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Maternal buffering beyond glucocorticoids: impact of early life stress on corticolimbic circuits that control infant responses to novelty

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Abstract

Maternal presence has a potent buffering effect on infant fear and stress responses in primates. We previously reported that maternal presence is not effective in buffering the endocrine stress response in infant rhesus monkeys reared by maltreating mothers. We have also reported that maltreating mothers show low maternal responsiveness and permissiveness/secure-base behavior. Although still not understood, it is possible that this maternal buffering effect is mediated, at least partially, through deactivation of amygdala response circuits when mothers are present. Here we studied rhesus monkey infants that differed in the quality of early maternal care to investigate how this early experience modulated maternal buffering effects on behavioral responses to novelty during the weaning period. We also examined the relationship between these behavioral responses and structural connectivity in one of the underlying regulatory neural circuits: amygdala-prefrontal pathways. Our findings suggest that infant exploration in a novel situation is predicted by maternal responsiveness and structural integrity of amygdala-prefrontal white matter depending on maternal presence (positive relationships when mother is absent). These results provide evidence that

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maternal buffering of infant behavioral inhibition is dependent on the quality of maternal care and structural connectivity of neural pathways that are sensitive to early life stress.

Keywords

Social buffering; maternal care; infant maltreatment; rhesus monkeys; uncinate fasciculus

Introduction

Social buffering, the ability of conspecifics to reduce an individual's stress response (both behavioral and physiological) in potentially threatening contexts, has been recognized for decades (Cohen & Wills, 1985; Hennessy, Kaiser, & Sachser, 2009). For infant mammals social buffering is uniquely provided by the mother and supports infant attachment to the caregiver, which is of vital importance for survival in altricial species (Gunnar, Hostinar, Sanchez, Tottenham, & Sullivan, 2015; Landers & Sullivan, 2012; Sanchez, McCormack, & Howell, 2015). Maternal presence buffers the endocrine and behavioral responses to threats in primate infants, including human and nonhuman primate species (Gunnar & Donzella, 2002; Gunnar et al., 2015; Sanchez et al., 2015). Recent elegant work in rodents has provided critical evidence of the neurobiological mechanisms of these effects. In rat pups maternal presence seems to act as an external regulator/switch that can attenuate fear responses via suppression of the infant glucocorticoid response and activation of the amygdala (Moriceau & Sullivan, 2006). Thus, maternal buffering of the infant stress response seems to work via regulation of neural circuits underlying emotional and stress reactivity, including amygdalaprefrontal cortex pathways. The ability of maternal signals (e.g. odor in rat dams) to regulate this aspect of infant brain function follows a specific developmental course, with decreased maternal effectiveness to reduce/buffer her infant's stress response coinciding with the infant's ability to independently locomote and leave the nest. Primate species also show maternal buffering of stress responses in infants and juveniles, which are thought to involve modulation of developing self-regulatory systems, including amygdala-prefrontal circuits (Gee et al., 2014; Gunnar et al., 2015; Gunnar and Sullivan, 2016; Sanchez et al., 2015; Tottenham 2015). The quality of maternal care can affect not only the buffering power of the mother's presence, as we have demonstrated for macaques (Sanchez et al., 2015), but can impact the way these self-regulatory systems mature, with some evidence suggesting accelerated maturation of self-regulatory processes under poor caregiving experiences, interpreted as an accelerated transition to the offspring's independent state, although also linked to enhanced emotional reactivity (Gee et al., 2013).

Among the behaviors used to examine how maternal presence modulates the offspring's emotional reactivity are exploration and fear behaviors in response to novelty. They form part of a construct related to fear termed behavioral inhibition, which involves decreased exploratory behavior and increased vigilance and approach towards the caregiver in response to novelty (Fox, Henderson, Marshall, et al., 2005). In rhesus monkeys differences in behavioral inhibition have been related to differences in the function of limbic brain regions and of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis, which is strongly controlled by the amygdala and its connections with the prefrontal cortex (Kalin, Larson,

Shelton, & Davidson, 1998). Human studies have demonstrated that individual differences in these same limbic brain regions are also associated with different emotional and temperamental types (Schwartz, Wright, Shin, et al., 2003; Davidson 2002). Interestingly these cortico-limbic, amydala-prefrontal circuits, and the white matter tracts connecting them, are sensitive to early life stress, including that associated with poor early caregiving and childhood maltreatment experiences (Eluvathingal, Chugani, Behen, et al., 2006; Govindan, Behen, Helder, et al. 2009; Kumar, Behen, Singsoonsud, et al., 2013).

Myelination of brain white matter (WM) is an important property of fiber bundles that increase connectivity through rapid axonal conductance between distant regions and subsequent behavioral responses (Fields, 2008; Thomason & Thompson, 2011; Zatorre, Fields, & Johansen-Berg, 2012). Brain WM undergoes massive developmental increases in volume during early life, due in part to increases in myelination, making this developmental process vulnerable to environmental influence during infancy and childhood (Deoni, Dean, O'Muircheartaigh, Dirks, & Jerskey, 2012; Dubois et al., 2014; Geng et al., 2012). There is also evidence that myelination is sensitive to glucocorticoids (Jauregui-Huerta et al., 2010), suggesting that elevated levels of these stress hormones due to chronic stress activations and alterations in maternal buffering of the stress response could affect the development of brain WM during infancy and childhood. Diffusion tensor imaging (DTI) is a powerful imaging modality for *in vivo* characterization of WM in the brain (Le Bihan, 2003). DTI is able to quantify water diffusion in the brain on a microscopic scale using a variation of a typical MRI magnetization sequence (Jones & Leemans, 2011). Diffusion properties along WM bundles can be quantified because myelinated axons restrict what would otherwise be non-directional diffusion (i.e. isotropic) of water, resulting in anisotropy (Le Bihan et al., 2001). These diffusion properties include fractional anisotropy (FA), calculated as the ratio of diffusion parallel to the fibers, to diffusion perpendicular to the fibers, and thus can be affected by either changes in diffusion perpendicular to the tract, which decreases with increased axonal myelination (Keller & Just, 2009; Zhang et al., 2009), or to changes in diffusion parallel to the tract, which increases with axonal density, caliber, and microtubular packing and organization (Kumar, Nguyen, Macey, Woo, & Harper, 2012). Thus, higher FA values could be interpreted as increased white matter tract integrity due to increased myelin or increased fiber tract organization. FA was selected in the current study because it increases across early development (Cohen et al., 2016; Deoni et al. 2012; Shi et al., 2013) and it has been associated with variations in behavior (Fields 2008; Howell et al., 2013; Scholz et al., 2009).

Given that amygdala-prefrontal circuits seem to be involved in the behavioral effects of maternal buffering (Gee et al., 2014; Tottenham 2015), the current study focused on the uncinate fasciculus (UF), a major WM tract connecting the prefrontal cortex and the amygdala (Schmahmann & Pandya, 2009; Schmahmann et al., 2007). This tract is involved in emotional regulation (Von Der Heide, Skipper, McFarlin, & Olson, 2013) and anxious temperament (Baur, Hanggi, and Jancke 2012; Kim and Whalen 2009). Reductions in the structural integrity of this tract have been implicated in affective disorders (de Almeida, Verscae, Mechelli, et al., 2009; Phan, Orlichenko, Boyd, et al., 2009; Tromp, Grupe, Oathes, et al., 2012). Previous work shows that social deprivation early in life, including lack of typical maternal care, reduces FA in the UF (Eluvathingal, Chugani, Behen, et al., 2006;

Govindan, Behen, Helder, et al. 2009; Kumar, Behen, Singsoonsud, et al., 2013). This suggests that alterations in this tract may contribute to psychopathology, and are caused by poor maternal care and impaired maternal buffering early in life, such as in situations of childhood maltreatment.

Childhood maltreatment, including physical abuse and neglect, is a much more pervasive issue than most people are aware. In 2013 there were approximately 700,000 substantiated victims of childhood maltreatment (US Department of Health and Human Services, 2014). Estimated lifetime cost (including medical care, child welfare, criminal justice, special education costs, and productivity losses) per victim of childhood maltreatment is estimated to be $210,012 (Fang, Brown, Florence, & Mercy, 2012). The high medical costs are not only due to the acute care needed as a direct result of maltreatment, but also to the poor long-term outcomes associated with this adverse early experience. These outcomes include increased incidence of psychopathology (e.g. anxiety and depression, post-traumatic stress disorder, PTSD), behavioral disorders, substance abuse, cognitive and language deficits and obesity (Danese & Tan, 2014; Gunnar & Quevedo, 2007; Teicher et al., 2003). Furthermore, there is some evidence supporting that the earlier the exposure to maltreatment the more severe the negative psychological and behavioral outcomes (Kaplow & Widom, 2007). This becomes especially important given that more than 25% of victims of maltreatment are under three years of age (US Department of Health and Human Services, 2014). Given the poor behavioral and health outcomes, and the number of children affected by this problem, it is critical to determine how these experiences get under the skin to affect the brain, physiology, and behavior.

Child maltreatment is not a uniquely human phenomenon, but has also been reported in wild and captive populations of nonhuman primates, including macaques, baboons, and marmosets (Brent, Koban, & Ramirez, 2002; Johnson, Kamilaris, Calogero, Gold, & Chrousos, 1996; Maestripieri & Carroll, 1998; Troisi & D'Amato, 1984; Sanchez, 2006; Howell, Neigh, & Sanchez, 2016). The current study focused on spontaneous infant maltreatment by macaque mothers as a translational animal model in which we are trying to identify biological mechanisms underlying the neurobehavioral consequences of early adverse experiences. Infant maltreatment in this model is comprised of (1) physical abuse in which the mother exhibits aberrant violent behaviors towards the infant (drags, crushes, sits on, or roughly grooms the infant) during the first 2-3 months of life, which triggers overt signs of distress in the infant (vocalizations, tantrums, etc.), and (2) high rates of infant rejection early in life, which is a physically undamaging behavior consisting of pushing the infant away when it solicits contact with the mother, but that also results in infant distress (i.e. tantrums, screams) (Maestripieri & Carroll, 1998; Sanchez, 2006; Howell, Neigh, & Sanchez, 2016). Studies using this model have identified socioemotional alterations and activation of the stress response comparable to those seen in maltreated children (Koch, McCormack, Sanchez, & Maestripieri, 2014; Maestripieri, 1998; McCormack, Sanchez, Bardi, & Maestripieri, 2006; McCormack, Newman, Higley, Maestripieri, & Sanchez, 2009; Sanchez, 2006; Sanchez et al., 2010; Howell, McCormack, Grand, et al., 2013). Delays in social development including delayed infant independence from the mother, less exploration and play have also been reported in this model (Maestripieri, 1998; Maestripieri, Jovanovic, & Gouzoules, 2000). These findings parallel long-term consequences found in humans with

histories of childhood maltreatment, supporting the construct validity of this model to gain understanding of the mechanisms underlying the poor developmental outcomes associated with this adverse early experience. Based on evidence that maltreatment can be perpetuated from generation to generation in humans and nonhuman primates, and that genetic and epigenetic mechanisms can result in behavioral traits associated with maltreatment (Franklin et al., 2010; Huizinga et al., 2006; Maestripieri, 2005; Tarullo & Gunnar, 2006), it is important to disentangle the role of early life stress experience from that of potential heritable phenotypes (e.g. higher stress and/or emotional reactivity). This was accomplished in the current study using a crucial experimental design involving cross-fostering of infants at birth and random assignment to either control mothers or mothers with a history of maltreating their infants.

The goals of the current study were to examine (1) the impact of infant maltreatment on behavioral inhibition of rhesus monkeys in response to novelty during weaning, (2) how maternal presence modulated behavioral inhibition, and (3) the role of amygdala-prefrontal circuits and specific aspects of the maltreatment experience on these behavioral responses. We examined behavioral inhibition of control and maltreated infants in response to a novel environment and stimuli, with and without maternal presence, using (1) the Free Play portion of the rhesus monkey Laboratory Temperament Assessment Battery (adapted from the human Laboratory Temperament Assessment Battery (Lab-TAB) (Goldsmith & Rothbart, 1991) by Dr. Judy Cameron's group at the Oregon National Primate Research Center, for use in 3-12 month old infant rhesus monkeys (Bethea et al., 2004; Williamson et al., 2003), and (2) the Alone Condition of the Human Intruder Paradigm (Kalin & Shelton, 1989). We hypothesized that infants that experienced maternal maltreatment early in life would be more behaviorally inhibited (i.e. would explore less and seek more close ventral maternal contact) when exposed to a novel environment, even if the mother was present. This is based on our previous findings that maternal presence is not effective in buffering the endocrine stress response in infant rhesus monkeys reared by maltreating mothers (Sanchez et al, 2015). We also hypothesized that weaker structural integrity of the UF in maltreated infants (suggesting weaker amygdalaprefrontal connectivity; quantified as FA using DTI) would predict higher behavioral inhibition during these novelty tasks. Based on our previous reports that maltreating mothers also show low maternal responsiveness and permissiveness (McCormack et al, 2015), we investigated whether these qualitative aspects of maternal care, in addition to abuse and rejection, are important predictors of infant behavioral reactivity.

Methods

Subjects

A total of 36 infant rhesus monkeys (*M. mulatta*) and their mothers were studied when the infants were 6 months of age (early weaning phase), with 18 raised by competent – control– mothers (9 male and 9 female) and 16 raised by maltreating mothers (12 males and 6 females). Animals were born and lived at the Yerkes National Primate Research Center (YNPRC) Field Station, Lawrenceville, GA, where they were socially housed for the entire study. Subjects lived in large social groups consisting of between 75 and 150 adult females, their sub-adult and infant offspring, and 2-3 adult males. Animal enclosures were

approximately 100ft × 100ft, and included a climate controlled indoor housing area. Standard high fiber, low fat monkey chow (Purina Mills Int., Lab Diets, St. Louis, MO) and seasonal fruits and vegetables were provided twice daily. Water was available *ad libitum*. All of the procedures described here were performed in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and Use of Laboratory Animals" and approved by the Emory Institutional Animal Care and Use Committee.

In order to disentangle the effects of early life experience from those of heritable phenotypes (e.g. emotional reactivity, behavioral inhibition) the current study used a critical experimental design involving cross-fostering of infants at birth and random assignment to either mothers with a solid history of competent –control– maternal care, or mothers with a history of infant maltreatment. Of the animals raised by control dams, 11 were the biological offspring of control mothers (7 males and 4 females) and 7 were born to maltreating mothers (2 males and 5 females). Of the animals raised by maltreating dams, 10 were the biological offspring of control mothers (7 males and 3 females), and 8 were born to maltreating mothers (5 males and 3 females). Subjects were matched for dominance rank (high, medium, low) and mothers were selected from different matrilines (i.e. they were unrelated individuals) whenever possible. Newborn monkeys were cross-fostered to an unrelated female on the first day of life (except for a small number of infants cross-fostered within 48-72h of birth) following modified published procedures (Maestripieri, 2005). Briefly, on the day of birth infants were taken from their biological mothers and placed with their foster mothers within 5 minutes of initial separation. These procedures had a high rate of adoption success (90%) if done within 48h of birth. In the rare cases where the foster dam did not immediately accept her foster infant, both dam and foster infant were moved to a quiet room and left in close proximity for a couple of hours. If that was unsuccessful the dam was lightly anesthetized with ketamine (5mg/kg, i.m.) and the foster infant was allowed to nurse (to increase endogenous levels of the maternal hormone oxytocin). If none of these strategies were successful, infants were returned to their biological mothers.

Behavioral observations of mother-infant interactions

Observations of mother-infant interactions were performed following birth and cross-fostering over the first 3 months of life to verify and quantify early maternal caregiving experienced by infants (e.g. control mothers did not maltreat their foster infants, and maltreating mothers did maltreat their foster infants; to capture rates of abuse and rejection received). Behavioral observations were collected in each mother-infant pair by four experienced coders from observation towers located at each social compound during the first three months postpartum using an adaptation of a well-established rhesus monkey ethogram (Altmann, 1962) and following previously published procedures (Maestripieri, 1998; McCormack et al., 2006; McCormack et al., 2009). Prior to data collection, inter-observer reliability was reached among the four coders, with percent agreements exceeding 90% and Cohen's Kappa exceeding 0.8. Each observation lasted 30 minutes and was performed on separate days 5 times per week in the first month postpartum, 2 times per week in the second month, and 1 time per week in the third month, for a total of 32 observations (16 hours) per mother-infant pair. This observation schedule was chosen to best document early maternal

care received by the infant, including the occurrence of infant maltreatment, as the frequency of physical abuse is highest in the first month and decreases steadily thereafter (Maestripieri, 1998; McCormack et al., 2006). Observations were completed between 7:00 and 11:00 AM, when the animals were most active. All animals in the group were locked outdoors during observation sessions.

Infant maltreatment was defined as physical abuse, which was co-morbid with early maternal rejection of the infant, following behavioral definitions, observation protocols and inclusion/exclusion criteria described in detail for this species in previous publications (e.g. Troisi & D'Amato, 1983; Maestripieri, 1998; McCormack et al., 2006; McCormack et al., 2009). Briefly, physical abuse was operationalized as at least three instances of violent maternal behavior directed towards the infant (dragging the infant by its tail or leg, crushing it against the ground, sitting/stepping on it, throwing it, roughly grooming it, or carrying the infant with an arm away from the body preventing it from clinging) during the first three months of life (Maestripieri, 1998; Maestripieri et al., 2006; McCormack et al., 2006, 2009). In addition, maltreating mothers also exhibited high rates of early infant rejection, operationalized as the mother preventing contact or infant access to her nipple by pushing the infant away or passively blocking her chest with her arm or twisting her torso away. Both abuse and rejection behaviors produce high levels of distress in the infant (Maestripieri, Jovanovic, & Gouzoules, 2000; McCormack et al., 2006; Sanchez, 2006).

In addition to the collection of frequency and severity of infant abuse and rejection by the mother, additional behavioral data was collected to measure mother-infant interactions and individual infant's and mother's behavior. Behaviors collected included affiliative behaviors (e.g. contact, proximity, grooming, play), aggressive behaviors (hit, bite, chase, threat), fear behaviors (e.g. screams, fear grimaces), anxiety-like behaviors (e.g. yawning, scratching, and body shakes- Maestripieri, Schino, Aureli, & Troisi, 1992; Troisi et al., 1991), normative mother-infant behaviors (e.g. time spent on dam's ventrum, cradle, groom infant), and general behavior (e.g. eating, drinking, passive) (see Supplemental Material for complete ethogram).

Instrument of Macaque Maternal Care (IMMC)

In addition to recording frequencies and durations of specific mother-infant behaviors in the social group, global aspects of maternal care were also assessed using observer rating scales of maternal care, the IMMC, adapted for use in rhesus monkeys by our group (McCormack et al., 2015). The global aspects of maternal care captured by this tool include maternal sensitivity/responsivity, protectiveness, permissiveness/secure-base behavior, and irritability. Briefly, the IMMC is a 22-item global rating instrument of the quality of maternal care created by adapting measures published in the Maternal Q-sort for macaques (Kondo-Ikemura & Waters, 1995), the Human Maternal Behaviors Q-Sort (Pederson & Moran, 1995), and the Free Play and Clean-Up task for children (Gardner et al., 1999; Pears & Ayres, 2000) (see McCormack et al., 2015 for details). Items in the IMMC were selected based on applicability to rhesus monkey mother-infant behavioral interactions and their coverage of a spectrum of maternal care qualities. Each item was scored by trained raters on a 5 point Likert scale, with 1 representing no behavior occurrence and 5 reflecting high

occurrence of the behavior. If the particular situation described in an item did not occur during the observation, then the event was scored as "N/A". The coders completed this 22-item IMMC instrument immediately following each 30 min focal behavioral observation described above.

Laboratory Temperament Assessment Battery (LabTAB)- Free Play

In order to examine the effects of maternal care on infant behavioral responses to novelty, including behavioral inhibition, the Free Play portion of the Laboratory Temperament Assessment Battery (LabTAB), developed by Dr. Judy Cameron at the Oregon National Primate Research Center (Bethea et al., 2004; Williamson et al., 2003), was used. This test was adapted for use with infant rhesus monkeys (3-12 months old) from the LabTAB originally developed for human children (Goldsmith & Rothbart, 1991). Just as in the human LabTAB, the mother is present during all phases of testing, but for the rhesus version she is anesthetized to control for differences in her response to the task that may affect the infant's responses. The rhesus infant LabTAB implemented in the current study was divided into two consecutive tests: Free Play (35 min in a novel room with toys) and the Remote Controlled Car (RCC: 2 min with a RCC being driven towards the infant). Only results from the Free Play test are presented here because the responses of infants to the RCC reached a ceiling effect, with no individual variability (data not shown). We were unable to test 3 of the subjects due to acute illness and equipment malfunctions, for a final sample of 33 infants.

To administer this test each mother-infant pair was removed together from their social group in the morning following standard procedures in our lab and the YNPRC that require prior training of the mothers to move on command from the outdoor enclosure to indoor caging. Each dam was anesthetized (6 mg telazol/kg BW, i.m.) prior to transport to the testing room. Mother and infant were transported together to the testing room (2.3m × 2.85m with two one-way windows for videotaping and monitoring) where the dam was placed in a car seat with a waterproof pad in the corner of the room (Fig. 1) with the infant comfortably placed on her ventrum. The testing room was equipped with a climbing structure (150cm × 125cm × 30cm) located opposite to the door and nine novel toys arranged in a semicircle (Fig. 1). The floor was marked with a 1-foot grid to assist in measuring infant locomotion and distance from the mother from video recordings. Cameras were located both inside the testing room and behind the one-way mirror to completely capture infant behavior. Videotaping began once the experimenters left the testing room and locked the access door. Both the mother and infant were monitored constantly through the one-way mirror throughout the test. On the rare occasion that the mother started to recover from anesthesia during the test, the test was paused and a ketamine supplement was administered.

Videotapes were coded using the two synchronized video recordings (inside and outside of the room) for the behaviors included in the LabTAB ethogram (Supplementary material) using The Observer XT 10 software (Noldus Information Technologies Inc, Netherlands). A single experimenter that had achieved intra- and inter-observer reliabilities of 80% with other trained observers and who was blind to group assignment coded all LabTAB videos. Frequencies and durations of behaviors were scaled for total test duration, and excluded periods where there was an outside disturbance or the infant was out of view. Proportion of

time spent exploring (sum of all exploration) and proportion of time spent on the mother's *ventrum* (*on ventrum*: indicative of behavioral inhibition) (Fox, Henderson, Marshall, et al., 2005; Kagan et al., 1987) are presented here as we predicted that they would be affected by early maternal care and buffering.

Human Intruder Paradigm - Alone Condition

The Human Intruder (HI) paradigm (Kalin & Shelton, 1989) was also used at 6 months of age as part of a more extensive series of studies to determine whether maltreated animals differ from control animals in the development of their behavioral responses to threat. In the current study we are only reporting results from the Alone condition so as to compare the modulation of the infants' behavioral responses to a novel environment without maternal presence with the infant's reactivity during the LabTAB when the mother is present. For the HI mother-infant pairs were accessed in their home compound as described previously on a different day than the LabTAB. The infant was separated from the mother and transported to a novel testing room where it was then transferred to a stainless steel testing cage with one side made of clear plexiglass to allow video recording. The entire test lasted approximately 35 minutes, and consisted of three 10-minute conditions (alone, profile [referred to as "no eye contact" in other publications], and stare) presented in the same order for all animals (Kalin & Shelton, 1989). Immediately following the test the infants were reunited with their mothers and returned to their social groups.

For this study the infants' behavior during the HI paradigm was video recorded and later coded using a detailed ethogram based on previous studies (Kalin & Shelton, 1989; Raper et al., 2013; Howell, Godfrey, Gutman, et al., 2013) (Supplementary material). Digital videos were coded using The Observer XT software (v10.5, Noldus, Inc., Netherlands) by one experimenter. Before coding the coder reached an average inter-rater reliability of Cohen's Kappa = 0.845 with another trained experimenter who coded a representative set of videos from similar studies with rhesus infants (Raper et al., 2013). For the current study we focused on total proportion of time exploring because this behavior represents similar constructs in both the LabTAB Free Play and HI Alone contexts (i.e. more behaviorally inhibited infants would explore less in both tasks), making it ideal to explore differences in responses to a novel environment that may be modulated by maternal presence.

Diffusion Tensor Imaging (DTI)

Diffusion-weighted images (DWI) were acquired at 6 months of age in 34 of the subjects (2 were not scanned due to acute illness) using a 3T Siemens scanner and 8-channel array coil. DWI data were collected with the following parameters: single-shot dual spin-echo EPI sequence with GRAPPA (R=3), voxel size: $1.3 \times 1.3 \times 1.3 mm^3$ with zero gap, 60 directions, TR/TE=5000/86 ms, 40 slices, FOV: 83 mm, b:0, 1000 s/mm^2, 12 averages, acquisition time: 75 min. All preprocessing and tensor reconstruction was performed using the DTIPrep software developed by the UNC Neuroimaging Research and Analysis Laboratories (NIRAL) (Oguz et al., 2014). Tensors were calculated to produce a diffusion tensor image (DTI) for each subject. Each DTI dataset was visually assessed for artifacts and was excluded if any artifact could not be corrected. A DTI atlas was built as described previously (Hart et al., 2010). Briefly, FA was calculated for each subject and each subject's data was

then nonlinearly warped into a standard space. The UF was tracked in each hemisphere using the 3D Slicer software (Fig. 2) (Fedorov et al., 2012). The UF was identified using anatomical landmarks as defined in previous work (Schmahmann & Pandya, 2009; Schmahmann et al., 2007). The deformation fields calculated during atlas building were used to transform the UF tracked in standard space into each subject's native space. FA was calculated at 0.3 mm intervals along the right and left UF for each subject in native space, and then averaged along the entire tract to produce a single value used for statistical analyses.

Statistical Analyses

Normality was assessed for each variable using the Shapiro-Wilk test as implemented in the IBM SPSS Statistics for Macintosh, Version 22.0 (Armonk, NY: IBM Corp). If data were not normally distributed a log transform was applied. If this did not result in normally distributed data, non-parametric statistical approaches were applied in subsequent analyses. A preliminary analysis was run to assess the effects of biological mother (control versus maltreating) on each dependent variable using one-way ANOVA (parametric for normally distributed data, non-parametric Kruskal-Wallis ANOVA for non-normally distributed data). Biological dam did not have a significant effect on any of the variables, and was thus excluded from further analyses. For normally distributed variables, a parametric 2×2 ANOVA was used to examine the effect of maternal care (control versus maltreating foster care groups) and sex (male, female) as the fixed factors. For variables where there were no significant effects of sex, this factor was removed from the statistical model. For non-normally distributed data, sex effects were first assessed using a nonparametric Kruskal-Wallis ANOVA. There were no significant effects of sex for any of the non-normally distributed data, thus sex was excluded from subsequent analyses for all of these variables. As a consequence, Mann-Whitney U tests were applied to data that was not normally distributed to investigate effects of maternal care (control versus maltreating).

We first examined the effects of maltreatment on the infants' behavioral inhibition during the LabTAB and HI tasks. The analysis of time spent *on ventrum* was unique to the LabTAB task, and was performed using a nonparametric Mann-Whitney U test because this behavior was not normally distributed. The effects of maltreatment on exploration were analyzed using a repeated measures ANOVA, with early maltreatment experience (control versus maltreatment) as the fixed factor and task (LabTAB, with dam, versus HI, without dam) as the repeated measure. Exploratory behavior was chosen as the dependent variable for this focused analysis because it is decreased in behaviorally inhibited individuals, including children, in response to novelty (Fox et al., 2005), allows us to compare it across both the LabTAB Free Play and HI Alone Condition as implemented here. No other behaviors that represented the same underlying construct/state in both tasks occurred at a high enough frequency in both tasks to directly compare the effect of mother's presence using this statistical approach. From previous analyses of maternal care in this infant maltreatment model we have evidence that maltreating mothers are not only abusive and neglectful, but are also less responsive and permissive (maltreating mothers had significantly lower responsivity ($F_{(1,37)} = 136.18$, P< 0.001) and permissiveness ($F_{(1,37)} = 40.42$, P< 0.001) than controls, McCormack et al., 2015). Thus, another goal of this study was to investigate the

relationships between specific qualitative aspects of maternal care that differ between maltreating and competent mothers (i.e maternal responsiveness, permissiveness, abuse rates, and rejection rates) and infant behavioral inhibition in response to a novel environment. We were also interested in the role of PFC-amygdala structural connectivity (quantified as FA of the UF) predicting behavioral inhibition, given the evidence that this WM tract is sensitive to stress, is involved in emotional/stress regulation and regulates behaviors altered in response to social buffering (i.e. fear behavior). A univariate ANOVA was also run to examine effects of maltreatment on FA in the UF. Exploratory analysis of covariance (ANCOVA) was used to examine whether aspects of maternal care that differ between control and maltreating mothers (sensitivity/responsivity, permissiveness/secure-base behavior, abuse, and rejection), and UF structural integrity (FA in both left and right UF) predict behavioral inhibition (i.e. time spent in close contact - *on ventrum* - with the dam during the LabTAB free play; exploration during the LabTAB and the Alone Condition of the HI). For exploration we applied a repeated measures ANCOVA design. For time spent on ventrum we applied a univariate ANCOVA.

Results

Time spent on mother's ventrum during the LabTAB free play task: Effect of early maltreatment experience

Transformation did not normalize the distribution of time spent *on ventrum*. Thus a nonparametric Mann-Whitney U test was applied to the untransformed data to detect differences between maternal care groups (control versus maltreating). A significant difference between proportion of time spent *on ventrum* between maltreated ($M = 0.62$, SEM = 0.07) and control ($M = 0.39$, SEM = 0.08) infants ($U = 72$, p = 0.021) was detected, with maltreated infants spending more time on their dams' ventrum than control infants during the test (Fig. 3).

Exploration with mother present (LabTAB Free Play) versus absent (HI Alone Condition): Effect of early maltreatment experience

The rmANOVA failed to detect significant main or interaction effects of maternal care on exploration during the LabTAB Free Play and HI Alone tests ($F_{(30,1)}=0.792$, p=0.381, $\eta p^2=0.026$). However, a main effect of maternal presence was detected with the proportion of time spent exploring increasing when the dam was not present (HI mean = -0.392, SEM = 0.03, or an average of 40% time spent exploring) as compared to when the dam was present (LabTAB mean = -1.116, SEM = 0.02, or an average of 7% time spent exploring) ($F_{(30,1)}=166.208$, p<0.001, $\eta_p^2=0.847$).

Effect of early maltreatment experience on FA in the UF at 6 months of age

The univariate ANOVA analysis failed to detect a significant main effect of maternal care on FA in either the left ($F_{(30,1)}=2.062$, p=0.162, $\eta_p^2=0.066$) or right UF ($F_{(30,1)}=0.233$, p=0.633, $\eta_p^2=0.008$).

Associations between aspects of maternal care, UF WM tract integrity, and behavioral inhibition with and without dam present

ANCOVA statistics were calculated using SPSS for Macintosh Version 22.0 (Armonk, NY: IBM Corp). When maternal responsivity, permissiveness, abuse rates, rejection rates, and FA in the UF, were included in the model the differences in exploration behavior based on maternal presence were no longer significant (see Table 1). A significant interaction was detected between maternal presence and FA in the left UF (Table 1), in which animals with higher FA in the left UF explored more when the dam was absent and less when she was present (Fig. 4). An additional significant interaction was detected between maternal presence and her responsivity (Table 1), with animals with highly responsive mothers exploring more when the dam was absent and less when she was present (Fig. 5). A univariate ANCOVA was used to examine the effects of maternal responsiveness, permissiveness, abuse and rejection, as well as of UF FA on time spent *on ventrum* during the LabTAB. None of these variables predicted time *on ventrum* (Table 2).

Discussion

Social buffering is a well-documented phenomenon, especially in the context of the mother-infant relationship, resulting in blunted fear and endocrine stress responses. The current study followed a group of rhesus monkey mother-infant pairs during the early weaning period (6 months of age) to investigate the effects of infant maltreatment on behavioral inhibition in response to novelty. Additionally, we investigated the ability of maternal presence to modulate (buffer) these behavioral responses to novelty based on histories of maternal care received early in life. We also investigated whether behavioral response to novelty, both with and without the dam present, was predicted by differences in (1) amygdala-prefrontal connectivity, measured as structural integrity of the UF, brain WM tract connecting amygdala with prefrontal cortex, and important for emotional regulation, and (2) specific aspects of maternal care that differ between control and maltreating mothers (specifically, maternal responsiveness, permissiveness, and abuse and rejection rates). By examining caregiving quality as a continuous measure we were able to make inferences regarding the specific aspects of maternal care that are relevant to maternal buffering. Our hypothesis that maltreated infants would be more behaviorally inhibited based on previous literature (Fox et al., 2005) was supported by the finding that they spent more time in close ventral contact with the mother in comparison to controls in response to a novel environment (LabTAB test) However, no differences in exploratory behavior were detected between maltreated and control infants based on whether or not the dam was present. Exploratory ANCOVA analysis revealed that increased structural integrity in prefrontal cortex-amygdala circuits (as measured by increased FA in the left UF) positively predicted increased exploratory behavior when the dam was absent (i.e. during the HI paradigm). Similar effects were detected for maternal responsivity in which higher maternal responsivity predicted higher levels of exploration in the absence of the mother. These results suggest that at this stage in development (i.e. weaning) high quality maternal care facilitates the self-regulatory role of amygdala-prefrontal circuits supporting exploratory behavior, particularly when the mother is not present. This also suggests that the relative impact of prefrontal-limbic circuits on infant behavior is sensitive to maternal presence, meaning the structural connectivity of

this WM tract predicts behavioral inhibition in infants in different ways when the primary caregiver is present versus when she is not.

In agreement with our hypothesis, infants that had experienced maltreatment spent a larger proportion of time in close ventral contact with the dam during free play in a novel environment as compared to those that had experienced competent maternal care. This can be interpreted as higher behavioral inhibition, at least based on the existing literature on this LabTAB task (Fox et al., 2005) and it is also consistent with previous work in this model. In a previous study maltreating mothers broke contact with their infants more frequently than competent, control, mothers, which resulted in maltreated infants breaking contact with their mothers less often than controls, suggesting an insecure attachment (McCormack et al., 2006). Thus, it is not surprising that during the LabTAB maltreated infants chose to spend more time in close ventral contact with their mothers despite being able to freely explore the novel room while using the mother as a secure base from which to explore. These group differences in secure-base behavior during the LabTAB are consistent with differences in global aspects of maternal care detected during naturalistic social observations in which maltreating mothers are less responsive to their infants, more irritable, and do not show the same secure-base attachment behaviors as control mothers (McCormack et al., 2015). These findings are also in agreement with reports that human children who have insecure attachments with their mothers are also less likely to explore novel environments (Calkins & Fox, 1992; Nachmias, Gunnar, Mangelsdorf, et al., 1996; Schieche & Spangler, 2005). These children also showed higher endocrine stress responses to novelty (Nachmias, Gunnar, Mangelsdorf, et al., 1996; Schieche & Spangler, 2005). There is evidence that a strong attachment is important when human infants start locomoting and exploring/ interacting with the environment (Ainsworth & Bell, 1970; Campos, Kermoian, & Zumbahlen, 1992). It is interesting to note that in the exploratory ANCOVA analyses, despite the difference in time spent *on ventrum* detected between maltreated and control infants during the LabTAB, specific aspects of the maternal care known to differ between control and maltreating mothers were not predictive of such a behavior. This suggests that there may be other aspects of the early experience that affect this behavior.

There is strong evidence that prefrontal-amygdala circuits are especially sensitive to early adversity involving the primary caregiver (Sanchez, McCormack, & Howell, 2015) and are important for emotional reactivity and behavioral responses to novelty. In the current study, although there were no differences in duration or frequency of exploratory behaviors between maltreated and control infants (with or without the dam present), there were differences in time spent on ventrum when the dam was present, which is a behavior related to increased behavioral inhibition in the literature (Fox et al, 2005). When the dam was absent FA in the left UF positively predicted exploratory behavior, suggesting an important role of the brain regions connected by this fiber bundle (i.e. prefrontal cortex and amygdala) in regulating these behaviors. However, when the dam was present, FA in the left UF did not predict exploratory behavior suggesting that prefrontal-amygdala circuits are not involved in this behavior in the presence of the dam. A potential explanation for these findings is that at this stage in development (i.e. weaning) the dam acts as an external regulator of the neural pathways that support exploratory behavior via buffering of the infant's fear and endocrine stress response, serving to bypass the need for the infant to use their prefrontal-amygdala

pathways to independently regulate/guide their behavior. Although we are underpowered to directly test this hypothesis, it is possible that aspects of the maltreatment experience interact with UF FA and maternal presence to predict exploratory behavior (i.e. this may be a three-way, complex, interaction effect).

In both humans and rhesus monkeys individuals with higher right/left prefrontal asymmetry show increases in negative affect (Kalin et al., 1998; Sutton and Davidson 1997). In a study combining the analysis of levels of the stress hormone cortisol with comparisons of brain asymmetry, Kalin et al. (1998) found that monkeys with greater left frontal lobe activation had lower levels of cortisol, while those with higher right activation had higher cortisol levels. These results might suggest that primates, both humans and non-humans, with greater left frontal lobe activation may also have lower rates of fearfulness, anxiety, and stress reactivity (both behavioral and hormonal). These reports, together with our findings of an association between higher exploration with higher structural integrity of the left UF support the idea of left amygdala-prefrontal laterality bias in the regulation of stress and fear responses. These findings need to be corroborated in future studies.

Based on work by Regina Sullivan and colleagues, we know much about infant attachment learning and maternal buffering of the stress response. Early in life in rodents, from approximately postnatal days 1 through 7, maternal presence buffers the glucocorticoid response to a stressor and subsequent amygdala activation, which suppresses the fear learning circuit (Moriceau & Sullivan, 2005; Moriceau & Sullivan, 2006). Interestingly, alterations in these circuits (specifically the amygdala) occur when pups are reared under low-bedding conditions in which the mother exhibits fragmented and unpredictable care (Moriceau, Shionoya, Jakubs, & Sullivan, 2009; Raineki, Moriceau, & Sullivan, 2010). Although we did not specifically target the amygdala, but the tracts that connect it with the prefrontal cortex, our findings build onto the rodent literature by suggesting that during the rhesus infant weaning period there is a decoupling of the uncinate fasciculus and the behavioral responses it regulates when the dam is present, as one would hypothesize if the mother, indeed, acts to suppress amygdala activation in response to threat. These results do not provide any insight into the underlying neural correlates of exploratory behavior in the presence of the dam, something that requires further investigation in future studies. Interestingly, our group has also shown that maternal presence doesn't buffer the cortisol response to a novel environment in maltreated infants, as opposed to infants reared by competent mothers (Sanchez et al., 2015), an effect that may be mediated by impaired attachment to the mother, as suggested by the current findings and in agreement with the human literature).

The current study has several limitations that must be considered and addressed in future investigations. Although it is reasonable to compare exploration in the LabTAB free play and HI alone tasks given that the same operationalization was used in both, these tasks are not perfectly comparable. The sample size used for these analyses is modest and thus lacks statistical power to investigate the more complex models needed to completely investigate possible relationships between maternal care and the links between the brain and behavior. Thus, some of the exploratory analyses presented here do not serve as definitive evidence of the roles of specific aspects of the early mother-infant experience. But they do suggest that

further investigations of the relationships between the quality of maternal care and brain WM development are important for elucidating potential mechanisms of maternal buffering. We also only have data from a single age, which limits extrapolation of these relationships to other developmental stages. Future work is needed to determine not only the developmental transitions, but the biological mechanisms by which maternal presence regulates the links between brain and behavior across development, and under what contexts this occurs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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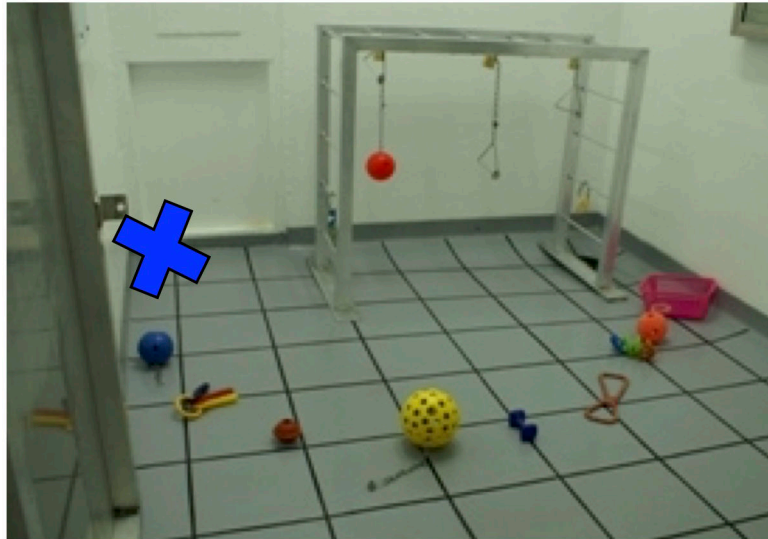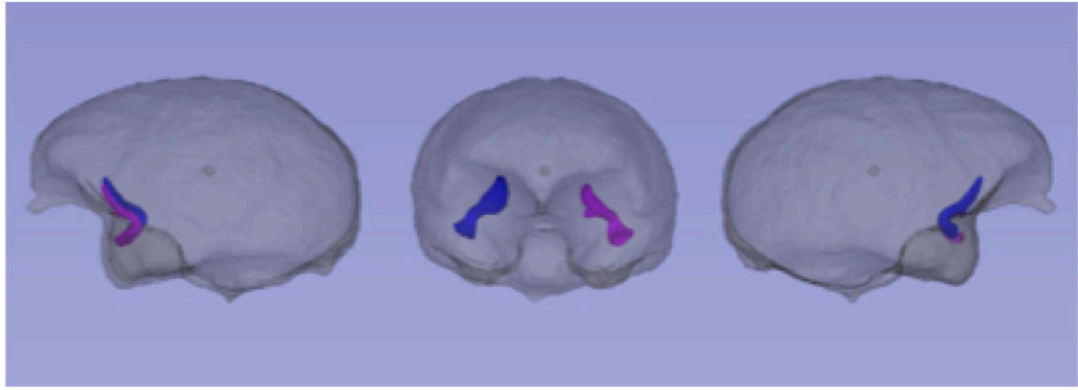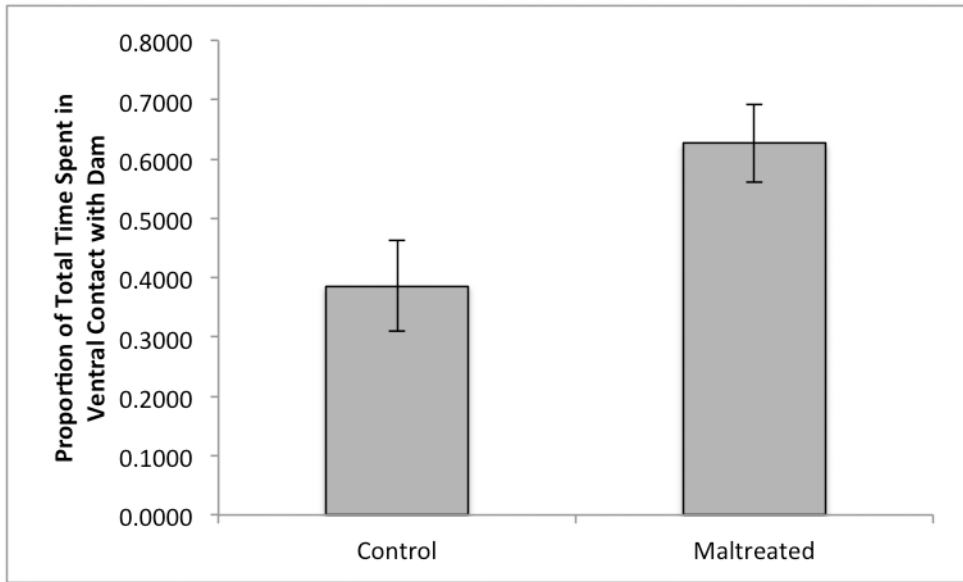
Figure 1.

Figure 2.

Figure 3.
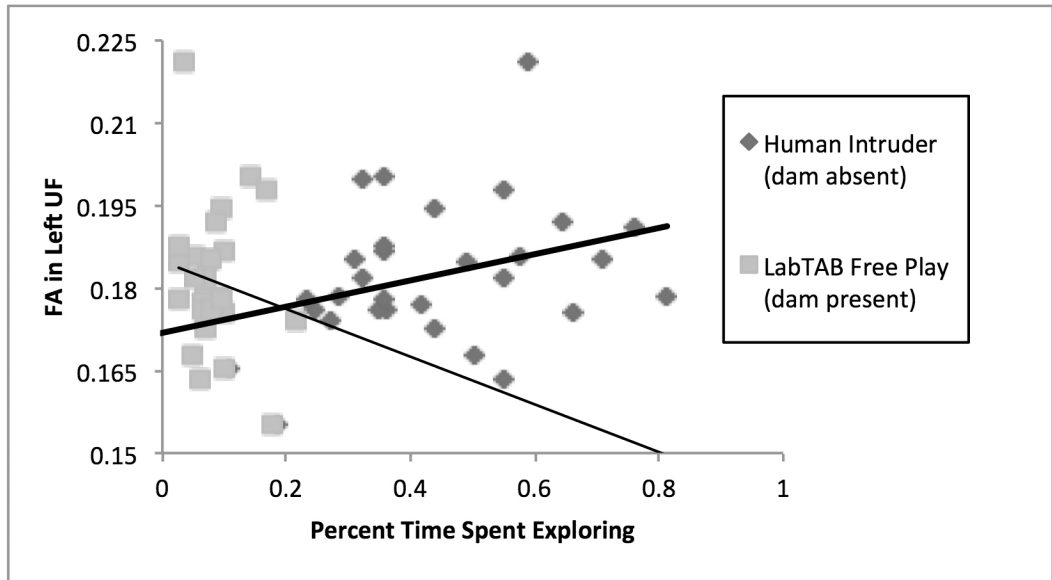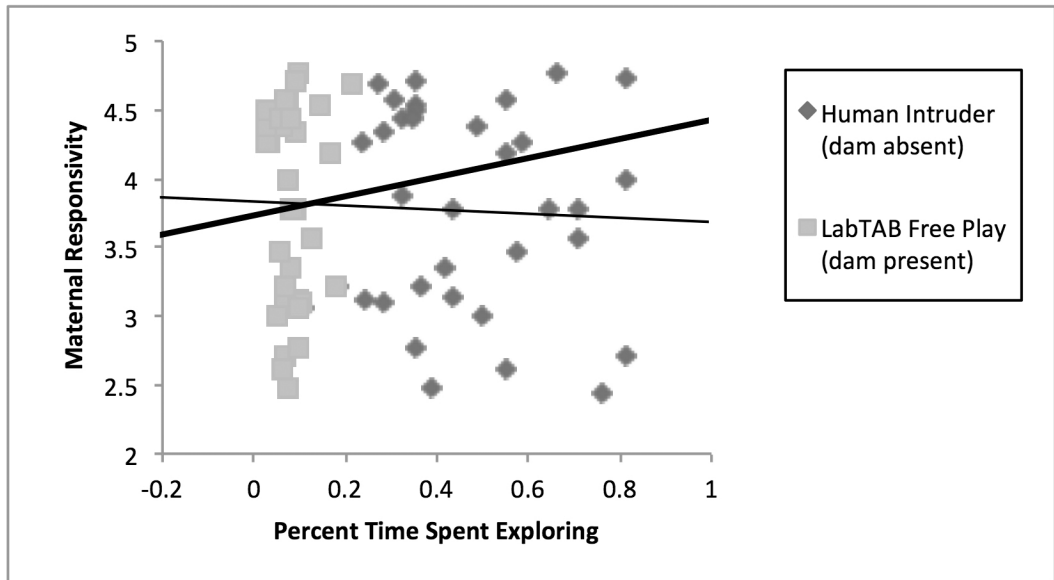
Figure 4.

Figure 5.

Table 1

Source	Sum of Squares	df	Mean Square	F	Partial Eta Squared
Between Subject					
FA in right UF	3.82E-09	1	3.82E-09	9.45E-08	4.50E-09
FA in left UF	0.001	1	0.001	0.014	0.001
Maternal responsivity	0.053	1	0.053	1.324	0.059
Maternal permissiveness	0.032	1	0.032	0.794	0.036
Abuse	2.18E-07	1	2.18E-07	0.000005	2.57E-07
Rejection	0.000004	1	0.000004	0.000106	0.000005
Error	0.848	21	0.04		
Within Subject					
Dam presence	1.00E-03	1	1.00E-03	0.033	0.002
Dam presence × FA in right UF	0.082	1	0.082	1.82	0.08
Dam presence × FA in left UF	0.214	1	0.214	4.756[**]	0.185
Dam presence × Maternal responsivity	0.19	1	0.19	4.225[**]	0.167
Dam presence × Maternal permissiveness	0.067	1	0.067	1.479	0.066
Dam presence × Abuse	0.058	1	0.058	1.285	0.058
Dam presence × Rejection	0.076	1	0.076	1.683	0.074
Error	0.945	21	0.045		

[**]
p 0.05

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Table 2

Source	Sum of Squares	df	Mean Square	F	Partial Eta Squared
FA in right UF	0.08	1	0.08	0.933	0.041
FA in left UF	0.082	1	0.082	0.953	0.042
Maternal responsivity	0.044	1	0.044	0.508	0.023
Maternal permissiveness	0.169	1	0.169	1.97	0.082
Abuse	0.194	1	0.194	2.261	0.093
Rejection	0.017	1	0.017	0.199	0.009
Error	1.889	22	0.086		

**
p 0.05