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Social Buffering By Unfamiliar Adult Males in Periadolescent Guinea Pigs: The Effects on HPA Axis Activity And Fos Induction In The Medial Prefrontal Cortex

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SOCIAL BUFFERING BY UNFAMILIAR ADULT MALES IN PERIADOLESCENT GUINEA PIGS (*CAVIA PORCELLUS*): THE EFFECTS ON HPA AXIS ACTIVITY AND FOS INDUCTION IN THE MEDIAL PRELIMBIC CORTEX

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ABSTRACT

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In the guinea pig, the ability of the mother's presence to buffer hypothalamic-pituitary-adrenal (HPA) axis activation in her young during exposure to stressful stimuli has been well documented. Under similar testing conditions, other conspecifics (littermates, other adult females) are less effective in doing so. The effect does seem to wane with age but is still present to a significant degree in offspring approaching adolescence. However, we recently observed that an unfamiliar adult male buffered HPA axis activation and increased Fos expression in the prefrontal cortex of preweaning infants exposed to a novel enclosure at both 60 and 120 minutes but did not buffer HPA axis activation in periadolescent guinea pigs tested at 60 minutes. Here, we found that an unfamiliar adult male buffered mean plasma cortisol levels in periadolescent guinea pigs exposed to a novel enclosure at 120 minutes. Social interactions between the adult male and periadolescent were observed with the periadolescent vocalizing less but exhibiting more passive behavior and locomotion than when alone. Fos values in the prefrontal cortex were lower than values measured in prior studies and were not affected by the presence of the male. In summary, unfamiliar adult males are, in fact, capable of buffering plasma cortisol responses in periadolescent guinea pigs if given a sufficient amount of time for

the effect to be detected. We found no evidence that this buffering was mediated by increased prefrontal activity.

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I. INTRODUCTION

All successful animal species possess an innate ability to deal with stressful stimuli. Without this adaptive mechanism, individuals would succumb to the demands of their environment and would be unable to successfully pass on genetic information from one generation to the next. Through years of evolutionary fine tuning, this stress response has come to provide animals with a means by which to escape potentially dangerous stimuli as well as to survive in the moments immediately thereafter. However, if activated for too long a period of time, this same adaptive mechanism can prove detrimental to an individual's long-term health and well-being. In order to maintain this delicate balance, various fail-safe mechanisms are essential and must be functioning in unison.

Hypothalamic-Pituitary-Adrenal Axis

In vertebrates, the amygdala is a central region of the brain that, through connections with other structures throughout the limbic system, plays a critical role in both memory and decision making, as well as the formation and processing of emotions. The amygdala is also a central node for initiation and maintenance of the stress response. During times of elevated stress, the amygdala receives input through upstream connections with various cortical association areas, the hippocampus, and olfactory bulbs. After processing this information, it then sends projections via the fornix to regions of the brain that execute the various components of the stress response. For example, it stimulates the parabrachial nucleus, which elevates respiratory drive, the

locus coeruleus, which releases norepinephrine throughout the brain, and the periaqueductal gray, a deeper nucleus of the midbrain, which triggers defensive behaviors such as freezing, running, and jumping. Meanwhile in the cortex, activation of the cingulate gyrus is associated with a fearful, negative affective state (Venkatraman et al., 2017). However, most notable of all of these downstream targets is the hypothalamus. Not only does the hypothalamus increase systemic sympathetic discharge, but it is also part of the hypothalamic-pituitary-adrenal axis (HPA axis). Upon excitation by the amygdala, the PVN of the hypothalamus releases corticotropin-releasing factor (CRF) onto the anterior pituitary gland. This allows local neuroendocrine cells to secrete adrenocorticotropic hormone (ACTH) into the blood stream, which triggers the zona fasciculata of the adrenal cortex to secrete cortisol into the blood stream. Cortisol then binds to intracytoplasmic receptors before being transported inside the nucleus. It is here that anti-inflammatory transcription factors are activated, and pro-inflammatory transcription factors are inhibited. This results in increased expression of IL-10, and decreased expression of IL-4, IL-5, and TNF-a by the cell. In these relative concentrations, inhibition of the immune system takes place and energy is conserved (Kox et al., 2014). These same signaling pathways are also responsible for the increased degree of gluconeogenesis in hepatocytes, which is essential for providing the glucose necessary for combatting stressful stimuli. Elevated levels of cortisol act as a feedback signal to activate the negative feedback mechanisms in the PVN and anterior pituitary gland. This negative feedback functions to inhibit further secretion of both CRH and ACTH, which, when functioning properly, lowers systemic cortisol and returns the system to normal. The development of this system is a highly sensitive process.

Repeated exposure to stressful stimuli experienced throughout development can result in varying degrees of receptor expression in adulthood (McEwen 2006). Infancy seems to be a particularly vulnerable time.

Chronic Stress and the Developing Brain

Vulnerability to stress during infancy is clearly illustrated in infants deprived of maternal care. The relationship between familial separation during infancy and depressive-like behavior observed in children has been well established in the literature dating back to the 1940s. At this time, newborn motherless infants were shown to demonstrate depressive behavior, insomnia, and in some cases developmental delay by as early as six months of age (Spitz and Wolf, 1946). The first two years of life seem to be the most critical in terms of proper amygdala development in humans. Infants who were subjected to repeated maternal separation at this time tended to have greater amygdala volume by the time they reached full maturity (Malter Cohen et al., 2013). This increased amygdala volume has been directly implicated with limbic hyper-plasticity and over-activation of the HPA axis (Radley et al., 2015). Follow up studies reinforced these findings and further demonstrated the necessity for the attachment figure, most typically the mother, to be available throughout infancy not only for proper psychological development but also for proper development of physiological systems, including the HPA axis (Rincón-Cortés and Sullivan, 2014). Nonhuman primates exposed to traumatic events early in life demonstrated overactivity of CRF secreting neurons throughout the remainder of their lives. This led to increased sensitivity of neuroendocrine cells in the anterior pituitary, which resulted in increased HPA axis activation and subsequent cortisol release. These chronically elevated plasma cortisol levels increased the

likelihood that subjects would suffer from recurring depressive-like behavior (Coplan et al., 1996).

Anxiety, schizophrenia, and other similar mood disorders have also been linked to increased sensitivity of the HPA axis. However, it has been proposed that this increased sensitivity is the result of increased expression of the glucocorticoid receptor secondary to epigenetic alterations in DNA methylation and histone configuration. An individual's early environment more than likely serves as a basis for these epigenetic alterations with repeated stressors resulting in increased susceptibility (Zannas and West, 2013). Regardless of the proposed mechanism, this upregulation of glucocorticoid receptors and the subsequent increased HPA axis sensitivity seems to be one common denominator in all of the aforementioned disorders. This relationship between an upregulation of glucocorticoid receptors and an increased sensitivity of the HPA axis underscores the need for negative feedback and other means by which HPA axis activity can be reduced. Another notable mechanism seems to involve the ability of various social partners to inhibit HPA axis activation through both their presence and interaction. This mechanism is known as social buffering (Hennessy et al., 2015).

Social Buffering between Pair-Bonded Partners

Species of the animal kingdom vary considerably in their gregariousness. Many mammals including tigers, hamsters, and orangutans spend their lives in solitude leaving their mothers around the time of puberty only coming into contact with one another for mating. Contrarily, some mammals, spend their lives in family groups or larger social groups. This can be said of most primates, rodents, and herd-living ungulates (Hennessy et al., 2009). Social buffering has most often been demonstrated in these more

gregarious species. When 3-year-old rhesus monkeys were taken from their home cage and placed in a novel environment with cage-mates, glucocorticoid levels were significantly reduced when compared to monkeys placed in a novel environment alone (Feng et al., 2011). In the monogamous yellow-toothed cavy, a species of wild guinea pig, similar results have been measured. Male subjects who had been removed from their home cage for weighing and later returned to their home cage alone showed significantly elevated glucocorticoid levels at 2 hours when compared to the group that was returned to their home cage in the presence of their female pair-mate (Adrian et al., 2008). Herd-living ungulates such as sheep are also capable of buffering HPA axis activity. When sheep were placed in a novel pen, glucocorticoid levels significantly increased if the sheep were isolated when compared to when sheep were placed in the same novel pen accompanied by other sheep. The same cannot be said of other ungulates such as goats. Glucocorticoid levels in these animals were elevated regardless of the presence of other goats in the novel environment (Lyons et al., 1993). These findings were not unexpected given that when threatened (i.e., stressed) sheep are more likely to flock than goats.

Social Buffering Between Mothers and Infants

It is clear that these species are all capable of social buffering to some degree. It is also clear that the social partner capable of buffering is species-specific and dependent on the social organization by which a species lives. Species whose members live in families or other social groupings often show attachment to whichever members serve the role of caregiver and to members with whom they spend the majority of their time. It is this attachment, or the degree of attachment, that appears to best predict the likelihood of partners buffering HPA axis activation (Hennessy et al., 2006a). For this reason, the

mammalian mother tends to buffer stress responses in her offspring to a stronger degree than most other social partners. Studies have shown that not only does the mother buffer stress responses in her offspring, but the presence of her offspring also seems to buffer the stress response in the mother. Squirrel monkey infants show a strong attachment to their mothers. This relationship is mutual with little to no interaction between the infant and the father. When the mother was separated from her infant and placed in a novel environment, her glucocorticoid levels significantly increased as would be expected in any animal experiencing a stressful situation. The same was true of the infant when it was separated from its mother. However, when they were placed in a novel environment in each other's company, glucocorticoid levels in both animals were significantly reduced (Wiener et al., 1987). Although mothers tend to be the prominent attachment figure in the lives of most offspring, this is not true of the titi monkey. In the monogamous titi monkey, fathers take on the role of primary caretaker which differs from most other species. Accordingly, infants seem to show a greater degree of attachment to the father when compared to the mother. When the father was taken from the home cage, cortisol levels of the infant became significantly elevated even in the presence of the mother. However, when the mother was taken from the home cage, and the infant remained in the presence of its father, plasma cortisol levels were significantly buffered and were much closer to baseline (Hoffman et al. 1995). Although social buffering most typically occurs between animals of the same species, interspecies buffering relationships have also been observed. That is, the presence of the human caretaker significantly reduced glucocorticoid levels in dogs that were being exposed to stressful stimuli (Tuber et al. 1996).

Hypothesized Neural Mechanisms of Social Buffering

Multiple, interconnected neural mechanisms seem to be responsible for eliciting social buffering, and these mechanisms seem to be dependent on specific social partners. One proposed mechanism involves the release of oxytocin in the paraventricular nucleus (PVN) of the hypothalamus as a mediator for buffering the effects of the HPA axis. Following a 1 hour immobilization period, monogamous female prairie voles were either isolated or placed with their male partners for recovery. Glucocorticoid levels were significantly reduced in the subjects placed with their male partners for recovery when compared to the subjects recovering in isolation. In these same subjects, PVN oxytocin levels were also significantly elevated when compared to the subjects recovering from immobilization. A subgroup of subjects recovering in the presence of their male partners were given either oxytocin or an oxytocin antagonist directly into their PVN. Subjects who received oxytocin infusions showed significantly decreased glucocorticoid levels when compared to subjects that received the antagonist. In fact, subjects that received the oxytocin antagonist showed reduced buffering even though they were still in the presence of their male partners (Smith and Wang, 2014). These findings suggest that the animal's own endogenous oxytocin is at least partially responsible for the buffering that occurs when female subjects recover in the presence of their male partners following a prolonged period of immobilization.

Inhibition of noradrenergic input to the PVN is also a proposed mechanism of buffering. Two-week-old infant rats show odor-aversion following odor-shock conditioning whenever they are not in the presence of their mother. This conditioning is mediated by elevated glucocorticoids. However, when their mother is present, the pups

show reduced corticosterone levels and an odor preference regardless of the fact that the odor is associated with a painful stimulus. When infant rats were given an intra-PVN norepinephrine antagonist infusion, they demonstrated odor preference with maternal absence. Contrarily, when infant rats were given an intra-PVN norepinephrine agonist infusion, they demonstrated odor aversion even in the presence of their mothers. These findings suggest that the rat mother buffers the corticosterone stress response in her pups through an inhibition of norepinephrine release on the PVN of the hypothalamus (Shionoya et al., 2007). Recent data suggest that the prefrontal cortex, most notably the prelimbic and infralimbic cortices, may also play a role in social buffering. Lesions in these areas have been shown to exacerbate the physiologic response to stressful stimuli, while activation has been shown to inhibit the HPA axis (Radley et al., 2006).

Social Buffering in Guinea Pigs

The guinea pig (*Cavia porcellus*) is a valuable model for studying social buffering in the laboratory setting. Infants and adolescents both show a strong attraction and motivation to be near their mother. However, mothers show little obvious interest in their litter (König, 1985). For instance, unlike rats and mice, guinea pig mothers do not retrieve their pups when they are separated. Mother-infant proximity is, therefore, maintained by the motivation of the pups to remain close to their mother's side. This simplifies the design of buffering studies as it allows us to study the effects of separation from an attachment figure without the confounding influence of deprivation of maternal care. Once the pups are separated from their mothers and placed in a novel environment, a full stress response typically manifests itself within the first two hours (Hennessy and Moorman, 1989; Hennessy and Ritchey, 1987). During a typical separation, pups first

display a period of increased activity and vocalization, which the literature refers to as the protest stage. This is followed by a period of passive-like behavior in which locomotion and vocalizations begin to decrease in frequency until subjects are motionless in a crouched posture with their fur erect, and their eyes closed. Visually, this passive-like behavior is suggestive of illness and is mediated by inflammatory signaling (Hennessy and Moorman, 1989, Hennessy et al., 2019). When the mother is placed with the subject in the testing cage none of these responses occur. There is no significant increase in physical activity or vocalizations. The passive phase is absent entirely, and subsequently cortisol levels remain within normal resting limits. This buffering effect of the HPA axis by the mother is not the result of the mother's interaction with her pups but rather her mere presence as an attachment figure to her offspring. This idea is supported by the fact that anesthetized mothers placed in the testing cage alongside test subjects reduce HPA axis activity as measured by cortisol levels (Hennessy and Ritchey, 1987). This buffering effect does wane with age but through early adolescence is always present to some extent (Hennessy et al., 2006b).

Although guinea pig mothers show the greatest ability at buffering the stress responses of her pups, other social partners are also capable. At about two weeks of age, unfamiliar adult females also reduce the cortisol response of pups albeit less consistently than the mother. Following weaning, unfamiliar females are as effective as is the mother (Graves and Hennessy, 2000; Hennessy et al., 2000; Hennessy et al., 2002a; Hennessy et al., 2002b). The presence of littermates in the testing cage consistently reduces some protest behaviors but has no effect on mean plasma cortisol levels in test subjects (Hennessy, et al., 2006b; Hennessy et al., 1995; Hennessy et al., 2015). Unfamiliar adult

males have also shown an ability to buffer the stress response. However, this effect has only been observed in pups prior to weaning. When exposed to a novel cage for 120 minutes in the presence of an unfamiliar adult male, preweaning pups showed reduced protest behaviors and mean plasma cortisol levels when compared with pups that were tested alone over the same time period (Hennessy et al., 2015). This reduction in mean plasma cortisol levels occurred even though adult males frequently interacted roughly with the pups. Both non-agonistic and agonistic behavior was demonstrated by the adult males. Non-agonistic behavior included sniffing, nose-to-nose, and anogenital investigation. Agonistic behavior included nipping, lunging, and kicking (Hennessy et al., 2015). A follow-up study exposed pups to a novel environment with unfamiliar adult males for 60 minutes. Like the previous study, subjects separated with unfamiliar adult males again showed significant reductions in protest behaviors and mean plasma cortisol levels when compared with subjects that were tested alone over the same time period. Both non-agonistic and agonistic behaviors were again measured and findings were the same as in the previous study. Mean plasma cortisol levels were significantly lowered regardless of the degree of agonistic behavior received from the stimulus animal (Hennessy, et al., 2018). In contrast, when unfamiliar adult males were separated with post-weaned "periadolescent" animals approximately 45 days of age no evidence of buffering could be detected at 60 minutes. Mean plasma cortisol levels were as high as when pups that were tested alone. However, vocalizations were reduced (Hennessy et al., 2002b).

Behavior between unfamiliar adult males and the test subjects is necessary for buffering to take place. When unfamiliar adult males were anesthetized, plasma cortisol

levels in pups at 60 minutes were as great as those measured in pups tested alone. Only subjects tested with conscious unfamiliar adult males showed significant reductions in plasma cortisol levels (Hennessy et al., 2018). The mechanism underlying the ability of unfamiliar adult males to buffer cortisol levels in periadolescent guinea pigs is currently not known but may involve the prefrontal cortex. Behavioral interactions with adult males increased fos expression in the prelimbic cortex of male and female pups, as well as in the infralimbic cortex (Hennessy et al., 2018). Because there is evidence indicating both of these regions modulate HPA axis activity through indirect connections with the PVN (Herman et al., 2005; Hostinar et al., 2014), this is a possible mechanism through which the unfamiliar adult male buffers HPA axis activity in preweaning pups. Recall that when an anesthetized mother was placed in the testing cage, so that no behavior was directed toward the pups, plasma cortisol levels of pups were significantly decreased when compared to plasma cortisol levels of pups tested alone (Hennessy and Ritchey, 1987). This difference in the effect of behavior on the buffering ability of the mother and unfamiliar adult male indicates that they produce buffering through differing mechanisms. Although the mechanism by which the guinea pig mother reduces HPA axis activation in her offspring has not yet been determined, it has been hypothesized that it involves inhibition of excitatory projections from the brainstem to the PVN as is the case in the preweaning rat (McKlveen, 2015).

Rational for Current Study

In summary, unfamiliar adult guinea pig males buffer the cortisol response of preweaning pups at both 60 and 120 minutes. Buffering requires social interaction that activates the prefrontal cortex. However, when unfamiliar adult guinea pig males are

placed in a testing cage with post-weaned periadolescent guinea pigs no buffering can be detected at 60 minutes. This finding is suggestive of two alternatives: an age effect or a time point effect. It is possible that the buffering effect of unfamiliar adult males diminishes with test subject age and that post-weaned periadolescent guinea pigs are no longer capable of being buffered by unfamiliar adult males. However, it is also possible that the buffering effect takes longer to become established in this older age group. Further, how the adult males interact with periadolescents or what their effect is on prefrontal activation has not been assessed. Therefore, the current study exposed post-weaned periadolescent guinea pigs to a novel environment for 120 minutes, both while alone and while accompanied by an unfamiliar adult guinea pig male. Following the 120-minute period, plasma cortisol levels were measured, and brain tissue was collected for staining and subsequent analysis of Fos induction. Protest and passive behaviors were again measured as in previous studies. Both non-agonistic and agonistic behaviors exhibited by the unfamiliar adult males were also measured.

II. METHODS

Animals and Experimental Conditions

Albino guinea pigs *(Cavia porcellus)* of the Hartley strain were bred in our laboratory. Following birth (Day 0), each mother and her litter were housed in a plastic cage (73.7 cm X 53.4 cm X 25.4 cm) with both a wire front and sawdust bedding. An opaque plastic shelter open on either end was located in the middle of the cage. Juveniles had no direct contact with the males of the colony, though it should be noted that mothers and juveniles were housed in the general colony room which contained adult males throughout their first 10 days of life. They were then relocated to a secondary colony room without males that was adjacent to the testing and observation rooms. As a result, the juveniles did have early exposure to the odors of all breeding males. All animals in both rooms were maintained on a 12:12 light/dark cycle (lights on 0700). Both water and guinea pig chow were available ad libitum. All procedures were approved by the Wright State University Animal Care and Use Committee.

Six male juveniles and six female juveniles were randomly assigned to each of the three conditions: Home Cage, Alone, and Adult Male. No more than one juvenile per litter was assigned to each condition in order to control for litter effects. In the Home Cage condition, juveniles remained with the mother until it was time to be sacrificed for both blood and brain collection. In the remaining two conditions, the juvenile was taken from the mother and was tested either alone or together with a conscious, unfamiliar adult male. Seven adult males were used with none being used more than four times.

Precaution was taken to ensure that the adult male used for testing was not the juvenile's father. Testing was conducted on day 46 (+/- 1 day).

Testing Procedure and Behavioral Scoring

For both the Alone and the Adult Male conditions, juveniles were taken from the home cage and were carried quietly via a carrying cage to the testing room where they were then placed into a clear, empty plastic cage (55 cm X 32 cm X 18 cm) under full room lighting. It should be noted that after testing approximately one-third of the subjects, the walls of the cage were heightened using Plexiglas to prevent subjects from attempting to escape. White tape was used to section the floor of the cage into four equal length regions. A trained observer then sat behind one-way glass and scored various pre-determined behaviors during minutes 0-30 and 90-120. These time intervals were chosen to capture behaviors of the active and passive stages of separation, respectively. The active behaviors were the number of whistle vocalizations (Berryman, 1976), as well as the movement (number of times lines were crossed) within the cage. The passive behaviors were the number of 1-min intervals that a juvenile exhibited either the crouched stance or lying down, eye-closure, or extensive piloerection. The measure of "full passive" was defined as the number of 1-min intervals that a juvenile exhibited all three passive behaviors.

The frequency of mild agonistic and non-agonistic interactions were recorded in the Adult Male condition, as in our previous studies (e.g. Hennessy et al., 2015). Non-agonistic interactions included nose-nose, as well as fur sniff and anogenital investigation by the adult male. Agonistic interactions included kick, nip, thrust/lunge/lift by the adult

male. See Table 1 for a full list of scored behaviors and their corresponding definitions. All behaviors with the exception of vocalizations were recorded on check sheets. A microphone located in the testing room transmitted vocalizations to the headphones of the observer who then tallied them with a handheld counter. Contact time between the adult male and the test subject was also recorded in the Adult Male condition. Testing cages were thoroughly cleaned with detergent following each test.

Blood Sample Collection and Cortisol Determination

Upon conclusion of testing, the juvenile was transferred to a nearby CO2 chamber until it was rendered unconscious. Approximately one milliliter of blood was then collected directly from the heart via cardiac puncture. Blood was collected at the same time each day (1400 - 1600 hours) in all conditions. Blood samples were collected in under 4 min from the time of completion of behavioral testing for both the Alone and Adult Male conditions and from the time of disturbance for the Home Cage condition. After collection, blood samples were immediately placed in vials containing heparin. Plasma was separated using centrifugation (3000 rpm for 15 min) and was then frozen until assayed with a radioimmunoassay kit routinely used in this laboratory (ImmuChem Coated tubes Cortisol RIA, MP Biomedicals, Orangeburg, NY). Samples were assayed in one run. The intra-assay coefficient of variation was measured to be 5.6%.

Brain Tissue Collection

Upon completion of the blood collection procedure, juveniles were given an overdose IP injection of Euthasol. They were then perfused via the left ventricle with 0.01% heparinized 1X phosphate-buffered saline (PBS) until there was no remaining blood return to the right atrium. This was then followed by an additional perfusion using

a 4% paraformaldehyde solution. Brains were then removed from the skull and submerged in a 4% paraformaldehyde solution for post-fixation. This was followed by submergence in a 30% sucrose solution for an additional 48 hours as a means of cryoprotection. The brains were then caudally blocked and coated in CRYO-OCT compound (Tissue Tek: Fisher Scientific, Pittsburgh, PA) before being flash frozen in -80°C isopentane for approximately 20 sec. They were then stored at -80°C before being sectioned into 16 micron coronal slices using a cryostat (Microm HM 550: thermo Scientific, Waltham, MA). Frozen sections were thaw-mounted onto gelatin-coated slides (Goal Seal UltraStick micro adhesion slides: VWR International, LLC, West Chester, PA) and stored at -20°C.

Fos Immunohistochemistry and Cell Quantification

All immunohistochemistry procedures were performed at room temperature except for the incubation of the primary antibody, which was performed at 4 °C. Two sections spaced ~ 16 μm were used for Fos analysis. The slides containing the tissue sections were removed from the − 20 °C freezer and allowed to thaw for 10 min. A PAP pen (Invitrogen, Frederick, MD) was used to encircle the sections on each slide, which were allowed to dry for 2–5 min. First, sections were washed three times in PBS/0.2% Triton then incubated in 0.5% peroxide solution for 5 min. Sections were washed twice with PBS/0.2% Triton, incubated in 10% normal goat serum (Sigma-Aldrich Co. LLC, St. Louis, MO) for 1 h, and then incubated overnight for ~12 h with a primary antibody for Fos (1:50; rabbit polyclonal IgG Santa Cruz Biotechnology, Inc., catalog number SC52, Santa Cruz, CA) in PBS/0.2% Triton. The sections were washed three times with PBS/0.2% Triton and then incubated for 1 h with biotinylated secondary antibody

(1:200; anti- rabbit polyclonal IgG BA-1000, Vector Laboratories, Burlingame, CA) in PBS/0.2% Triton. The sections were washed three times with PBS and then incubated in an AB enzyme reagent (Vector Laboratories) for 30 min, after which they were washed three times in PBS and then incubated in diaminobenzidine solution with nickel intensification (Vector Laboratories) for 8 min. The sections were washed in distilled water and coverslipped with Permount mounting medium and were allowed to dry overnight and stored at room temperature.

Fos was quantified within borders of the PVN traced with the aid of DAPI staining. For the prelimbic and infralimbic cortex, Fos was measured in equal-sized fields of the regions as illustrated in Figure 1. Images were analyzed by counting the number of Fos positive pixels per area using Image J v.1.47. This was accomplished by selecting a pixel range just above the background threshold and below any artifact. The number of pixels within that range was counted and then summed across the range. All quantification was performed blind to the condition of the animal.

Data Analysis

Cortisol and Fos were analyzed with analyses of variance (ANOVAs) with Condition and Sex as between subject variables. For Fos, side of the brain was treated as a repeated measure. Post-hoc tests were conducted with follow-up ANOVAs (for significant interaction) and multiple paired-comparisons with the Newman-Keuls procedure. Because of lack of normality for many of the behavioral measures, and for consistency in reporting, non-parametric tests were used for all non-social behaviors. Comparisons between two groups were conducted with the Mann-Whitney U test. Effects across multiple groups were assessed with the Kruskal-Wallis test, with

significant results followed by Dunn-Bonferroni paired comparisons. For all non-social behavioral measures, we first assessed sex differences before conducting any tests of differences between or among conditions. For social behaviors, data from males and females were compared with t-tests. Data are represented as means and standard errors of the means for measures examined with ANOVA or t-test and as medians and semi-interquartile ranges for measures subjected to non-parametric analysis. Except as noted, a two-tailed value of $p < 0.05$ was used for determining significant effects. For two females tested alone, blood sampling exceeded 4 min. However, their values fell within the range of scores for the group, and so were included in the analysis.

III. RESULTS

Behavior

Mann-Whitney U Tests were first conducted comparing the number of vocalizations, line crossings, and full passive response scores between sexes in both the Alone condition and the Adult Male condition. These showed that sex had no effect on any of these behaviors in either condition (p's > 0.05). Male and female data were then pooled, and Mann-Whitney U Tests were conducted comparing the number of vocalizations, line crossings, and full passive response scores between the Alone and the Adult Male conditions, which can be seen in Table 2. Juveniles in the Alone condition exhibited more vocalizations ($p < 0.005$) but fewer line crossings ($p < 0.01$) and number of minutes in which the full passive response was observed ($p < 0.05$) than did juveniles in the Adult Male condition.

A t-test revealed that male juveniles ($\overline{x} = 18.38$, SE = 3.11) engaged in fewer non-agonistic interactions than did female juveniles ($\overline{x} = 37.63$, SE = 7.28); $t(14) = -2.43$, $p < 0.05$. There was no sex difference for the mildly agonistic interaction. The difference between contact time with the adult male was also not significant between males ($\overline{x}$ = 2,639.6 sec, SE = 243.7 sec) and females ($\overline{x}$ = 2,985.2 sec, SE = 284.3 sec).

Cortisol

Cortisol levels were analyzed using analysis of variance (ANOVA). Levene's test indicated heterogeneous sources of variance ($p < 0.01$), so values were subjected to square root transformation prior to ANOVA. ANOVA yielded effects for both

Condition, $F(2,46) = 25.42$, $p < .001$, and Sex, $F(1, 46) = 11.33$, $p < 0.001$ (females > males, as is typical for guinea pigs). Post-hoc Newman-Keuls test showed that plasma cortisol levels of juveniles in the Alone condition were greater than those of juveniles of either the Home Cage or Adult Male conditions (p's < 0.05). Plasma cortisol levels of juveniles in the Alone condition were nearly double those of juveniles in the Home Cage and the Adult Male conditions (Fig. 2; raw values shown for ease of interpretation). These findings show that the presence of the unfamiliar, adult male significantly reduced the plasma cortisol volume of juveniles.

Fos

Fos expression in the left and right pre and infralimbic cortices was analyzed using analysis of variance (ANOVA), which revealed no effect of Group or Sex. Nonetheless Fos expression was always numerically greater in the Alone and Adult Male conditions than in the Home Cage condition, ANOVA data is included in Table 3.

IV. DISCUSSION

When periadolescent guinea pigs were isolated in a novel environment for 120 minutes they exhibited frequent vocalizing and line crossing followed by prolonged periods of passive behavior. Although vocalization is typically studied in preweaning pups and is associated with maternal separation, increased vocalizing was expected as animals continue calling in isolation for weeks post-weaning (Pettijohn, 1979). However, when periadolescents were tested in the presence of an unfamiliar adult male, vocalizations were significantly reduced. In typical separations, vocalizations and line crossings increase and decrease in unison, but this was not the case in animals tested with adult males. While vocalizations decreased, line crossings increased. That is, test subjects showed more locomotion when they were in the presence of unfamiliar adult males than when alone. Although increased vocalizing and line crossing and reduced vocalizing are often associated with the protest stage, it is likely that the increased line crossing observed with adult males is not representative of this protest behavior but rather reflects the interaction between the two animals.

Both non-agonistic and agonistic behaviors demonstrated by unfamiliar adult males toward test subjects were consistent with all previous studies, infant and periadolescent alike, with one exception. Periadolescent females engaged in significantly more non-agonistic interactions with the adult males than did periadolescent males. Although females of this age are not typically considered to be sexually mature, the

increased amount of sniffing, nose-to-nose contact, and anogenital investigation demonstrated by the adult males could be the response of adult males reacting to females approaching sexual maturity. These behaviors, common to the guinea pig, are demonstrated during investigation of unfamiliar individuals and are also seen prior to copulation (Hennessy et al., 2018). However, agonistic behaviors, including but not limited to nipping, lunging, and kicking are less typical and can be observed when animals become aggressive in an attempt to assert dominance. These agonistic behaviors could be responsible for the prolonged passive responses observed in juveniles tested with adult males.

Similar findings can be observed in infant rats separated in enclosures directly adjacent to enclosures containing unfamiliar adult males. Infanticide is a common occurrence in the rat species, so infants cannot be placed in the same enclosure as fully mature adults. In the presence of the adult males, infants emitted significantly fewer ultrasonic vocalizations and were immobile for longer periods of time when compared to the controls (Wiedenmayer et al., 2003). Although present during infancy, this effect begins to diminish by the time rats approach sexual maturity. Locomotion and ultrasonic vocalizations increase as adolescents begin to challenge adults for breeding rights with the females as they are no longer at an increased risk for infanticide (Wiedenmayer et al., 2003). Although adult male guinea pigs do not kill pups or juveniles, adult males may still pose a threat, leading to reduced vocalizations and increased passivity. Alternatively the decrease in vocalizations in subjects separated with adult males may have been the result of the adult distracting the young. This is further supported by the fact that subjects also demonstrated increased locomotion when compared with animals separated

in isolation as they tended to follow the adult male but would also retreat upon being approached. Unlike rats, guinea pig males are known to buffer serum cortisol levels in infants. The same can now be said for guinea pigs approaching adolescence, although a longer time interval is required for this buffering to be detected (60 vs 120 minutes). Even in the presence of this buffering effect, subjects continued to demonstrate full passive responses at higher rates than subjects separated in isolation with persistently elevated serum cortisol levels. This dissociation raises the question as to whether or not these responses are interconnected or rather the result of multiple neural mechanisms operating independently of one another. Evidence from the current study supports the latter. The increased passivity was likely the result of persistent agonistic and non-agonistic stimulation from the adult toward the young. It appears that the protest stage never took place because the subject was preoccupied with the advances of the adult, and serum cortisol levels continued to be buffered as unfamiliar adult males have been found to serve as adequate social buffering partners in the guinea pig species.

The ability of the unfamiliar adult male to serve as a social buffering partner in guinea pig offspring is an unusual finding in that adult males do not interact with offspring in any degree throughout development as mothers serve as primary caretakers (Graves and Hennessy 2000; Hennessy and Ritchey 1987). Moreover, this buffering is still present regardless of the degree of agonistic and non-agonistic behavior toward the adolescents. Although these behaviors seemed to increase time spent by subjects in the full passive response, serum cortisol levels remained significantly reduced when compared to subjects tested in isolation and approached levels measured in the control group. These findings are similar to findings from past experiments in which infant

guinea pigs were similarly tested with unfamiliar adult males. These animals also showed decreased serum cortisol levels while simultaneously spending more time in the full passive response when compared with animals tested in isolation. This was the case in infants tested at both 60 and 120 minutes (Hennessy et al., 2015). However, significant decreases in serum cortisol levels in periadolescent guinea pigs were not detected in subjects tested at 60 minutes but could be detected at 120 minutes as is shown in the current study (Hennessy et al., 2002b).

This age-related difference could suggest a diminished buffering response in the older animals that requires a longer time period to activate. It could also suggest that the mechanism responsible for buffering the HPA axis in infants could differ from the mechanism responsible for buffering the HPA axis in periadolescents. When guinea pigs are raised in large, multi-familial social groups, it is typical of offspring to interact with both adult males and adult females. Interacting with the adult males has been shown to radically reduce serum cortisol levels in young males at early sexual maturity. This effect, which can be seen at about 4 months of age, has been theorized as being essential for reducing aggression and preventing younger, sexually mature males from challenging larger, more mature adults for breeding rights (Sachser et al., 2013). As previously mentioned, this mechanism most likely differs from the buffering mechanism that has been detected in developing offspring prior to maturity, as these animals pose no significant threat to the breeding patterns of the adult males. To date, no potential mechanism has been found, but it is likely that the mechanisms differ between the two age groups.

Increased activity in the prelimbic and infralimbic cortices has been hypothesized as a potential mechanism responsible for suppression of HPA axis activation. Increased number of behavioral interactions between juvenile guinea pigs and unfamiliar adult males has been associated with elevated expression of fos in these regions (Hennessy et al., 2018). Ablating these regions in rats significantly increased HPA axis activity and subsequent plasma cortisol response to stressful stimuli, while stimulating these regions decreased HPA axis activity (Radley et al., 2006). This suggests that prelimbic activation could play a role in the inhibition of excitatory input to the PVN, thereby decreasing HPA axis activation. However, findings from the current study do not seem to support this hypothesis. When comparing the conditions, there was no significant difference in the expression of fos in either the prelimbic or infralimbic cortices. That is to say, one would expect to find increased fos expression in subjects tested with the unfamiliar adult males, but this was not the case. However, fos values across all conditions tended to be much lower when compared with values measured in prior studies. This may have been the result of an age effect, as the current study tested periadolescent guinea pigs and the previous study pups. However, current findings should be considered preliminary until replicated.

In summary, the results of the present study demonstrate that an unfamiliar adult male guinea pig is capable of buffering the cortisol response in guinea pig offspring approaching adolescence. However, 120 minutes is necessary to detect a significant effect when 60 minutes is all that is required in infants. This finding suggests that the ability of the unfamiliar adult male to buffer the stress response is age dependent in that a longer period of time is necessary to detect an effect in older animals. This reduction in

cortisol levels is present even though subjectively the animals spend a significantly increased amount of their time demonstrating a full passive response. This suggests duration of passiveness is not dependent on serum cortisol levels and that the two effects are likely the result of differing mechanisms.

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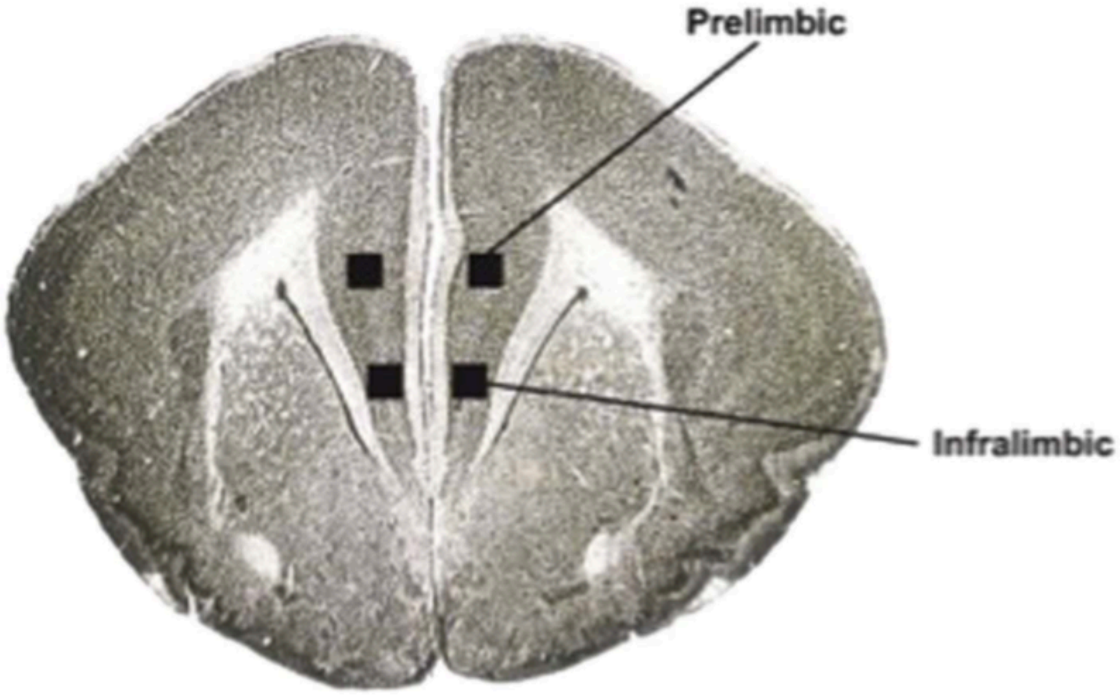

Figure 1. Approximate location of regions selected for analysis of Fos staining in prelimbic and infralimbic cortex. Section displayed is 14.2 mm anterior to the intraaural axis. Adapted from *Stereotaxic Atlas of the Forebrain of the Guinea Pig (*Luparello, 1967)

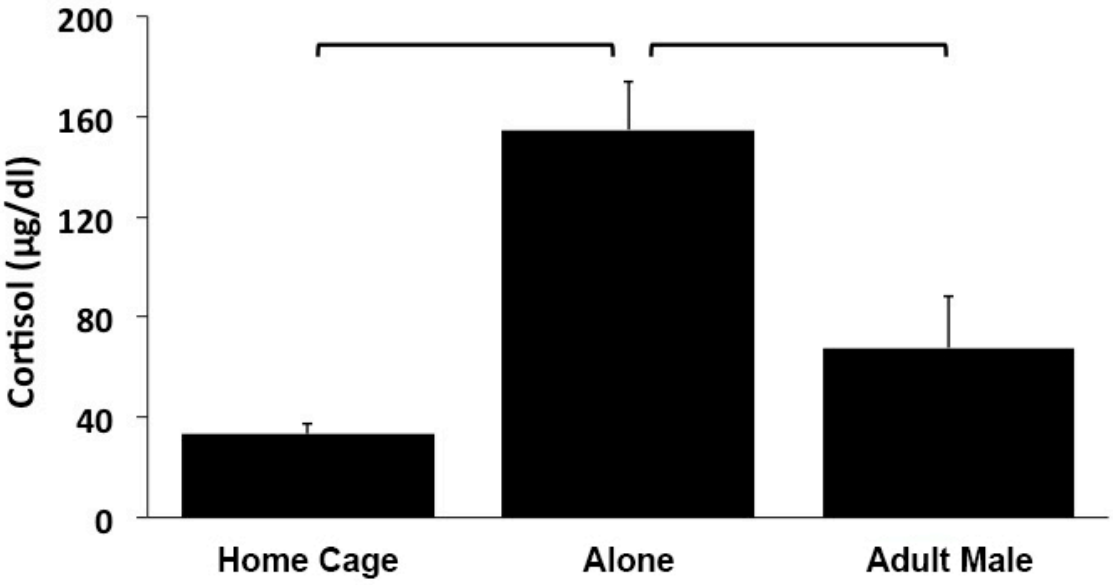

Figure 2. Mean plasma cortisol levels of periadolescent guinea pigs in the three conditions. Vertical lines represent standard errors of the means. Horizontal lines above histograms indicate conditions that differ significantly from each other by at least $p < 0.05$.

Table 1.

Behavior definitions

Behavior	Definition
Active	
Vocalization	High pitched "whistles" (F)
Passive	
Crouch	Hunched stance with feet tucked beneath body (1-min)
Lie	Trunk supported by cage floor (1-min)
Eye-close	Closure of one or both eyes for at least 1 s (1-min)
Piloerection	Piloerection over at least half of the body surface (1-min)
Full Passive	Occurrence of eye-close, piloerection, and either crouch or lie (1-min)
Non-agonistic social	
Nose-nose	Physical contact between noses of the two animals (F)
Anogenital Investigation	Partner places nose in contact or within 1 cm of pup's anogenital region (F)
Fur Sniff	Partner lifts section of pup's fur with snout (F)
Agonistic	
Kick	Partner kicks or attempts to kick pup (F)
Nip	Partner nips or attempts to nip pup (F)
Thrust/lunge/lift	Partner jabs head or makes short run at pup or uses snout to lift pup (F)

Note. F = frequency of behavior; 1-min = number of 1-min intervals in which behavior occurred.

Table 2.

Median (Semi-interquartile range) numbers for behavioral comparisons of Alone and Adult Male periadolescents.

	Alone	Adult Male
Vocalization	40.0 (87.7)	0.0 (1.1)*
Line Crossings	2.5 (25.5)	67.5 (24.50)**
Full Passive	0.0 (1.7)	4.5 (5.2)***

Note. * $p < 0.01$ vs Alone. ** $p < 0.01$ vs Alone. *** $p < 0.05$ vs Alone.

Table 3.

Mean number of Fos positive cells in the prelimbic and infralimbic cortices.

	Home Cage	Alone	Adult Male
Left Prelimbic Cortex	17.1 ± 3.9	29.1 ± 10.4	25.7 ± 13.1
Right Prelimbic Cortex	19.6 ± 4.3	26.5 ± 9.1	25.4 ± 6.9
Left Infralimbic Cortex	15.7 ± 4.1	17.1 ± 13.5	20.9 ± 5.4
Right Infralimbic Cortex	14.0 ± 3.3	21.7 ± 2.9	15.6 ± 4.0