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Early life stress affects cerebral glucose metabolism in adult rhesus monkeys (*Macaca mulatta*)

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Abstract

Early life stress (ELS) is a risk factor for anxiety, mood disorders and alterations in stress responses. Less is known about the long-term neurobiological impact of ELS. We used [^{18}F]-fluorodeoxyglucose Positron Emission Tomography (FDG-PET) to assess neural responses to a moderate stress test in adult monkeys that experienced ELS as infants. Both groups of monkeys showed hypothalamic-pituitary-adrenal (HPA) axis stress-induced activations and cardiac arousal in response to the stressor. A whole brain analysis detected significantly greater regional cerebral glucose metabolism (rCGM) in superior temporal sulcus, putamen, thalamus, and inferotemporal cortex of ELS animals compared to controls. Region of interest (ROI) analyses performed in areas identified as vulnerable to ELS showed greater activity in the orbitofrontal cortex of ELS compared to control monkeys, but greater hippocampal activity in the control compared to ELS monkeys. Together, these results suggest hyperactivity in emotional and sensory processing regions of adult monkeys with ELS, and greater activity in stress-regulatory areas in the controls. Despite these neural responses, no group differences were detected in neuroendocrine, autonomic or behavioral responses, except for a trend towards increased stillness in the ELS monkeys. Together, these data suggest hypervigilance in the ELS monkeys in the absence of immediate danger.

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Keywords

Early life stress; rearing; HPA axis; monkey; PET; social brain

1. Introduction

Considerable evidence suggests that stress during critical developmental periods significantly increases an individual's risk for developing persistent mood and anxiety disorders later in life (Glaser, 2000; Heim & Nemeroff, 2001; Sánchez et al., 2001). These effects are hypothesized to be mediated by alterations in the neurobiological circuits involved in emotional and neuroendocrine regulation, including the hypothalamic-pituitary-adrenal (HPA) axis, which has long been implicated in the etiology of stress and affective disorders (Caldji et al., 2001; Gold et al., 1998; Nemeroff, 1996; Plotsky & Meaney, 1993; Sanchez et al., 2001), and corticolimbic circuits, including the prefrontal cortex (PFC), amygdala (AMYG), hippocampus (HIPP), and anterior cingulate cortex (ACC) (De Bellis et al., 2000; Matthew et al., 2003). Together, these regions are responsible for coordinating appropriate behavioral, neuroendocrine and autonomic responses to salient and potentially threatening emotional stimuli and in regulating attention to these stimuli (Posner & Rothbart, 1998). Early life stress (ELS) adversely affects the neurodevelopment of these systems by increasing an individuals' sensitivity to relevant stimuli, particularly those signaling potential threat, lowering the threshold to respond to these stimuli, and causing serious impairments in both the short- and long-term regulation of stress and emotional reactivity (Bremner & Vermetten, 2004; Kaufman & Charney, 2001; Sánchez et al., 2001; Sánchez, 2006).

Functional MRI (fMRI) studies have shown abnormalities in the function of the prefrontal cortex (PFC), amygdala (AMYG), and hippocampus (HIPP) in adults with histories of childhood maltreatment (a form of ELS), and volume loss in these regions is commonly reported using structural MRI (Bremner et al., 1997; Bremner, 2003; Stein et al., 1997; Vythilingam et al., 2002). The effects of this type of ELS in children and adolescents often present differently than adults. Children with ELS-related posttraumatic stress disorder, for example, show increased grey matter volume in the middle-inferior and ventral PFC, but decreased grey matter volume in the dorsal PFC which was correlated with the severity of their functional impairments on a standardized scale (Richert et al., 2006). Overall brain volume, white matter and corpus callosum volume may also show a reduction in children exposed to maltreatment-related ELS (De Bellis et al., 1999; Kaufman & Charney, 2001; Teicher et al., 2003). Intriguingly, similar alterations in cortical white matter and interhemispheric connectivity have been detected in juvenile rhesus monkeys with ELS related to alterations in early social environment, such as isolation-rearing in infancy (Sánchez et al., 1998). The limited number of studies on functional changes in brain activity in children and adolescents with ELS underscores the need for further research in this field (Chugani et al., 2001).

Developing longitudinal animal models is essential for studying the causes, consequences and potential treatments of deficits associated with ELS. Previous maternal deprivation models have highlighted the importance of the mother-infant relationship in the development of typical socioemotional behavior (Harlow et al., 1965; Harlow et al., 1971; Sanchez et al., 2001), but they are extreme and do not adequately account for more common forms of early adverse care-giving which also has devastating consequences. More recently, several alternative ELS models have been developed in macaque monkeys that keep infants in their social environment (Andrews & Rosenblum, 1994; Coplan et al., 1996). These typically involve maternal variable foraging demands, e.g, VFD (Coplan et al., 1996;

Jackowski et al., 2011), or brief (3-6 hour), but repeated, mother-infant separations between 3-6 months of age (Sánchez et al., 2005). Early life stress due to the variable foraging demand paradigm in bonnet macaques resulted in reduced corpus callosum size that correlated with fearful behavior when the animals were adults, and reduced volume in the hippocampus and middle and inferior temporal lobe gyri (Jackowski et al., 2011). The short-term effects of repeated separations on infants include delayed social development and an initial sensitization of the HPA-axis resulting in cortisol elevations that can persist into the juvenile period, long after the separations have terminated. This initial HPA-axis sensitization was followed by a flattened diurnal cortisol rhythm in juveniles resulting from lower than normal morning cortisol levels, particularly among females, and an elevated acoustic startle response, suggesting heightened anxiety (Sanchez et al, 2005). Moreover, high cortisol separation responses in infants were associated with higher anxiety and lower basal cortisol levels as juveniles; that is, a “more flattened” daytime rhythm of cortisol (Sánchez et al., 2005).

Previous studies by our group and others have confirmed that monkeys exposed to ELS showed short-term alterations in socioemotional behavior and neuroendocrine function. To further investigate the long-term effects of ELS on neural processing using this monkey model, we used FDG-PET to examine differences in the regional cerebral glucose metabolism (rCGM) of ELS and control monkeys in response to a mild stressor (i.e., being removed from their home room and placed alone for 45 minutes in a familiar testing room). Only a handful of studies to date have utilized functional neuroimaging techniques to examine the neural bases of behavior in nonhuman primates (Ichise et al., 2006; Kalin et al., 2005; Rilling et al., 2001). Kalin and colleagues, for example, identified several brain regions using FDG-PET including the dorsal ACC, thalamus, and dorsal raphe nucleus that correlated with the regulation of anxiety-related freezing behavior in monkeys (Kalin et al., 2005). This study placed subjects in an immediately threatening situation by confronting them with a strange intruder. Rilling and colleagues examined rCGM using FDG-PET in monkeys either separated or together with their mother. The acutely stressful maternal separation was associated with increased activity in right dorsolateral PFC, posterior ventral temporal regions, and decreased activity in left dorsolateral PFC (Rilling et al., 2001). The goal of the present study was to determine the long-term effects of ELS on neural responses to a moderate stressor, in parallel with behavioral and physiological responses. Instead of provoking subjects with an intense, threat-related stressor, we used a manipulation deemed to be moderately stressful, analogous to the challenges of a typical day. Based on the data described above, we expected to find hyperactivation in specific corticolimbic brain regions involved in emotion and stress responding, including PFC, HIPP, AMYG and ACC (Kalin et al., 2005; Rilling et al., 2001). We also anticipated that ELS monkeys would show increased behavioral, HPA axis and autonomic reactivity in response to the test manipulation compared to control monkeys.

2. Materials and Methods

2.1. Subjects

Thirteen adult rhesus monkeys between 6 and 7 years of age were tested. Six (3 male and 3 female) of these had been exposed to ELS (see protocol below) and 7 were controls (3 males and 4 females). The monkeys were born into social groups at the Yerkes National Primate Research Center (YNPRC), Lawrenceville GA. They had been participants in a larger, longitudinal study to examine the effects of early adverse experience on HPA axis function, socioemotional behavior and neurodevelopment. The results of this study, including short-term effects of ELS on behavior, HPA axis function, and acoustic startle response, have been published elsewhere (Sánchez et al., 2005). In brief, the ELS protocol began when the infants were 3 months old and continued for 3 months. It consisted of repeated mother-infant

separations of systematically varied durations (lasting 0.5, 3.0 or 6.0 hours) following a counterbalanced design and pseudo-random schedule. Separations were scheduled once per day, 2-3 times per week, resulting in a total of 36 separations per infant over a 90 day period. Separations were performed by removing the mother from the social group; the infant remained in the social group. At the end of each separation, mothers were returned to their social group and reunited with their infants. Control individuals were half-siblings (1 year age difference) living in the same social group, but they did not experience the maternal separations. Assignment of infants to the control or ELS conditions was done following a counterbalanced design. At 18 months of age both the ELS and control monkeys were transferred to the YNPRC main campus where they were pair-housed in a manner that counterbalanced their early rearing experience, e.g., control-control, control-ELS, or ELS-ELS pairs. All studies described here were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Emory University Institutional Animal Care and Use Committee.

2.2. Procedures (testing in adulthood)

The control and maternally-separated (ELS) animals were tested as adults to examine the longterm effects of ELS on neural responses to a moderate stressor. This was done using FDG-PET to examine differences in regional cerebral glucose metabolism, in parallel with behavioral and physiological responses exhibited by the animals. The moderate stressor consisted of removing these individuals from their home cage and cage mate and placing them in a nearby testing room (adjacent building). Each subject had been tested in this room during a previous 1-month study of heart rate habituation. This consisted of them being placed in this room once a week, for an 1 hour period (4 hours total exposure) while resting heart rate was monitored. There were no perturbations performed during this period. The experimental design and timeline of events for this PET-FDG study are illustrated in Figure 1.

Prior to this study, all of the subjects had undergone extensive training and habituation to all procedures used in the PET-FDG study, including handling, i.m. injections, and awake blood sampling from the saphenous vein in order to limit reactivity to procedures which could potentially confound the behavioral, physiological or neural responses measured during the 45 min testing period.

2.2.1. Heart Rate Telemetry—One year prior to this study, all subjects underwent surgery to implant a telemetric transmitter for recording heart rate (CTA-D70, 20 cc volume, 5×5 cm, Data Science International, www.transomamedical.com). The transmitters were surgically placed into a pocket created between the internal and external abdominal oblique muscles by the veterinary staff at the YNPRC. The lead wires were routed to the chest, following the guidelines published by DSI. After implantation, each transmitter was tested for functionality in the operating room before finalizing the surgical procedures. The transmitters' power was controlled via radio frequencies, enabling it to be turned on and off as needed to preserve battery life.

As described in Figure 1, at the beginning of the testing session, the subject was removed from its home cage and taken to an adjacent, familiarized room for testing. Once in the testing room, the subject was placed alone in a testing cage that had been modified for the attachment of the telemetry receiver behind the squeeze mechanism at the rear of the cage. This receiver was connected to the main data collection unit in an adjacent room so that experimenters could operate the equipment while remaining out-of-site during the testing session. Once the monkey was in the testing cage, the transmitter was turned on and the ECG (electrocardiogram) signal was identified. Heart rate was sampled continuously

throughout the testing session at 1000 Hz. The ECG segments were recorded for one minute epochs, every 5 minutes, for the duration of the 45 minute FDG uptake period (see Fig. 1). This produced 10, one-minute ECG segments for analysis. These files were extracted from DSI as ascii files and converted for analysis using Mindware HRV 2.16 (www.mindwaretech.com). This software provides a beat-by-beat editing feature to correct aberrant beats. Its analysis features include heart rate (HR/bpm, beats per minute collected over a range of 40-350 bpm) and RSA (respiratory sinus arrhythmia). RSA provides a measure of variability in heart rate due respiration, and is an index of parasympathetic vagal tone related to arousal modulation (Cacioppo et al, 1996). Cardiac data representing HR and RSA were analyzed separately using repeated measures ANOVAs and polynomial contrasts where the within-subjects factor was time (10 levels), and rearing group (control, ELS) and sex were the between-subject factors. Significance level was set at $p<0.05$, two-tailed.

2.2.2. Behavioral Recording and Analysis—Subjects were video recorded during the 45 minute test session (FDG uptake period; see Fig. 1). The video camera was connected to a monitor in the adjacent observation room so that experimenters could ensure the monkey was calm and not engaging in excessive movement, vocalizations, or self-biting. If such behavior occurred, the session was terminated. Behavior was analyzed later from the video tapes using an established ethogram (see Table 1). Behaviors were scored using a one/zero sampling method during 10 second time blocks throughout the 45 minute session by two raters blind to the rearing condition of the monkeys (inter-rater reliability > 0.7 for individual behaviors). The maximum frequency for a behavior was 270, occurring in every 10 second block during the 45-minute test period. Behaviors were averaged across rearing group and any single behavior with low occurrence (<5% toral frequency) was excluded from analysis. Group differences across the remaining behavioral categories were analyzed using ANOVA with rearing condition (control vs. ELS) and sex as the between-subject factor. Significance level was set at $p<0.05$, two-tailed.

2.2.3. Plasma Sample Acquisition and Analysis—As shown in Figure 1, a pre-test blood sample (3 ml) was drawn from the saphenous vein of each subject after being quickly transferred from their home room to the experimental testing room. This was a baseline sample, collected within an average of 5 min (range: 4-9 min) from initial disturbance (when experimenter first entered the room where the subject was housed). At the end of the 45 minute testing session, a post-test blood sample (3 ml) was collected. In each case (pre- and post-test samples), the blood was quickly transferred to pre-chilled plastic tubes containing ethylenediaminetetraacetic acid (EDTA) and placed on ice until the end of the testing session when they were centrifuged at 12,000 rpm for 10 minutes at 4° C. The resulting plasma aliquots were stored at −80° C until assayed. Stress-induced cortisol elevations are detectable in plasma after 10 minutes of the onset of the stressor, so for the analysis of the pre-test baseline samples, it was important to include only pre-test samples that were acquired within less than 10 minutes from the initial home room disturbance (i.e., experimenter enters the home room to box the subject) (Buono et al., 1986; Sapolsky, 1982). The average time to get the pre-test samples was 5 min. Samples acquired later than 10 minutes from the initial disturbance were excluded from the analysis. The average time to get the pre-test samples was 5 min (range: 4-9 min).

Plasma concentrations for cortisol were assayed in duplicate 10 ul aliquots by RIA using commercially available kits (Diagnostic Systems Laboratoies, Webster TX). The sensitivity of this assay was 1.25 ug/dl and intra- and inter-assay coefficients of variation were < 10%. Additionally, ACTH plasma levels were assayed in duplicate 200 ul aliquots by a two-site IRMA method using commercial kits (Nichols Institute Diagnostics, CA). The sensitivity of the assay was 1 pg/ml and inter-and intra-assay coefficients of variation were < 6%. These data were analyzed using repeated measures ANOVAs where time point (pre- vs. post-test)

was the within-subject factor and rearing condition (control vs. ELS) was the between-subject factor. Significance level was set at $p<0.05$, two-tailed.

2.2.3. PET FDG Protocol and Procedure—Although subjects had been habituated to the awake blood draw procedure, they were left undisturbed for 5-minutes after the pre-test (baseline) blood sample had been acquired (see Figure 1). After this acclimation period, the experimenter re-entered the testing room and administered a single intramuscular injection of 15 mCi [^{18}F]-FDG (in 2-3 ml volume), and the ECG recording protocol was initiated. This marked the beginning of the 45-minute test session, corresponding to the optimal time before [^{18}F]-FDG uptake begins to asymptote in the brain (Rilling et al., 2001). At the end of the 45 minute period, the subject was quickly anesthetized using an intramuscular injection of Telazol (4-5 mg/kg, i.m.) and a post-test blood sample (3ml) was quickly taken. The subject was then transported to the Yerkes' Imaging Center where it received a 3D whole-brain scan (Siemens microPET Focus 220). For scanning, the subject was placed supine on the scanner bed, with its head positioned at standardized coordinates and secured using a soft elastic wrap. Propofol anesthesia (10 mg/kg/hr) was administered by veterinarians and the anesthesia, heart rate, blood oxygenation levels and respiration were monitored throughout the scanning period. An initial transmission scan was performed using a Co-57 point source. The attenuation image was reconstructed, segmented into air, tissue (water) and bone and then the segments were replaced with the appropriate 511 keV attenuation coefficients. Attenuation correction factors were determined by fore projecting this image. Emission data were collected for 15 min in list mode. The data were then rebinned into a 3D sinogram (span 3). Scatter was estimated using the single scatter source method of Watson and colleagues (1996) with slice by slice consistency constraints. Emission images were reconstructed iteratively using the 3DRP algorithm supplied by the manufacturer at a resolution of $2\times2\times2$ mm^3 and implemented on a 20 node PC cluster.

2.2.4. MRI Scan Acquisition & Template—Magnetic resonance imaging (MRI) scans were acquired from all subjects prior to PET scans for co-registration and tracing of the Regions of Interest (ROI) in the structural MRI images. Scans were acquired at the YNPRC Imaging core using a 3T Siemens scanner and a T1-weighted MPRAGE sequence (TR= 2300 ms, TE = 4.4 ms, TI=1100 ms, flip angle = 8, 3 signals averaged) with a $0.6 \times 0.6 \times 1.0$ mm voxel size. Subjects were anesthetized directly in the home cage using Telazol (4-5 mg/kg, i.m.) and transported to the scanner. Anesthesia was maintained during the scan with intravenous propofol (10 mg/kg/hr) administered by veterinary staff and monitored continually throughout the 30 minute scan. Subjects were positioned prone on the scanner bed and their head was fitted inside a standard human knee coil. After a short orientation scan, the T1 sequence was started. These images were reconstructed into a 3D volume and the skull was stripped from each image by hand using MRIcro (http://www.sph.sc.edu/comd/rorden/mricro.html) to reveal only brain tissue. The stripped MRI scans were then used for co-registration the PET scans before analysis. Additionally, a custom rhesus monkey MRI template was created from 16 adult rhesus monkeys using nonlinear registration algorithms in FSL (http://www.fmrib.ox.ac.uk/fsl/). This consisted of the 13 monkeys in this study, plus 3 additional monkeys that did not receive PET scans (8 control and 8 ELS subjects in total). First, all subjects' T1-weighted MRI scans were AC-PC aligned via rigid registration and subjected to bias field correction, noise reduction and contrast enhancement. Then, initial affine linear alignments were made to a preexisting linear macaque template made in our lab using MRI images from a different set of monkeys. This earlier template was unbiased in that all subjects' images were deformed an equal amount. Using the initial affine alignments, a linear template was then constructed using the MRI images from the current subject pool. After this initial alignment, a second set of nonlinear alignments was calculated between each subject's undeformed T1 image and the

pre-existing linear template using FSL (http://www.fmrib.ox.ac.uk/fsl/). In the last step, a nonlinear template was produced by averaging the nonlinearly aligned images. By forming the average in this way, all subjects contributed equally to the final standard, with no explicit biases in the amount of deformations applied to any one subject. Figures 2A and 2B show an illustration of this template. The final voxel resolution of the MRI template was 0.5 mm isotropic.

2.2.5. PET Image Processing and Data Analysis—Each subject's PET scan was first co-registered to their own skull-stripped MRI using SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/). The MRIs were then converted to binary masks and applied to the co-registered PET scans to effectively strip away non-brain information. Each stripped and co-registered PET scan was then normalized to its average whole-brain activity so that regional cerebral glucose metabolism (rCGM) could be compared across subjects. These images were then spatially normalized to the rhesus monkey MRI template (see Figures 2A and 2B). The normalized scans were then submitted to a voxel by voxel, whole brain analysis using SPM5 where rearing condition (control, ELS) was the between-subject factor. The resolution of the normalized PET scans was 2 × 2 × 2 mm (original PET resolution, not upsampled to the MRI template's), making each voxel approximately 8 mm^3. Following previous studies in nonhuman primates (Kalin et al., 2005), independent t-tests were then used to identify group differences in rCGM in the whole brain analyses using a more conservative probability value ($p < 0.005$, two-tailed, uncorrected). Given the small sample size in this experiment, a more stringent p-value is acceptable given that alternative methods for controlling for multiple comparisons, such as the False Discovery Rate correction, would increase the type II error to unacceptable levels.

2.2.6. ROI Tracing Procedures and Anatomical Definitions—Next, a region-of-interest (ROI) analysis was used to examine group differences in a select number of brain regions known to be affected by early stress (e.g. Damsa et al., 2008). These bilateral ROIs included amygdala (AMYG), medial and dorsolateral prefrontal cortex (mPFC and DLPFC), anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), and the hippocampus (HIPP) (see Figure 2C). Each ROI was traced manually on the rhesus monkey MRI template in the coronal plane after first realigning the brain into stereological AC-PC space. The anatomical identification of ROI boundaries was guided by macaque atlases (Paxinos et al, 2000; Saleem & Logothetis, 2006), and more detailed anatomical conventions for ROI landmarks for the rhesus monkeys (Bertolino et al., 1997; Mathew et al., 2003; Sánchez et al., 1998; Zola-Morgan and Squire, 1985). When applicable, right and left ROIs were traced separately. An outline of representative ROIs can be seen overlaid on a coronal slice of the rhesus monkey template (Figure 2C). Prefrontal cortex (PFC): The posterior boundary of the PFC was defined as the slice rostral to the most anterior extension of the genu of the corpus callosum (CC), following MR protocols for rhesus monkey published by our group (Sánchez et al., 1998) and in human studies (Castellanos et al., 1996; De Bellis et al., 1999; Giedd et al., 1999; Giedd et al., 1995 ; Giedd et al., 1996; Rosenberg et al., 1997). Anterior cingulate cortex, medial prefrontal cortex, orbitofrontal cortex and dorsolateral prefrontal cortex were manually traced in this slice and in 5 additional slices rostral to it (total: n=6 slices), both in the right and left hemispheres. This region overlaps primarily with prefrontal cortex in rhesus monkeys, excluding 50% of area 25 (Barbas, 1995; Goldman-Rakic, 1987). Obitofrontal cortex (oPFC) was segmented between gyrus rectus and lateral orbital gyrus, dorsolateral PFC (dlPFC, area 46) between the superior and inferior frontal sulci, medial PFC (mPFC) between cingulate sulcus and gyrus rectus (Paxinos et al., 2000); and the anterior cingulate cortex (ACC) was segmented following previous MR methods (McCormick et al., 2005), where the borders of the ACC were defined by the cingulate gyrus. Amygdala (AMYG) and hippocampus (HIPP): Both structures were traced on

coronal slices with the aid of sagittal and axial confirmation. The amygdala is a difficult structure to trace due to diffuse boundaries, particularly at its most rostral and dorsal levels. We approached this technical problem by defining clear anatomical rules/criteria with the caveat that we excluded tissue in the most rostral, and caudal amygdaloid aspects. Based on previous published protocols (Greenberg et al., 2005), the round shape of the structure was traced from the ventrolateral white matter tract to the boundary with the dorsomedial CSF boundary. The hippocampus appears more caudal and ventral to the amygdala, and served as the most posterior border for the 5 slices selected for drawing this ROI. In contrast to AMYG, the MR protocols used provided a strong contrast between HIPP gray and white matter, so that the hippocampal formation (including Cornu Ammonis -CA1 to CA4-, dentate gyrus and subiculum) was successfully traced following neuroanatomical landmarks defined for the primate (Rosene and Van Hoesen, 1987) and previously used for rhesus monkey MR studies by our group (Sánchez et al., 1998) and in human studies (e.g.. Rusch et al., 2001). Briefly, the HIPP was drawn in 10 coronal slices from the most rostral limit of the midbody where CSF delineated the dorsal and ventral (and sometime lateral) boundaries and white matter delineated the medial (and sometimes ventral) borders. Because the selection of ROIs was limited and hypothesis driven, these analyses set the level of criteria for rejecting the null hypothesis at $p<0.05$ (two-tailed).

2.2.7. Correlations Between ROI Brain Activity and Behavior—Pearson product moment correlations were conducted to determine the relationship between the mean metabolic brain activity in each ROI and group differences in behavioral responses to the moderate stress. Log transforms were used to improve the normality of the behavioral values prior to analysis. All p-values were set at $p< 0.05$ (two-tailed).

3. Results

3.1. Heart Rate Variability

Cardiac data (HR and RSA) were acquired from all subjects and analyzed separately using repeated measures ANOVAs and polynomial contrasts using time (10 levels) as the within-subject cariable and rearing group as the between-subject factor. A significant main effect of time was found for HR ($F(9,63) = 5.08$, $p< 0.001$) which was significantly linear ($F(1,7) = 22.52$, $p< 0.002$). HR slowly decreased over the 45 minute period (see Figure 3). A significant main effect of time was also found for RSA ($F(9,63) = 2.71$, $p< 0.02$). RSA significantly increased over time but this was not significantly linear (see Figure 3). No other main effects or interactions were detected.

3.2. Behavioral Analysis

One testing session had to be terminated because the subject was self-biting. Overall, subjects were extremely quiet during the test sessions. No subject, for example, was observed to exhibit piloerection, aggressive lunging, open mouth threats, teeth grinding, freezing, self-scratching, yawning or bared-teeth displays during the 45 minute study period. Moreover, self-groom, cage shake, scalp lift, aggravated stereotypic behaviors, urination/defecation, lipsmack, present, threat bark, scream and coo occurred in fewer than 5% of the total sampling blocks and were, therefore, excluded from the analyses due to low occurrence. After these adjustments, only five individual behaviors remained. These were analyzed using ANOVAs where rearing group and sex were the fixed factors. No significant group or sex differences were found for the following behaviors: manipulate object ($F(1,11) = 0.09$, $p= 0.77$), stationary/alert ($F(1,11) = 2.21$, $p= 0.17$), stereotypic behaviors ($F(1,11) = 3.02$, $p= 0.11$), or movement ($F(1,11) = 0.25$, $p= 0.63$). There was, however, a trend toward a significant group effect for sitting quietly ($F(1,11) = 4.48$, $p= 0.06$). Table 2 lists the means (±SEM) for these behaviors, indicating that ELS (maternally-separated) monkeys spent

more time sitting quietly than control monkeys. The SEM values in the table indicate considerable individual variability in these behaviors within each group of animals, contributing to the failure for group differences to reach statistical significance.

3.3. HPA axis measures

Blood samples could not be obtained from one ELS monkey (Cf) and the pre-test blood samples from two ELS subjects (Fw and Ee) and one control subject (Dr) were acquired more than 10 minutes after the initial home cage disturbance and, therefore, were not included in the analysis. For the resulting samples, the average time from the initial disturbance until the collection of the pre-test blood sample was 5.6 (SEM= 1.08) minutes for the control and 4.0 (SEM= 0.41) minutes for the ELS monkeys. ACTH and plasma cortisol analyses were performed on the remaining 4 ELS and 5 control subjects. A significant main effect of time (pre- vs post-test sample), was found for both ACTH ($F(1,7) = 8.13$, $p < 0.03$), and cortisol ($F(1,7) = 91.97$, $p < 0.001$), but there was no significant main effect for group, or group x time interaction effects. Figure 4 illustrates the mean (±SEM) ACTH and cortisol values for both groups, which were significantly elevated at the end of the stress session, in comparison to baseline levels. There were only 3 males remaining in the analysis; so, sex was not included in the model due to small sample size.

3.4. PET Whole Brain Analysis: Group Differences in Cerebral Glucose Metabolism

Results of the whole brain analysis revealed several regions of activation that differed significantly between groups. Independent t-tests were used to assess the nature of these activations by contrasting control versus ELS subjects and vice versa. These activations are illustrated on the rhesus monkey template (see Figure 5). All brain images are shown in neurological convention, in which the right side of image corresponds to the right hemisphere. Six regions showed significantly greater activation in the ELS compared to control monkeys. Figure 5A illustrates significant bilateral activations in the upper and lower banks of the anterior STS, regions TPO and TEa (left = 24 mm^3, right = 40 mm^3), respectively, and the right putamen (32 mm^3). Figure 5B illustrates activation in the left inferotemporal cortex, region TEpv (24 mm^3) and two smaller areas in the left pretectum and right thalamic nuclei centered around the intermedullary lamina that consisted of only one voxel (8 mm^3). Finally, Figure 5C illustrates a large cluster of activation in the upper bank of the right posterior STS, region TPO (32 mm^3). This region may also have some activation extending into the lower bank, region TEO, but it avoids the depth of the sulcus, area PGa.

3.5. PET ROI Analysis and Behavioral

Results of the ROI analysis revealed significantly greater activation in the left hippocampus ($t = 1.93$, $p < 0.05$) of control compared to ELS monkeys, while ELS monkeys showed significantly greater activation in right orbitofrontal cortex ($t = 3.32$, $p < 0.01$). These activations are overlaid on the rhesus monkey MRI template shown in Figure 6.

A significant correlation was found between quiet sitting and mean metabolic brain activity in the OFC ROI for the control group, $r = -0.91$, $p < 0.01$, but not the ELS group, $r = 0.49$, $p = 0.27$. The correlation between quiet sitting and mean metabolic brain activity in the HIPP ROI showed a trend towards significance for the control group, $r = 0.72$, $p = 0.11$, but not the ELS group, $r = 0.02$, $p = 0.96$.

4. Discussion

This study revealed several important findings related to the long-term effects of early life stress (ELS) in rhesus monkeys. The specific stress manipulation that was performed on

these monkeys as adults was moderate by design. Subjects were removed from their home cage/home room and placed alone in a familiar testing room. As opposed to evoking an extreme stress response, or confronting the subject with an immediately threatening stimulus (e.g., a strange intruder), the purpose of this manipulation was to model the moderately stressful situations that may be encountered daily. The testing room was familiar to the subjects, as they had been exposed to it on various occasions prior to this study (i.e., 60 minutes once per week for 4 weeks) and the animals had been extensively trained and habituated to the handling, awake blood sampling, and i.m. injection procedures several months before testing started. Despite previous exposure to this room, both groups of animals exhibited heightened HPA-axis activity in response to this manipulation, as demonstrated by elevated plasma cortisol and ACTH levels measured at the end of the 45 min testing session. However, contrary to our predictions there was no effect of rearing group on either basal levels or stress-induced elevations of these hormones. Both groups of subjects responded to this moderately stressful situation with similar HPA-axis activation.

Changes in HR and RSA were measured as a means for evaluating the subject's ability to self-regulate their autonomic arousal during the 45-minute moderately stressful test session. The pattern of results indicates that the animals exhibited an initial increase in autonomic arousal (elevated HR and low RSA) that confirms the reported HPA axis activations. These measures, however, showed gradual acclimation to the testing condition, with no evidence of group differences suggestive of affective dysregulation (Porges et al., 1994). The finding of self-regulation of autonomic arousal was not unexpected as the test manipulation was only moderately stressful and subjects were never under immediate threat, or provocation. In contrast to the physiological changes, subjects showed very little evidence of behavioral emotional reactivity during the 45-minute period (e.g., few vocalizations, anxiety- or fear-like behaviors, little frustration or aggressive behaviors). There was only a trend ($p= 0.06$, two-tailed) for the ELS monkeys to spend more time in a quiet sitting position compared to the control monkeys.

Despite the lack of significant group differences in behavioral or physiological reactivity to the stress session, there were significant effects of the ELS experience on neural responses. The results of the PET analyses revealed several interesting differences between rearing groups. The whole brain analysis, conducted to reveal voxels that were significantly different at a threshold of $p< 0.005$ (uncorrected), provided a descriptive picture of group differences in rCGM. Overall six brain regions revealed hyperactivation in the ELS compared to control monkeys, including bilateral anterior STS, right posterior STS, right putamen, left pretectum, right thalamus, and left inferotemporal cortex. No region was significantly more active in control compared to ELS monkeys at this significance threshold.

Anterior STS, regions TEa and TPO (Damsa et al., 2008), is involved with both auditory and visual processing in primates. Rostral TEa, for example, has auditory projections to ventral orbital and ventral medial frontal cortices (Seltzer & Pandya, 1989) and amygdala (Stefanacci & Amaral, 2002), regions important for social information processing including the appraisal and response to threat (Davis, 1998; LeDoux, 2000). Posterior STS, caudal region TPO (Seltzer & Pandya, 1989), is a polysensory region receiving inputs from visual, auditory and somatosensory cortices (Seltzer & Pandya, 1978; 1989). The excitatory inputs from prefrontal and parietal cortices to TPO are overlapping, suggesting that this region can maintain the segregation of sensory information, perhaps helping individuals to divide attention to different sensory inputs (e.g., sound from one location but visual information from another) (Seltzer et al., 1996). Moreover, inferotemporal cortex, region TEpv, has reciprocal connections to the rostral, lower bank of STS (Saleem et al., 2000) and is additionally involved in sensory information processing. Previous studies have shown that ELS populations, such as maltreated children with PTSD, have larger superior temporal

gyrus volume compared to control children (De Bellis et al., 2002), and peer-reared rhesus monkeys also exhibit alterations in temporal structures, as measured by PET (Ichise et al., 2006). A more recent paper has also reported structural alterations in the middle and inferotemporal cortex in VFD-reared bonnet macaques using MRI (Jackowski et al., 2011). Greater activation of these regions in ELS compared to control monkeys suggests a general problem with sensory information processing, perhaps indicating hypervigilance, when no external stimuli are present. This is supported by the trend for increased “stillness” (sitting quietly) displayed by the ELS group in our study. A similar behavioral profile has been described as “behavioral hyporeactivity” in bonnet macaques that had experienced ELS through the VFD paradigm (Rosenblum et al, 2001). This hypervigilant state may lead to disrupted information processing in situations when the monkeys are required to extract important, relevant information from external stimuli.

The striatum is a region critical for the integration of sensorimotor information, where decisions about actions are informed by both learned stimulus contingencies and their predicted reward value (Pasquereau et al., 2007; Shultz, 2005). Striatal neurons, for example, respond to both the sensory aspect of a conditioned stimulus, e.g. whether it’s a tone or a shape, and the action predicted by that stimulus, e.g. press or release a lever (Shultz, 2005). Thus, greater activity in this region in the ELS compared to control monkeys may reflect general problems with sensorimotor integration, deciding which motor outcomes are appropriate based on previously learned cues. This may cause monkeys with ELS to respond inappropriately in social situations that could put them at risk of aggression or harm from conspecifics. Interestingly, alterations in striatal regions (or its connections with frontal, temporal and thalamic areas) have been previously reported in other ELS macaque models (variable-foraging demand, peer-rearing) using structural MRI or PET neuroimaging techniques (Coplan et al., 2010; Ichise et al., 2006) .

ROI-based analyses revealed two significant group differences. First, the ELS monkeys had significantly greater activation in OFC compared to control monkeys. This was concentrated along the medial portion of OFC. These frontopolar, limbic regions are highly interconnected with medial temporal cortex, amygdala, hippocampus, and striatum and are involved in emotional regulation, inhibitory control of behavior and goal-oriented behavior. Thus, greater activation in the OFC of the ELS monkeys could be related to higher emotional reactivity and an overall hyperactivation in the sensory information processing regions described above (Cavada et al., 2000; Machado & Bachevalier, 2003; Sanchez et al., 1998; Spinelli et al., 2009). Increased glucose metabolism in the OFC has been reported previously in rhesus monkeys using similar PET-FDG approaches in response to relocation and threat stress (Kalin et al., 2008). Moreover, lesions of the OFC in monkeys reduces fear-related behaviors (Kalin et al., 2007), supporting our interpretation that the greater OFC activation detected in our ELS monkeys may reflect elevated stress reactivity, although these physiological measures showed no overall group differences.

Significantly greater activity was detected in the left hippocampus of control compared to ELS monkeys. This structure has a tonic inhibitory effect on the HPA-axis (Dedovic et al., 2009). Thus, the hippocampus of control monkeys could have been more actively engaged in the stress-regulation processes. Previous reports of reduced hippocampal volume in post-pubertal macaques that had experienced early life stress (VFD paradigm; Jackowski et al., 2011), and in human studies of adults with early adverse experiences (Bremner et al., 1997; Stein et al., 1997; Driessen et al., 2000; Vythilingam et al., 2002) suggest that early stress can result in hippocampal atrophy and hypofunctionality, providing support for our interpretation. The mean metabolic activity in the OFC was correlated with the monkeys’ behavior during the test situation. For the control monkeys, mean glucose metabolism in the OFC was negatively correlated with quiet sitting. Thus, rather than the hypervigilant

stillness displayed by the ELS monkeys, they seemed to respond to the moderately stressful situation with a more active emotion regulation strategy. Neither the findings in OFC nor HIPP activity can be explained by differences in overall stress response, as no differences in peripheral stress hormone levels were found between the two groups. An interesting follow-up study would be to examine the recovery of these hormones to baseline levels to more accurately assess negative feedback on the HPA-axis. However, the experimental design of this study did not permit an assessment of HPA-axis recovery as monkeys were anesthetized for the PET scanning protocol after the end of the stress test.

5. Conclusions

Despite finding no significant group differences in either the behavioral or physiological reactivity to the experimental condition, there were significant effects of the ELS experience on neural responses at both the whole brain and ROI levels. Compared to controls, the ELS monkeys exhibited higher metabolic activity in brain regions involved in emotion regulation and sensory processing. These regions included the orbitofrontal cortex, superior temporal sulcus, thalamus, putamen and inferotemporal cortex. Lower metabolic activity was reported in the hippocampus. This would suggest overall heightened vigilance in the ELS group in response to social separation and placement in a familiar testing room, despite no significant group differences in the behavioral, neuroendocrine and autonomic responses.

Early life stress (ELS) adversely affects the neurodevelopment of stress-response systems in both humans and monkeys by increasing the individuals' sensitivity to relevant stimuli, particularly those signaling potential threat (Heim & Nemeroff, 2001; Sanchez et al., 2001; Sanchez & Pollak, 2009). Using a moderately stressful manipulation (removal of subjects from their home cage and placement in a familiar testing room), the present results support a heightened sensitization in adult monkeys that experienced ELS as infants. Both the ELS and control groups showed HPA-axis stress-induced activations to the testing session, indicating that, although moderate, this experimental condition was stressful. In terms of neural activity, however, the ELS monkeys exhibited greater cerebral glucose metabolism (CGM) in orbitofrontal cortex, superior temporal sulcus, putamen, thalamus and inferotemporal cortex, and lower CGM in hippocampus, compared to control monkeys. These results indicate hyperactivity in emotional and sensory processing regions in the ELS monkeys, and lack of appropriate stress-regulation. Behaviorally, however, the only notable difference between the groups was that the ELS monkeys engaged in more quiet sitting than the control monkeys. This was an unexpected result, as it was hypothesized that the ELS animals would exhibit higher behavioral reactivity during the test compared to control monkeys, including increased levels of fear, anxiety, and stress-related behaviors. However, because the stressor was moderate in nature, one cannot rule out that while sitting quietly, the ELS monkeys were also more intensely attending to their environment, with increased auditory vigilance. Hyperactivity in emotion-regulation and sensory processing regions in combination with elevated quiet sitting behavior suggests an environmental hypervigilance in the absence of any immediate danger or threat, or other measurable cue. This is also consistent with the findings of others who have reported behavioral hyporeactivity in bonnet macaques in response to the VFD model ELS (Rosenblum et al., 2001). It is unclear whether this neural pattern is related to heightened sensitivity of these neural circuits (overreading potential threats) or a dysregulation in the ability of monkeys with ELS to appropriately filter relevant (or irrelevant) information from the environment. A question for future studies is whether more intense stressful or threatening manipulations would produce a similar, but stronger, pattern of neural activity and a concomitant hyperactivation of behavioral and physiological responses.

In summary, our findings suggest persistent effects of early life stress in the form of repeated maternal separation during infancy on the function of neural circuits involved on emotional and sensory processing in adult macaques. Although these findings are interesting, they also need to be interpreted with caution due to the limitations of our small sample sizes and the multiple comparisons performed, particularly in the whole brain analysis. Further studies are needed to replicate these findings and to extensively examine the functional (behavioral, physiological) correlates of the neurobiological differences detected.

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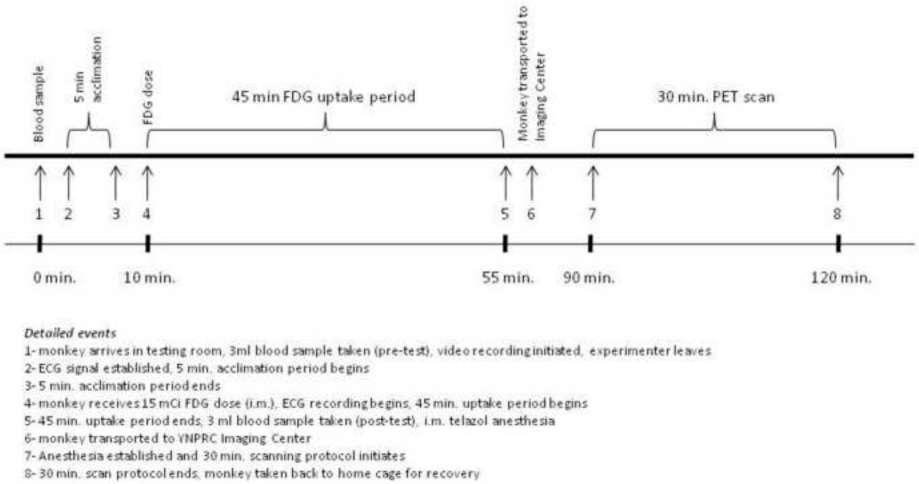

Figure 1.
Timeline of events during the PET experimental protocol.

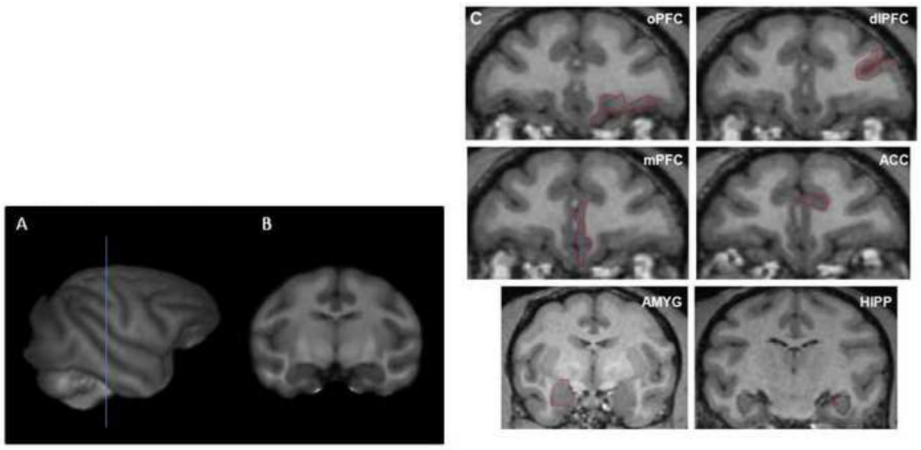

Figure 2.

(A) 3D reconstruction of the nonlinear rhesus monkey MRI template showing right side, and **(B)** representative coronal slice at plane indicated the line in A. These images are in neurological convention where the left side of the image is the left hemisphere. **(C)** Examples of ROIs traced –in redon coronal slices of the rhesus monkey MRI template. Abbreviations: ACC=anterior cingulate cortex, AMYG=amygdala, dlPFC=dorsolateral PFC, HIPP=hippocampus, mPFC=medial PFC, oPFC=orbitofrontal cortex, PFC=prefrontal cortex.

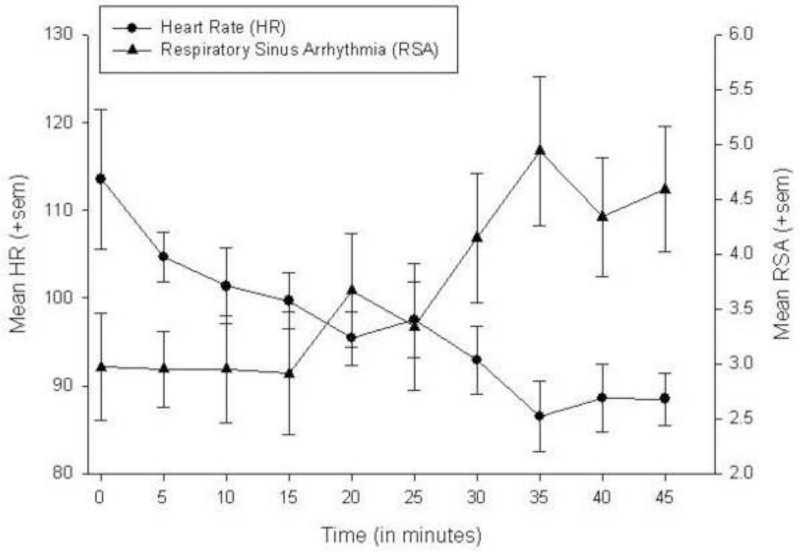

Figure 3.

Mean heart rate (HR) and respiratory sinus arrhythmia (RSA) measured at 1 minute intervals during the 45 min stress test, which was also the [^{18}F]-fluoro-deoxy-glucose (FDG) uptake period.

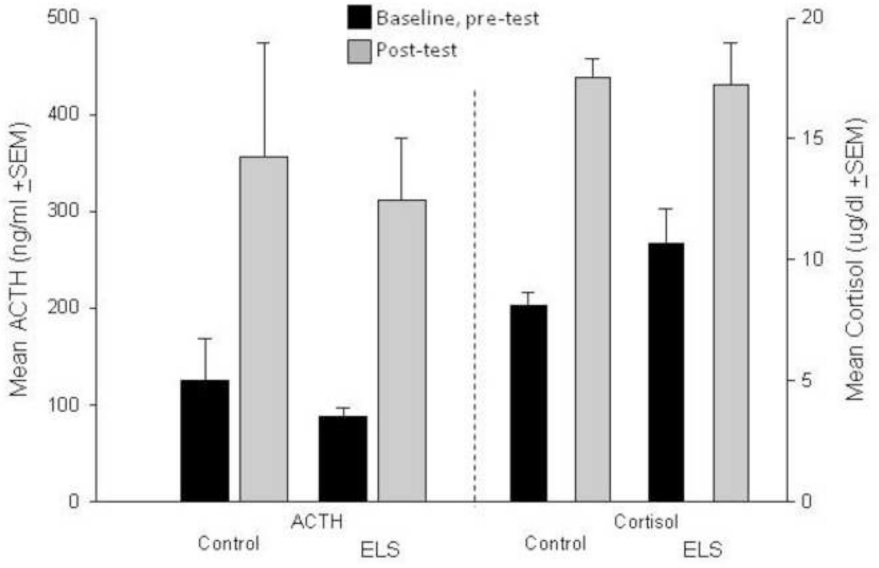

Figure 4.
Mean cortisol and ACTH responses, measured pre- and post-test.

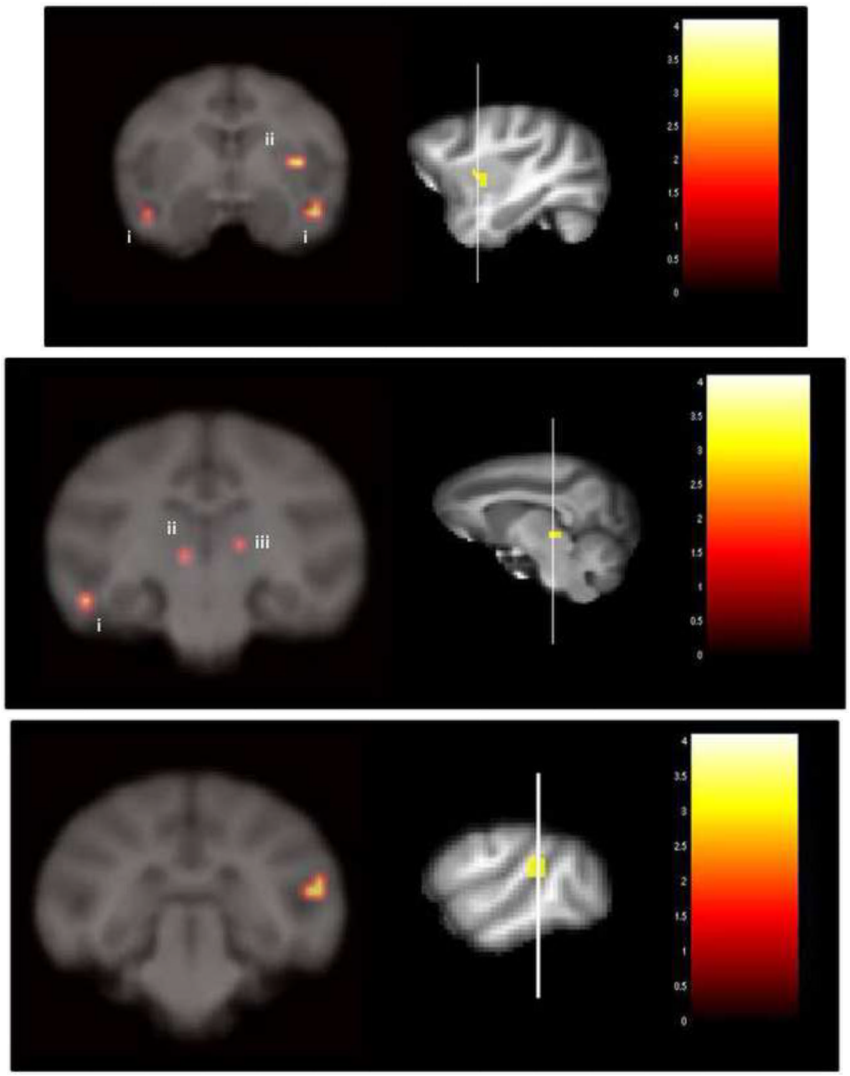

Figure 5.

Results of the Whole Brain analysis showing: **(A)** significantly greater [^{18}F]-fluorodeoxyglucose (FDG) uptake –reflecting increased glucose metabolism- in ELS (maternally-separated) monkeys than in Controls in bilateral upper and lower banks of anterior STS , rostral TPO and TEa, respectively (i), and right putamen (ii); **(B)** significantly greater [^{18}F]-FDG uptake in ELS than in Control animals in left inferotemporal cortex –area TEpv- (i), left pretectum (ii), and right thalamus in the region of the intermedullary lamina (iii); **(C)** significantly greater glucose metabolism in the right posterior dorsal STS (area TPO) of ELS animals as compared to Controls.

Abbreviations: STS=superior temporal sulcus; TEa=ventral bank of STS; TEpv= posterior ventral portion of inferotemporal cortex; TPO=dorsal bank of STS. $p<0.005$.

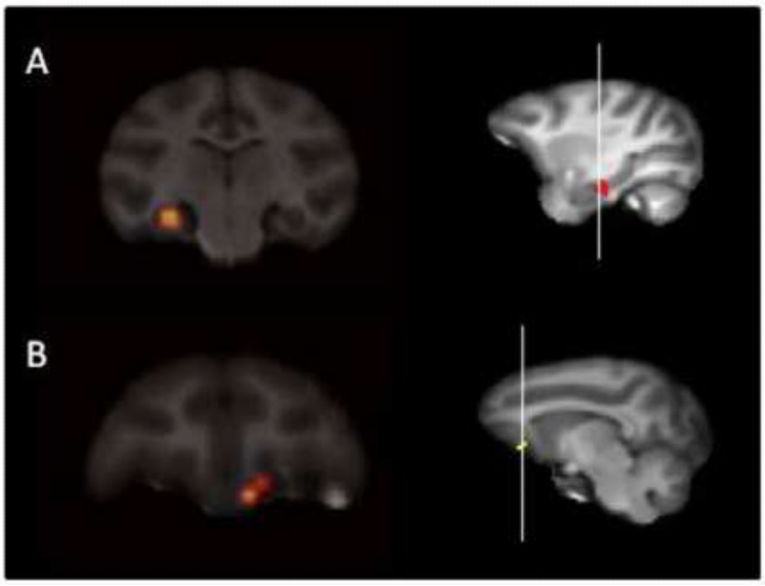

Figure 6.

Results of the Region of Interest (ROI) analysis showing significantly greater [^{18}F]-fluorodeoxy-glucose (FDG) uptake –reflecting increased glucose metabolism- in **(A)** left hippocampus of Control versus ELS monkeys ($t= 1.93$, $p< 0.05$) and **(B)** in right orbitofrontal cortex of ELS animals, as compared to Controls ($t= 3.32$, $p< 0.01$).

Table 1

Ethogram designed to measure the behavior of solitary rhesus monkeys in a test cage. Behaviors were scored every 10 seconds for 45 minutes using a one/zero sampling technique.

Behavior	Description
Manipulate object	subject handles an object in its cage, such as an enrichment toy or cage lock
Self Groom	subject grooms parts of its own body
Sitting quietly	subject is in a stationary neutral state, not otherwise engaged and not sleeping
Stationary alert	subject is in a stationary state but with head up and appears to be attentive to environment
Ambulation	locomotive movement by animal over two steps in a direction that is not consistent with stereotypy
Aggressive lunge	a forward, lowered body motion indicating threat
Open mouth threat	Open mouth facial expression, teeth covered, typically occurs with lowered head posture
Cage shake	subject holds bars on cage and shakes it violently
Piloerection	obvious occurrence of hair bristling making the animal appear bigger
Scalp lift/ears back	facial expression where brow over forehead is raised and ears flatten back against the head (if occurs with lip smack, only code lip smack)
Bared-teeth display	subject retracts lips to expose upper and lower teeth in a grin
Lip-smack	subject rapidly smacks lips together, typically occurs with scalp lift
Stereotypy	subject engages in a repetitive behavior at least 3 times, including pacing, spinning and/or jumping
Aggravated stereotypy	same as stereotypy only it involves a more aggravated behavior and rough movements
Teeth grind	subject grinds teeth by horizontally mashing the jaws together
Freezing	subject remains motionless, typically occurs with crouched posture
Scratch	rough scratching, not self-groom
Yawn	self-explanatory
Deficate/urinate	self-explanatory
Present	subject presents hindquarters to outside of the cage
Bark	subject makes threat bark vocalization
Scream	subject makes scream vocalization
Coo	subject makes coo vocalization

Table 2

Mean behaviors (±SEM) exhibited during the 45 minute testing procedure by monkeys that experienced Early Life Stress (ELS) and Control monkeys.

Behavior Category	Mean (±SEM) ELS	Mean (±SEM) Control
Manipulate object	28.43 (10.57)	24.67 (6.16)
Sit quietly	97.14 (21.59)	35.00 (19.18)
Stationary/alert	70.14 (11.00)	104.00 (21.13)
Stereotypies	24.00 (15.00)	79.00 (29.52)
Movement	40.13 (15.17)	11.64 (4.75)