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Angiotensin-II Receptor Blocker and Its Effects on Depressive-Like Behavior During Maternal Separation

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ANGIOTENSIN-II RECEPTOR BLOCKER AND ITS EFFECTS ON DEPRESSIVE-LIKE BEHAVIOR DURING MATERNAL SEPARATION

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Evan David Xanthos ENTITLED Angiotensin receptor blocker and its effects on depressive-like behavior during maternal separation. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science of Anatomy.

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Abstract

Xanthos, Evan D. M.S. Anatomy Program, Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2015. Angiotensin-II receptor blocker and its effects on depressive-like behavior during maternal separation.

Maternal separation has been shown to promote the onset of adult depression. This early life stressor produces a biphasic response marked by an active "protest" phase followed by a passive "despair" phase in humans and other species. In infant guinea pigs, active phase behaviors include increased locomotion and distress vocalizations, whereas the passive phase is marked by depressive-like behaviors including a crouch stance, eye closure and piloerection. Separation elicits physiological responses including increased core body temperature and proinflammatory cytokine expression suggesting fever. Angiotensin-II receptor blockers (ARB's) inhibit inflammation both peripherally and centrally following peripheral administration. The goal of the present study is to determine the effects the ARB blocker Losartan (Cozaar) on passive behavior and sensitization in the guinea pig. Subjects were separated into four groups: Losartan (LSEP), Vehicle (VSEP), Separated (SEP) and Undisturbed (UND). Peripheral administration was provided to LSEP and VSEP groups before the first test. Although the results of this study provided evidence of sensitization in guinea pig pups, Losartan did not decrease depressive like behavior or sensitization upon repeated maternal separations

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Introduction

Attachment Theory In Humans

Attachment is an affectional tie between two individuals, typically a mother and infant, that remains over time and in spite of temporary separations (Ainsworth and Bell, 1970). Common attachment behaviors displayed by infants include maintenance of proximity to the attachment figure (ensuring the possibility of nourishment and protection), use of the attachment figure as a "safe base" from which to explore the environment, and distress upon separation from the attachment figure. During brief separation, behaviors such as crying or other vocalizations function to bring the mother back to the infant and thus re-establish contact. After reuniting with the mother following the period of separation, distress behaviors are diminished and the infant's behavior returns to normal.

While most maternal separations are brief in duration, longer-term or frequent separations may have profound consequences on infant behavior and well-being. Rene Spitz (Spitz, 1946) examined the chronic effects of maternal deprivation in human infants. Spitz worked with children who had been subjected to prolonged hospital stays at an early age. Because of isolation that was intended to maintain sanitary conditions and reduce the spread of disease, these infants experienced interruptions in contact with their mothers for an extended period of time (e.g., months). He documented that over time many of the children exhibited a

series of behavioral and emotional changes that he termed “anaclitic depression” due to maternal deprivation. These effects included reduced interest in socializing, profound sadness, lack of motivation and extended periods of immobility.

While Spitz’s work with hospitalized children documented a pathological endpoint to prolonged maternal deprivation in human children, John Bowlby examined the process by which such phenomena occur. He outlined three phases of the separation response (Bowlby, 1952): protest (related to separation anxiety), despair (related to grief and mourning) and denial attachment (presumably reflecting a Freudian defense mechanism). In the “protest” phase, which is the first to occur, the infant exhibits distress vocalizations that are paired with increased motor activity. The function of these behavioral changes is believed to be attempts by the infant to reunite with its mother. The second phase “despair” is a stage of passive behavior that corresponds largely to Spitz’s anaclitic depression. The stage is characterized by decreased vocalizations and motor activity; infants instead may lie down and assume a hunched posture (lying in fetal position) or engage in various soothing behaviors (self-clasping or rhythmic rocking). The third and final phase, “denial attachment,” was thought to be a defense mechanism acquired by the infant against their mother. Some defense behaviors include not acknowledging the mother when she is in the room and no longer crying when the mother leaves the room. Because of Bowlby’s work on early human separation, several investigators in

the decades that followed researched attachment in the laboratory with nonhuman primates in order to more precisely understand attachment processes.

Attachment in Non-Human Primates

While Bowlby's work was limited by the nature of human subjects research, others examined the behavioral and physiological sequelae of maternal separation in non-human primates. Kaufman and Rosenblum (1967) described an initial phase of separation in infant macaques monkeys that consisted of increased locomotion, which was complemented by intense vocalizations (e.g., cooing or shrieking). They suggested this initial (distress) phase was aimed at restoring contact with the infant's mother. Within several hours to days following the distress phase, the monkeys spiraled into "despair". During despair the monkeys' behavior became quiet, with a hunched posture and withdrawal from social interaction. Infants were difficult to rouse and socially withdrawn. Observers related this appearance to being physically ill (Kaufman and Rosenblum, 1967; Mineka and Suomi, 1976). Thus, the behavioral effects of maternal separation in non-human primates were similar to what was previously described in human children, though in monkeys, the third stage did not occur.

Indeed, prolonged maternal deprivation produced a syndrome of a depressed and socially withdrawn infant. While the examination of the effects of

maternal separation in non-human primates provides a good model for human behavior, such experiments are costly to pursue and ethically challenging. Many common laboratory rodents, however, do not exhibit similar responses to maternal separation, making them unsuitable as a substitute for primates.

Attachment and Separation Responses in Guinea Pigs

Infant guinea pigs display a strong maternal attachment similar to that of nonhuman primates and unlike that of other laboratory rodents such as rats and mice (Hennessy, 2003). Another the similarity to nonhuman primates is the active/passive, two-stage response to maternal separation, which is exhibited more rapidly after separation (within hours as opposed to days) than in the macaque monkeys. Following separation and placement in a novel environment, infant guinea pigs exhibit elevations in circulating levels of the stress hormone cortisol (Hennessy, Long, Nigh, Williams and Nolan, 1995), increased distress vocalizations (Berryman, 1976; Hennessy, Long, Nigh, Williams and Nolan, 1995), and increased locomotor activity (Hennessy, Long, Nigh, Williams and Nolan, 1995). After about an hour, vocalizations and locomotor activity subside, and the animal transitions to a "passive" phase characterized by extensive piloerection, prolonged crouch (a hunched-back posture in which all four feet are drawn under the body) and prolonged eye-closure (Hennessy et. al., 1995.)

Behaviors during the passive phase in infant guinea pigs are similar to behaviors exhibited during illness, echoing the comparison of an appearance of physical illness in monkeys (Kaufman and Rosenblum, 1967) and children (Spitz, 1946). Physical illness is accompanied by activation of the acute phase response (Dantzer et. al., 2008; Hart et. al., 1988), which includes the release of proinflammatory cytokines that, in turn, induce changes in immune and endocrine parameters as well as passive behavioral responses. Interestingly, many of these same changes occur during stress (Maier and Watkins, 2000). Psychological stressors such as fear, uncertainty, and lack of control over the environment could potentially trigger many of these systemic changes to occur, which would ultimately result in a physically ill appearance.

Lipopolysacchride (LPS), an active inducer of the acute phase response, triggers increases in crouch, piloerection and eye-closure in separated guinea pig pups mimicking the behavioral responses seen during separation (Hennessy et al., 2004). Separation also elicits physiological responses of the acute phase response: increased proinflammatory cytokine expression and increased core temperature, suggesting fever (Hennessy et al., 2007b; Schneider et al., 2012). Additionally, passive behaviors induced by maternal separation, or triggered by proinflammatory drugs, can be blocked by a variety of anti-inflammatory compounds. To date, three known anti-inflammatory agents, alpha-melanocyte stimulating hormone (alpha-MSH)

(Schiml-Webb, et.al., 2006), indomethacin, a cyclooxygenase-2 inhibitor (Hennessy et.al., 2007a), and interleukin-10 (IL-10) (Perkeybile et al., 2009) have been shown to reduce the levels of passive behavioral responses during a 3-hr separation in infant guinea pigs. Because alpha-MSH did not reduce HPA activity, it is suggested that these drugs worked to block the proinflammatory consequences of stress rather than buffer the psychological stressor of maternal separation. These findings strongly indicate that proinflammatory processes are a mediator of the behavioral responses to maternal separation guinea pigs.

Role of Sensitization

Psychological reactions induced by stressors have been associated with various forms of psychopathology. Episodes of adult depression have been observed to coincide with, or follow, periods of severe, prolonged stress (Bonde, 2008; Caspi et al., 2003). Stressors early in life, such as separation from the maternal figure (Robertson & Bowlby, 1952), extended hospital stays (Spitz, 1946), or loss of parents (Spitz, 1946) have been correlated with increased susceptibility for acquiring depressive disorders at a later age (Agid et al., 1999; Bernet & Stein, 1999). These lasting effects have been hypothesized to involve a sensitization process in which the early stress (e.g., traumatic childhood events) increases the responsiveness to later challenges in life (e.g., induces depressive symptomology

after significant stressors); it is hypothesized that these early events alter neural or physiological set points to make the systems respond in an exaggerated fashion at a later point in time. One possibility is that this process involves a sensitization of proinflammatory cytokines. Cytokines are known to induce depressive reactions (Dantzer et. al., 2008). Proinflammatory cytokines can cause a reduction in serotonin synthesis and increase in hypothalamic-pituitary-adrenal activity, both of which are potential mediators of depression (Miura et al., 2008).

The passive behavioral response in guinea pigs sensitizes with repeated separations. Pups increased passive behavior during a second 3-hour separation when compared to the first 3-hour separation 24 hrs earlier (Hennessy et al., 2011). Central infusion of interleukin-10 (IL-10; a cytokine with anti-inflammatory properties) before an initial separation blocked the increase in passive behavior during separation the following day (Hennessy et al., 2011). These results suggest that proinflammatory activity not only mediates the initial response to separation, but also may contribute to the sensitization of the behavioral response to later separations. In another study, behavior and core temperature of guinea pig pups of different ages were examined during separations on two consecutive days, and then again 10 days after the first separation. Enhancement of passive behavior with repeated separations occurred across a 20-day age range and persisted for at least 10 days following the initial separation (Schneider et. al., 2012). Behavioral effects of repeated

separation were accompanied by corresponding changes in thermogenic response. Core temperature showed a more rapid elevation and subsequent decline during separations up to 10 days following the first separation experience (Hennessy et al., 2010; Schneider et al. 2012; Yusko et. al. 2011). That is, core body temperature also showed sensitization, with greater and faster elevations during later separations.

The involvement of proinflammatory factors in sensitization suggests that suppression or reduction of the proinflammatory response during early life stress, such as maternal separation, might be therapeutic in reducing the risk of adult-onset depression. Unfortunately, centrally acting anti-inflammatory agents like those we have used in our guinea pig model often must be delivered directly to the brain; this is not feasible with humans. Recently, interest has grown in the possibility of modulating aspects of the proinflammatory stress response via angiotensin II receptor blockers (ARB's) following the finding that peripherally administered drugs aimed at blocking the angiotensin-II cascade are capable of inhibiting inflammation both peripherally and centrally (Saavedra et. al., 2011). ARBs are both easily accessible and widely used for various medical treatments. They are extremely safe pharmacological substances with few side effects. A peripherally administered ARB might therefore be quite useful in reducing the long-term risks associated with childhood traumas or stressors.

The Role of ARBs

As one of the final products produced by the renin-angiotensin system, angiotensin II plays a vital role in regulating fluid balance and blood pressure. Renin secretion from the kidneys can be triggered following activation of baroreceptors in the heart by a drop in arterial blood pressure, which can be associated with a loss of blood volume (due, for instance, to hemorrhage or dehydration). Renin reacts with angiotensinogen, which is constantly circulating in blood, to form angiotensin I. Angiotensin I combines with angiotensin converting enzyme (ACE) to produce the most biologically active form of the angiotensin group, angiotensin II. The reason this hormone is categorized as the most active of the group is a direct result of its prohypertensive and water conserving effects. Angiotensin II immediately causes vasoconstriction, which directly increases blood pressure. Vasoconstriction is made possible through decreasing the release of bradykinin (which is typically associated with vasodilatation).

Angiotensin II also signals the posterior pituitary (neurohypophysis) to release arginine vasopressin (AVP). Vasopressin, also known as antidiuretic hormone (ADH), provides additional support to vasoconstriction and maintenance of blood pressure in spite of lost fluid volume. AVP increases water absorption at the collecting ducts of the nephron. As a result, decreased urine flow to the bladder occurs, resulting in diminished urinary output. In addition, aldosterone is released

from the zona glomerulosa of the adrenal cortex and causes the kidneys to increase reabsorption of sodium ions and water, promoting osmotic physiology in the cellular system.

The development of ARBs has been aimed at decreasing hypertension and salt-retention, but recently the use of ARBs as general anti-inflammatory agents has grown, especially when considering their ability to be administered peripherally but to act centrally when administered peripherally. ACE inhibitors, which prevent the conversion of angiotensin I to angiotensin II in body tissues, are commonly prescribed to patients diagnosed with hypertension to reduce the amount of fluid retention by the kidney and thus lower blood pressure by reducing blood volume. As angiotensin II continues to circulate throughout the arterial system, it activates AT1 receptors in central circumventricular organs lying outside as well as inside the blood brain barrier (Saavedra et. al., 2011). It appears that this activation triggers peripheral inflammation to occur. ARBs therefore have many therapeutic effects outside of their more common clinical application pertaining to hypertension.

In addition to the direct physiological effects of ARBs, there are several lines of evidence suggesting that altering the angiotensin II system can influence emotional behavior. In patients suffering from depression, ACE inhibitors, such as Captopril, produced mood elevation (Zubenko and Nixon, 1984). These clinical findings led to the investigation of these effects using animal models. The ACE

inhibitor Captopril had powerful anxiolytic-like effects in rats, mice, and marmosets in a variety of tests (Costall et. al., 1990). Both AT1 and AT2 receptor subtypes exist in the CNS. The AT1 receptor subtype plays a crucial role in the modulation of hypertension (Timmermans et. al., 1993). This receptor plays a pivotal role within the CNS, especially the circumventricular organs and blood vessels, however, it is unclear which of these receptors might be responsible for the effects of ACE inhibitors on the neurochemical systems involved with emotional behavior (Barnes et. al., 1991).

The effects of ACE inhibitors on mood may be related to their effects on reducing inflammation, therefore, they have the potential to act on behavior in the guinea pig model in a manner similar to other anti-inflammatory agents. ARBs cause a decrease in the secretion of pro-inflammatory cytokines (Sanchez-Lemus, 2009). This anti-inflammatory effect may contribute to therapeutic conditions such as elevated mood disorders (Saavedra et al., 2010).

Losartan is an antagonist to the AT1 receptor subtype. Thus, the laboratory wanted to investigate the effects of Losartan on passive behavior and core temperature during separation in the guinea pig. The dose of Losartan chosen for the experiment was 20 mg/kg, b.w. This dose was chosen based on preliminary observations that this dose of losartan appeared to reduce passive behavior in the first few subjects tested. The present study examined whether losartan

administered before an initial separation would reduce the sensitization of passive, depressive-like behavior observed during later separations. Body temperature was measured to determine effects of losartan on sensitization of fever, and therefore, indirectly on the sensitization of proinflammatory activity. The study also examined vocalizations, the primary response of the initial "protest" phase of separation. Cortisol was measured to assess effects on the stress-responsive hypothalamic-pituitary-adrenal system. Finally, locomotor activity was monitored to determine its relation to changes in body temperature. The study employed the paradigm of Schneider et. al. (2012) in which separations occur on two consecutive days and then again 10 days after the first.

Methods

Subjects

Albino guinea pigs (Hartley strain) were bred in our laboratory. Following birth (Day 0), the mother and her litter were housed in an opaque cage (73cm x 54cm x 24cm) supplied with wood chip bedding; chow and water were available *ab libitum*. The lighting system was maintained at a 12 hour cycle beginning at 7 am with the lights turning off at 7 pm. All procedures were approved by Wright State University's Laboratory Animal Care and Use Committee. Prior to experimentation, pups were kept with their mother and littermates with disturbances only occurring during periodical cage cleaning.

Design

The pups were assigned to one of 4 groups with a total of 6 males and 6 females per group: Losartan Separation (LSEP), Vehicle Separation (VSEP), Separated Only (SEP) and Undisturbed (UND). No more than one pup from a litter was assigned to any single condition. A total of 3 behavior tests were completed between Days 17-31. Each test was 3 hr in length. At the end of the third test, the pup was euthanized and a blood sample was collected to determine cortisol levels.

Surgery

Between Days 14-17, a telemetry probe (PD4000 Emitter from Mini-Mitter Company, Bend, OR) was surgically implanted into the abdominal cavity of pups in the LSEP, VSEP and SEP groups under aseptic conditions. Guinea pigs were pretreated with atropine (0.05 mg/kg to reduce secretory activity) and 3-5% isoflurane was used as anesthesia throughout the procedure. Heart rate and blood oxygen were recorded periodically throughout the surgical procedure to ensure the animal's well-being. Buprenorphrine (0.05 mg/kg), was administered subcutaneously immediately following surgery and again 24 hours later to control pain. Animals were weighed daily post-op and gained weight steadily following surgery. A minimum of three days recovery was required between surgery and the first behavioral test.

Procedure

The first separations occurred between Days 17 and 21 (day of birth = Day 0), during the preweaning phase. The final separation occurred between Day 27 and 31, which is after the typical age at weaning (Day 25). However, guinea pig young continue to show robust responses to maternal separation for weeks after weaning. Thus, a series of three maternal separations took place for the LSEP, VSEP and SEP

groups. Approximately 24 hours after the first separation, the second separation took place. The third and final separation occurred ten days following the first.

Subjects in the LSEP and VSEP conditions were injected subcutaneously with either 20 mg/kg b.w. of losartan or an equal volume of saline vehicle, respectively on three occasions at 24-hour intervals, with the last injection occurring 1 hour before the first separation. Subjects in the SEP group did not receive injections prior to separation. UND subjects never underwent telemetry surgery, injections, or separation tests. At the time of the first separation, the pup was placed in a clean transport cage and quietly taken from the home cage to a nearby testing room where it was placed in a clear plastic cage isolated from its mother or siblings. The testing cage was empty with a bright light above and was of different dimensions (47 x 24 x 20 cm) than the home cage (73 x 54 x 24 cm) creating a novel environment for the pups. A plastic lid was placed over the cage to prevent the guinea pig from escaping during the test. The testing cage was placed on a receiver platform that detected signals correlated with body temperature and movement from the implanted telemetry. Cages were cleaned thoroughly with water and detergent between tests.

Temperature data were collected using Vital View (Mini-Mitter) software run by a remote computer. Data were processed by the software as mean values for each 3-min interval. Movement of the animal was also detected with the telemetry

system in 3-min bins across the 3-hr test. The primary purpose of assessing activity was to evaluate whether any observed changes in body temperature were associated with physical exertion.

Behaviors

Behaviors were noted during the Minutes 0-30, 60-90 and 150-180 (as in other studies, e.g., Schneider et al., 2012; Yusko et al., 2012) by a single observer behind a one-way glass wall. The total number of "whistle" vocalizations (Berryman et.al, 1976) were counted using a hand counter. These vocalizations were transmitted by a microphone positioned above the cage to the observer wearing a headset on the other side of the glass. The passive behaviors that were measured were crouch (hunched stance with feet tucked beneath the body), eye-close (complete or near complete sustained closure of the eye for greater than 1 second), piloerection (over most of the body), and lying down (limbs tucked under body with the cranial and ventral or flank region touching the bottom of cage). These were scored in a one-zero fashion each during each 1-minute interval. Additionally, intervals of "full passive" responding were also assessed; for this, the pup needed to exhibit all 3 (piloerection, eye-close, and either crouch or lying down) individual responses during the minute.

Blood collection and hormone analysis

Immediately after a pup's final separation or removal from the home cage, it was exposed for 1 min to CO2 and decapitated. Trunk blood was collected on heparin and centrifuged for 20 min at 1935g. All samples were collected within 2 min of the onset of disturbance. Separated plasma was stored at -20 degrees celcius until assayed. A commercial cortisol kit (Siemens "Coat-a-Count") was use with plasma diluted 1:5. Intra-assay variability was calculated to be <3%.

Data Analysis

Due to large number scores of zero, behavioral data other than movement violated assumptions for parametric tests. Therefore, these data were analyzed using non-parametric tests. Preliminary analyses revealed sex differences on several measures, therefore behavioral analyses were conducted separately for males and females. Vocalizations and full passive behaviors of LSEP, VSEP and SEP groups were compared using the Kruskal-Wallis and Mann Whitney U tests. Friedman analysis of variance was used for within subject comparisons of these measures over days of testing. For core temperature and activity counts, data across five consecutive 3-min bins were averaged to generate scores for 15-min time blocks. These data were analyzed with analysis of variance (ANOVA) procedures.

Results

Passive Behavior and Vocalizations

For crouch (Fig. 1), there were no effects for condition or day of testing for males. For females, however, crouching increased by Day 10 in the Saline and LSEP groups (p's < 0.05), but no change over days was observed in the SEP condition.

No changes across days or among groups for eye-close was noted for either males or females (Fig. 2). Piloerection (Fig. 3) exhibited no significant changes across days or differences between treatment conditions for males. In contrast, for females, piloerection increased across days within the SEP and LSEP groups, reaching the highest values by Day 10 (p's < 0.05). In addition, SEP females exhibited more piloerection on Day 2 of testing relative to LSEP females ($p < 0.05$). Lying down was rarely observed and so not analyzed separately. Instances of the full-passive response, in which all three passive behaviors were exhibited simultaneously within a 1-min interval (Fig. 4), exhibited no significant changes across days of testing. Moreover, treatment condition did not alter pups' likelihood of displaying the full-passive response to maternal separation (Fig. 4). There were no significant effects of the day of testing or treatment condition on the number of distress vocalizations pups emitted during separation.

Temperature

Because of heterogeneity of variance, temperature scores were transformed prior to analysis. However, neither a square root transformation, nor a logarithmic transformation could equate variances. These effects appeared due to differences between male and female subjects. Therefore, separate 3 (Group) x 3 (Day) x 12 (Bin) repeated measures ANOVAs were performed for each sex (Figure 7). This approach resulted in acceptable levels of heterogeneity. For males, there was only an effect of Day, $F(2, 28) = 8.58$, $p < 0.01$. Similarly, the ANOVA for females also yielded only a significant effect of Day, $F(2, 30) = 4.47$, $p < 0.05$. As seen in Figure 6 for both males and females, the highest core temperature was observed on Day 10. Although there was no significant interaction of Day by Bin, Figure 7 indicates that the increase in temperature over days appears to have resulted primarily from a more-rapid increase in temperature on Days 2 and 10 than on Day 1.

Movement

The movement score was examined in a 3 (Group) x 2 (Sex) x 3 (Day) x 12 (Bin) repeated measure ANOVA using the Huynh-Feldt correction for sphericity. There were significant effects for Day, $F(2, 60) = 24.91$, $p < .001$, Bin, $F(11, 330) = 17.84$, $p < 0.001$, and Day x Bin, $F(8.7, 261.4 = 5.39$, $p < 0.001$. Because the primary interest in the movement measure was to determine if exertion during movement

could account for differences in temperature, the Day effect (which was significant for temperature) was examined further. As seen in Figure 8, the lowest movement score occurred on Day 10, the day of the highest temperature. Therefore, increased temperature during the last day cannot be accounted for by increased movement.

Cortisol

For cortisol, a 4 (Condition) X 2 (Sex) ANOVA yielded main effects for Condition, $F(3,40) = 14.74$ $p < 0.001$, and Sex, $F(1, 40) = 13.27$, $p < .001$ (females > males). Newman-Keuls paired comparisons showed all three groups undergoing separation (SEP, VSEP, and LSEP) had higher plasma cortisol levels than the pups in the UND condition.

Discussion

The data presented here partially replicates previous work that showed sensitization of passive, depressive-like behavior in the guinea pig pup, meaning that passive behaviors are enhanced on subsequent separations (Schneider et. al., 2012). However, in the present study sensitization was limited and occurred only in female pups. Crouching behavior in the VSEP and LSEP groups and piloerection in the SEP and LSEP groups all reached highest values by Day 10. This indicates that passive behaviors were, in part, sensitized by previous experiences. One reason why a full replication of behavior sensitization did not occur may have been that, unlike earlier studies, the LSEP and VSEP groups received a series of three injections prior to the first day of testing. This disturbance may somehow have interfered with the sensitization process.

Hennessy et.al. (2011) found that "sensitization" was decreased via centrally administered IL-10, which possesses anti-inflammatory properties. This result suggests that proinflammatory activity not only mediates the initial response to separation, but also may contribute to the sensitization of the behavioral response with repeated separations. In the present study, there was only one significant finding across groups. On Day 2, piloerection by females was exhibited more in the SEP group than in the LSEP group. Because this was the only significant effect of many comparisons across groups, it likely represents a spurious finding. The inability of Losartan to decrease passive behavior might be attributed to the dose administered to the pups. This dose was tested on the first

few pups. Because results appeared to be positive, it was chosen for the full experiment. However, the initial positive trend was not maintained as testing proceeded. If effects of Losartan are to be examined further, several doses should be used. It is also possible that Losartan needs to be administered over a longer time period, or that it has an exceptionally rapid half-life in guinea pig pups. In addition, losartan may reduce certain elements of inflammation, but not those important for the behavioral effects observed here.

In the present study, core body temperature increased (i.e. sensitized) over days. This increase in body temperature, however, was not accompanied by an increase in motor behavior; rather, motor behavior declined over the days of testing. The increase in core body temperature appears reflective of true fever, which is an element of the acute phase response that is mediated by proinflammatory cytokine activity. The temperature data provides additional evidence that sensitization involves a greater and more rapid febrile response upon repeated separation of guinea pig pups.

There was only one significant finding for blood plasma cortisol levels. Each of the three separated groups exhibited levels of cortisol as compared to the UND group. However, Losartan appeared to have no effect on cortisol as there were no differences between the LSEP and VSEP groups.

Although the passive responses of crouch, eye-closure and piloerection undergo a sensitization process with repeated separations, vocalizations do not. This is not unexpected since these vocalizations are considered an anxiety-like, rather than

depressive-like, response. In fact, the inhibition of guinea pig separation vocalizations is commonly used as a preclinical screen for proposed anxiolytic compounds (e.g., Zhang et. al. 2011; Varty et. al., 2008). Thus, results reported here suggest that early attachment disruption may have differing effects on the development of anxiety disorders and major depression.

The goal of the current study was to determine if peripherally administered Losartan, an ARB blocker, could decrease passive responses and the sensitization process in isolated guinea pig pups. Peripherally administered drugs are essential if this model is to be applied to human depression. However, the peripherally administered losartan produced no significant reduction in passive behavior or its sensitization during repeated maternal separations. It must be concluded that multiple losartan injections do not reduce depressive-like behavior in guinea pig pups upon repeated separations at least with the specific procedures of the present experiment.

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Appendix

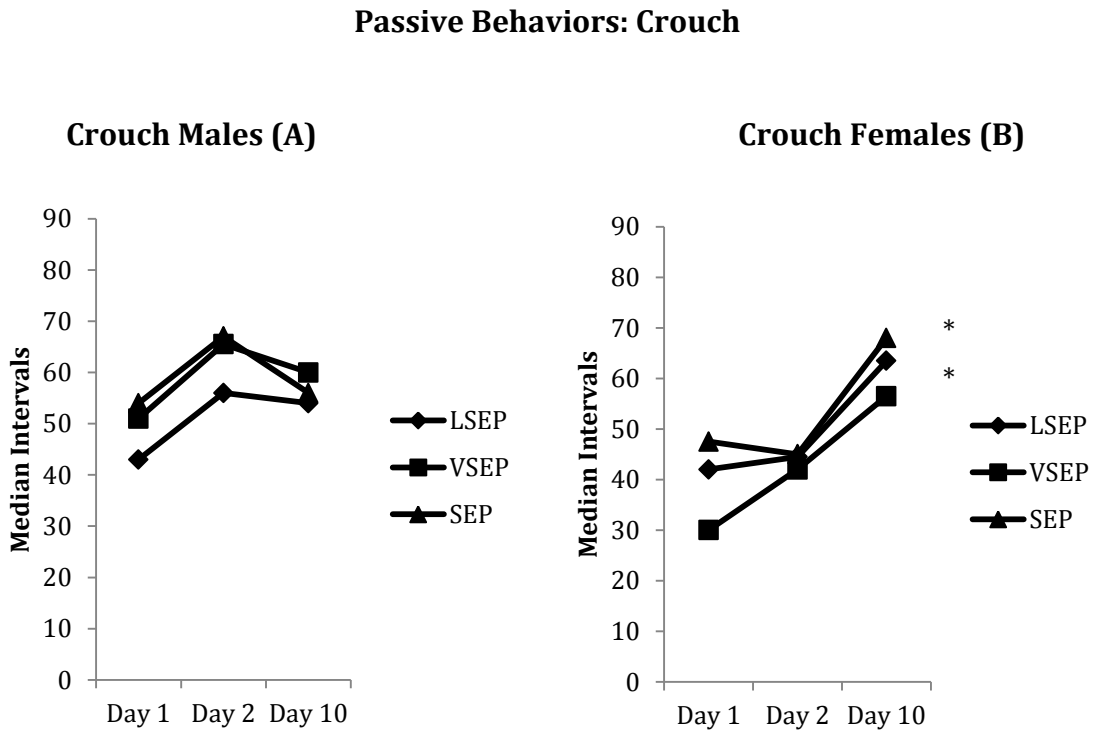
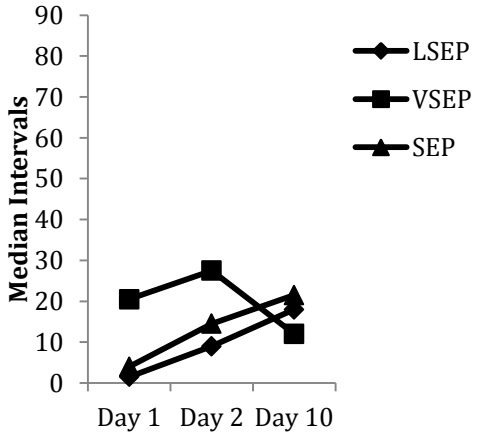


Figure 1. Median number of 1-min intervals that crouch was observed during each of the three tests for males (A) and females (B).*$p < 0.05$ for change over days

Passive Behavior: Eye Closure

Eye Closure Males (A)



Eye Closure Females (B)

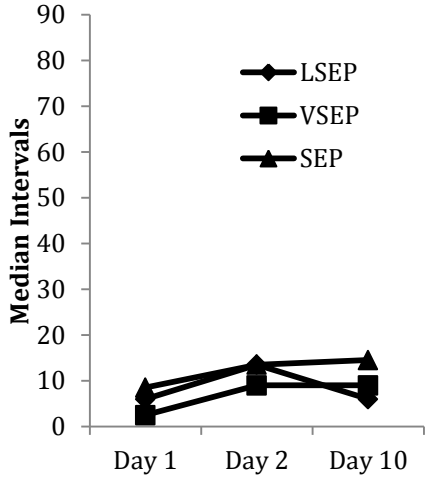


Figure 2. Median number of 1-min intervals that eye closure was observed during each of the three tests for males (A) and females (B). *$p < 0.05$ for change over days

Passive Behavior: Piloerection

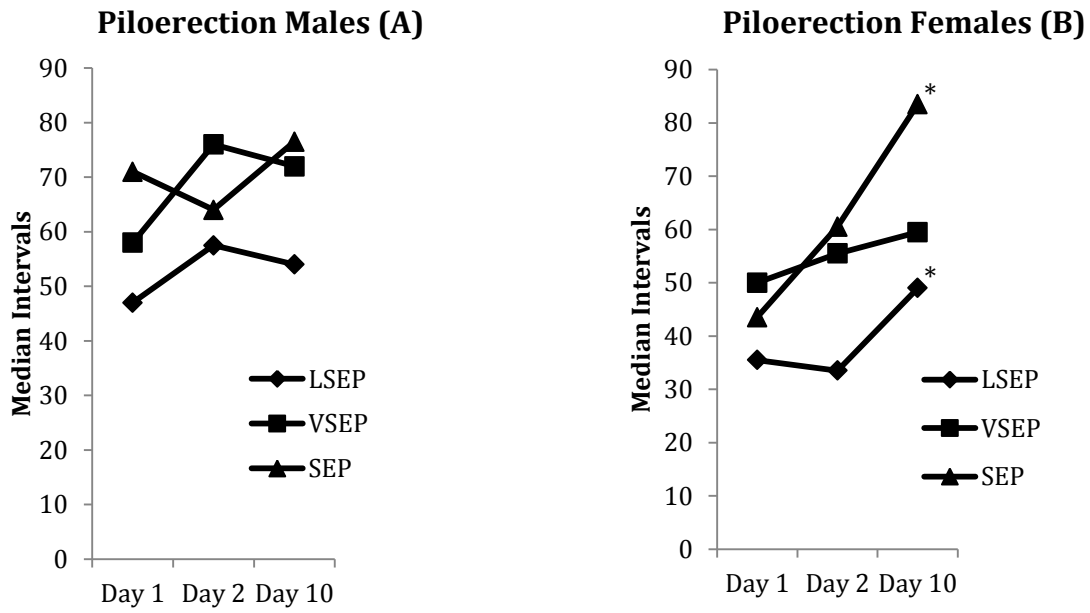


Figure 3. Median number of 1-min intervals that piloerection was observed during each of the three tests for males (A) and females (B). *$p < 0.05$ for change over days

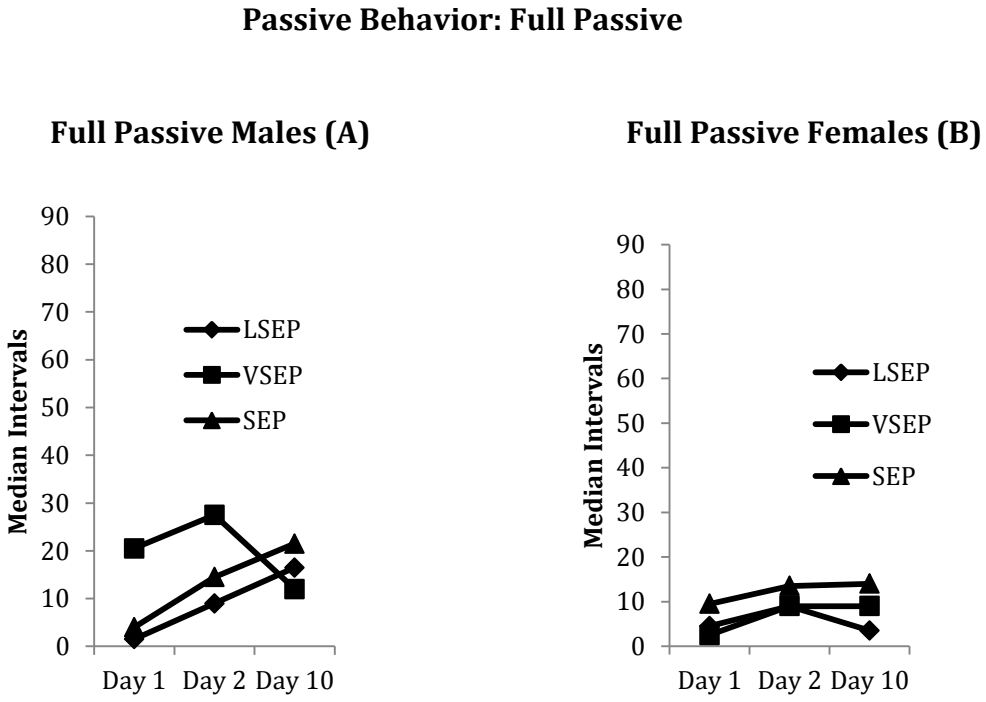


Figure 4. Median number of 1-min intervals that full passive behaviors was observed during each of the three tests for males (A) and females (B). *p < 0.05 for change over days

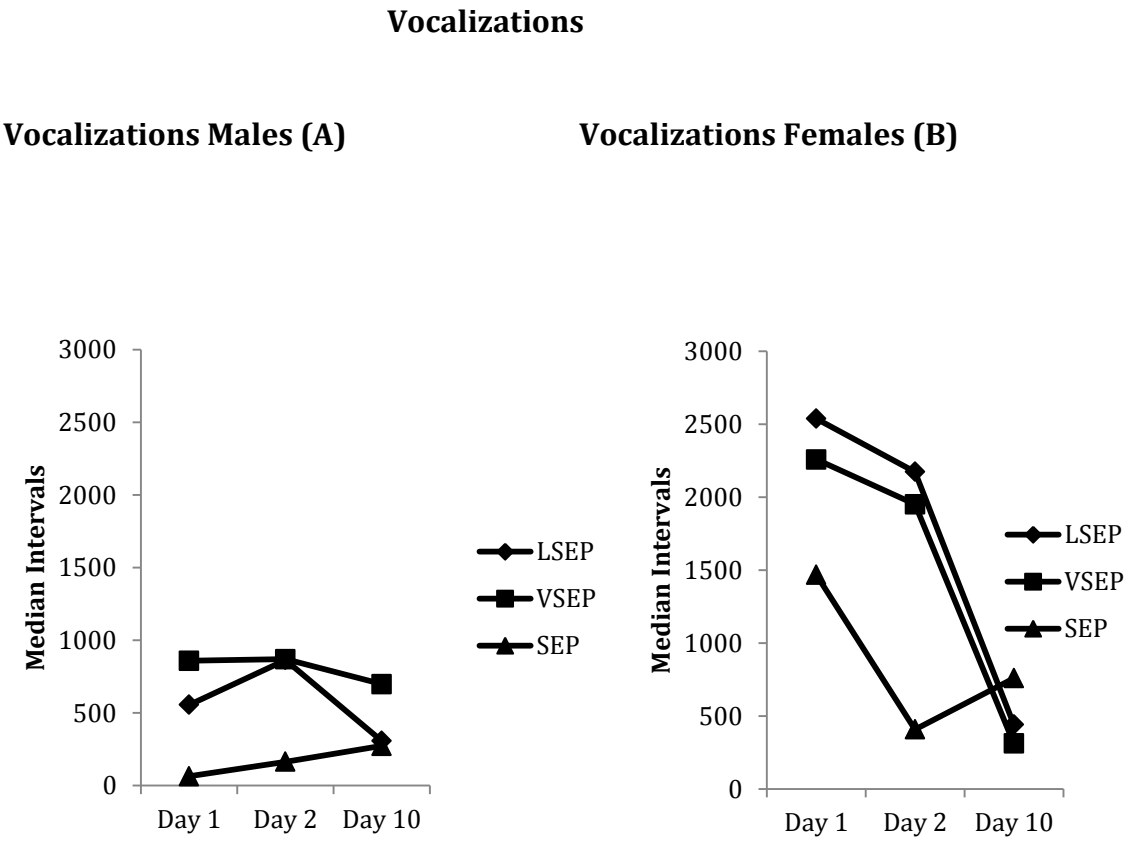


Figure 5. Median number of 1-min intervals that vocalizations was observed during each of the three tests for males (A) and females (B).

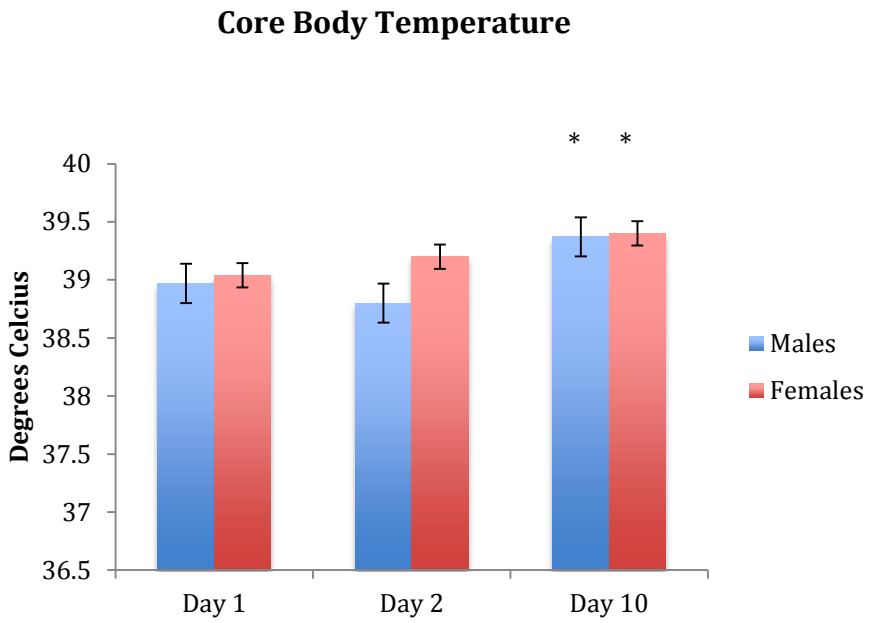


Figure 6. Mean core body temperature of both males and females observed across three separations. * p's < 0.05 for change across days of separation.

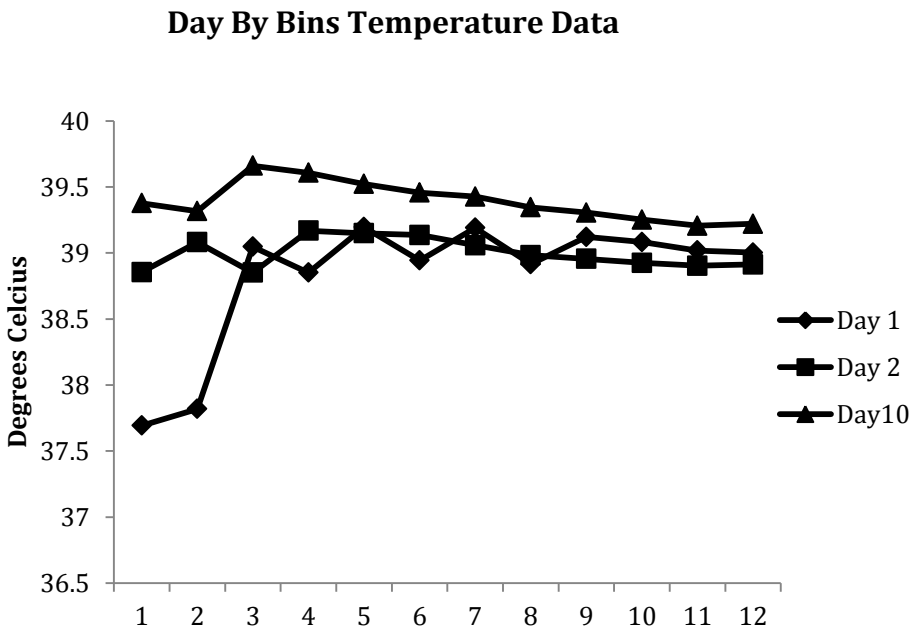


Figure 7. Mean temperature of guinea pigs during the three tests in each 15-min bin.

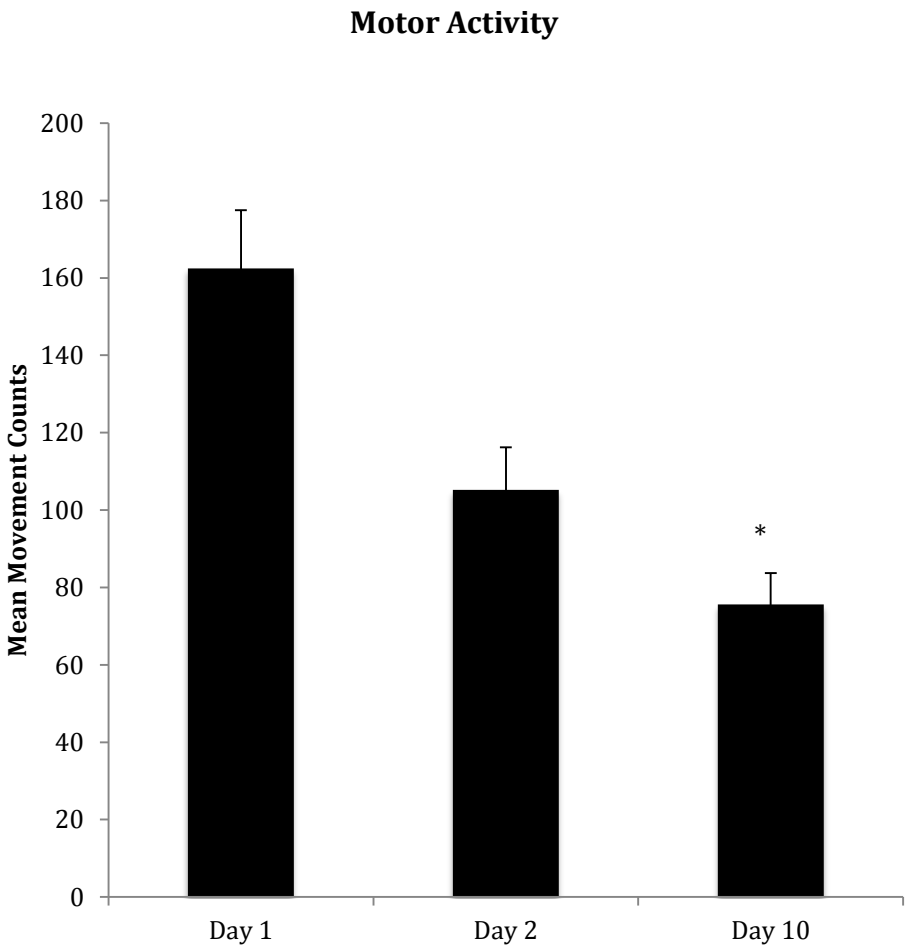


Figure 8. Mean movement counts observed across three separation tests. Vertical lines represent standard errors of the means. *p < 0.05 for ???

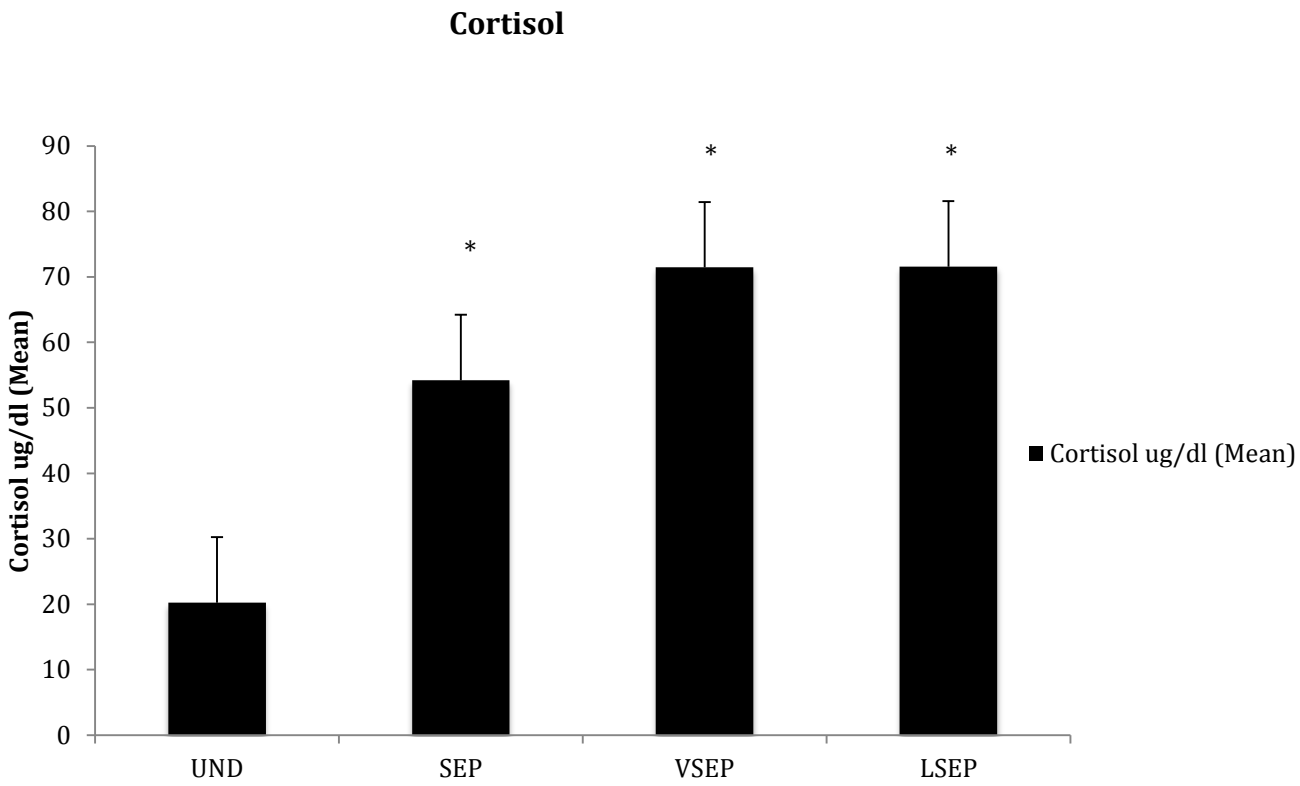


Figure 9. Mean plasma cortisol (ug/dl) for each of the four groups. Vertical lines represent standard errors of the means. *$p < 0.05$ versus UND.