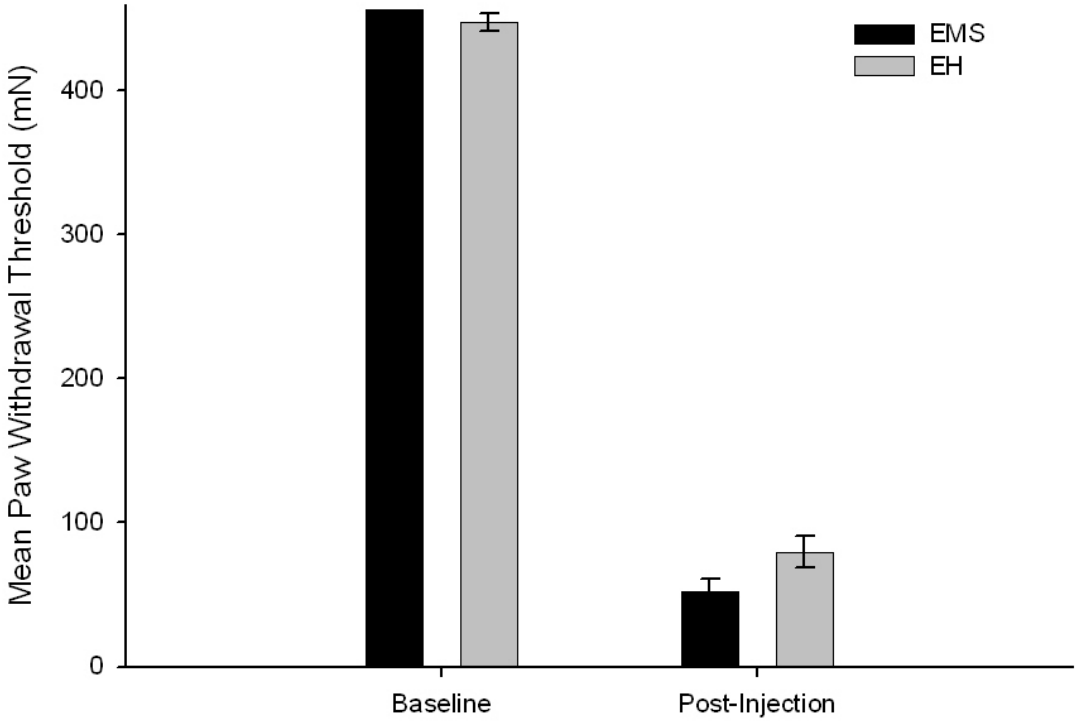

Figure A.8 Mean Paw Withdrawal Threshold Values at Baseline and Four Hours Post-Carrageenan Injection (±SEM). There were no differences between maternal separation animals and early handling animals in mechanical thresholds, but the means were significantly lower than baseline values for both groups four hours following the carrageenan injection.

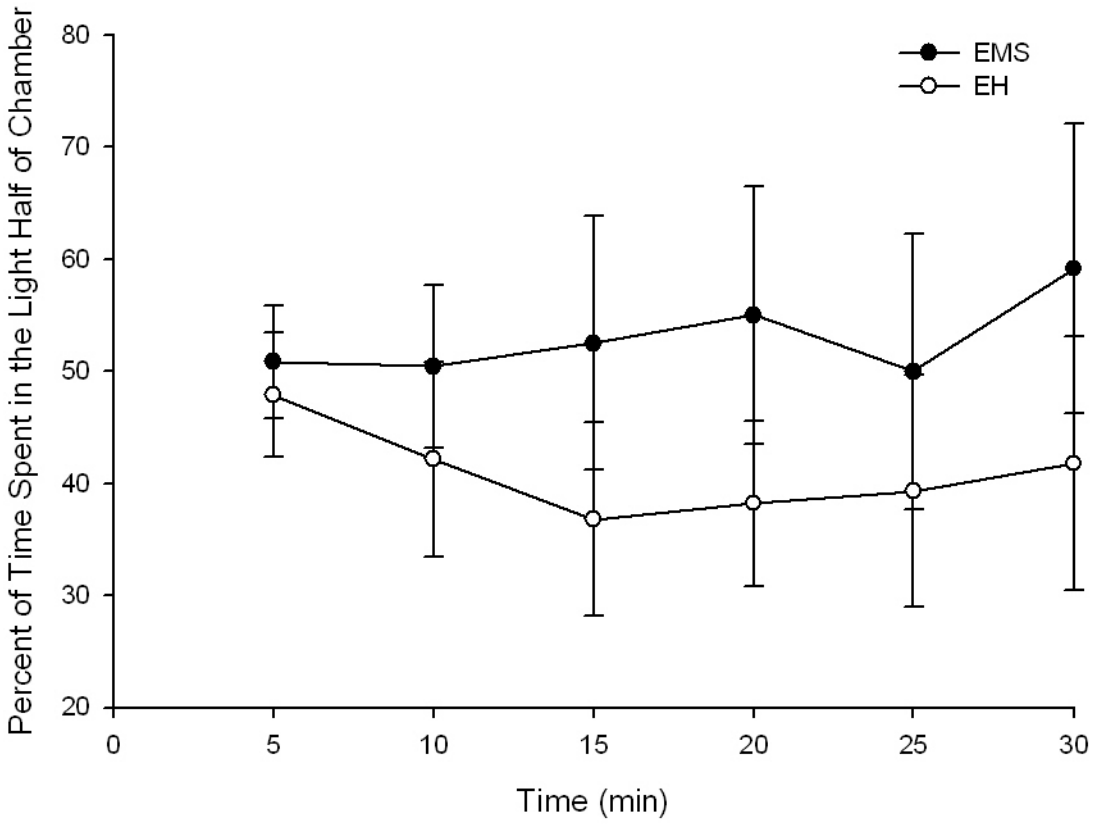

Figure A.9 Percentage of Time Spent in the Light Side of the PEAP Chamber (±SEM). Maternal separation animals tended to spent more time on the light side of the chamber, but this trend was not significant.

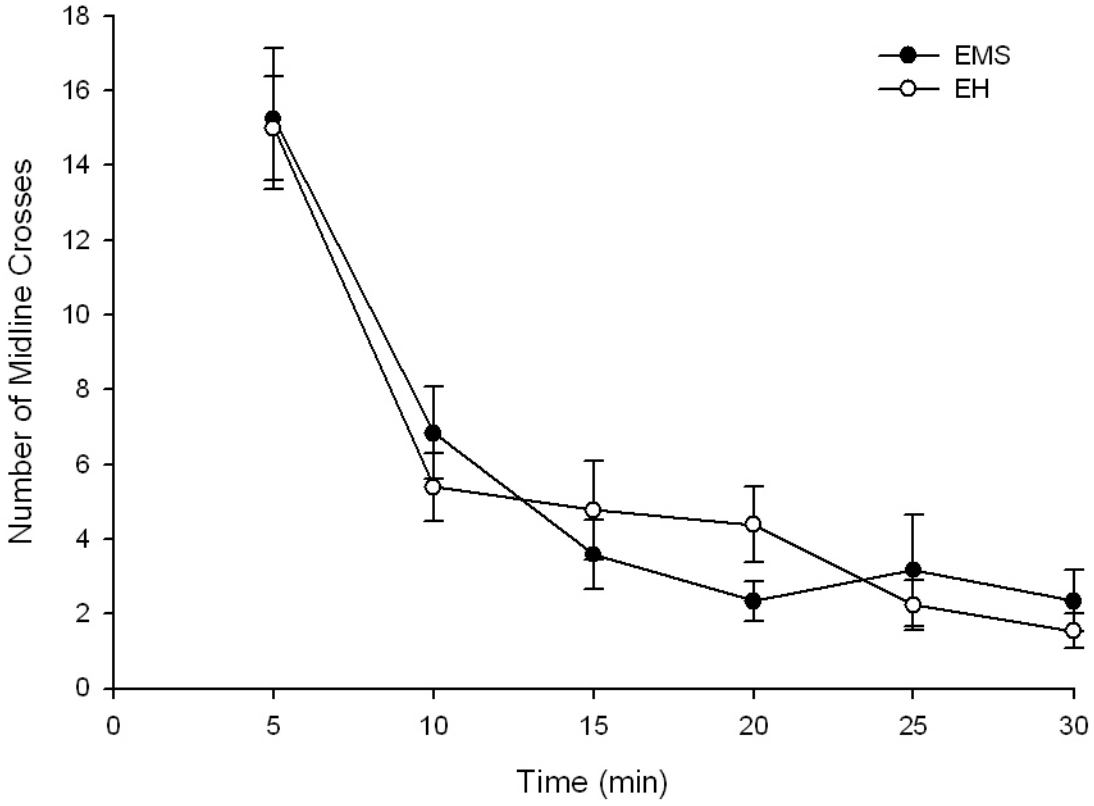

Figure A.10 Number of Midline Crosses in the PEAP Chamber (±SEM).

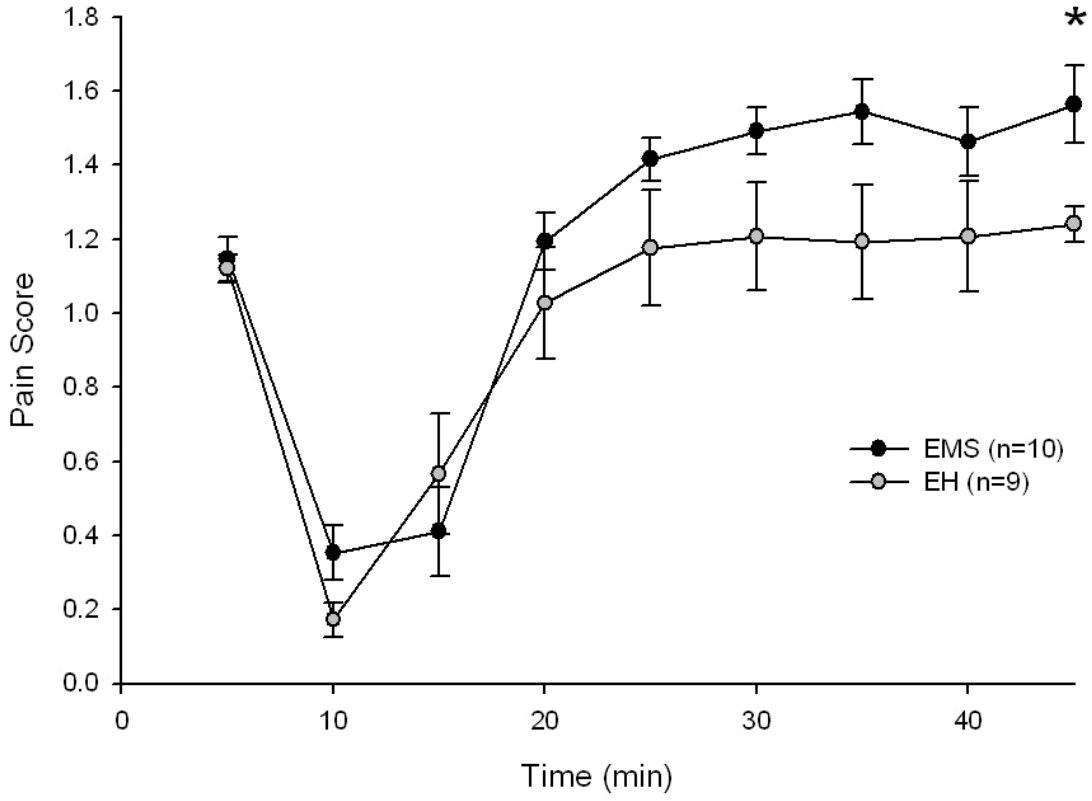

Figure A.11 Formalin Test: Composite Pain Score (±SEM). Maternal separation animals demonstrated significantly higher pain scores during the tonic phase of the formalin test compared to early handled animals. * *p<.05*

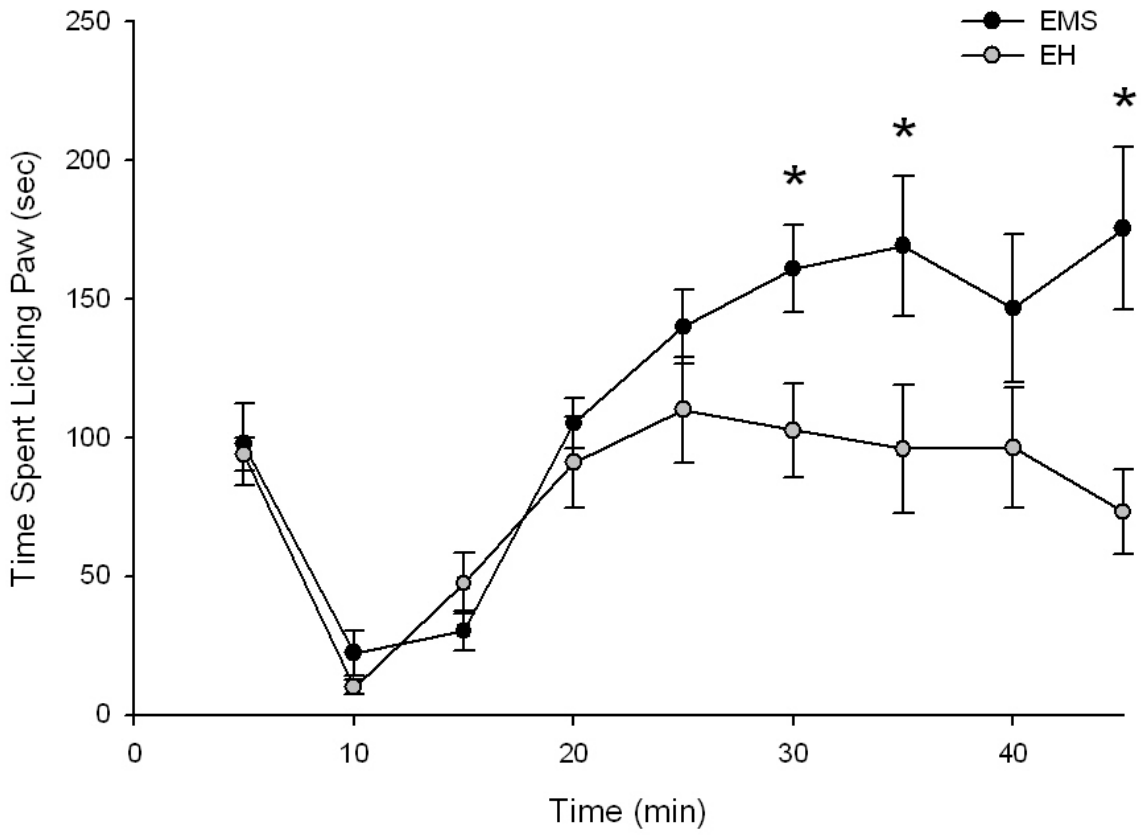

Figure A.12 Formalin Test: Time Spent Licking Paw (±SEM). Maternal separation animals spent significantly more time licking the injected paw during the tonic phase of the formalin test compared to early handled animals. * *p<.05*

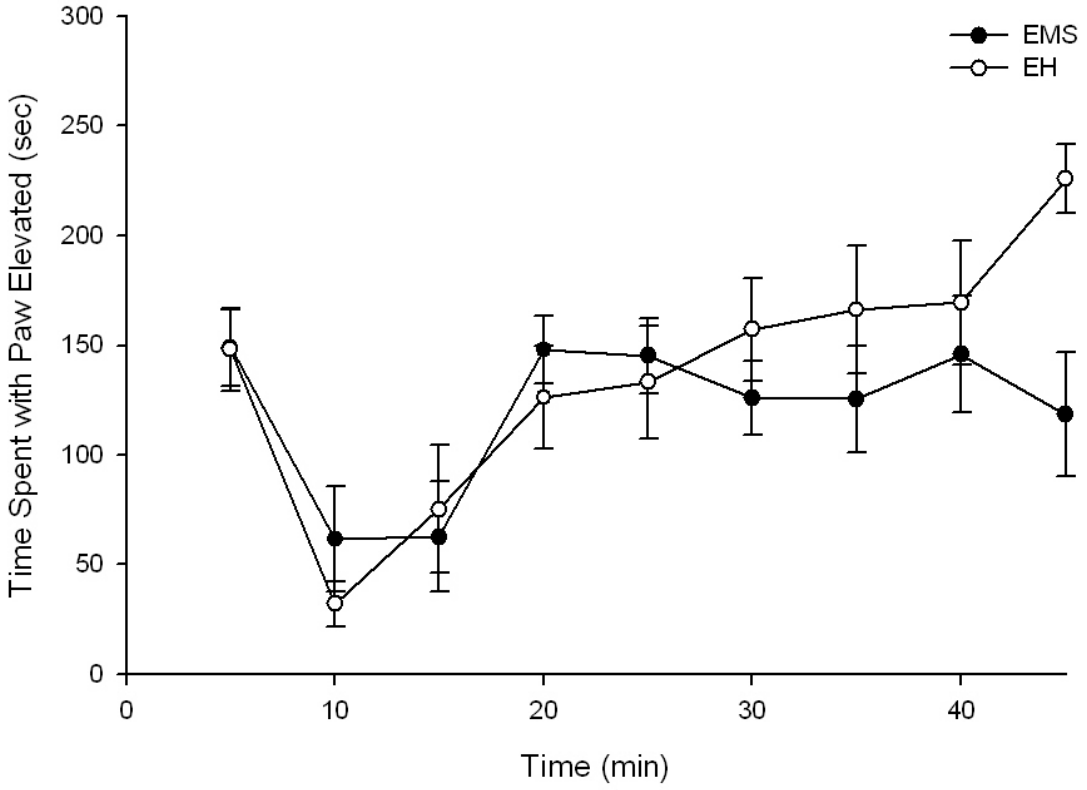

Figure A.13 Formalin Test: Time Spent with Paw Up. There were no significant differences between the two groups in time spent with the injected paw elevated.

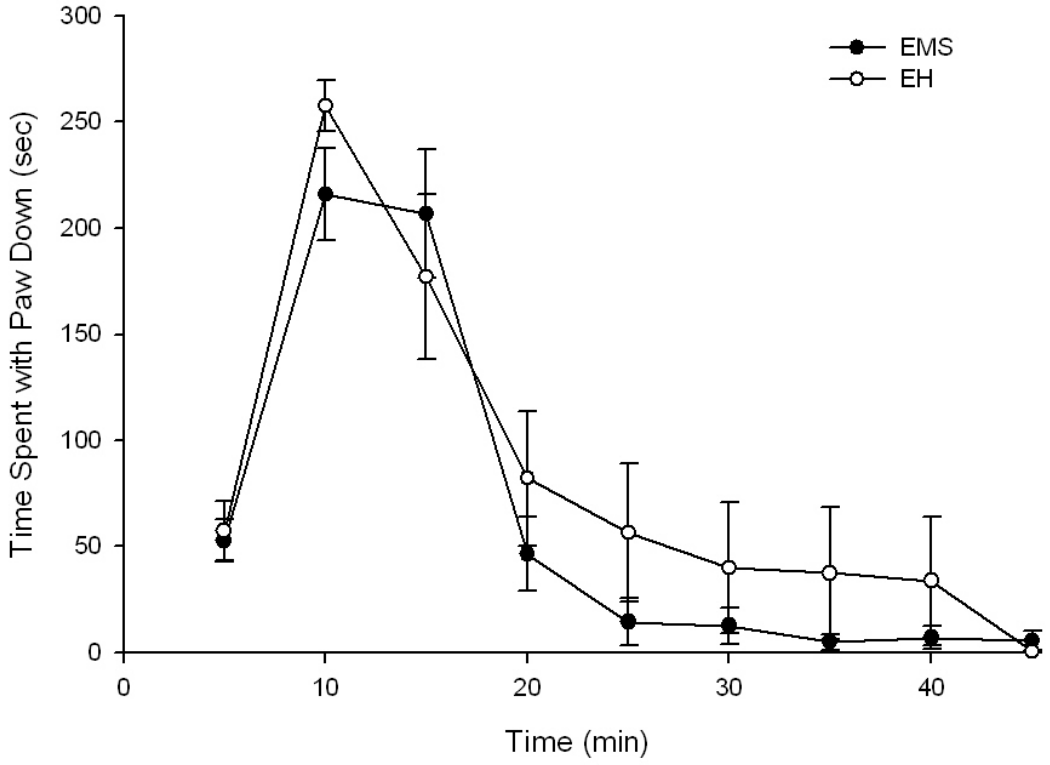

Figure A.14 Formalin Test: Time Spent with Paw Down. There were no significant differences between the two groups in time spent with the injected paw down.

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BIOGRAPHICAL INFORMATION

Megan L. Uhelski was born in Lansing, MI on April 26, 1984. She completed her undergraduate education at Baylor University in Waco, TX, earning a Bachelor of Science degree in Psychology in 2006. She is currently working toward a doctoral degree in Health Psychology at the University of Texas at Arlington under the mentorship of Dr. Perry N. Fuchs. Her research focuses on behavioral analysis of various acute and chronic pain states and includes work with pharmacology and developmental aspects of pain processing. She is interested in studying the relationship between stress and pain mechanisms, as well as novel pharmacotherapy for chronic pain disorders.