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An Investigation of the Effects of Maternal Separation and Novelty on Central Mechanisms Mediating Pituitary-Adrenal Activity in Infant Guinea Pigs (*Cavia porcellus*)

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Abstract

In mammalian species in which the young exhibit a strong filial attachment (e.g., monkeys, guinea pigs), numerous studies have shown that even brief separation from the attachment figure potentially elevates circulating concentrations of glucocorticoids and adrenocorticotrophic hormone (ACTH). However, effects of separation on central regulation of this stress response are not known. Therefore, we investigated central mechanisms mediating pituitary-adrenal activation during maternal separation and novelty exposure in guinea pig (*Cavia porcellus*) pups. Corticotropin-releasing factor (CRF) mRNA expression in the hypothalamic paraventricular nucleus (PVN), and plasma cortisol and ACTH levels, were elevated only during separation in a novel environment. C-Fos activity was elevated in the medial amygdala (MeA) and reduced in the bed nucleus of the stria terminalis (BNST) during novelty exposure, regardless of separation. On the other hand, c-Fos activity was elevated in the PVN during separation, regardless of novelty exposure. These results demonstrate independent and combined effects of separation and novelty in regions of the guinea pig CNS that regulate pituitary-adrenal activity. Moreover, they suggest that a pathway from MeA to BNST to PVN mediates responses to novelty in the guinea pig pup, as in the adult rat, though inputs from other cell populations appear required to fully account for the HPA activity observed here.

Keywords

bed nucleus of the stria terminalis; maternal separation; medial amygdala; paraventricular nucleus of the hypothalamus

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Stressors experienced during early life can have particularly profound and lasting consequences. In humans, disruption of normal filial attachment processes by separation, abuse, or neglect is a major predisposing factor for the later development of depression and anxiety disorders (Agid et al., 1999; Brown, Harris, & Copeland, 1977; Gillespie & Nemeroff, 2007; Gilmer & McKinney, 2003; Gold, Goodwin, & Chrousos, 1988a, 1988b; Heim & Nemeroff, 1999; Takeuchi et al., 2002). Although much remains to be learned about how these early stressors exert their effects, long-term changes in the activity of hormones of the hypothalamic-pituitary-adrenal (HPA) axis, particularly corticotropin-releasing factor (CRF) and glucocorticoids, are likely to play a critical role (Coplan et al., 1996; Gillespie & Nemeroff, 2007; Heim, Newport, Mletzko, Miller, & Nemeroff, 2008; Teicher, Andersen, Polcari, Anderson, & Navalta, 2002). In species of nonhuman primates in which the young exhibit a strong filial attachment, separation procedures can have long-term effects (Capitanio, Rasmussen, Snyder, Laudenslager, & Reite, 1986; Young, Suomi, Harlow, & McKinney, 1973) and even brief separation from the attachment figure potently and reliably elevates circulating concentrations of glucocorticoids and adrenocorticotrophic hormone (ACTH; Coe, Mendoza, Smotherman, & Levine, 1978; Hennessy, 1997; Hoffman, Mendoza, Hennessy, & Mason, 1995; Smotherman, Hunt, McGinnis, & Levine, 1979; Wiener, Johnson, & Levine, 1987).

Among laboratory rodents, the guinea pig appears to be particularly useful for such studies. Guinea pig pups exhibit evidence for a primate-like attachment process (Hennessy, 1997; Jäckel & Trillmich, 2003; Pettijohn, 1979; Porter, Berryman & Fullerton, 1973) and there are many similarities in the response of guinea pigs and nonhuman primates to separation procedures (Hennessy, 2003). Of particular interest here, brief separation of infant guinea pigs from their mothers elevates plasma levels of cortisol and ACTH (Hennessy & Moorman, 1989; Hennessy & Ritchey, 1987; Hennessy, Tamborski, Schiml, & Lucot, 1989). This response is selective in that separation from an equally familiar sibling under the same conditions does not increase cortisol levels (Ritchey & Hennessy, 1987). However, the environment in which the separation occurs is crucial for determining the effect on pituitary-adrenal activity. Plasma levels of cortisol and ACTH are greatly elevated if the guinea pig pup is isolated in a novel cage, but not if it is placed into the novel cage together with its mother (Hennessy & Moorman, 1989; Hennessy et al., 1989). On the other hand, if the infant is left in the home environment during maternal separation, there is no elevation of cortisol levels (Hennessy & Moorman, 1989; Wewers, Kaiser, & Sachser, 2003). Although peripheral measures of pituitary-adrenal activity during separation have been studied extensively in developing guinea pigs, direct measurement of CRF activity in the hypothalamus of separated guinea pigs has yet to be made. Further, little is known about which neural structures regulate HPA responses during separation or how the interaction of separation and novelty is represented in the activity of central mechanisms controlling the HPA axis, although studies with adult rats have suggested several forebrain regions that might be involved.

One area consistently implicated in governing HPA responses is the amygdala. This structure is well-known to be involved in the interpretation of fearful stimuli and to mediate behavioral and physiological responses to stressors (LeDoux, 2000), including HPA

responses (Davis, 2000; Van de Kar, Piechowski, Rittenhouse, & Gray, 1991). Evidence indicates that “processive” or psychological stressors (e.g., novelty, restraint) route information to the HPA system primarily by way of the medial amygdala (MeA). The MeA appears to activate the paraventricular nucleus (PVN) to increase the release of CRF in the median eminence, resulting in subsequent ACTH and cortisol release but does so indirectly. The MeA has very few direct projections to the PVN (Canteras, Simerly, & Swanson, 1995; Herman et al., 2003). Rather, information appears to be relayed primarily through the bed nucleus of the stria terminalis (BNST) by way of inhibitory GABAergic axons that terminate in the BNST, and an inhibitory GABAergic signal from the BNST to the PVN (Canteras et al., 1995; Dunn, 1987; Herman, Cullinan, & Watson, 1994; Herman et al., 2003; Herman, Ostrander, Mueller, & Figueiredo, 2005; Prewitt & Herman, 1998). That is, the MeA appears to activate the HPA axis as a result of a two-neuron sequential GABAergic circuit, producing disinhibition of the PVN by removal of GABAergic tone and resulting in release of CRF in the median eminence (Cullinan, Helmreich, & Watson, 1996; Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Cullinan, Herman, & Watson, 1993; Cullinan, Ziegler, & Herman, 2008; Dong, Petrovich, & Swanson, 2001; Herman & Cullinan, 1997; Ju & Simerly, 1989; Ju & Swanson, 1989; Prewitt & Herman, 1998; Swanson & Petrovich, 1998; Ziegler & Herman, 2002).

The current experiments were designed to further our understanding of the central mechanisms regulating pituitary-adrenal activity during conditions of separation in the guinea pig. Because of the importance of separation occurring in a novel environment for pituitary-adrenal activation in this species, we examined the independent and combined effects of separation and novelty. As noted above, the work with rats suggests that novelty influences the PVN through connections from the MeA and BNST. As maternal separation, like novelty, is a psychological stressor, we hypothesized that effects on the PVN would occur through these same connections. In Experiment 1, we assessed the effect of several durations of maternal separation in a novel environment on CRF mRNA in the PVN. Experiment 2 compared the independent effects of separation and novelty with their combined influence on PVN CRF mRNA. Experiment 3 used c-Fos protein to assess cellular activity in the MeA, BNST, and PVN during separation and/or novelty exposure.

General Method

Subjects and Housing Conditions

Infant male guinea pigs (*Cavia porcellus*) of the Hartley strain, were gleaned from our breeding colony. No more than one male from a litter served as a subject in any condition. Males were housed in opaque plastic cages (73 cm × 54 cm × 24 cm) with their mothers and siblings until the day of testing on Postnatal Day 16 (±1), which is roughly a week before the typical age of weaning. The colony room was maintained on a 12-hr light–dark cycle (lights on at 0700) with food and water available ad libitum. All procedures were approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC).

Brain Tissue Collection

Samples were collected by rapid decapitation immediately following removal from the home cage (undisturbed home cage controls) or the test cage between 1100 and 1400 to minimize circadian variation. In preparation for cryostat sectioning, brains were removed from the skull following decapitation within 5 min, frozen in isopentane at -30°C to -40°C for 45 s and stored at -80°C . Brains were sectioned coronally in 14 μm sections. Brain regions were determined by extrapolation from the guinea pig atlas of Luparello (1967) for the MeA, BNST, and PVN (see Figure 1). Frozen sections were thaw mounted onto gelatin-coated slides (Gold Seal UltraStick micro adhesion slides: VWR International, LLC, West Chester, PA) and stored at -80°C .

In Situ Hybridization for CRF mRNA

Brain preparation—One series of slides for each animal was stained with Cresyl Violet and coverslips were mounted with Permount (Fisher Scientific Ltd., Ottawa, Canada) to accurately locate the PVN. Cresyl violet-stained sections were observed from a Q-imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope (Carl Zeiss Canada Ltd, Toronto, Canada). A second series of slides for each animal were used for the in situ hybridization procedure.

Oligonucleotide probes and labeling—Oligonucleotide probes to measure CRF mRNA were synthesized at Integrated DNA Technologies, Inc. (Coralville, IA) 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 (negative control; 5'-TCC ACC TTC TCC GGG AAG TCT TGG AGA TGG CCA GGG CCG AGC AGT-3'; Go et al., 2001). Probes were 3' tail labeled with ^{33}P -dATP (Perkin Elmer Inc., Waltham, MA) using terminal deoxytransferase (Promega Corporation, Madison, WI) as per supplier protocol. Probes were purified using Roche DNA G-25 Sephadex Columns (Roche Diagnostics, Indianapolis, IN).

Sections were thawed (30 min) and went through prehybridization as follows: 3.7% paraformaldehyde (20 min), 1 $\times$ PBS (10 min) twice, 0.1M triethanolamine-hydrochloride (TEA) + 0.25% acetic anhydride (AA; 10 min), 2 $\times$ 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 sulfate, Superpure formamide, 20 $\times$ SSC, 50 $\times$ 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., Oakville, Canada)). Sections were incubated at 42°C in 50% formamide humidified containers. Hybrislips were removed and slides were washed in 2 $\times$ SSC (30 min) twice, 2 $\times$ SSC (40°C , 15 min), 1 $\times$ SSC (40°C , 15 min), 1 $\times$ SSC/50% formamide (40°C , 30 min), 1 $\times$ SSC (room temperature, 10 min), 0.5 $\times$ 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., Rochester, NY) 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 densitometric 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 four sections of tissue) was selected to be processed by in situ hybridization to obtain a representation for the region of interest. Once processed, the two 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. Four readings per animal (two from the left half and two 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 determined to be within linear range of detectability, as measured by calibration curves from ARC 146–14C standards (American Radiolabeled Chemicals, St. Louis, MO).

Data analysis—Data were analyzed with one-way (Time–Experiment 1) or two-way (Separation $\times$ Novelty–Experiments 2 and 3) analysis of variance (ANOVA) procedures. *F* tests (planned comparison, Experiment 1) or simple effects tests (Winer, 1971, Experiments 2 and 3) were used to follow significant ANOVA results. For Experiments 2 and 3, the four groups included in the ANOVA were compared to an undisturbed control with Dunnett's procedure. In Experiment 2, cortisol data were subjected to a square root transformation because of heterogeneity of variance (raw data are presented in the figure). For Experiment 3, "side" of brain was included as a factor in preliminary ANOVAs. Because there were no significant main or interaction effects for this factor for any of the measures, data from the two sides were pooled for ANOVAs reported here. For the undisturbed control group, there was a significant effect of side for one area, the paraventricular nucleus, $t(1, 27) = 4.36, p < .05$. However, Dunnett's comparison of the undisturbed group to the four groups included in the ANOVA showed an identical pattern of results regardless of whether sides were analyzed separately or the data were pooled. Only the pooled data are reported here. A probability level of $p < .05$ was accepted throughout.

Experiment 1

Experiment 1 was designed to determine if 15 to 240 min of separation in a novel environment would elevate CRF mRNA expression in the PVN of guinea pig pups as well as to choose a time point for study in later experiments.

Method

Subjects were divided among six conditions. Pups either remained in their home cages (undisturbed home cage control condition) or were separated in a novel environment for either 15, 30, 60, 120, or 240 min (separation/novel 15–240 conditions). In the separation

conditions, the pup was transported in a carrying cage from the colony room to the adjoining test room (~10 s), where it was placed into a clear, empty, plastic cage (47 × 24 × 20 cm) located on a table under full room lighting. A clear plastic lid was placed over the cage to prevent the animal from escaping. On termination of the test, the brain was removed (<5 min) for later analysis of CRF mRNA levels. The cage and lid were cleaned with detergent prior to each use.

Results

As seen in Figure 2, the peak CRF mRNA response occurred at 120 min. The ANOVA yielded a significant main effect of condition, $F(5, 17) = 3.44$, $p < .05$. CRF mRNA was greater in the 120 separation/novel condition than in the undisturbed home cage control condition ($p < .05$). Examples of coronal sections from brains of animals in the various conditions are shown in Figure 2.

Experiment 2

In Experiment 1, separation in a novel environment elevated CRF mRNA expression in the PVN of guinea pig pups at 120 min. Experiment 2 examined the independent and combined effects of separation and exposure to novelty on CRF mRNA expression at the 120-min time point. Plasma levels of cortisol and ACTH were determined for comparison with the CRF mRNA measure.

Method

Experimental conditions—There were five conditions in Experiment 2: an undisturbed home cage control condition and four conditions comprising a 2 (mother, separation) × 2 (home, novel) factorial design. In these four conditions, all littermates were first taken to a separate room out of auditory range. Both the mother and the pup were then removed from the home cage for ~10 s and either both returned to the home cage (mother/home), the pup returned to the home cage and the mother taken to a different room (separation/home), the pup and the mother both placed into the novel environment in the test room (mother/novel), or the pup placed into the novel environment alone in the test room and the mother returned to the home cage (separation/novel). At the termination of the 2-hr test, blood samples and brains were quickly collected for analysis of hormones (cortisol, ACTH) and CRF mRNA levels, respectively.

Blood sampling and hormone determination—Trunk blood collected in heparinized tubes was centrifuged to separate plasma, which was frozen (−80 °C) until assayed with standard radioimmunoassay kits (cortisol: Siemens Diagnostics, Los Angeles; ACTH: Phoenix Pharmaceuticals, Inc., Burlingame, CA). For each assay, all samples were assayed in a single run. Intra-assay coefficients of variation were 6% for cortisol and 10% for ACTH.

Results

Cortisol—The 2 (separation) × 2 (novelty) ANOVA yielded significant main effects for separation, $F(1, 36) = 11.94$, $p < .01$; and novelty, $F(1, 36) = 17.02$, $p < .01$; as well as the Separation × Novelty interaction, $F(1, 36) = 21.79$, $p < .01$. Follow-up comparisons

indicated that separation produced an increase in cortisol levels only in the novel environment (mother/novel vs. separation/novel, $p < .01$; Figure 3). Furthermore, Dunnett's test revealed that cortisol levels were greater only in the separation/novel condition ($p < .01$) compared to those in the undisturbed home cage control condition.

ACTH—The 2×2 ANOVA yielded a significant main effect for separation, $F(1, 33) = 8.11$, $p < .01$, and a Separation $\times$ Novelty interaction, $F(1, 33) = 3.98$, $p = .05$. Post hoc tests showed that separation increased ACTH only when separation occurred in the novel environment ($p < .05$; Figure 3). Dunnett's test revealed that only the separation/novel condition elevated ACTH concentrations over the undisturbed home cage control condition ($p < .01$).

PVN CRF mRNA—The 2×2 ANOVA yielded significant main effects for separation, $F(1, 22) = 26.69$, $p < .01$; and for novelty, $F(1, 22) = 21.10$, $p < .01$; as well as a significant Separation $\times$ Novelty interaction, $F(1, 22) = 6.98$, $p < .05$. As was the case for the peripheral hormone measures, CRF mRNA was increased by separation when it occurred in the novel environment ($p < .01$; Figure 3) but not the home cage. Dunnett's test showed that CRF mRNA was elevated over the undisturbed home cage control condition only in the separation/novel condition ($p < .01$). A representative coronal section from one subject in each condition can be seen in Figure 3.

Experiment 3

In Experiment 2, CRF mRNA in the PVN and plasma cortisol and ACTH all showed a similar pattern of response. In each case, separation produced a significant response, but only when it occurred in a novel environment. Experiment 3 was designed to begin to elucidate inputs to the PVN that could account for the observed pattern of response. Measures of c-Fos protein were made to assess the activity of two regions thought to drive activation of CRF neurons in the PVN during exposure to psychological stimuli: the MeA and BNST. For comparison, c-Fos activity in the PVN also was examined.

Method

Experimental conditions—The experimental conditions were the same as for Experiment 2.

Immunofluorescence for c-Fos immunoreactive cells—Brain tissue for the immunofluorescence procedure used here and the in situ procedure of Experiment 2 were obtained from sequential alternating sections. Immunofluorescence labeling was performed at room temperature. The slides (Gold Seal) containing the tissue sections were removed from the -80°C freezer and allowed to thaw for 30 min. A pap pen (Invitrogen, Frederick, MD) was used to encircle the sections on each slide; marks were allowed to dry for 15 min. 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 double distilled water and a lid on top) and washed in $1\times$ phosphate buffered saline (PBS)/0.2% Triton $\times$ 100 (TX) for 1 hr. Slides were incubated in normal goat serum (1:10 PBS/TX) for 1 hr to minimize nonspecific binding.

Subsequently, all sections were incubated with a primary antibody for c-Fos (1:50; rabbit polyclonal IgG Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 1 hr (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 on third wash) in PBS. Sections were then incubated with Cy2 immunofluorescence-conjugated secondary antibody (1: 750; Alexa fluor 488 donkey anti-rabbit IgG Invitrogen) for 1 hr. The sections were washed three times (5 min each plus an additional 10 min on third wash) in PBS and then cover slipped with Prolong Gold Antifade Reagent (Invitrogen).

Cell quantification—Immunofluorescent stained tissue was visualized using an Olympus BX51 fluorescent microscope (Olympus America Inc., Melville, NY), and images were captured using an RT Slider Spot camera (Diagnostic Instruments Inc., Sterling Heights, MI). The density of c-Fos immunoreactive cells was quantified using Image-Pro Plus 5.1 software program (Media Cybernetics, Bethesda, MD). A cell was considered to contain c-Fos if its nucleus contained characteristic staining and could be distinguished clearly from background. 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 performed twice and a mean value obtained that represented the cell density for each animal.

Results

MeA c-Fos protein—The 2×2 ANOVA yielded a significant main effect for novelty, $F(1, 21) = 10.11$, $p < .01$. The density of c-Fos immunoreactive cells was greater in the novelty conditions than the home conditions (see Figure 4). The Dunnett's test revealed that the density of c-Fos immunoreactive cells was greater only in the separation/novel condition than in the undisturbed home cage control condition ($p < .05$).

BNST c-Fos protein—The 2×2 ANOVA yielded a significant main effect for novelty, $F(1, 19) = 5.27$, $p < .05$. The density of c-Fos immunoreactive cells was lower in the novelty conditions than in the home conditions (see Figure 4). The Dunnett's test indicated that the density of c-Fos immunoreactive cells was lower only in the mother/novel ($p < .05$) and the separation/novel ($p < .01$) conditions in comparison to the undisturbed home cage control.

PVN c-Fos protein—The 2×2 ANOVA yielded a significant effect for separation, $F(1, 21) = 22.53$, $p < .01$. The density of c-Fos immunoreactive cells was greater in the separation conditions than the mother conditions (see Figure 4). The Dunnett's test showed that the density of c-Fos immunoreactive cells was greater only in the separation/home ($p < .01$) and the separation/novel ($p < .01$) conditions compared to the undisturbed home cage control condition.

Discussion

In recent years, it has become increasingly clear that disruption of early attachment relations is a major predisposing factor for later development of anxiety and depressive disorders (Gillespie & Nemeroff, 2007; Gold et al., 1988a, 1988b). Moreover, hormones of the HPA axis are thought to be primary mediators of these outcomes (Heim et al., 2008; Teicher et al.,

2002). There have been numerous studies of central effects of maternal separation in rats and mice—species that show little evidence for a specific filial attachment process—and there also have been many studies examining peripheral changes in ACTH and glucocorticoid levels during separation in species in which the young display good evidence of a specific attachment (Hennessy, 1997; Wiener et al., 1987). However, the present study appears to be the first to directly assess central mechanisms mediating pituitary-adrenal responses in preweaning infants during conditions of maternal separation in a species in which evidence for a specific filial attachment is strong.

Our finding that infant male guinea pigs separated from their mothers for 2 hr in a novel environment showed elevated pituitary-adrenal activity is consistent with previous results (Hennessy & Moorman, 1989). Plasma cortisol and ACTH levels increased when infants were separated in the novel but not in the familiar environment, suggesting that cues from the home environment appeared to prevent an adrenal response to separation. Further, no increases in cortisol or ACTH 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 and ACTH levels that were no different from values in the undisturbed control condition. Although only males were used in the present study, previous experiments have found male and female guinea pigs to show similar patterns of cortisol and ACTH responses across these experimental conditions (Hennessy & Moorman, 1989; Hennessy et al., 1989).

As predicted, we observed changes in PVN CRF mRNA activity when infants were separated from their mothers in the novel environment, with the clearest activation evident at 2 hr. Peak levels of CRF mRNA have been reported to occur between 2 and 4 hr 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 due to release during the experimental manipulation (Dallman, 2000). The finding that 2 hr of exposure to the experimental conditions resulted in CRF mRNA activity in the PVN that paralleled the activity of the pituitary-adrenal hormones suggests that increased CRF activity may be sufficient to explain the elevations of cortisol and ACTH without involvement of arginine vasopressin. It has been reported that it is during exposure to repeated psychological stressors, not acute stressors (Wotjak et al., 1996), that vasopressin is most often coreleased with CRF to stimulate release of stored ACTH (Rivier & Vale, 1983).

The density of c-Fos immunoreactive cells was measured in the MeA and BNST to assess 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, c-Fos activity was significantly increased above undisturbed levels only in the separation/novel condition, suggesting that separation still enhanced 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 once again that it was novelty, not separation, that affected the BNST, though in a direction opposite to that seen in the MeA. Further, there were 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. The results suggest that in the guinea pig, as in the rat (Herman et al., 2003, 2005), the MeA sends inhibitory projections to the BNST, and these neurons are activated by novelty.

The density of c-Fos immunoreactive cells was measured in the PVN for comparison with cellular activity in the MeA and BNST. Intriguingly, we found that c-Fos activity in the PVN was elevated in those conditions involving separation, regardless of the novelty of the environment. This discrepancy with findings in the MeA and BNST can perhaps be better understood by considering expected results for individual conditions. Activity observed in the MeA and BNST would lead us to expect an increase in c-Fos activity in the PVN in both the separation/novel and mother/novel conditions. For separation/novel, the results confirmed this expectation. However, there was no corresponding increase in c-Fos activity in the mother/novel condition. If in the guinea pig, as in the rat, the MeA–BNST circuit drives PVN CRF activity, there appears to be two possible explanations for this result. One possibility is active inhibition. There is evidence that the medial prefrontal cortex (mPFC) and ventral subiculum inhibit HPA activity during exposure to psychological stressors in rats (Cullinan et al., 2008). Moreover, in juvenile rhesus monkeys placed into a novel environment with or without their mothers, positron emission tomography indicated that activity in the left prefrontal cortex was correlated with both presence of the mother and lower circulating cortisol levels (Rilling et al., 2001). 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 (Cullinan et al., 2008). 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; Hurley, Herbert, Moga, & Saper, 1991; Sesack, Deutch, Roth, & Bunney, 1989) that project to the PVN to directly inhibit CRF release. In the mother/novel condition of our study, it is possible that stimuli associated with the presence of the mother activated the mPFC and/or ventral subiculum to inhibit HPA activity during the mother/novel condition.

A second possible explanation 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 the guinea pig. 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 norepinephrine (NE) input to the hypothalamus originating from the A2 cell group in the region of the nucleus of the solitary tract. In infant rats, the mother's ability to buffer the corticosterone response during exposure to electric shock was correlated with NE input from the A2 group to the PVN (Shionoya, Moriceau, Bradstock, & Sullivan, 2007). Further, in a previous study (Harvey, Moore, Lucot & Hennessy, 1994), separation in a novel environment, but not exposure to the novel environment together with the mother, increased NE turnover in the anterior

hypothalamus. Thus, it is possible that NE may have played a role in increasing HPA activity in the separation/novel condition in the current study.

In addition to increased PVN c-Fos activity in the separation/novel condition, there was increased activity in the separation/home condition, in which no HPA activation was observed. Whereas activity of CRF neurons likely accounts for most of the increased c-Fos activity in the separation/novel condition, it appears that some other cell population(s) was activated in separation/home. In addition to CRF, there are a number of other neurotransmitter cell groups in the PVN including those releasing AVP, angiotensin II, cholecystokinin, neurotensin, GABA, enkephalin, and oxytocin (Chadwick & Marsh, 1992). Of the possibilities, oxytocin neurons seem the most likely to have increased c-Fos activity in the separation/home condition. Other studies have shown that oxytocin activity increases in response to some social stimuli and some stressors (Uvnäs-Moberg, 1998). In other rodents, release of oxytocin is related to affiliative behavior (Insel, 1992; Carter, 1998). For example, when oxytocin was injected into the PVN in infant rats, the vocalization response that pups emit when separated from their mothers was selectively decreased, an effect that mimics the presence of the mother (Insel, 1992). We find it interesting that 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 home cage, it is possible that cues from this environment (e.g., maternal odor) in the context of separation may have caused an increase in activity of oxytocin neurons that might account for the increase in c-Fos activity.

There are limitations of this initial examination of the central circuitry mediating pituitary-adrenal activity during maternal separation in guinea pigs that raise questions for future study. For instance, although the time point chosen for comparison of CRF expression in the various conditions in Experiment 2 was based on the results of Experiment 1's time course study of pups in the separation/novel condition, one cannot be sure that some increase in CRF expression or other endocrine measures in the remaining disturbance conditions might have been observed at some other time point. In Experiment 3, investigation of c-Fos activity in additional brain regions would help establish the specificity of the results obtained. It would also be instructive to determine how quickly the various responses return to resting levels following reunion with the mother in the home cage. Finally, because the conclusions for Experiment 3 are based on corresponding activation in different brain regions, direct manipulative studies, for instance in which the GABA_A receptor agonist muscimol is administered to the MeA or BNST to examine downstream effects, would provide a stringent test of the circuit driving HPA activity in separated guinea pig pups that is hypothesized here.

In sum, the present study provides a first glimpse of the central circuitry mediating HPA activity in the guinea pig. The results 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 (c-Fos activity in PVN), only to novelty (c-Fos activity in MeA and BNST), or only to the combination of the two (CRF mRNA in PVN). The findings suggest a pathway from the MeA to BNST to PVN that mediates

responses to novelty exists in the guinea pig, as in the rat, though other inputs appear required to fully account for the HPA activity observed here.

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Figure 1.

Areas examined including the PVN, MeA, and BNST, located at approximately 9.8 mm, 9.8 mm, and 11.0 mm anterior to the interaural axis, respectively. PVN = paraventricular nucleus; MeA = medial amygdala; BNST = bed nucleus of the stria terminalis. Adapted from *Stereotaxic Atlas of the Forebrain of the Guinea Pig*, (pp. 44, 38), by T. J. Luparello, 1967, Basel, Switzerland: Karger. Copyright 1967 by Karger. Adapted with permission.

Figure 2.

Left panel: Semiquantitative measurements of corticotropin-releasing factor (CRF) mRNA hybridization signal in the paraventricular nucleus (PVN) of male infants exposed to a novel environment alone in each of six conditions in Experiment 1: undisturbed home cage control (home), and exposure the novel environment for 15, 30, 60, 120, or 240 min. Vertical lines represent standard error of the mean (* $p < .05$, compared to the home condition; $n = 3$ to 4 animals per group). Right panel: CRF mRNA expression in the hypothalamic 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: home and exposure the novel environment for 15, 30, 60, 120, or 240 min.

Figure 3.

Top left and right panels: Mean plasma cortisol and adrenocorticotropin (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 (* $p < .05$. ** $p < .01$. $n = 10$ animals per group, cortisol; $n = 8$ to 10 animals per group, ACTH). Bottom left panel: Semiquantitative measurements of corticotropin-releasing factor (CRF) mRNA hybridization signal in the paraventricular nucleus (PVN) of male infants in each of five conditions in Experiment 2: HC, MH, MN, SH, and SN. Vertical lines represent standard errors of the means (** $p < .01$; $n = 6$ to 7 animals per group). Bottom right panel: CRF mRNA expression in the hypothalamic 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, MH, MN, SH, SN.

Figure 4.

Top two panels: Mean density of c-Fos immunoreactive cells in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) in the combined novel conditions (separation/novel and mother/novel) and the combined home conditions (separation/home and mother/home; left panels) and in each of the five individual conditions (right panels) in Experiment 3: Undisturbed home cage control (HC), mother/home (MH), mother/novel (MN), separation/home (SH), and separation/novel (SN). For the left panels, $n = 12$ to 13 (MeA) and 11 to 12 (BNST) animals per group; for the right panels, $n = 6$ to 7 (MeA) and 5

to 7 (BNST) animals per group. Vertical lines represent standard errors of the means. Comparisons in right panels are with the undisturbed home cage control. * $p < .05$. ** $p < .01$. Bottom panel: Mean density of c-Fos immunoreactive cells in the PVN in the combined separation conditions (separation/novel and separation/home) and the combined mother conditions (mother/novel and mother/home; left panel) and in each of the five individual conditions (right panel) in Experiment 3: HC, MH, MN, SH, and SN. For the left panel, $n = 12$ to 13 animals per group; for the right panel $n = 6$ to 7 animals per group. Vertical lines represent standard errors of the means. Comparisons in right panel are with undisturbed home cage control. * $p < .05$. ** $p < .01$.