Central Mechanisms Regulating Pituitary-Adrenal Activity in Infant Guinea Pigs (Cavia porcellus) during Exposure to Psychological Stressors: Independent and Combined Effects of Maternal Separation and Novelty

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CENTRAL MECHANISMS REGULATING PITUITARY-ADRENAL ACTIVITY IN INFANT GUINEA PIGS (CAVIA PORCELLUS) DURING EXPOSURE TO PSYCHOLOGICAL STRESSORS: INDEPENDENT AND COMBINED EFFECTS OF MATERNAL SEPARATION AND NOVELTY.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT


Separation from the maternal attachment figure, particularly when it occurs in a novel or threatening environment, reliably increases pituitary-adrenal activity in a number of species, and is thought to increase later susceptibility to psychopathology in humans. However, little is known about the central mechanisms mediating these effects. Therefore, I investigated cortisol and ACTH levels in plasma, corticotropin-releasing factor (CRF) mRNA expression in the hypothalamic paraventricular nucleus (PVN), and c-Fos activity in a circuit [medial amygdala (MeA)- bed nucleus of the stria terminalus (BNST)] thought to drive pituitary-adrenal activity during exposure to psychological stressors, as well as in the PVN. Measurements were taken in 16 (+/-1)-day-old infant guinea pigs (Cavia porcellus) during maternal separation, exposure to a novel environment, and exposure to both separation and a novel environment. Levels of plasma cortisol and ACTH, and PVN CRF mRNA expression were elevated only when animals were exposed to both separation and novelty. The putative MeA-BNST circuit was activated during exposure to novelty regardless of whether or not the animals were separated, and c-Fos activity in the PVN was elevated during separation regardless of whether infants were in a novel environment. In sum, there were effects due only to separation; others due only to novelty; and some due to both. These results suggest that CRF activity in the PVN can account for changes in pituitary-adrenal activity during
separation in a novel environment. Further, it appears that the effect of novelty on the
hypothalamus is mediated at least in part by a circuit from MeA to BNST to PVN.
However, other factors appear to mediate activation of the PVN during separation from
the attachment figure in a familiar environment, and the lack of activation in the PVN
when the attachment figure is present in a novel environment.
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DEDICATION

I dedicate this work first and foremost to my beautiful mother Donna for all of her generous support, friendship, kindness, guidance and dedication to my well-being throughout my entire life. Her strength, perseverance and insightfulness have always been an inspiration to me. I also dedicate this work to my brother Jon for all of his support, friendship, kindness, strength, and dedication to my well-being throughout my life. In addition to my mother, he is one of the most caring, generous and intuitive people that I know. I am truly blessed to have these wonderful people as my family.
I. Introduction

The presence of specific social partners can have salubrious effects on the well-being of an individual (Unchino, 2006; Von Holst, 1998). These effects can be clearly observed during times of stress. Whereas the absence of these partners during stressful events can have negative consequences or deleterious effects on health, the presence of a companion can appreciably reduce these effects. This is particularly true for important social partners where there is a strong attraction or affiliation. This dissertation examined the central mechanisms underlying the effect of a social partner on the response of the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA Axis

The HPA axis is the primary stress-responsive neuroendocrine system (Sapolsky, Romero, & Munck, 2000). The activity of the system consists of a cascade of three classes of hormones. The neurons in the medial parvocellular paraventricular nucleus (mpPVN) of the hypothalamus synthesize and release corticotropin-releasing factor (CRF) into the hypophyseal portal system. In addition to functioning as a hormone, CRF also can act as a neurotransmitter, and is synthesized and released from extrahypothalamic neurons to affect other regions of the brain. Hypothalamic CRF is the primary secretagogue stimulating release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. The action of CRF can be enhanced by arginine vasopressin (AVP) from hypothalamic neurons released into the portal blood supply (Aubry, Bartanusz, Jezova, Belin & Kiss, 1999). ACTH is cleaved from a larger molecule
proopiomelanocortin (POMC) in the intermediate lobe of the pituitary and typically co-released with beta endorphin, another cleavage product of POMC. ACTH is released into the general circulation and arrives at the adrenal cortex where it produces and releases glucocorticoids, primarily cortisol and corticosterone, the percentage of which varies with the specific species in question.

The system shows a basal level of activity that demonstrates circadian rhythmicity. Under the influence of pacemaker cells in the suprachiasmatic nucleus, peak circulating basal hormone levels correspond roughly with the onset of the active behavioral phase. Importantly for the functioning of the system, exposure to any of an assortment of noxious or arousing stimuli increases drive to the PVN and thereby increases circulating levels of each of the three classes of HPA hormones. These hormones have widespread actions that assist the animal in combating the effects of environmental challenges (Sapolsky et al, 2000).

These actions are initiated by the binding of glucocorticoids to their receptors. There are two classes of receptors: the high affinity mineralocorticoid receptors (MR) and the lower affinity glucocorticoid receptors (GR). The MRs are largely saturated during the peak of the circadian rhythm, whereas actions of higher levels of glucocorticoids seen following exposure to stressors are primarily mediated by GRs. The occupation of GRs in the hippocampus, frontal cortex and hypothalamus also acts to inhibit further activity of CRF neurons. This negative feedback seems to prevent protracted high levels of glucocorticoids from adversely affecting various biological systems (De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998).

*Psychological Stressors*
Although the HPA axis responds to a wide variety of aversive stimuli it appears to be particularly sensitive to psychological stressors (i.e. those that produce no actual physical harm or challenge). Psychological stressors include novelty, unpredictability or inability to control environmental events. Novelty exposure is a germane example of a potent psychological stressor. For instance, when an animal is placed in a novel environment and exposed to electric shock, elevations of glucocorticoid levels often are no greater than when the animal is exposed to the chamber in absence of shock (Friedman, Ader, Grota & Larson, 1967; Ader, 1970). In this situation, the exposure to the novel environment appears to be a more salient activator of the HPA axis than the shock exposure. Further, the glucocorticoid response has been found to be more sensitive to differences in the novelty of a test environment than to variations in the level of electric shock presented in a test environment (Hennessy & Levine, 1978). Even what could be considered a mild stressor such as exposure to a novel odor in the home environment is capable of producing an HPA response. For example, when infant mice were exposed to clean bedding for only 15 min, they showed increased levels of plasma cortisosterone (D’Amato, Puglisi-Allegra, Patacchioli, Cigliana, Maccari & Angelucci, 1992).

Psychological stressors such as exposure to novelty may be potent at activating the HPA axis because they may signify increased probability of impending environmental challenge (e.g. appearance of a predator). These notions are incorporated in the recent conceptualization of allostasis (McEwen, 1998) in which animals routinely activate neurohormonal processes in preparation for, or anticipation of, an impending aversive event. Dividing stressors into either psychological or physical classes is a useful
means of organizing stimulators of the HPA axis, but there is a long-standing realization
that these distinctions are not absolute. The predominant difference between a
psychological stressor and a physical stressor is the particular brain level where stimulus
information is processed, with psychological stimuli, which require more interpretation,
presumably being processed at higher brain levels (Mason, 1975).

Separation from Attachment Figures

One potent psychological stressor is separation from an attachment figure.
Attachment involves an intense attraction for a specific social figure and typically the
term is used in the context of the relationship infants of some mammalian species show
for their mother. The attraction for the attachment object is characterized by an
emotional component, demonstrated by the calming influence of the presence of the
attachment object and the distressful response to its removal. Brief separation from the
attachment figure has been found to reliably activate the HPA axis (Hennessy, 1997).
Infant New World squirrel monkeys show elevated cortisol levels when separated from
their mother for a short period of time (Coe, Mendoza, Smotherman, & Levine, 1978;
Wiener, Johnson, & Levine, 1987). Behavioral changes also are observed (e.g. increased
vocalizations) during the separation period. However, with repeated separations,
behavioral changes rapidly subside while cortisol elevations persist for much longer.
Indeed, when infants were tested over eighty different occasions, cortisol levels remained
high across the separations while behavioral changes rapidly habituated and had long ago
subsided by the time of the final test (Hennessy, 1986). These effects can be seen well
past the age of weaning in infants that have been continuously housed with their mothers
until testing. Not just any social partner is capable of influencing HPA activity. When
mothers were removed from a group-housed environment, infant squirrel monkeys exhibited cortisol elevations, irregardless of whether a familiar lactating female or “aunt” cared for the infant during the separation period (Coe et al., 1978). These data illustrate the specificity of the infant’s response; it is specific to the mother: other familiar social partners typically are unable to reduce the infant’s HPA response and compensate for the absence of the attachment object.

Infants of a different species of nonhuman primate, Old World rhesus macaques, show elevated HPA activity to maternal separations similar to squirrel monkey infants. When infants were isolated in a novel environment for 3h, greater elevations in cortisol were observed than when infants were placed with their mothers in a novel environment (Smotherman, Hunt, McGinnis & Levine, 1979). If infant rhesus were separated from their mothers by simply removing the mother from the home cage environment for 30 min or 3 h, they exhibited cortisol elevations at both time points. In all, non-human primates show robust HPA axis effects demonstrating the strength and enduring nature of the infant’s response to being separated from a figure to which it shows an intense attraction, namely its mother.

Sometimes infants will show an intense attraction to social partners other than the mother. For example, infant titi monkeys prefer to spend the majority of their time with the father. The infant leaves its father only when it is ready to nurse. It will cling to the mother for the duration of the nursing period and then immediately return to cling to the father (Mendoza & Mason, 1986). Infants show cortisol elevations to being separated from the father for short periods of time (Hoffman, Mendoza, Hennessy, & Mason,
1995). Their HPA responses are specific to the presence or absence of the father: the infant titi does not show a cortisol response when only separated from its mother.

Non-human primate infants display changes in HPA activity when separated from an attachment object in the short-term. They also show long-term effects of early stress on HPA activity in adulthood. For example, when infant bonnet macaques are exposed to adverse maternal rearing conditions they show persistently elevated CRF levels as adults (Coplan, Andrews, Rosenblum, Owens, Friedman, Gorman, & Nemeroff, 1996). Moreover in humans, exposure to early life stress, notably disruption of attachment relationships, appears to predispose individuals to depression and other forms of psychopathology later in life (Gold, Goodwin, & Chrousos, 1988a,b). In sum, disruption of attachment processes early in life can have long-lasting effects (Hammen, Davila, Brown, Ellicott, & Gitlan, 1992) that may be mediated by hyperactivity of CRF and other components of the HPA axis (Coplan et al, 1996; Gillespie & Nemeroff, 2007).

The Guinea Pig

One rodent species in which these ideas have been explored is the guinea pig (*Cavia porcellus*). Guinea pigs are born precocially; that is, they are fully furred, with eyes and ears open and can locomote within minutes. Weaning occurs around postnatal day 25, but infants are capable of eating solid food and drinking water within a day of birth (Schiml & Hennessy, 1990). Maternal care is passive and minimal; it is the infant that initiates interaction with the mother (Schiml & Hennessy, 1990; Hennessy & Jenkins, 1994). Further, it is the infant’s strong attraction for its mother that maintains the interaction and characterizes the filial attachment.
Developing guinea pigs show evidence for the primary measures of attachment as defined in non-human primates. There is evidence indicating that an infant guinea pig recognizes and prefers cues specific to its own mother relative to other social partners. Infants were able to discriminate, and showed a preference for, the urine of their mother in comparison to that of other lactating females that were unfamiliar and related, unfamiliar and unrelated or familiar and unrelated (Jäckel & Trillmich, 2003). Even following weaning, guinea pig pups have been found to prefer spending the majority of their time with the mother relative to other social partners. Periadolescent animals given free access to separate chambers in a modified living environment in which they could spend time with their mother, an unfamiliar adult male, an unfamiliar adult female, or alone, spent the vast majority of their time with their mother (Hennessy, Young, O’Leary, Maken, 2003).

Infant guinea pigs will explore novel environments if the mother is present and appear to use her as a secure base. When an infant was placed in an open-field environment in the presence of the mother, it explored more than when it was isolated in the environment. The infant alternated between spending time in exploration of the chamber and spending time in the proximity of its mother (Porter, Berryman & Fullerton, 1973). Similarly, non-human primate and human infants show increases in exploration of a novel environment when the mother is present with them (Harlow, 1961; Ainsworth & Bell, 1970).

Infants will exhibit distress when separated from an attachment object. One behavioral indicator of distress observed in infants is increased vocalizations. Distress vocalizations have been described as “whistle calls” in the guinea pig (Berryman, 1976).
Isolation in a novel environment increases vocalizations that are substantially reduced if the attachment object is present. When an infant was in the presence of its mother, vocalizations were reduced by 98% in comparison to being alone in a novel environment (Pettijohn, 1979). Other unfamiliar and familiar social partners are not as effective at reducing the infant’s vocalizations. If an unfamiliar female was present with the infant, vocalizations were reduced by only 50% (Pettijohn, 1979). In a different study, infants were placed in a novel environment with either their anesthetized mother or an anesthetized unfamiliar lactating female. Infants vocalized less when in the presence of the mother than in the presence of the unfamiliar female. In fact, there was no difference in levels of vocalizations when the infant was in the presence of the unfamiliar female as compared to when it was alone in the novel environment (Hennessy & Ritchey, 1987). Even an equally familiar sibling is not as effective as the attachment object at reducing vocalizations. While vocalizations were reduced in the presence of the sibling, the presence of the mother reduced the infant’s vocalizations significantly more than the sibling and in fact almost completely eliminated them (Ritchey & Hennessy, 1987).

In addition to behavioral measures there are also physiological responses to separation in guinea pigs. Brief separation reliably activates the HPA axis (Hennessy & Ritchey, 1987; Hennessy & Moorman, 1989; Hennessy, 1997). If an infant is isolated in a novel environment, it exhibits cortisol elevations that are significantly reduced if the mother is present. Eventually, cortisol elevations will wane during separations from the mother. When infants were isolated, cortisol levels were increased at 30 and 90 min. However, by 180 min, levels decreased but were still higher than when the mother was with the infant (Hennessy & Moorman, 1989). In addition to changes in cortisol levels,
changes in ACTH also are observed. If an infant was isolated for 30 or 90 minutes, ACTH was significantly elevated (Hennessy, Tamborski, Schiml, & Lucot, 1989). However if the mother was present with the infant in the novel environment, ACTH levels were significantly reduced. Although release of the initial hormone of the HPA axis, corticotropin-releasing factor (CRF), also presumably increases, it has not been measured.

The presence of the mother is an important factor in modulating the infant’s pituitary-adrenal activity. However, it is in the context of a novel environment that maternal separation has its greatest effect. When infants were left in the home environment during maternal separation, no elevations of cortisol levels were observed regardless of whether the infants were isolated (Hennessy & Moorman, 1989) or left with other conspecifics (Wewers, Kaiser & Sachser, 2003). Thus, cues from the home environment appear to inhibit the increase in pituitary-adrenal activity that is observed when infants are separated from their mothers in a novel environment.

The mother’s ability to influence the infant’s HPA axis hormones continues well past weaning, but does not last indefinitely. When infants continuously housed with their mother were tested at postnatal day 50 either with their mothers or isolated in a novel environment, they showed cortisol reductions when with the mother. However, by around postnatal day 100 (when a guinea pig is considered to be an adult), cortisol levels were no lower when with the mother than when isolated in the novel environment (Hennessy, Nigh, Sims, & Long, 1995).

The effect of the attachment object is relatively specific to the mother; other social partners are not as effective at reducing the infant’s HPA activity. If the infant was
placed in the novel environment with either an anesthetized unfamiliar female or its conscious or anesthetized mother, only the mother was able to reduce its cortisol levels (Hennessy & Ritchey, 1987). Even another sibling was unable to reduce the infant’s cortisol levels when with it in the novel environment (Ritchey & Hennessy, 1987). In all, guinea pigs show evidence for filial attachment and biobehavioral responses to maternal separation in a fundamental way akin to non-human primates.

Although pituitary-adrenal activity during brief separations from an attachment object has been extensively studied in developing guinea pigs, little is known about how the brain may regulate pituitary-adrenal activity during these separations. In addition, it is not known how the attachment object affects the brain to modulate pituitary-adrenal activity during brief exposure to a novel environment, or how the presence of the home environment reduces the response during separations, in other words, how the interaction of separation and novelty is represented in central mechanisms controlling the HPA axis.

*Central Activity during Psychological Stress*

There is some evidence to support the role of brain norepinephrine (NE) in regulating HPA activity during separations from an attachment figure. When infant guinea pigs were separated from their mothers and placed in a novel environment, they showed increased levels of hypothalamic NE and plasma cortisol in comparison to those pups that were separated in the home environment (Harvey, Moore, Lucot & Hennessy, 1994). While not providing a direct link between NE activity in the PVN and HPA responses, this is suggestive of an important role that central noradrenergic input may have in regulating HPA activity in this situation. However, there is research showing other neurotransmitter activity and neural circuitry in higher brain regions during
exposure to psychological stressors that may play a role in behavioral responses, release of CRF, and activation of the HPA axis to separation.

Although, there are various brain regions that appear to be involved in HPA regulation during exposure to psychological stress, only a few of these have been consistently implicated, including the medial prefrontal cortex (mPFC), amygdala, and BNST. Some regions contain specific subregions that can have their own very distinct effect on HPA activity (Herman et al, 2003, 2005). The specific type of stressor plays a significant role in determining which specific subregion is activated and its impact on HPA activity. Although psychological stressors and physical stressors both activate the HPA axis in a similar manner to cause the release of CRF, ACTH and cortisol (Sawchenko et al., 1996), they often appear to take different routes in order to initiate activation of the HPA axis (Emmert, & Herman, 1999; Pacák et al., 2001; Sawchenko et al., 1996). In contrast to many psychological stressors, information regarding physical stressors is thought to be relatively directly relayed to the PVN via brainstem catecholaminergic projections (Cunningham and Sawchenko, 1988; Herman & Cullinan, 1997; Herman et al., 2003; Swanson and Sawchenko, 1983; Cunningham, Bohn, and Sawchenko, 1990). The following sections will focus mainly on higher brain regions that have been implicated during exposure to psychological stressors.

The mPFC integrates and interprets emotionally relevant stimuli. It has been shown to selectively modulate the HPA axis during exposure to a psychological stressor (Herman et al, 2003; Diorio, Viau, & Meaney, 1993; Figueiredo et al, 2003) and not a physical stressor (Diorio et al., 1993; Figueiredo et al, 2003). More specifically, it appears to be the dorsal subregions comprising the anterior cingulate and prelimbic
components that may inhibit HPA activity (Diorio et al, 1993; Figueiredo et al, 2003; Herman et al., 2003). When these areas were lesioned, ACTH and cortisol levels were enhanced during exposure to psychological stressors (Diorio et al, 1993; Figueiredo et al, 2003).

The amygdala is involved in the interpretation of fearful stimuli and plays a role in the generation of behavioral and physiological responses to stressors (LeDoux, 2000). It is an important functional region that modulates the activity of the HPA axis during exposure to brief stressors (Davis, 2000). The amygdala will indirectly activate the mpPVN to increase the release of CRF in the median eminence, resulting in subsequent ACTH and cortisol release. Different regions of the amygdala appear to play distinctive roles in HPA axis activation depending upon the type of stressor involved (Herman et al, 2003, 2005). Until recently, most studies examined the effect of the central nucleus of the amygdala (CeA) in controlling HPA responses to psychological stressors. CeA lesioning experiments demonstrated altered ACTH and cortisol responses to psychological stressors (Beaulieu, DiPaolo, & Barden, 1986; Beaulieu, DiPaolo, Cote & Barden, 1987; Prewitt, & Herman, 1994; Roozendaal, Koolhass & Bohus, 1991; Roozendaal et al., 1992). However, during exposure to a psychological stressor, when only cell bodies and not the axons of the CeA were lesioned, HPA responses were unaffected (Herman & Prewitt, 1997). Further, an anterograde tracing experiment showed that axons of the medial nucleus of the amygdala (MeA) partially go through the CeA and past the medial portion of the CeA (Gray, Carney, & Magnuson, 1989). It is possible then that it was the lesioning of MeA efferents and not a disruption in CeA
activity that affected HPA output during exposure to psychological stressors in previous studies (Dayas, Buller & Day, 1999).

Measurements of c-fos activity, and lesioning or stimulation of the MeA have all been used as procedures to determine whether the MeA is involved in regulation of HPA activity during exposure to psychological stressors. Stimulation of the MeA has been found to activate the HPA axis (Dunn & Whitner, 1986; Herman & Cullinan, 1997). In contrast to the CeA, several studies have shown that c-fos activity is more consistently and robustly induced in the MeA in response to psychological stressors, including novelty (Dayas et al., 1999; Emmert & Herman, 1999) than all of the other amygdaloid nuclei, (Campeau, & Watson, 1997; Cullinan, Herman, Battaglia, Akil & Watson, 1995; Dayas et al., 1999; Li & Sawchenko, 1998). The MeA shows significantly less c-fos activity in response to physical stressors, such as cytokine injections (Sawchenko et al., 1996), ether stimulation (Emmert & Herman, 1999), or hemorrhage (Thrivikraman, Su & Plotsky, 1997). Lesions of the MeA result in a decrease in HPA activity in response to psychological stressors (Dayas et al., 1999). Unlike the CeA, lesions of the MeA will reduce c-fos activity in the PVN in response to restraint.

This is not to say that the CeA is uninvolved in regulating the HPA axis. Although the CeA shows little c-fos activity during exposure to stimuli such as novelty, restraint, or air-puff startle (Cullinan et al., 1995; Emmert et al., 1999; Sawchenko et al., 1996; Thrivikraman, Nemeroff, & Plotsky, 2000) it does respond to stressors such as hemorrhage, cytokine infusions, and lithium chloride injections by displaying increased levels of c-fos activity (Sawchenko et al., 1996; Thrivikraman et al, 1997; Yamamoto, Sako & Iwafune, 1997). Further, while lesions of the CeA do not result in a reduction
of ACTH levels or c-fos in the PVN after exposure to restraint (Dayas et al., 1999; Prewitt & Herman, 1998) lesions do reduce ACTH levels and the number of c-Fos positive PVN CRF neurons after IL-1β injections (Xu, Day, & Buller, 1999). Therefore, the CeA seems to be involved in regulating HPA responses to physical stressors.

The BNST functions to integrate and relay information from the MeA, the mPFC, and the hippocampus, to the mpPVN during exposure to psychological stressors (Herman et al., 1997). The MeA has very few direct projections to the PVN (Herman et al., 2003; Canteras, Simerly, & Swanson, 1995); information is instead primarily relayed through the BNST (Herman et al., 2003, 2005). During exposure to psychological stressors the MeA most likely increases HPA activity through its connection with the BNST (Canteras, Simerly, & Watson 1995; Herman et al., 1994) by way of an inhibitory GABAergic axon that terminates in the BNST (Canteras, Simerly, & Watson 1995; Dunn, 1987; Herman, Cullinan, & Watson, 1994; Prewitt & Herman, 1998) and an inhibitory GABAergic signal from the BNST to the mpPVN (Dunn, 1987; Herman et al., 1994). In sum, the MeA appears to activate the HPA axis as a result of a two-neuron sequential GABAergic circuit (Dong, Petrovich, & Swanson, 2001; Cullinan, Helmreich, & Watson 1996; Cullinan, Herman & Watson, 1993; Swanson & Petrovich, 1998) resulting in disinhibition of the mpPVN by removal of GABAergic tone (Herman et al., 1997; Cullinan, Ziegler, & Herman, 2008; Prewitt & Herman, 1998), resulting in release of CRF from the median eminence (Cullinan et al., 1995; Ju & Simerly, 1989; Ju & Swanson, 1989; Ziegler & Herman, 2002). The MeA-BNST-PVN sequential GABAergic circuit (Cullinan et al., 2008; Herman et al., 2003, 2005; Feldman, Conforti, and Saphier, 1990) appears to be involved in regulating HPA activity following novelty,
swim, restraint, but not IL-1β injection (Cullinan et al., 2008; Herman et al., 2003, 2005; Xu et al., 1999). There is evidence to support the role of the CeA-BNST-PVN circuit involvement in regulating HPA activity following IL-1β injection and hemorrhage, but not novelty, swim, restraint, or footshock (Cullinan et al., 2008; Herman et al., 2003, 2005; Cullinan, Hemreich, & Watson, 1996; Day & Akil, 1996; Sawchenko et al., 1996).

Relevance for Health

Neuropsychiatric diseases such as major depression and anxiety disorders have been linked to dysfunction in the amygdala and HPA axis (Arborelius, Owens, Plotsky, & Nemeroff, 1999; Drevets, 1992, 2000; Imaki et al., 1996). The amygdala and the BNST have been shown to modulate HPA activity particularly during exposure to some psychological stressors. The psychological stressor of early separation from an attachment object and other forms of disruption of attachment relations may be the most cited predisposing factors for later-life depression and anxiety (Agid et al., 1999; Brown, Harris & Copeland, 1977; Gillespie, & Nemeroff, 2007; Gilmer, & McKinney, 2003; Heim, & Nemeroff, 1999; Takeuchi, et al., 2002). Yet, the specific brain regions that control HPA activity during attachment object separation has received little experimental attention. The opportunity to study brief separation from an attachment figure in a relevant animal model and the resulting impact on central activity may provide information that could lead to a better understanding of neuropsychiatric diseases in humans. Specifically, the study of central activation during brief exposure to attachment object separation is a critical beginning point in the understanding of how subsequent chronic dysregulation of these circuits can have long-term detrimental consequences for health, potentially leading to disease.
Summary and Integration

HPA activation is a prominent response to early separation from an attachment object. Pituitary-adrenal activity has been extensively studied but changes in central circuitry specific to separation from an attachment object have not been demonstrated. Studies of the effects of other psychological stressors in rats suggest the circuitry that may be involved in the HPA responses during separation from an attachment object. Based upon these studies we would expect to see changes in the anterior cingulate mPFC, MeA, BNST, and mpPVN. To begin to study these questions we limited our investigation to the amygdala and BNST, two regions most proximal to the PVN.

Nearly all of the earlier work on the neural circuitry regulating the HPA axis was performed on rats and mice. The present study provides an opportunity to examine this circuitry in a distantly related rodent. The guinea pig is an excellent rodent model to study central activity during separation from an attachment object. In particular, infants show robust elevations in pituitary-adrenal activity when briefly separated from their mother in a novel environment. However, these changes are not observed if the mother is present with the infant in a novel environment. Similarly, separation effects do not occur if the pup remains in the familiar home environment. Thus, manipulations of the social and physical environment were used in the current experiments as a means of testing predictions about changes in central activity that specifically regulate HPA activity during separations.

The current experiments were designed to further our understanding of the neural mechanisms involved in regulating pituitary-adrenal activity during separation from an attachment object, exposure to a novel environment, and exposure to both separation and...
a novel environment. These two psychological stressors are each a potent stimulus for HPA activation in certain circumstances but may involve similar or different neural circuitry. I designed the experiments to tease apart the influence of these two psychological stressors on neural activity. In Experiment 1, I examined levels of CRF mRNA in the PVN and the time course of any activation of this response in infants during maternal separation in a novel environment. I did this in order to determine if it was possible to observe increased levels of CRF mRNA in the condition that produces the greatest pituitary-adrenal response. And further, if I was able to observe changes in levels, I wanted to determine the time course of its activation to inform subsequent experiments. Next, Experiments 2 and 3 were designed to analyze the effects of novelty without maternal separation, maternal separation without novelty, and the combined effects of novelty and separation. In Experiment 2, I measured output at each level of the HPA system. I examined levels of CRF mRNA in the PVN when the mother was either present or absent and the pup was either in the home cage or novel environment. These manipulations were designed to determine whether the mother and the familiar environment would inhibit CRF mRNA activity in a manner that would parallel pituitary-adrenal activity observed during these procedures. In addition to looking at CRF mRNA in the PVN, I measured plasma ACTH and cortisol as indicators of pituitary-adrenal activity. In Experiment 3, I investigated c-Fos protein levels in the MeA, BNST and PVN in the same animals used in Experiment 2. The purpose of this experiment was to investigate and distinguish the effects of separation and novelty on central activity in brain regions that may modulate HPA activity based upon data from other studies emphasizing psychological stressors.
II. General Methods

Subjects and Housing Conditions

Infant male guinea pigs (Cavia porcellus) of the Hartley strain, were gleaned from guinea pigs bred in our laboratory. No more than one male from a litter served as a subject in a condition. Males were housed in opaque plastic cages (73 cm X 54 cm X 24 cm) with their mothers and siblings until the day of testing on postnatal day 16 (+/-1). The colony room was maintained on a 12-hr light-dark cycle (lights on at 0700) and food and water were available ad libitum. All procedures were approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC).

Blood Sampling and Hormone Determination

Blood samples were collected by rapid decapitation immediately following removal from the home cage (home cage controls) or the test cage between 1100h and 1400h in order to minimize circadian variation. Trunk blood was collected on heparin for later cortisol and adrenocorticotropin (ACTH) analysis. Plasma was separated by centrifugation and frozen (-80°C) until assayed with standard radioimmunoassay kits.

Brain Tissue Collection

In preparation for cryostat sectioning procedures, brains were removed from the skull following decapitation within 5 min, frozen in isopentane at -30°C to -40°C for 45 seconds and stored at -80°C.

Radioimmunoassay (RIA)
Blood samples were centrifuged for 20 minutes at 3200 rpm at 4 °C. Plasma was then transferred to 1ml Eppendorf tubes and stored at -80 °C until assayed.

**ACTH.** Plasma ACTH levels were measured using ACTH RIA kits (Pheonix Pharmaceuticals, Inc., Burlingame, CA, USA) with \(^{[125]}\)I human ACTH. The ACTH antibody cross-reacts 100% with human ACTH\(_{1-39}\), human ACTH\(_{1-24}\) and rat ACTH; 31% with human ACTH\(_{7-38}\); 1% \(\beta\)-endorphin; but not with human or rat corticotropin-releasing-factor (CRF), \(\alpha\)-melanocyte-stimulating hormone (\(\alpha\)-MSH), human or canine \(\alpha\)-atrial natriuretic peptide (\(\alpha\)-ANP\(_{1-28}\)), human B-type natriuretic peptide (BNP-31), or Met-Enkephalin. The minimum detectable ACTH concentration was 10 pg/ml and the intra-assay coefficient of variation was 10%.

**Cortisol.** Plasma cortisol levels were measured using the Coat-A-Count Cortisol RIA kit (Siemens Diagnostics, California, USA) with \(^{[125]}\)I human cortisol. The cortisol antiserum is highly specific for cortisol and cross-reacts 100% with hydrocortisone (cortisol), 0.26% with deoxycorticosterone, 0.02% with progesterone, 0.03% with aldosterone, 0.94% with corticosterone, and 0.98% with cortisone. The minimum detectable cortisol concentration was 0.2 \(\mu\)g/dL and the intra-assay coefficient of variation was 6.4%.

**In situ Hybridization**

Levels of CRF mRNA were determined by using an in situ hybridization technique that was performed in the laboratory of Dr. Joanne Weinberg at the University of British Columbia, Vancouver, BC, Canada.

**Brain preparation.** Brains were sectioned coronally in 14\(\mu\)m sections. Frozen sections were thaw-mounted onto gelatin-coated slides (Gold Seal UltraStick micro
adhesion slides: VWR international, LLC Catalog #: 62404-539) and stored at -80 ºC. One series of slides for each animal was stained with Cresyl Violet and coverslips were mounted with Permount (Fisher Scientific Ltd., ON, Canada) in order to accurately locate the brain area of interest to be included in the in situ hybridization procedure. Cresyl violet stained sections were observed from a Q-imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope.

*Oligonucleotide probes and labeling.* Oligonucleotide probes were used to measure CRF mRNA in the PVN. Oligonucleotide probes were synthesized at integrated DNA Technologies, Inc., as follows: antisense CRF (5’-ACT GCT CGG CCC TGG CCA TCT CCA AGA CTT CCC GGA GAA GGT GGA-3’) (Go, Lingas, Wheeler, Irwin, & Matthews, 2001) and CRF sense (5’-TCC ACC TTC TCC GGG AAG TCT TGG AGA TGG CCA GGG CCG AGC AGT-3’) (Go, K.S., et al. 2001) was used as negative control. Probes were 3’ tail labeled with 33P-dATP (Perkin Elmer, Massachusetts, USA) using terminal deoxytransferase (Promega, Wisconsin, USA) as per supplier protocol. Probes were purified using Roche DNA G-25 Sephadex Columns (Roche Scientific, IN, USA)

Sections were thawed (30 min) and went through prehybridization as follows: 3.7% paraformaldehyde (20 min), 1 x PBS (10 min) twice, 0.1M triethanolamine-hydrochloride (TEA)+ 0.25 % acetic anhydride (AA) (10 min), 2 x SSC (5 min), dehydrated through a graded series of ethanol, chloroform (5 min) followed by 100% ethanol, and then air dried (60 min). Probes were diluted (probe activity for CRF: 500,000 cpm/section) in 50% hybridization buffer (DEPC water, 10% dextran sulphate, Superpure formamide, 20 x SSC, 50x Denhardt’s solution, 25 mg/ml yeast tRNA, 1M
sodium phosphate buffer (pH 7.4) and 200 µl of aliquoted solution was applied to each slide and covered with hybrislips (Sigma-Aldrich Canada Ltd., ON, Canada). Sections were incubated at 42 °C in 50% formamide humidified containers. Hybrislips were removed and slides were washed in 2 x SSC (30min) twice, 2 x SSC (40 °C. 15 min), 1 x SSC (40 °C, 15 min), 1 x SSC/50% formamide (40°C, 30 min), 1 x SSC (Room temperature, 10 min), 0.5 x SSC (Room temperature, 10 min). Sections were dipped briefly in distilled water five times then plunged into 75% ethanol (agitated for 5 min), then air dried overnight. Sections for CRF were exposed to Kodak Biomax MR film (Eastman Kodak Co., New York, USA) for 6 weeks for Experiment 1 and for 6.5 weeks for Experiment 2. Slides were developed with Kodak D-19 developer and fixed with Polymax T fixer.

_Densitometric analysis._ Images were captured from in situ hybridization autoradiographs by using Epson Perfection 1650 scanner and Photoshop 7.0 software. Semiquantitative densometric analyses were performed using Scion Image for Windows version Beta 4.0.4 (Scion, Frederick, MD). A selection of slides, typically every tenth slide (containing 4 sections of tissue) was selected to be processed by in situ hybridization to obtain a representation for the region of interest. Once processed, the 2 sections that showed the largest area of the PVN, were analyzed by obtaining the mean optical density (OD) as determined by Scion Image. Signal was measured by outlining clusters of labeled neurons and measuring the mean OD. The average of the 4 readings/animal (2 from the left half and 2 from the right half of the brain) were averaged to represent the total CRF mRNA levels of each animal. Background density readings were obtained from areas immediately adjacent to the areas of interest, and were
subtracted from the density readings to determine the final mRNA expressions. All measured signals were analyzed to determine to be within linear range of detectability, as measured by calibration curves from ARC 146-14C standards (American Radiolabeled Chemicals, Missouri).

**Immunofluorescence**

The immunofluorescence technique was performed in the laboratory of Dr. David Cool at Wright State University, Dayton, Ohio, USA. The density of c-Fos immunoreactive cells was determined using the immunofluorescence technique that allows for the visualization and quantitative measure of cellular proteins in brain tissue. This technique relies upon the use of two antibodies: a primary antibody used to recognize the c-Fos protein in the tissue and an immunofluorescence-conjugated secondary antibody that recognizes the primary antibody. The primary and secondary antibodies used in this procedure were made from two different species: rabbit polyclonal and donkey anti-rabbit, respectively.

Immunofluorescence labeling was performed at room temperature. The slides (Gold Seal) containing the tissue sections were removed from the -80 ºC freezer and allowed to thaw for 30 min. A pap pen (Invitrogen catalog #008899) was used to encircle the sections on each slide and allowed to dry for 15 minutes. Solutions were applied to and removed from the encircled sections with a pipette during each step in the procedure. Slides were placed into a humidifier chamber (large plastic container with bench paper saturated with dd water and a lid on top) and washed in 1X phosphate buffered saline (PBS)/.2% Triton X 100 (TX) for 1 hr. Slides were incubated in normal goat serum (1:10 PBS/TX) for one hour to minimize non-specific binding. Subsequently,
all sections were incubated with a primary antibody for c-Fos (1:50; rabbit polyclonal IgG Santa Cruz Biotechnology, Inc, California, USA) for one hour (except for one slide incubated in normal goat serum that was used as a negative control) and then washed three times (5 min each plus an additional 10 min) in PBS. Sections were then incubated with Cy2 immunofluorescence-conjugated secondary antibody (1:750; Alexa fluor 488 donkey anti-rabbit IgG Invitrogen catalog # A21206) for one hour. The sections were washed 3 times (5 min plus 10 min) in PBS and then cover slipped with Prolong Gold Antifade Reagent (Invitrogen Catalog# P36651A).

Cell quantification. Immunofluorescent stained tissue was visualized using an Olympus BX51 fluorescent microscope, and images were captured using a RT Slider Spot camera (Diagnostic Instruments Inc.). The density of c-Fos immunoreactive cells was quantified using Image-Pro Plus 5.1 software program. A cell was considered to contain c-Fos if its nucleus contained characteristic staining and could be distinguished clearly from background. Brain regions were determined by extrapolation from the guinea pig atlas of Luparillo (1967) for the MeA, BNST and PVN (Fig.1). Coronal sections for each animal from comparable rostrocaudal levels (one section per animal for each brain region) were chosen for counting. Each left and right side was individually counted and then averaged. Such counting was repeated twice and a mean value obtained that represented the cell density for each animal.

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Figure 1 about here

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III. Experiment 1: CRF mRNA Expression in the PVN

Method

Experiment 1 comprised of subjects divided among six conditions. There was a home cage control group, and groups separated in the novel environment for either 15 min, 30 min, 60 min, 120 min, or 240 min. Animals were transported in a carrying cage from the colony room to the adjoining test room (~10sec). The subject was placed in a clear, empty, plastic cage (47 x 24 x 20 cm) located on a table under full room lighting that served as the novel environment test chamber. A clear plastic lid was placed over the cage to prevent animals from escaping the test chamber. The cage and lid were cleaned with detergent prior to each use. Upon termination of the test the infant was transported (<10s) to a lab bench and the brain was removed (< 5 min) for later analysis of CRF mRNA levels.

Data were expressed as means +/- SEM and were analyzed by a one-way analysis of variance (ANOVA). Student Newman Keuls tests were used to analyze all possible pair-wise comparisons.

Results

To determine the peak response of CRF mRNA in the PVN when animals were exposed to a novel environment alone, levels were measured in the Undisturbed Home Cage Control condition or at 15, 30, 60, 120 or 240 min of exposure to the novel environment. The peak CRF mRNA response occurred at the 120 min time point. A one-way analysis of variance (ANOVA) yielded a significant main effect; $F(5, 17) = 3.44$, $p <$
0.05. Student Newman-Keuls paired comparisons revealed that CRF mRNA was greater in the 120 min condition than in the home cage control and 15, 30 and 60 min conditions (Fig. 2), and no other comparisons were significant. Examples of coronal sections illustrating the conditions are shown in Fig. 3. Accordingly, I chose the 120 min time point to serve as the time point of exposure to the social and environmental conditions in Experiment 2.
IV. Experiment 2. Plasma Cortisol and ACTH Responses and CRF mRNA Expression in the PVN during Exposure to the Social and Environmental Manipulations.

Method

Experiment 2 comprised of subjects divided among five conditions including an Undisturbed Home Cage Control condition, and four disturbance conditions comprising a 2 (Separation, Mother) x 2 (Home, Novel) between-subjects design. In these four disturbance conditions both the mother and the pup were removed from the home cage for ~10 sec. They were then either returned to the home cage for two hours (Mother/Home), or the pup was returned to the home cage without the mother for the two hours (Separation/Home), the pup and the mother were both placed in the novel environment test chamber for two hours (Mother/Novel), or the pup was placed in the novel environment alone for two hours and the mother returned to the home cage (Separation/Novel). All littermates in the Home conditions, and mother and littermates in the Separation conditions, were removed from the home cage and placed in a room out of auditory range during these procedures. At the termination of the two-hour test, pups were then quickly decapitated, blood samples obtained, and brains rapidly removed and frozen, for later analysis of cortisol, ACTH and CRF mRNA levels.

A 2 x 2 ANOVA was performed to examine the main and interactive effects of novelty and separation. Because of heterogeneity of variance, cortisol data were subjected to a square root transformation in order to equate variances; however, raw data are presented in the figures. Student-Newman-Keuls tests were used to follow-up
significant interactions of the ANOVA. Next, a one-way ANOVA followed by Dunnett’s procedure for comparison to a control was used to determine which of these four conditions involving disturbance (Mother/Home, Mother/Novel, Separation/Home, Separation/Novel) elevated levels of cortisol, ACTH or CRF mRNA over baseline levels.

Results

Cortisol. A 2 (Separation) x 2 (Novelty) ANOVA yielded significant effects for Separation, $F(1,36) = 11.94, p < 0.01$, and for Novelty, $F(1,36) = 17.02, p < 0.01$. Plasma cortisol levels were greater in the Separation conditions than the Mother conditions and greater in the Novelty conditions than in the Home conditions. However, these main effects were qualified by a Separation X Novelty interaction, $F(1,36) = 21.79$, $p < 0.01$. A Student-Newman-Keuls test revealed that cortisol was greater in the Separation/Novel condition than in any other disturbance condition ($p’s < 0.01$) and no differences were found between the other pairs of conditions. Plasma cortisol levels were increased only when infants were separated in the novel not the home environment, indicating that the effects of separation was dependent upon novelty. To determine which individual conditions produced increases over resting levels, a one-way ANOVA for cortisol yielded a significant main effect of Condition: $F(4,45) = 16.10, p < 0.01$. Dunnett’s test revealed that cortisol levels were greater in the Separation/Novel condition ($p < 0.01$) than in the Undisturbed Home Cage Control condition (Fig. 4) and no other comparison to the Undisturbed Home Cage Control was significant.
**ACTH.** A 2 (Separation) x 2 (Novelty) ANOVA yielded a significant effect for Separation, \(F(1,33) = 8.11, p < 0.01\). Plasma ACTH was greater in the Separation conditions than in the Mother conditions. However, these main effects were qualified by a Separation X Novelty interaction, \(F(1,33) = 3.98, p = 0.05\). A Student-Newman-Keuls test revealed that ACTH was greater in the Separation/Novel condition than in the Mother/Novel \((p < 0.05)\), Separation/Home \((p < 0.05)\), and the Mother/Home \((p < 0.01)\) conditions. No differences were found between the other pairs of conditions. Plasma ACTH levels were increased only when infants were separated in the novel not the home environment, indicating that the effects of separation were dependent upon novelty. A one-way ANOVA for plasma ACTH yielded a significant effect: \(F(4, 41)= 4.68, p < 0.01\). Dunnett’s test revealed that ACTH was greater in the Separation/Novel condition \((p < 0.01)\) than the Undisturbed Home Cage Control condition and no other comparisons to the Undisturbed Home Cage Control were significant (Fig. 5).

**PVN CRF mRNA.** A 2 (Separation) x 2 (Novelty) ANOVA yielded significant effects for Separation, \(F(1,22) = 26.69, p < 0.01\), and for Novelty, \(F(1,22) = 21.10, p < 0.01\). PVN CRF mRNA was greater in the Separation conditions than the Mother conditions and greater in the Novelty conditions than the Home conditions. However, these main effects were qualified by a Separation X Novelty interaction, \(F(1,22) = 6.98, p < 0.05\). A Student-Newman-Keuls test revealed that CRF mRNA was greater in the
Separation/Novel condition than in any other disturbance condition (p’s < 0.01) and no differences were found between the other pairs of conditions. PVN CRF mRNA levels were increased only when infants were separated in the novel not the home environment, indicating that the effects of separation was dependent upon novelty. A one-way ANOVA for PVN CRF mRNA yielded a significant main effect, $F(4, 28) = 13.16$, $p < 0.01$. Dunnett’s test revealed that CRF mRNA was greater in the Separation/Novel condition ($p < 0.01$) than the Undisturbed Home Cage Control condition and no other comparisons to the Undisturbed Home Cage Control was significant (Fig.6). An example coronal section from one subject in each condition can be seen in Figure 7, reflecting that the animals in the Separation/Novel condition exhibited the greatest expression of CRF mRNA in comparison to the other groups.

Figures 6 and 7 about here
V. Experiment 3: Density of c-Fos immunoreactive Cells in the MeA, BNST and PVN, during Exposure to the Social and Environmental Manipulations.

Method

Experiment 3 used the same subjects and conditions as Experiment 2. Brain tissue for the immunofluorescence procedure used here and the in situ procedure of Experiment 2 were obtained from sequential alternating sections.

A 2 x 2 ANOVA was performed to examine main and interactive effects of novelty and separation. Next, a one-way ANOVA followed by Dunnetts procedure for comparison to a control was used to determine which of these four conditions involving disturbance (Mother/Home, Mother/Novel, Separation/Home, Separation/Novel) increased the density of c-Fos immunoreactive cells over baseline levels.

Results

MeA c-Fos protein. A 2 (Separation) x 2 (Novelty) ANOVA yielded a significant main effect for Novelty, $F(1,21) = 10.11, p < 0.01$. The density of c-Fos immunoreactive cells was greater in the Novelty conditions than the Home conditions (Fig. 8). No other effects were significant. A one-way ANOVA yielded a main effect, $F(4,27) = 3.78, p < 0.05$. A Dunnett’s test revealed that the density of c-Fos immunoreactive cells was greater in the Separation/Novel condition than in the Undisturbed Home Cage Control condition ($p < 0.05$) and no other comparisons to the Undisturbed Home Cage Control were significant (Fig.9). Representative photomicrographs of each of the conditions can be seen in Figure 10.
**BNST c-Fos protein.** A 2 (Separation) x 2 (Novelty) ANOVA yielded a significant main effect for Novelty, $F(1,19) = 5.27$, $p < 0.05$. The density of c-Fos immunoreactive cells was less in the Novelty conditions than the Home conditions (Fig. 11). No other effects were significant. A one-way ANOVA yielded a significant main effect, $F(4,25)=5.235$, $p < 0.01$. A Dunnett’s test revealed that the density of c-Fos immunoreactive cells was less in the Mother/Novel ($p < 0.05$) and the Separation/Novel ($p < 0.01$) conditions in comparison to the Undisturbed Home Cage Control and no other comparisons were significant (Fig. 12). Representative photomicrographs of each of the conditions can be seen in Figure 13.

**PVN c-Fos protein.** A 2 (Separation) x 2 (Novelty) ANOVA yielded a significant effect for Separation, $F(1,21) = 22.53$, $p <0.01$. No other effects were significant. The density of c-Fos immunoreactive cells was greater in the Separation conditions than the Mother conditions (Fig.14). A one-way ANOVA yielded a significant main effect, $F(4,27) = 10.39$, $p < 0.01$. A Dunnett’s test revealed that the density of c-Fos immunoreactive cells was greater in the Separation/Home ($p < 0.01$) and the Separation/Novel ($p <0.01$) conditions than in the Undisturbed Home Cage Control.
condition and no other comparisons were significant (Fig. 15). Representative photomicrographs of each of the conditions can be seen in Figure 16.

Figures 14, 15 and 16 about here
VI. Discussion

Studies using the domestic guinea pig have shown this species to be an interesting and effective animal model to study attachment-like bonds and social buffering effects during infancy. Infants show patterns of pituitary-adrenal responses during separations from an attachment figure and buffering effects when a partner is present not unlike what has been observed in non-human primates. Pituitary-adrenal activity is enhanced during maternal separation and attenuated when the maternal attachment figure is present during exposure to a psychological stressor. Pituitary-adrenal activity has been extensively studied during conditions of separation and novelty but little is known about the neural mechanisms involved in regulating activity during these events. In the current study, I investigated the independent and combined effects of maternal separation and exposure to novelty on HPA and neural activity in the MeA, BNST, and PVN. I examined CRF mRNA in the PVN to detect the activity of CRF neurons in this region of the hypothalamus. In the rat, the MeA and BNST are two regions thought to drive activity in the PVN during exposure to psychological stressors. I investigated c-Fos activity in these brain regions to examine whether they also regulated PVN activity in the guinea pig during exposure to the social and environmental manipulations of my study. This is the first experiment in which the independent and combined effects of separation and novelty on any central measure have been investigated in the guinea pig. It is also the first time that the activity in the MeA and BNST have been examined in response to psychological
stressors in this species, and the first time that CRF activity in the PVN has been confirmed under the conditions of separation and novelty in guinea pig pups.

When infant males were separated from their mothers in the novel environment for two hours they showed elevated pituitary-adrenal activity consistent with what has been found previously (Hennessy & Moorman, 1989). Plasma cortisol levels increased when infants were separated in the novel but not in the familiar environment. Cues from the home environment prevented an adrenal response to separation. Further, no increases in cortisol were found when the mother was with the pup in the novel environment. The presence of the mother in a novel environment resulted in cortisol levels that were no different than the levels of cortisol when the infant was with the mother in the familiar environment or basal cortisol levels in the undisturbed control condition. This is noteworthy because novelty is one of the most reliable stimuli for activation of the pituitary-adrenal axis. ACTH shows a shorter time course of activity compared to the time course of cortisol. When infant guinea pigs were exposed to the combined stressors of separation and novelty in a different study, levels of plasma ACTH reached a high of about 90 pg/ml at thirty minutes (Hennessy, Tamborski, Schiml, & Lucot, 1989). In our present study, plasma levels were found to reach a high of about 20 pg/ml at two hours in the Separation/Novel condition. Because plasma ACTH elevations typically peak and decline before cortisol elevations, the lower levels here were to be expected. Although overall levels of ACTH were reduced for all conditions at two hours, there still was a significant increase when the infant was separated in the novel environment, but no increase in the other conditions as compared to basal levels.
As predicted, we were able to observe changes in PVN CRF mRNA activity when infants were separated from their mothers in the novel environment. The time course of CRF mRNA activity showed that the largest activation occurred at two hours. Peak levels of CRF mRNA have been reported to occur between two and four hours in rats (Dent, Okimoto, Smith, & Levine, 2000) and our results are consistent with these findings. Increased levels of CRF mRNA most likely indicate that additional CRF peptide was being made to compensate for the losses (Dallman, 2000) due to release during the experimental manipulation.

Following two hours of exposure to the experimental conditions, CRF mRNA activity in the PVN paralleled the activity of the pituitary-adrenal hormones. We found the greatest activation of CRF mRNA in the Separation/Novel condition at the same time we observed increased levels of cortisol and ACTH. Therefore, the increase in CRF mRNA activity at this time is sufficient to explain the patterns of cortisol and ACTH without any involvement of AVP. If levels of CRF mRNA had been the same when the infant was separated in either the familiar or novel environment, but cortisol levels had been greater in the novel condition, then it would be likely that AVP was involved in potentiating CRF release, but that is not what I found. It has been reported that it is during exposure to repeated psychological stressors, not acute stressors (Wotjak, Kubota, Liebsch, Montkowski, Holsboer, Neumann, & Landgraf, 1996), that AVP is most often co-released with CRF to stimulate release of stored ACTH (Rivier & Vale, 1983). The release of AVP enhances CRF’s effect on ACTH release (Antoni, Holmes, Makara, Karteszi, & Laszlo, 1984; Plotsky, 1988) and it is thought to occur by upregulation of the CRF1 receptor in the pituitary (Aguilera, Rabadan-Diehl, & Nikodemova, 2001).
summary, separation did not have an effect when the infant was in the familiar environment, and novelty did not have an effect when the infant was with the mother, on CRF mRNA levels in the PVN. The familiar environment and the presence of the mother appeared to completely buffer the infant’s HPA response to separation and novelty, respectively.

C-Fos protein is an inducible transcription factor that is a marker of increased cellular activity. C-Fos activity was observed in this study in order to measure activation in the brain regions of interest. The onset of the appropriate stimulus will cause c-Fos protein levels to accumulate in the cell nucleus. When increased levels are detected, it is an indication that the specific distribution of neurons has been activated by the experimental manipulation. Levels typically peak at several hours after the onset of a stimulus (Morgan, Cohen, Hempstead, & Curran, 1987; Sonnenberg, Mitchelmore, Macgregor-Leon, Hempstead, Morgan & Curran, 1989) and brain tissue is usually collected at two to three hours (Kaczmarek, & Robertson, 2002). Exposure to the experimental conditions at the two hour time point in the current study then appears to have been an appropriate time point. Measurements of c-Fos activity have been useful in delineating the circuitry involved in regulating HPA activity in other species. We observed c-Fos activity in the brains of the same subjects that we used to measure CRF mRNA in the PVN.

The density of c-Fos immunoreactive cells was measured in the MeA and BNST to measure input to the PVN. In the MeA, there were more c-Fos immunoreactive cells in the Mother/Novel and the Separation/Novel conditions relative to the Mother/Home and Separation/Home conditions, indicating that it was exposure to the novel
environment that primarily stimulated cellular activity in the MeA regardless of the mother’s presence. Although the Mother/Novel and the Separation/Novel conditions did not statistically differ, the density of c-Fos immunoreactive cells was still somewhat greater in the Separation/Novel condition. Further, when comparing each of the four experimental conditions to the Undisturbed Home Cage Control, c-Fos activity was significantly increased only in the Separation/Novel condition, indicating that it was exposure to the combination of both separation and novelty that was necessary to stimulate more cellular activity in the MeA. Thus while overall it was novelty rather than separation that drove activity in the MeA, separation still appeared to enhance novelty’s effect.

In the BNST there were significantly fewer c-Fos immunoreactive cells in the Mother/Novel and the Separation/Novel conditions relative to the Mother/Home and the Separation/Home conditions, signifying that it was novelty, not separation, that affected the BNST, though in the opposite direction than in the MeA. When comparing each of the four experimental conditions to the Undisturbed Home Cage Control, the BNST contained significantly fewer c-Fos immunoreactive cells in the Mother/Novel and the Separation/Novel conditions than the Undisturbed Home Cage Control, indicating that cellular activity in the BNST was inhibited relative to basal levels. There was no difference in cellular activity found between the Undisturbed Home Cage Control and either the Mother/Home or the Separation/Home conditions. Novelty was key in the effects on the BNST like it was in the MeA. The results suggest that the MeA sends inhibitory projections to the BNST in the guinea pig, and these neurons are activated by novelty.
The density of c-Fos immunoreactive cells was measured in the PVN in order to correlate with cellular activity in the MeA and BNST during exposure to the experimental conditions. c-Fos activity was increased in the Separation/Novel condition but not in the Mother/Novel condition. In the Separation/Novel condition, activity in the MeA and BNST would lead us to expect an increase in c-Fos activity in the PVN, and the results confirmed our prediction. However, there was no corresponding increase in c-Fos activity in the Mother/Novel condition. This finding was not as would be expected based upon the MeA and BNST results and past research with rats. If in the guinea pig, as in the rat, the MeA-BNST circuit drives PVN CRF activity, there seems to be two general possibilities as to why no c-Fos activity occurred in the PVN in the Mother/Novel condition. One possibility is active inhibition of HPA activity. There are other regions of the brain that have been observed to inhibit HPA activity during exposure to psychological stressors in rats. There is evidence that higher level supralimbic regions of the brain including the mPFC, and ventral subiculum inhibit HPA activity. Glutamatergic efferents from the ventral subiculum appear to connect with the GABAergic neurons of the perinuclear PVN region that directly inhibit release of PVN CRF. Similarly, the mPFC appears to inhibit HPA activity during exposure to psychological stressors (Figueiredo, Bruestle, Bodie, Dolgas, & Herman, 2003) through glutamatergic efferent projections to GABAergic neurons in the peri-PVN region (Herman et al., 2005; Sesack, Deutch, Roth, & Bunney, 1989; Hurley, Herbert, Moga, & Saper, 1991) that project to the PVN to directly inhibit CRF release. In the Mother/Novel condition of our study, we observed an increase in c-Fos activity in the MeA but no corresponding increase in HPA activity that would be predicted if the HPA axis was regulated solely by activity in the
MeA-BNST-PVN circuit. It may be that the signal from the MeA was inhibited before it reached the PVN, or inhibited at the level of the PVN because there was no corresponding increase in c-Fos activity in the PVN in this condition. Stimuli associated with the presence of the mother may have activated the mPFC and/or ventral subiculum to inhibit HPA activity during the Mother/Novel condition in the present study.

A second possible reason for the lack of c-Fos activity in the PVN in the Mother/Novel condition is that MeA-BNST activity may be necessary but not sufficient to drive HPA activity in these conditions. That is, another excitatory input may have combined with input from the MeA-BNST circuit to drive PVN CRF activity in the Separation/Novel but not in the Mother/Novel condition. One possibility is NE input to the hypothalamus originating from the A2 cell group in the nucleus of the solitary tract region. In infant rats, the presence of the mother was sufficient to buffer the corticosterone response to shock exposure and the corticosterone response was correlated with NE input from the A2 group to the PVN (Shionoya, Moriceau, Bradstock & Sullivan, 2007). Further, in a previous study in our lab, when infant guinea pigs were separated in a novel environment, NE turnover was increased in the anterior hypothalamus and was correlated with increased levels of plasma cortisol (Hennessy, et al., 1994). It is possible that NE may have played a role in increasing HPA activity in the Separation/Novel condition in the current study.

In the Mother/Home condition, as in the Separation/Novel condition, c-Fos activity in the PVN confirmed our prediction. That is, there was no change over basal level in cellular activity in MeA, BNST or in any of the measures of the HPA axis. The correlation between c-Fos activity in the PVN and c-Fos activity in the MeA and BNST
is consistent with the idea that the MeA-BNST circuit may be involved in regulating PVN activity during exposure to this experimental condition. In the Separation/Novel and the Mother/Home conditions then, the putative MeA-BNST-PVN two neuron GABAergic circuit that may regulate HPA activity is sufficient to explain the pattern of HPA activity in these two conditions.

In addition to increased PVN c-Fos activity in the Separation/Novel condition there was also increased activity in the Separation/Home condition. The activity in the Separation/Home condition was not predicted based upon activity in the MeA-BNST circuit in this condition. CRF activity may account for all or most of the activity in the Separation/Novel condition but it appears that some other cell population was activated in the Separation/Home condition. In addition to CRF, there are a number of other neurotransmitter cell populations in the PVN including AVP, angiotensin II, cholecystokinin, neurotensin, GABA, enkephalin and oxytocin (Chadwick, & Marsh, 1992). Of the possibilities, oxytocin seems to be the most likely candidate for the identity of the c-Fos cells that increased in the Separation/Home condition. Other studies have shown that oxytocin activity increases in response to some social stimuli and some stressors. In other rodents, release of oxytocin in the PVN is related to affiliative behavior (Insel, 1992). For example, when oxytocin was injected into the PVN in infant rats, the vocalization response that pups emitted when separated from their mothers was selectively decreased, an effect that mimicks the presence of the maternal attachment figure (Insel, 1992). Interestingly, when infant guinea pigs are separated from their mothers, relatively few vocalizations occur if pups are left in the home environment (Hennessy & Moorman, 1989). In our study, when the infant was separated in the
familiar environment, it is possible that cues from this environment (i.e. maternal odor) may have caused an increase in activity of oxytocin neurons that might account for the increase in c-Fos activity.

The circuitry mediating HPA activity has not previously been examined in the guinea pig. The results from our study indicate that separation and novelty have independent and combined effects in regions of the guinea pig CNS that regulate pituitary-adrenal activity. There were effects due only to separation; others due only to novelty; and some due to both. The results provide evidence the MeA-BNST pathway exists in the guinea pig like in the rat, but that other factors also appear to be involved. While the results of the current study certainly suggest a role for the MeA-BNST circuit in regulation of HPA activity, more work needs to be done to provide direct experimental evidence for this connection. One possibility would be to temporarily inactivate the MeA with the GABA<sub>A</sub> receptor agonist muscimol and observe the effects at each level of the HPA axis. A closer investigation of the circuitry involved in regulating HPA activity during the conditions of the present study is a goal for future research.
Fig. 1. Areas in which the c-Fos labeled cell nuclei were counted. PVN, MeA, and BNST located at approximately 9.8 mm, 9.8 mm and 11.0mm anterior to the interaural axis, respectively.
Fig. 2. Semi-quantitative measurements of CRF mRNA hybridization signal in the PVN of male infants exposed to a novel environment alone. Vertical lines represent standard error of the mean corrected gray level measured from autoradiographic images. * p < 0.05 compared to the 120 min condition; n= 3-4 animals per group.
Fig. 3. CRF mRNA expression in the hypothalamic paraventricular nucleus (PVN) of infant males. Autoradiographic images showing CRF mRNA hybridization signal in the PVN of one representative animal in each of six conditions in Experiment 1: Undisturbed Home Cage Control (A), and exposure the novel environment for 15 (B), 30 (C), 60 (D), 120 (E), or 240 (F) min.
Fig. 4. Mean plasma cortisol levels of infant males in each of five conditions in Experiment 2: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard errors of the means. ** p < 0.01 compared to the SN condition; n= 10 animals per group.
Fig. 5. Mean plasma ACTH levels of infant males in each of five conditions in Experiment 2: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard errors of the means. *p < 0.05 and ** p < 0.01 compared to the SN condition; n= 8-10 animals per group.
Fig. 6. Semi-quantitative measurements of CRF mRNA hybridization signal in the PVN of male infants in each of five conditions in Experiment 2: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard errors of the means. ** p < 0.01 compared to the SN condition; n= 6-7 animals per group.
Fig. 7. CRF mRNA expression in the hypothalamic paraventricular nucleus (PVN) of infant males. Autoradiographic images showing CRF mRNA hybridization signal in the PVN of one representative animal in each of five conditions in Experiment 2: HC (A), MH (B), MN (C), SH (D), SN (E).
Fig. 8. Mean density of c-Fos cells in the MeA in the combined Novel conditions (Separation/Novel and Mother/Novel) and the combined Home conditions (Separation/Home and Mother/Home). Vertical lines represent standard errors of the means. ** p < 0.01; n= 12-13 animals per group.
Fig. 9. Mean density of c-Fos cells in the MeA of male infants in each of the five conditions in Experiment 3: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard error of the mean. * p < 0.05; n= 6-7 animals per group.
Fig. 10. Photomicrographs of coronal sections showing c-Fos protein stained cells in the MeA of one representative animal in each of five conditions in Experiment 3: HC (A), MH (B), MN (C), SH (D), SN (E).
Fig. 11. Mean density of c-Fos cells in the BNST in the combined Novel conditions (Separation/Novel and Mother/Novel) and the combined Home conditions (Separation/Home and Mother/Home). Vertical lines represent standard errors of the means. ** p < 0.01; n= 11-12 animals per group.
Fig. 12. Mean density of c-Fos cells in the BNST of male infants in each of five conditions in Experiment 3: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard error of the mean. * p < 0.05; ** p < 0.01; n= 5-7 animals per group
Fig. 13. Photomicrographs of coronal sections showing c-Fos protein stained cells in the BNST of one representative animal in each of five conditions in Experiment 3: HC (A), MH (B), MN (C), SH (D), SN (E).
Fig. 14. Mean Density of c-Fos cells in the PVN in the combined Separation conditions (Separation/Novel and Separation/Home) and the combined Mother conditions (Mother/Novel and Mother/Home). Vertical lines represent standard errors of the means. ** $p < 0.01$; $n= 12-13$ animals per group.
Fig. 15. Mean density of c-Fos cells in the PVN of the hypothalamus of male infants in each of five conditions in Experiment 3: Undisturbed Home Cage Control (HC), Mother/Home (MH), Mother/Novel (MN), Separation/Home (SH), and Separation/Novel (SN). Vertical lines represent standard error of the mean ** p < 0.01; n=6-7 animals per group.
Fig. 16. Photomicrographs of coronal sections showing c-Fos protein levels in the PVN of the hypothalamus of one representative animal in each of five conditions in Experiment 3: HC (A), MH (B), MN (C), SH (D), SN (E).
VII. References


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