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Felicia Lombardi April 12, 2016

Effects of Social Rank and Delayed Puberty on Brain Structural Development of Juvenile Female Rhesus Macaques: Associations with Behavior and Stress Physiology

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Abstract

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During adolescence the brain is undergoing growth and remodeling, in parallel with changes in behavior and stress reactivity. Estradiol (E2) has organizational effects on the brain, influencing a broad range of cellular processes that result in gross morphological changes. During this time, changes are also occurring in the individual's social environment as he/she transitions from parental dependence to a more independent state. Non-human primates exhibit a complex social structure characterized by a matrilineal dominance hierarchy, and thus provide an ideal model to study the effects of E2 and social experience on the adolescent brain and the resulting behavioral and physiological changes. Here, we used socially housed female rhesus macaques to investigate the combined effects of delayed puberty, via E2 suppression, and social status in intermediate-ranking animals on brain structural development during adolescence. We focused primarily on prefrontal cortex (PFC), amygdala (AMYG), and temporal-parietal-occipital (TPO) region of the superior temporal sulcus (STS) due to their implications in processing socioemotional stimuli.

Structural MRI scans were collected at pre-puberty (22.64 ± 1.19 months) and peri-puberty (32.95 ± 1.15 months) in 21 juvenile female rhesus macaques. One cohort of animals received chronic Lupron injections to delay puberty. We examined volumes of total intracranial volume (ICV), grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF), as well as AMYG, PFC GM and WM, and TPO GM. Measures of stress physiology, and social behavior were collected to identify functional correlates of the brain structural effects. Results showed significant effects of rank on PFC GM, with larger volumes correlating with higher social rank.

In addition, suppression of E2 by Lupron treatment resulted in smaller GM and WM volumes, both total and in PFC, as well as in smaller ICV and AMYG volumes, relative to control subjects. In addition, structural changes in GM, total and in TPO, were predictive of behavioral changes, while AMYG and PFC GM were predictive of stress neuroendocrine measures. Altogether, these results provide evidence that intermediate-ranking animals are susceptible to the interactive effects of E2 and social experience that correspond to changes in behavior and stress neuroendocrine function during adolescence.

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Introduction

The evolution of the primate brain is thought to be associated with the demands of living in a complex social environment, a theory proposed by Dunbar, known as the "social brain hypothesis" (Dunbar 1998). Initial support for this hypothesis came from analysis of the relationship between social group size and neocortex size in anthropoid primates, which exhibit a strong positive correlation. Additionally, this hypothesis is supported by the finding that grooming clique size is positively correlated with neocortex size in primates (Kudo and Dunbar 2001). In social groups of the size found in troops of rhesus monkeys (*Macaca mulatta*), members inevitably encounter competition and aggression. Grooming cliques represent a type of alliance, allowing members to prevent aggressive encounters without losing the benefits of living in groups, such as decreased susceptibility to predation and enhanced ability to locate and access food sources (Alexander 1974). Dunbar states that this finding "can be seen as a crucial basis for primate sociality" (Dunbar 1998).

The social structure of rhesus macaque colonies consists of large, multimale, multifemale groups characterized by philopatry in which the males disperse and the females remain in their natal groups, forming matrilineal dominance hierarchies (Greenwood 1980, Melnick, Pearl et al. 1984). Female offspring adopt the rank of their mother and this rank remains relatively stable over the course of their lifetime (Smuts 1987).

Recognition of individuals appears to be the basis of the hierarchical organization of rhesus monkey social groups. Murphy and collaborators demonstrated that monkeys could be trained to avoid a shock associated with another monkey and that this was not a generalized response, providing evidence that individual recognition occurs in rhesus monkeys (Murphy, Miller et al. 1955). Learning to recognize individuals within the social group begins in the first few weeks of

life, which is when an infant monkey is able to distinguish his/her mother from other females (Rowell 1974). Beyond the ability to distinguish between members of the social group, and eventually learn where their family and themselves are in the overall social hierarchy, juvenile rhesus monkeys spend a substantial amount of time playing with other young monkeys. It is during this partner play that the young rhesus monkey learns and practices many of the behaviors and facial expressions involved in social communication, initially based on previous observation of adult encounters (Rowell 1974).

Current research suggests that these complex social behaviors and relationships are supported by a distributed neural network involved in the processing of social information. Brain regions implicated in this network include the prefrontal cortex (PFC), amygdala (AMYG), and superior temporal sulcus (STS) (Bickart, Wright et al. 2011, Lewis, Rezaie et al. 2011, Sallet, Mars et al. 2011, Powell, Lewis et al. 2012, Noonan, Sallet et al. 2014). Studies in individuals with autism spectrum disorder (ASD) provide evidence for the STS as a key cortical area of the "social brain" in humans (Ohnishi, Matsuda et al. 2000, Boddaert, Chabane et al. 2004). Brain abnormalities in individuals with ASD include decreased blood flow in the temporal lobe, including STS (Ohnishi, Matsuda et al. 2000), decreased grey matter (GM) density in the STS (Boddaert, Chabane et al. 2004), and less activation in STS during social tasks (Castelli, Frith et al. 2002).

Within the STS, the temporal-parietal-occipital (TPO) region is of particular interest because many neurons in this region are polysensory; that is, they show a greater response to a combination of sensory modalities including visual, auditory, and/or somatosensory stimuli compared to when each of these stimuli are presented alone (Bruce, Desimone et al. 1981, Baylis, Rolls et al. 1987). The TPO has overlapping connections with unimodal association areas

involved in these three sensory modalities (Barnes and Pandya 1992), suggesting that the TPO is involved in high-level integration of sensory information. Perception of biological motion, defined as "motion patterns characteristic of living organisms in locomotion," (Johansson 1973) is one such function that requires the integration of sensory information. Experiments in humans employing functional magnetic resonance imaging (fMRI) found areas of the upper bank of the STS, including the TPO region, that were activated by biological point light animations, in which the form and motion of a walking body is defined only by the movement of light patches attached to the head and major joints (Peuskens, Vanrie et al. 2005). In macaques, Oram and Perrett found that cells of the superior temporal polysensory area (STPa), which includes TPO (and another region, PGa), showed selectivity to the moving light displays that represented social forms (Oram and Perrett 1994). Thus, TPO is important in processing and integrating socially relevant stimuli in both humans and macaques.

The social status of an individual monkey in the hierarchy and the size of its social network are two major aspects of social experience in rhesus macaques. There is evidence that the structure of brain regions involved in socioemotional processing is specifically affected by social status and/or social network size, at least in animals housed in small social units (Sallet, Mars et al. 2011, Noonan, Sallet et al. 2014). Sallet and collaborators have reported a positive correlation between social network size and GM volume in mid-STS, rostral PFC (rPFC), inferior temporal gyrus, temporal pole, and AMYG (Sallet, Mars et al. 2011) In addition, rPFC GM density correlated positively with social rank (Noonan, Sallet et al. 2014). Similarly, in humans social network size has been positively associated with the size of brain regions that regulate socioemotional function, including larger GM volumes of orbital PFC, ventromedial

PFC, and AMYG and regions of the temporal cortex important for social cognition, such as the STS (Bickart, Wright et al. 2011, Lewis, Rezaie et al. 2011, Powell, Lewis et al. 2012).

In the linear dominance hierarchy typically formed by socially housed female rhesus monkeys, the subordinates are under constant harassment by more dominant animals and have less control over their environment and access to resources (food, males). Social subordination has been shown to impair reproductive development (Wilson, Gordon et al. 1986, Zehr, Van Meter et al. 2005, Wilson and Kinkead 2008), immune function (Paiardini, Hoffman et al. 2009), and cardiovascular health (Kaplan and Manuck 1999) in adult female rhesus macaques. In addition, subordinate monkeys exhibit symptoms of chronic psychological stress, including increased emotional reactivity and elevated basal cortisol levels (Michopoulos, Reding et al. 2012). Thus, social subordination is viewed as a constant psychosocial stressor, at least in adult animals (Shively 1998, Kaplan and Manuck 2004, Sapolsky 2005, Wilson, Fisher et al. 2008).

Although the subordinate phenotype is not as well understood in juvenile female macaques, there is evidence that it results in delayed puberty (Wilson et al. 1986; Wilson et al, 2013; Zehr et al. 2005) associated with increased emotional reactivity (Wilson et al, 2013). The neurobiological effects and how they emerge during development is the intense focus of current research. The existing evidence suggests that there are already brain structural differences related to social rank in pre-pubertal juvenile females. For example, social status differences have been detected using DTI in tracts connecting prefrontal, sensory processing, motor and association brain regions involved in socioemotional and sensory processing (Howell et al, 2014). Structural differences in these tracts, likely involving myelin, were linked to increased emotional reactivity in subordinates. In addition, our lab is examining the brain structural differences between the two extremes of the hierarchy: the most dominant and most subordinate animals in the group

(Godfrey et al, in preparation). The dominant animals were defined as those monkeys whose social rank was within the upper 30%, while the subordinates were defined as those monkeys whose social rank was within the bottom 40% of all monkeys living in their social group. Because subordinate female macaques experience delayed puberty, our lab is also examining whether brain structural effects of social subordination are due to social stress and/or to effects of delayed exposure to E2 during adolescence. For this, in a study of brain structural effects using MRI preceding mine, in addition to comparing the extreme social ranks in the hierarchy (dominants versus subordinate animals) undergoing spontaneous puberty, our lab examined the effect of pubertal delay (via treatment with Lupron, a drug that delays puberty through suppression of estrogen (E2)) and its interaction with social stress on neurodevelopment of brain regions involved in socioemotional processing, including the PFC, hippocampus, and AMYG (Godfrey et al, in preparation**).** Preliminary findings suggest that social subordination stress resulted in larger volume of the right AMYG, while exposure to low E2 levels in the Lupron-treated animals appeared to reduce this increase in AMYG volume in subordinate, but not in dominant animals, and resulted in increases in total brain and PFC volumes. Therefore, it appears that pubertal timing and social rank play important roles in neurodevelopment in dominant and subordinate animals. However, it is unclear how the social environment experienced by intermediate ranking female monkeys influences their brain development, particularly during the pubertal transition.

In this study, I hypothesize that the intermediate ranking individuals have a more complicated array of social relationships to navigate, in contrast to dominants or subordinates. For example, the top-ranking individual of the group is dominant over all other individuals and acts accordingly, whereas an intermediate ranking individual has to recognize and remember

those monkeys both more dominant and more subordinate in rank compared to them and behave differently in encounters with each type of these individuals. The intermediate-ranking individual, thus, must navigate more complex and nuanced series of relationships and needs to exhibit more behavioral flexibility compared to either the more subordinate or more dominant ranking monkeys within the same social group. A second argument in this proposal is that these experiences may therefore cause structural changes in the brain, especially during puberty and adolescence when the females experience significant changes in their social behavior as they become more mature and independent, and their brains undergo marked reorganization in cortical and limbic regions, many of which are involved in socioemotional processing (Sisk and Zehr 2005).

Adolescence is a period characterized by the transition from parental dependence to independence and involves changes in brain organization and behavior that are influenced by hormones and experience (Casey, Duhoux et al. 2010). Puberty, which occurs during the adolescent period, is characterized by the transition to sexual maturity. Adolescence is a sensitive period during development in which the brain exhibits reorganization and plasticity (Boyce and Ellis 2005, Steinberg 2005). Using structural MRI (sMRI) as a way to non-invasively examine brain structure, researchers have characterized changes in cortical GM and white matter (WM) during the adolescent period. WM increases throughout adolescence, whereas GM exhibits an inverted U-shaped curve in which the timing of peak volume varies across different brain regions (Giedd and Rapoport , Paus 2005, Sisk and Zehr 2005), followed by a decline caused in part by synaptic pruning, and remodeling (Markham, Mullins et al. 2013). For example, in humans GM in the STS reaches a peak at about 16 years of age and then steadily declines (Toga, Thompson et al. 2006). The changes in WM are mainly due to increases in myelination (Paus, Collins et al.

2001), whereas the cellular mechanism for the changes observed in GM are less well-understood, but are believed to be due, at least in part, to synaptic pruning (Bourgeois and Rakic 1993, Huttenlocher and Dabholkar 1997, Markham, Mullins et al. 2013). Other neural mechanisms responsible for brain changes during adolescence include neurogenesis, apoptosis, axonal growth, proliferation or retraction of dendritic spines, and synapse elimination (Goldstein, Kurz et al. 1990, Benes, Taylor et al. 2000, Nuñez, Lauschke et al. 2001, Pinos, Collado et al. 2001, Cunningham, Bhattacharyya et al. 2002). For example, synapse elimination has been shown to occur in PFC during adolescence in both humans and monkeys, in parallel with emerging behavioral changes (Mrzljak, Uylings et al. 1990, Bourgeois, Goldman-Rakic et al. 1994, Huttenlocher and Dabholkar 1997, Selemon 2013). In rodents, dendritic pruning has also been demonstrated in regions of the AMYG during adolescence (Zehr, Todd et al. 2006). While it is known that estrogens and androgens influence these cellular processes (Galea, Spritzer et al. 2006, MacLusky, Hajszan et al. 2006), a causal role of these hormones on the above changes in PFC or amygdala structure has not been demonstrated.

Gonadal hormones have both transient activational effects and long-term organizational effects on the nervous system (Phoenix, Goy et al. 1959, Gotz and Dorner 1976, Sisk, Schulz et al. 2003, Schulz, Richardson et al. 2004, Sisk and Zehr 2005, Zadran, Qin et al. 2009, Galvin and Ninan 2014). Activational effects refer to those effects of gonadal hormones that modify the acute function the cells in which they bind to receptors and alter signaling pathways. Organizational effects of gonadal hormones that occur during adolescence include neural structural changes, such as those processes mentioned above that are responsible for changes in brain structure during adolescence (e.g synaptic pruning, remodeling, myelination) (Schulz, Richardson et al. 2004, Sisk and Zehr 2005). These changes are generally considered permanent

or they program other biological systems, resulting in gross anatomical changes in cortical and subcortical structure (Sisk and Zehr 2005). Puberty-related increases in gonadal hormones are thought to be responsible, at least in part, for adolescent-related brain changes. However, this has not been demonstrated in humans, as it would be necessary to be able to manipulate the hormones.

Due to these limitations in studies involving human subjects, research on the *causal* effects of gonadal hormones on neural remodeling have been performed in rodents and non-human primates(Murphy and Segal 1996). In a study by Hajszan and collaborators, both androgens and E2 were shown to increase the number of medial PFC spine synapses in castrated male rats. However, data were collected in adulthood, and the effects may be different during adolescence. (Hajszan, MacLusky et al. 2007). In ovariectomized female rhesus monkeys, E2 replacement resulted in significant increases in spine numbers in PFC in young monkeys aged 6 to 8 years (Tang, Janssen et al. 2004). Altogether, this evidence is consistent with reports in the literature that reductions in cortical GM volume are due to synaptic pruning (Tang, Janssen et al. 2004, Hajszan, MacLusky et al. 2007, Markham, Mullins et al. 2013). In females, E2 has neurotrophic and neuroprotective effects in the AMYG and PFC and increases both cell survival and growth (Lee and McEwen 2001). While puberty in females is characterized by a surge in E2, the pre-pubertal period is also capable of secreting biologically active concentrations of E2, both in girls (Apter, Butzow et al. 1993, Norjavaara, Ankarberg et al. 1996) and female macaques (Pohl, deRidder et al. 1995, Wilson, Bounar et al. 2013). Previous work in our lab has demonstrated that even suppressing E2 secretion pre-pubertally in female rhesus monkeys results in marked alterations in brain structure, including development of cortical WM tracts (Godfrey et al., 2012; Godfrey et al., 2013a; Godfrey et al., 2013b). Thus, some of the brain structural

changes that take place during adolescence, including those that are the focus of this study, could very likely be due to the action of gonadal hormones.

In this study, we pharmacologically delayed puberty in half of the subjects in order to dissociate age-related maturational effects from puberty-related organizational effects of gonadal hormones on the brain and the resulting changes in behavior. Pubertal delay was achieved by administering Lupron Depot (leuprolide acetate), which is a gonadotropin-releasing hormone (GnRH) analog. GnRH acts on receptors in the anterior pituitary to stimulate release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In females, FSH and LH stimulate the ovaries to synthesize and release female gonadal hormones, E2 and progesterone (P), which have diverse effects in the body including development of the female reproductive system and secondary sex characteristics during female adolescence (Kugelberg 2013). Lupron acts as an agonist at pituitary GnRH receptors (Wilson, Meethal et al. 2007). During typical development, increased pulsatile secretion of GnRH by the hypothalamus is crucial in the initiation of puberty (Terasawa and Fernandez 2001). Initially, Lupron administration results in a sharp increase in LH, FSH, and gonadal hormones within three days of the initial treatment. Within two to four weeks of chronic administration of Lupron, desensitization of pituitary GnRH receptors results in suppression of LH, FSH, and gonadal hormones and thus, delay of puberty (Wilson, Meethal et al. 2007).

The goal of this study was to examine the effects of pubertal timing on the structural development of cortico-limbic brain areas (PFC, TPO, AMYG), social behavior, and stress physiology, specifically in intermediate-ranking female rhesus monkeys. For this, sMRI was used to non-invasively examine brain structural changes throughout the pubertal transition, by scanning the animals at two ages: the first at roughly 10 months prior to the start of puberty

(pre-puberty; 22.64 ± 1.19 months) and the second at the typical puberty onset for female rhesus monkeys (peri-puberty; 32.95 ± 1.15 months). In one cohort of monkeys, Lupron was administered chronically in order to pharmacologically delay puberty. Social behavior and stress physiology data were also collected at the two target ages to assess the functional correlates of brain structural differences. For this behavioral observations of the animals in their social group were performed to examine social (affiliative, agonistic), anxiety-like and play behaviors. In addition, stress neuroendocrine function was examined via collection of measures of hypothalamic-pituitary-adrenal (HPA) axis activity, including basal cortisol secretion, stress reactivity and glucocorticoid negative feedback through the dexamethasone (dex) suppression test.

The overall hypothesis is that juvenile female intermediate ranking rhesus macaques are subjected to a rich social experience in which they have to navigate complex and ambiguous social relationships involving not only very high and very low ranking animals in their groups, but also recognizing more nuanced social status differences with other middle-ranked animals. I hypothesized that this results in strong rank-related structural differences in regions of the brain involved in socioemotional processing, including the PFC, AMYG, and TPO that emerge during adolescence. An additional hypothesis is that puberty-induced increases in E2 play a crucial role in brain development and corresponding changes in social behavior and stress physiology.

This study design enabled testing of the following specific hypotheses:

(1) From pre-puberty to peri-puberty, females in both the Lupron and control groups will experience normative brain structure developmental changes, including an increase in total brain and WM volume, as well as a volume increase in regions involved in socioemotional processing, such as AMYG. Previous studies in rhesus monkeys and humans have demonstrated increases in

total brain and WM volume and an increase followed by a subsequent decrease in GM volume, including GM in the PFC (Giedd and Rapoport , Knickmeyer, Styner et al. 2010). In addition, AMYG has been shown to increase in rhesus monkeys during adolescence, with the right AMYG showing greater increases than the left (Payne, Machado et al. 2010).

(2) Lupron-treated subjects will show delayed developmental changes (e.g. delayed GM loss and WM increases) in these regions compared to controls, based on evidence of the influence of E2 on brain developmental processes during adolescence, particularly on synaptic pruning and myelination (Paus, Collins et al. 2001, Schulz, Richardson et al. 2004, Tang, Janssen et al. 2004, Sisk and Zehr 2005, Hajszan, MacLusky et al. 2007, Markham, Mullins et al. 2013). Preliminary work in our lab with the extreme groups in the social hierarchy (dominants, subordinates) also supports this hypothesis, as Lupron treatment-induced pubertal delay resulted in smaller AMYG volumes relative to controls. In addition, Lupron-treated controls did not show a significant increase in total intracranial volume (ICV), while control subjects did, indicating delays in overall brain growth (Godfrey et al, in preparation).

(3) Lupron-treated individuals will exhibit less anxiety-like behavior in the social group, based on previous research suggesting an increase in anxiety associated with earlier onset of puberty (Hayward, Killen et al. 1997, Schulenberg J. 1997, Graber, Seeley et al. 2004, Reardon, Leen-Feldner et al. 2009). To date, no other studies have looked specifically at intermediate-ranking rhesus monkeys. By examining the effect of rank within the intermediates as a continuous variable, I hypothesize that rank will predict an increase in GM volume of the regions previously mentioned to be involved in socioemotional processing (AMYG, PFC, TPO), as higher rank within this group is thought to correspond to greater success in navigating the social hierarchy (Utevsky and Platt 2014). I also hypothesize that higher rank will be associated with

lower HPA axis activity, based on findings of higher cortisol levels and impaired HPA axis function in socially subordinate animals (Michopoulos, Reding et al. 2012).

Methods

Subjects

Data were collected on 21 juvenile female rhesus macaques (*M. mulatta*) living in four social groups at the Yerkes National Primate Research Center Field Station (Lawrenceville, Georgia) at two different ages: pre-puberty (22.64 ± 1.19 months) and peri-puberty (32.95 ± 1.15 months). Social groups consisted of 2-3 adult males and 30-60 adult females with their juvenile offspring and were housed in an outdoor area of approximately one acre with attached climate-controlled indoor areas. Groups had been established for over five years before the start of this study. Animals were fed Purina monkey chow (Purina Mills Int., Lab Diets, St. Louis, MO, USA), a low-fat, high-fiber diet supplemented with fresh fruits and vegetables and had continuous access to water. All procedures were approved by the Emory University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for Care and Use of Laboratory Animals".

Social Rank

Each subject's social rank was determined during monthly 30-minute focal observations clustered around each of the two imaging ages and during group checks, as previously described (Embree, Michopoulos et al. 2013). Briefly, it was based on the result of dyadic agonistic interactions (Bernstein 1976), consisting of competitions between a subordinate animal, defined as one who produces an unequivocal submissive behavior, such as a flee, withdrawal, fear grimace or scream, to the other animal in response to an approach or aggressive act by the more

dominant animal. Therefore, social rank is determined based on the subordinate, rather than dominant, animal's behavior. In order to calculate each subject's relative rank, we divided her rank by the total number of monkeys in her group, excluding animals less than 12 months old (e.g. 0.2 would rank over 0.6). Animals with a relative rank below 0.3 and above 0.6 were classified as intermediate. Although social rank is very stable in these groups, females were excluded from the analysis if their rank changed from pre-puberty to peri-puberty in such a way that they were no longer considered part of the intermediate range.

Experimental Procedures

Lupron Administration

One group of females (n=13) was treated with Lupron (Tap Pharmaceuticals), a GnRH receptor agonist, to delay pubertal onset through Lupron action preventing increases in E2 during development (Golub, Styne et al. 1997, Wilson, Chikazawa et al. 2004). Females received an injection once a month (.75 μg/kg/mo, I.M.) from 16.38 ± 0.27 months (before the pre-puberty assessments) through 32.57 ± 0.27 months, spanning the interval from pre-puberty through post-menarche in this species (Wilson, Bounar et al. 2013). The other group of females (n=8) did not receive Lupron treatment, serving as a Control group that reached puberty spontaneously. Control subjects reached menarche at 32.95 ± 3.98 months and had their first ovulation at 37.39 ± 5.09 months, whereas Lupron-treated subjects reached menarche at 41.02 ± 1.91 months and had their first ovulation at 45.38 ± 1.6 months.

Structural MRI

Image Acquisition: One day prior to the scan, each subject was transported from the YNPRC Field Station to the YNPRC Imaging Center. Using a 3T Siemens Magnetom TRIO

system and an 8-channel phase array coil, T1-MRI and T2-MRI scans were acquired for each subject at pre-puberty (22.64 ± 1.19 months) and again at peri-puberty (32.95 ± 1.15 months). The first MRI scan in these animals occurred prior to menarche in both control and Lupron-treated subjects, while the second MRI scan was obtained after the occurrence of menarche in most control animals, but before the occurrence of menarche in all Lupron-treated animals. T1-MRI Scans were acquired using a 3D magnetization prepared rapid gradient echo (3D-MPRAGE) parallel imaging sequence (GRAPPA (R=2); TR=3000ms, TE=3.51ms, TI=950ms; FOV=96mm; Averages=6; voxel size=0.5x0.5x0.5mm^3). A T2-MRI scan was collected in the same direction as the T1 (TR/TE=7900/125ms, voxel size=0.5x0.5x1.0mm^3, 10 averages) in order to assist in the identification of GM, WM, and CSF by improving contrast of the borders between these tissue classes, and aid in the delineation of regions of interest (ROIs) (Rapisarda, Bergman et al. , Knickmeyer, Styner et al. 2010). Subjects were scanned under isoflurane anesthesia (1-1.2% to effect, inhalation) following induction with telazol (5mg/kg, I.M.) and intubation to ensure lack of motion artifacts. Animals were fitted with an oximeter, electrocardiograph, rectal thermometer and blood pressure monitor. Additionally, dextrose/NaCl (0.45%) was administered intravenously in order to maintain hydration during scanning. All subjects were scanned in the same supine placement and orientation on an MRI-compatible heating pad, using a custom-made head holder with ear bars and a mouth piece. A vitamin E capsule was taped to the right temple to indicate the right side of the brain. After scanning, subjects were monitored until full recovery and then returned to their social groups.

Image Processing and Analysis: Structural data were analyzed using AutoSeg (version 2.6.2), a software pipeline that performs automatic brain tissue structural segmentation of GM, WM, and cerebrospinal fluid (CSF) tissue classes, as well as cortical lobar parcellations and

subcortical parcellations, including selected ROIs, using and atlas-based automatic approach (see Figure 1). First, subjects' images were corrected for bias field inhomogeneity. AutoSeg employs N4-ITK bias field correction, which corrects image inhomogeneity due to RF coil imperfections that result in gradual variations in the image intensities, which reduces the performance of tissue classification and segmentation algorithms. Next, structural images of each subject in native space were nonlinearly registered to population-based T1- and T2-MRI atlases using BRAINSFit (Styner M. 2007, Short, Lubach et al. 2010, Howell, Grand et al. 2014). Warps produced during this step are used to generate probabilistic maps of the cortical and subcortical ROIs. Automatic segmentations and parcellations produced by AutoSeg were manually adjusted to ensure accuracy in the delineation of the tissue segmentations and ROIs, following published neuroanatomical landmarks (Amaral and Bassett 1989, Paxinos, Huang et al. 2000, Sallem and Logothetis 2006). ROI volumes were corrected for total intracranial volume (ICV), in order to control for group and age effects on ICV (Figure 3). Volumes were determined for total GM, WM, and CSF, as well as GM and WM of cortical regions, and total volume for subcortical regions. Total ICV was arithmetically calculated by adding total GM, WM, and CSF (both in the ventricles and subarachnoid space) volumes. The regions of interest for this study were AMYG, PFC, and TPO region of STS. Although both GM and WM volumes were determined for the cortical PFC ROI, we were only able to measure TPO GM volume, due to technical limitations. The AMYG volume was defined following macaque anatomical landmarks (Amaral & Bassett, 1989; Price et al., 1987; Reding et al., in preparation) with the anterior boundary defined by rostral periamygdaloid cortex, the ventral border defined by the CSF, the ventrolateral boundary defined by WM and the ventromedial boundary defined by the rhinal fissue (Howell, Grand et al. 2014). The PFC was defined using CSF at the surface of the brain as the lateral and anterior

boundaries, the interhemispheric fissure as the medial boundary and the arcuate sulcus as the posterior boundary (Knickmeyer, Styner et al. 2010). CSF in the anterior PFC and the arcuate sulcus in the posterior PFC were used to define the superior boundary. The inferior boundary, moving rostral to caudal, was defined by the CSF, the sylvian fissure, and the arcuate sulcus. The boundaries of the TPO region of the STS were defined laterally by the tip of the central sulcus, posteriorly by the lateral fissure, inferiorly by the STS, and superiorly by white matter (Seltzer and Pandya 1978, Paxinos, Huang et al. 2000)

Behavioral Observations

Both at pre- and peri-puberty, 30-minute focal behavioral observations were conducted monthly on each subject from towers above the animal group housing. Observations clustered around each of the two imaging ages and were performed using an established rhesus monkey ethogram (Altmann 1962, Maestripieri, McCormack et al. 2006) in order to code and quantify the frequency and duration of affiliative (groom, groom solicit, present, proximity), anxiety-like (self-scratch, yawn, body shake) and play (chase, self-play, wrestle) behaviors. Behavior was coded in real time, using a notebook computer and a custom-designed program (WinObs; (Graves and Wallen 2006)), which records the actor, behavior, recipient and the time and/or frequency for each behavior. Inter-observer reliability, calculated as % agreement on frequencies and durations of behaviors, was greater than 92%. Frequencies and durations of behaviors were averaged across observation sessions per age.

Stress Physiology Assessments

Basal and Stress-induced Cortisol levels: Serum cortisol levels were measured at pre-puberty (21.27 ± 0.81 months) and peri-puberty (29.62 ± 0.75 months) to examine HPA axis basal activity and stress reactivity. For this, a baseline blood sample was collected from the

saphenous vein in the awake animal in under 10 minutes from disturbance and at 10 am for each subject to control for diurnal variations in cortisol levels, following previously published protocols (McCormack, Newman et al. 2009, Arce, Michopoulos et al. 2010). Immediately following blood sample collection, the subject was transported to a novel behavioral testing room and placed in a testing cage for 30 minutes. Another blood sample (post-stress) was obtained immediately after to measure stress-induced increase in cortisol secretion. This brief social separation results in elevated serum cortisol through activation of the HPA axis, and is considered a potent stressor in monkeys (Arce, Michopoulos et al. 2010).

Dexamethasone (Dex) Suppression Test (Shively 1998, Jarrell, Hoffman et al. 2008): Dex Suppression Test was used to assess glucocorticoid negative feedback at pre-puberty (17.15 ± 1.98 months) and again at peri-puberty (33.55 ± 1.46 months). At 10am, a baseline blood draw was collected, and then subjects were administered Dex (0.25mg/kg I.M.) at 6pm on the same day. At 10am the following day, another blood sample was collected to measure glucocorticoid negative feedback, characterized by reduced (suppressed) levels of circulating cortisol in the blood as a result of the synthetic glucocorticoid binding to glucocorticoid receptors (GR) in the pituitary, hypothalamus and limbic brain regions to cause negative feedback (Ulrich-Lai and Herman 2009) Dex suppression is the ability of Dex to suppress typical cortisol secretion in the morning following administration of the drug.

Cortisol Assays: Blood samples were centrifuged and serum aliquoted and stored at -80C until assayed using a commercially available radioimmunoassay (RIA) kit (Beckman-Coulter/DSL, Webster, TX) at the Yerkes Biomarkers Core Lab.

Statistical Analyses

Analyses were first performed to determine the effects of social rank on each of the measures of interest (volumetric, behavioral and physiological) using Pearson correlation since relative rank is a continuous variable for this data set. When relative rank had a significant effect, it was included as a covariate in further analyses; otherwise, it was dropped from the overall statistical models.

Total ICV and brain WM, GM and CSF volume data was analyzed using a repeated measures analysis of variance (RM ANOVA) with Lupron treatment as the fixed factor and scan age as the repeated measure. Volumetric ROI data (PFC GM & WM, AMYG and TPO GM volume), was analyzed using a RM ANOVA with Lupron treatment as the fixed measure and scan age and hemisphere as repeated measures. Following detection of significant effects in the RM ANOVA pair-wise comparisons were performed using Fischer's LSD *post hoc* analyses with Bonferroni correction for multiple comparisons. Effect sizes were calculated for each significant main or interaction effect as partial η^2. Results with a $p<0.05$ were considered significant.

A step-wise multiple regression analysis was performed to determine whether the structural MRI measures were predictive of the behavioral and stress physiology measures. Predictive variables included volumes of each of the ROIs, subdivided by hemisphere, and total ICV, WM and GM, in addition to Lupron treatment and relative rank. Each of the predictive variables was analyzed separately for each of the two ages. Three subjects were excluded from analyses because they were identified as outliers. If more than one model was identified at a significance level of $p<0.05$, the model that accounted for the most variance was selected,

provided the condition index did not exceed 30 (to exclude multicollinear models). The behavioral and stress physiology dependent variables used in this analysis are listed in Table 1.

Results

Structural MRI Data

Analyses were first performed to determine the effects of social rank on each of the brain ROIs. Significant correlations were found between social rank and left PFC GM volume at pre-puberty ($r=-0.474$, $p=0.03$), as well as right and left GM PFC volume at peri-puberty (right: $r=-0.544$, $p=0.011$; left: $r=-0.574$, $p=0.007$), as shown in Figure 2. Therefore, social rank was included as a covariate in further analyses of PFC GM volume at both pre-puberty and peri-puberty. No other brain regions showed significant effects of social rank.

Total Intracranial Volume (ICV)

Analysis of total ICV (defined as total WM + total GM + total CSF –which included both CSF in the ventricles and subarachnoid space-) revealed a significant main effect of age ($F_{1,19}=21.111$, $p=0.000198$, $\eta^2_{partial}=0.526$; Figure 3). *Post hoc* analyses revealed greater volumes at peri-puberty than at pre-puberty, indicating an increase in ICV with age. A significant age by Lupron interaction effect was found ($F_{1,19}=11.007$, $p=0.00362$, $\eta^2_{partial}=0.367$), where Lupron-treated subjects had greater total ICV at peri-puberty relative to pre-puberty ($p=3.803\times10^{-6}$), while there was no significant age change for control subjects, indicating that the interaction effect was driven by smaller total ICV in Lupron-treated subjects at pre-puberty. No other main or interaction effects were found for Lupron or age.

Total Grey Matter (GM) Volume

There was a main effect of age, where total GM was larger at pre- than peri-puberty, indicating that total GM decreased with age ($F_{1,19}$=4.698, p=0.0431, $\eta^2_{partial}$=0.198; Figure 4). However, *post hoc* analyses indicated that total GM decreased with age only in control subjects. Lupron-treated subjects showed a significant increase in total GM volume from pre- to peri-puberty. A significant age x Lupron treatment interaction was also identified ($F_{1,19}$=35.975, p=0.00000901, $\eta^2_{partial}$=0.654). *Post hoc* analyses indicated that the effect of age was driven by differences in volume at pre-puberty, where control subjects had significantly larger total GM volumes (p=0.00417), whereas at peri-puberty there were no significant differences between the groups.

Total White Matter (WM) Volume

A main effect of age was identified, where total WM volume increased with age ($F_{1,19}$=136.727, p=4.0084x10^{-10}, $\eta^2_{partial}$=0.878), for both control and Lupron-treated subjects (Figure 5).

Total Cerebrospinal Fluid (CSF) Volume

A main effect of age was identified, where CSF volume increased from pre- to peri-puberty ($F_{1,19}$=31.694, p=00.00001987, $\eta^2_{partial}$=0.198), for both control and Lupron-treated subjects (Figure 6).

ICV-Corrected Grey Matter (GM) Volume

Significant main effects of age ($F_{1,19}$=77.647, p=3.875x10^{-8}, $\eta^2_{partial}$=0.803) and Lupron ($F_{1,19}$=9.435, p=0.00628, $\eta^2_{partial}$=0.332) were identified for total GM volume, with greater total GM volumes at pre-puberty than at peri-puberty, with GM volume decreasing with age, and

greater GM volumes in control relative to Lupron-treated subjects (Figure 7). A significant age by Lupron interaction effect was also found ($F_{1,19}$=15.259, p=0.000949, $\eta^2_{partial}$=0.445), with greater GM volumes in control subjects relative to Lupron-treated subjects at pre-puberty (p=0.000241), but no significant differences between the groups at peri-puberty, indicating that the effect of age was driven by the volumetric differences at pre-puberty. No other main or interaction significant effects were found for age, Lupron treatment.

ICV-Corrected White Matter (WM) Volume

A significant main effect of age ($F_{1,19}$=32.284, p=0.0000178, $\eta^2_{partial}$=0.630) was found for total WM volume, with greater WM volumes at peri- than at pre-puberty, indicating an increase in WM volume with age (Figure 8). A significant age by Lupron treatment interaction effect was found ($F_{1,19}$=9.246, p=0.00673, $\eta^2_{partial}$=0.327). *Post hoc* analyses indicated that total WM was significantly greater at peri-puberty than at pre-puberty for both control (p=0.0000240) and Lupron-treated subjects (p=0.046). No other main or interaction effects of age or Lupron treatment were found.

ICV-Corrected Cerebrospinal Fluid (CSF) Volume

Significant main effects of age ($F_{1,19}$=23.927, p=0.000101, $\eta^2_{partial}$=0.557) and Lupron treatment ($F_{1,19}$=12.380, p=0.00230, $\eta^2_{partial}$=0.395) were found for total CSF volume, which increased with age, and there were greater CSF volumes in Lupron-treated subjects compared to controls (Figure 9). There were significant differences between the groups at pre- (p=0.00264), but not at peri-puberty, indicating that the effect of cohort was driven by greater CSF volumes in Lupron-treated subjects at pre-puberty. In addition, pair-wise comparisons revealed that CSF volume increased with age in both control (p=0.000341) and Lupron-treated (p=0.0283) subjects No other main or interaction effects of age or Lupron-treatment were found.

Prefrontal Cortex GM Volume

As described above and in Figure 2, a significant correlation was found for social rank and left PFC GM volume at pre-puberty (r=-0.474, p=0.03), as well as right and left PFC GM volume at peri-puberty (right: r=-0.544, p=0.011; left: r=-0.574, p=0.007), with greater PFC GM volumes in higher-ranked relative to lower-ranked subjects. A significant main effect of cohort was also identified, with greater PFC GM volumes in control than Lupron-treated subjects ($F_{1,17}$=13.854, p=0.00169, $\eta^2_{partial}$=0.449), as shown in Figure 10. No other main or interaction effects of age, Lupron treatment or hemisphere were found.

Prefrontal Cortex WM Volume

A significant main effect of age was found for PFC WM volume ($F_{1,19}$=36.698, p=7.942x10^{-6}, $\eta^2_{partial}$=0.659), with greater PFC WM volumes at peri- than at pre-puberty indicating an increase with age (Figure 11). Significant age by Lupron ($F_{1,19}$=13.186, p=0.00178, $\eta^2_{partial}$=0.410) and age by Lupron by hemisphere ($F_{1,19}$=5.089, p=0.0361, $\eta^2_{partial}$=0.211) interaction effects were also detected. *Post hoc* analyses revealed that control subjects had greater PFC WM volumes relative to Lupron-treated subjects at peri-puberty (p=0.045), but there were no significant differences between the groups at pre-puberty. PFC WM was significantly greater at peri-puberty compared to pre-puberty in control subjects (p=6.440x10^{-6}), but there was no significant difference in PFC WM volume between both ages for Lupron-treated subjects. This indicates that the age-related increase of PFC WM volume was driven by the control group. In addition, PFC WM volume was significantly greater in the right hemisphere of control subjects relative to Lupron-treated subjects at peri-puberty (p=0.023), but not in the left hemisphere. No other main or interaction effects were found for age, Lupron treatment or hemisphere..

Amygdala Volume

Significant main effects of Lupron treatment ($F_{1,19}$=5.418, p=0.031, $\eta^2_{partial}$=0.222) and hemisphere ($F_{1,19}$=7.526, p=0.013, $\eta^2_{partial}$=0.284) were found for AMYG volume, with greater AMYG volumes in control relative to Lupron-treated subjects, and greater AMYG volumes in the right hemisphere relative to the left (Figure 12). No other main or interaction effects were found for age, Lupron treatment or hemisphere.

Temporal-Parietal-Occipital (TPO) Region of STS GM Volume

Significant main effects of age ($F_{1,19}$=14.321, p=0.001, $\eta^2_{partial}$=0.430) and hemisphere ($F_{1,19}$=16.059, p=0.001, $\eta^2_{partial}$=0.458) were identified for the TPO region of the STS GM volume, with greater volumes at pre- than peri-puberty, indicating a decrease in volume with age, and in the right than in the left hemisphere (Figure 13). No other main or interaction effects were found for age, Lupron treatment or hemisphere.

Multiple Regression Analysis

The results of the multiple regression analysis are shown in Table 1, which examined whether the neural measures are predictive of relevant social behaviors and measures of stress neuroendocrine function (specifically, HPA axis activity) at pre- and peri-puberty. The statistical model also included the main factors examined in the study: Rank and Lupron treatment. Each model was examined for outliers using Cook's Distance, and for multicollinearity using tolerance, VIF, eigenvalue and condition index.

Two social behaviors, wrestle and anxiety-like behavior, were predicted by brain ROIs, although only at peri-puberty. Thus, bigger total ICV at peri-puberty predicted more time

involved in wrestle behavior (p=0.041, adj. R^2=0.421; Figure 14). In addition, bigger right TPO GM volume at peri-puberty predicted less anxiety behavior (p=0.016, adj. R^2=0.232; Figure 15).

In addition, three cortisol measures, baseline cortisol, as well as the changes in cortisol from baseline following Dex administration and after separation stress, were predicted by brain ROIs. Left PFC GM at pre-puberty was predictive of baseline cortisol levels at pre-puberty, where larger PFC GM volume predicted lower baseline cortisol levels (p=0.017, adj. R^2=0.228; Figure 16). Left PFC GM volume predicted the change in cortisol following Dex administration at peri-puberty, where greater PFC GM volume predicted a less negative change in cortisol levels, indicating less cortisol suppression by Dex (p=0.029, adj. R^2=0.195; Figure 17). Right AMG volume predicted the change in cortisol from baseline following separation stress at peri-puberty, where greater AMYG volume predicted a greater increase in cortisol (p=0.039, adj. R^2=0.163), as shown in Figure 18.

Discussion

The principal goal of this study was to determine how social rank and pubertal timing in intermediate-ranking female rhesus macaques affect the development of cortico-limbic areas involved in the processing of socioemotional stimuli, specifically the PFC, AMYG, and TPO region of the STS. In addition, the functional correlates of these brain structural effects were also examined, focusing on relevant aspects of social behavior and stress physiology measures (specifically HPA axis basal, stress and negative feedback function) controlled by the target brain regions.

To address these questions, structural MRI scans were collected in control and Lupron-treated intermediate-ranking female juvenile rhesus monkeys before puberty and again at the

typical time of pubertal onset in this species (peri-puberty). The results from this study show that PFC GM volume increased with increasing rank, although rank did not predict any other brain structural measures, or had any interaction effects with the other factors studied (effects of Lupron treatment, age or brain laterality effects). As expected, total ICV increased with age, but it was driven by the Lupron-treated subjects, which had significantly lower ICV at pre-puberty relative to controls and had a very steep increase from pre- to peri-puberty. The smaller total ICV in Lupron-treated subjects at pre-puberty appears to be due to smaller total GM volumes in the Lupron-treated subjects relative to control subjects at the age. Total GM decreased with age in control subjects, but increased with age in Lupron-treated subjects. When ICV-corrected, both control and Lupron-treated subjects showed a decrease in GM volume with age, which is consistent with previous sMRI studies in rhesus macaques, with larger GM volumes in control subjects than Lupron-treated subjects at pre-puberty and no significant differences at peri-puberty. Also consistent with the literature, total WM increased with age in both Lupron-treated and control subjects, with control subjects showing a greater increase from pre- to peri-puberty. Total CSF volume also increased with age, particularly in Lupron-treated subjects. Developmental cortical GM decreases were detected in TPO, accompanied by increases in WM volume in PFC. Conversely, PFC GM and AMYG volumes did not show developmental changes in either of the groups. And finally, Lupron-treated animals show an overall reduction in ROI volumes, and showed slowed or delayed development, including smaller PFC GM, right PFC WM, and AMYG volumes.

Functional correlates of these brain structural changes were also examined and results showed interesting age-related effects: (1) total ICV volume was associated with less play wrestle behavior at peri-puberty; (2) greater PFC GM volume was associated with lower baseline

cortisol, but only at the first age; (3) larger right AMYG volume predicted a greater stress-induced cortisol response, but only at peri-puberty; (4) greater right TPO GM volume predicted less anxiety-like behavior, although only at peri-puberty. Altogether, these findings support an association between bigger PFC GM volumes and success in navigating the social hierarchical structure, as well as a strong role of puberty-induced E2 elevations on brain structure development, particularly in female cortico-limbic circuits involved in social and emotional regulation.

As summarized in the introduction, the regions selected for this study are involved in the processing and control of social and emotional behavior, and social cognition. The human PFC, in particular, is involved in theory of mind, which is the ability of an individual to attribute mental states to him/herself and to others (Premack and Woodruff 1978, Maat, van Haren et al. 2016). This same study showed that smaller PFC volume was associated with poorer recognition of social stimuli, specifically angry faces (Maat, van Haren et al. 2016). There is also evidence of PFC involvement in more complex aspects of social behavior, including navigation of group social structures, both in humans and nonhuman primates. Studies in humans show that increases in GM volume in a region of PFC, the ventromedial PFC, predicted increases in both high-level mentalizing capacities and social network size (Lewis, Rezaie et al. 2011). In rhesus monkeys rostral PFC GM density has been positively correlated with social network size, and anterior and dorsal PFC GM positively correlated with both social network size and social status (Noonan et al., 2014; Sallet et al., 2011). Findings from the present study are consistent with this literature, as they show that PFC GM volume positively correlates with rank even in the intermediate-ranking rhesus monkeys. These results support the hypothesis that success in navigating the

social hierarchy relies on bigger size of brain regions involved in social cognition, with the PFC playing a critical role in this process.

Consistent with previous studies in both humans and nonhuman primates, the developmental changes reported here involve increases in total ICV and total brain WM and CSF volumes from pre- to peri-puberty, in parallel with a decrease in total brain GM with age (De Bellis, Keshavan et al. 2001, Malkova, Heuer et al. 2006, Knickmeyer, Styner et al. 2010, Scott, Grayson et al. 2015). In a review of longitudinal structural MRI studies in children and adolescents, total WM was reported to increase with age through young adulthood, while total GM volume follows an inverted U-shaped curve, in which GM volume shows a pre-pubertal increase that peaks at approximately 6-7 years and shows a continual decline during adolescence (Giedd and Rapoport 2010). However, the developmental trajectories reported for different brain areas are region-specific, with GM volumes peaking at different times depending on the brain region, both in humans (De Bellis, Keshavan et al. 2001, Gogtay, Giedd et al. 2004, Lenroot and Giedd 2006, Giedd and Rapoport 2010, Aubert-Broche, Fonov et al. 2013) and non-human primates (Malkova, Heuer et al. 2006, Knickmeyer, Styner et al. 2010, Payne, Machado et al. 2010). While MRI measures change in volume, morphology, and density of brain structures, a limitation of this technique is that it does not address the underlying cellular or molecular mechanisms responsible for these changes. Neuronal changes in GM may include neurogenesis, synaptogenesis, and changes in neuronal morphology; whereas WM changes may be due to changes in the number of axons, axon diameter, the packing density of fibers, axon branching, and myelination (Zatorre, Fields et al. 2012). Ultimately, histological studies are required to explain the neurobiological and cellular mechanisms underlying the developmental, rank, and Lupron effects reported in this MRI study.

Although no significant changes in PFC GM volume were detected between the two ages studied (pre- and peri-puberty), in this study, PFC WM increased with age. In a study of the developmental trajectory of rhesus monkey cortical brain maturation (Knickmeyer, Styner et al. 2010), including both males and females, PFC WM also increased until approximately 45 months, while PFC GM followed an inverted U-shaped developmental trajectory with peak volume at approximately 40 months of age. Visual inspection of the PFC GM graph shows a leveling off between 20 and 30 months, which agrees with the finding of no significant difference in PFC GM between these two ages in my study. In rats, PFC also undergoes an increase, followed by a decrease in volume during adolescence (Markham, Mullins et al. 2013). This decrease during adolescence has been shown to be mainly due to synaptic pruning, for example, during the peri-adolescent period in rats there is extensive pruning in the PFC (Andersen, Thompson et al. 2000). In monkey and human studies, synaptic pruning occurs in the PFC throughout adolescence and results in a decrease in synaptic density (Bourgeois and Rakic 1993, Huttenlocher and Dabholkar 1997, Zecevic and Rakic 2001, Blakemore and Choudhury 2006).

Previous research in rhesus monkeys (Knickmeyer, Styner et al. 2010) found a linear developmental increase in AMYG volume during adolescence in both males and females. In humans, AMYG volume increases with age in males, but not significantly in females (Giedd, Vaituzis et al. 1996). In this study, no significant change in AMYG volume was detected from pre- to peri- puberty, which could potentially be due to the narrow developmental time between scans compared to the study by Knickmeyer and collaborators, which studied these animals between the ages of 10 and 64 months. In addition we found greater AMYG volumes in the right compared to the left hemisphere, which is consistent with laterality effects reported in humans

(Giedd, Vaituzis et al. 1996) and rhesus monkeys (Knickmeyer, Styner et al. 2010, Payne, Machado et al. 2010).

In children, the temporal lobe GM volume, including the STS, is one of the latest cortical structures to peak, doing so at approximately 16 years and then steadily declining (Giedd, Blumenthal et al. 1999, Lenroot and Giedd 2006, Toga, Thompson et al. 2006). In rhesus monkeys, Knickmeyer and collaborators found that GM in temporal auditory and temporal visual cortices continues to increase from 10 months to 64 months of age. In contrast, results from the current study showed a decrease in TPO GM volume from pre- to peri-puberty. Again, this discrepancy could be due to the narrow developmental time window in this study, relative to the study by Knickmeyer and colleagues (Knickmeyer, Styner et al. 2010). In addition, TPO GM exhibited laterality effects, with right hemisphere volume greater than left. This is consistent with asymmetries found in the temporal lobe and superior temporal gyrus in human adolescents (Giedd, Vaituzis et al. 1996).

In addition to the social rank and age effects discussed above, we found robust effects of Lupron-treatment on brain measures, suggesting that the suppression of E2 secretion during the peri-pubertal period and/or the pubertal delay have widespread impact on the female brain development. Female puberty is characterized by elevations in E2 and P levels circulating in the blood, and it typically takes place between 27 and 36 months of age in socially housed female macaques (Wilson, Gordon et al. 1984). E2 receptors are present in a variety of brain structures, including rodent hypothalamus, hippocampus, AMYG, cerebral cortex (including PFC), midbrain, and brainstem (McEwen 2001, Wang, Hara et al. 2010). Distribution of the estrogen receptor in the female macaque brain is also high in regions of the temporal lobe, although seem to be expressed primarily in the subcortical hippocampus and AMYG regions, with no evidence

of E2 receptors in the STS region (Gundlah, Kohama et al. 2000). This lack of E2 receptors in STS could explain the lack of Lupron treatment effects on TPO GM. Overall, Lupron-treatment resulted in smaller GM volumes and WM volumes (both total and in the PFC), as well as in smaller ICV and AMYG volumes, relative to untreated control subjects. These results suggest that E2 plays an important role regulating the development of both brain GM and WM during adolescence, although, as described in the introduction, the underlying mechanisms may be different (i.e., E2 seems to facilitate synaptic pruning, leading to decreases in GM, while at the same time activating myelination, leading to increases in WM). Now, interesting age-specific effects of Lupron were detected, with impact on WM volume only evident at the latest age, potentially reflecting blunted myelination processes in Lupron-treated animals in comparison to rapid growth in the control animals (Paus, Zijdenbos et al. 1999, Barnea-Goraly, Menon et al. 2005, Snook, Paulson et al. 2005, Shi, Short et al. 2013). Conversely, the effects of Lupron-treatment that led to reduced GM and AMYG volumes were evident at both ages, or just at pre-puberty, suggesting that even lower than the typically low E2 levels during pre-puberty (Pohl, deRidder et al. 1995, Wilson, Bounar et al. 2013) had robust neural structural effects. The effects of low E2 on smaller GM and AMG volumes are likely due to actions on dendritic arborizations, or synaptic pruning, at least based on the evidence that the reduction in cortical GM volumes reported during adolescence is due to the synaptic pruning (Huttenlocher 1979, Huttenlocher and Dabholkar 1997, Zatorre, Fields et al. 2012, Markham, Mullins et al. 2013). Previous studies have provided evidence for E2's modulatory role during development on brain areas that express E2 receptors. The AMYG is sexually dimorphic with differences emerging during the pubertal transition, indicating that E2, along with testosterone, has an important role in the structural changes occurring during this critical period of development. Specifically, E2 promotes dendritic

shaft synapse formation in the rat AMYG postnatally (Nishizuka and Arai 1981). The results from the present study provide further evidence that estrogen plays a critical role in the normal developmental trajectory of PFC and AMYG. A study performed in ovariectomized rodents found reduced spine density in PFC (Chisholm and Juraska 2012). In addition, ovariectomized female rats had less dendritic arbor in PFC compared to controls (Kolb and Stewart 1991). Thus, E2 appears to alter the number of synaptic spines and dendritic branches, but whether it is due to inhibition of synaptic and/or dendritic pruning or facilitation of spine synapse/dendrite proliferation is unknown (Chisholm and Juraska 2012). There is evidence of a connection between spine density and GM volume (Huttenlocher 1979, Huttenlocher and Dabholkar 1997, Zatorre, Fields et al. 2012, Markham, Mullins et al. 2013), suggesting that this could be a potential mechanism for the smaller PFC GM volumes found in Lupron-treated subjects in the present study.

Findings from the multiple regression analysis suggest very interesting associations between brain structural measures and behavioral and physiological correlates, while also accounting for rank and Lupron-treatment factors. Greater left PFC GM volume predicted lower baseline cortisol levels at pre-puberty and a weaker glucocorticoid negative feedback (Dex suppression) at pre-puberty, but no effects of either rank or Lupron treatment. The primate PFC is a target for glucocorticoid action due to the high levels of expression of glucocorticoid receptors (GR), which mediate glucocorticoid negative feedback to the HPA axis (Sanchez, Young et al. 2000, Kern, Oakes et al. 2008). Both excitatory (Wang, Rao et al. 2005) and inhibitory (Diorio, Viau et al. 1993) effects of PFC on HPA axis function have been reported in the literature, depending on the specific sub-region studied (Herman and Cullinan 1997), most evidence suggest a top-down inhibitory control of the PFC over HPA axis basal function, stress-

induced reactivity and glucocorticoid negative feedback (Ulrich-Lai and Herman 2009). An inhibitory role of PFC on HPA axis glucocorticoid negative feedback could explain our results which showed that greater left PFC GM predicted less suppression of cortisol by Dex, and thus weaker glucocorticoid negative feedback response. Also consistent with our findings, a study on children with posttraumatic stress disorder reported a negative association between pre-bedtime cortisol levels and left ventral PFC GM volumes (Carrion, Weems et al. 2010). While my study did not assess specific sub-regions of PFC, altogether my findings and this literature provide support for an inhibitory effect of PFC on HPA axis baseline and glucocorticoid negative feedback function.

Larger right AMYG volumes predicted greater stress-induced cortisol responses, although only at peri-puberty. The AMYG plays a critical role in mediating the stress response, as well as being sensitive to chronic or severe stress. Thus, studies in rodents showed that chronic stress increases the excitability of neurons in the lateral AMYG (Rosenkranz, Venheim et al. 2010). In humans, a study in chronically stressed children showed that they had greater AMYG volumes, especially on the right, relative to non-stressed controls (Mehta, Golembo et al. 2009). Consistent with previous literature, I found that bigger right AMYG volumes are associated with increased stress responses, suggesting that the control animals show greater stress reactivity. The AMYG has been shown to have excitatory effects on HPA axis function, with stimulation of specific nuclei of the AMYG increasing corticosterone secretion in rats (Dunn and Whitener 1986). Lesion studies also provide evidence for an excitatory role of the AMYG on HPA axis response to stress. For example, lesions of the central nucleus of the AMYG decrease corticosterone responses to restraint and fear conditioning (Van de Kar,

Piechowski et al. 1991). My findings are consistent with the literature in providing support for an excitatory effect of the AMYG on HPA axis function in response to stress.

Total ICV, as well as GM volume in right TPO was predictive of behavior, but only at peri-puberty. Greater ICV predicted more play wrestle behavior, and larger TPO GM predicted less anxiety-like behavior. Both AMYG and STS are involved in social cognition (Adolphs 2001, Amaral, Schumann et al. 2008, Bickart, Wright et al. 2011, Lewis, Rezaie et al. 2011, Sallet, Mars et al. 2011, Powell, Lewis et al. 2012, Noonan, Sallet et al. 2014). Functional imaging studies have shown that STS is activated by moving eyes and mouths in faces, as well as biologically-related motion, such as hand and whole body motion (Oram and Perrett 1994, Peuskens, Vanrie et al. 2005). In one theory of the components of social cognition, the STS is involved in the perceiving and evaluating stimulus features, and this information is relayed to the AMYG. The AMYG, with projections to the motor cortex, basal ganglia, hypothalamus, and brainstem, then drives socially- and emotionally-related behaviors (Adolphs 2001). Area TPO is a specific region within STS that receives input from auditory, visual, and somatosensory areas (Barnes and Pandya 1992, Seltzer, Cola et al. 1996) and has reciprocal connections with AMYG (Amaral and Insausti 1992). In a fMRI study of AMYG connectivity, the presentation of dynamic fearful facial expressions enhanced responses in dorsal temporal areas, including STS. STS is therefore sensitive to fearful stimuli presumably via input from the AMYG, which may control sensitivity of cortical areas, such as STS, to emotionally relevant stimuli (Furl, Henson et al. 2013). In this study, TPO GM volume predicted less anxiety-like behavior at peri-puberty. This fits with the evidence presented above, as well as previous findings of the AMYG-STS connections, and points to the TPO as a specific sub-region of the STS important for the social and emotional stimuli processing and relaying that information to areas such as the AMYG,

which in turn modulates the activity of the STS to focus on decoding stimuli relevant to fear and anxiety behaviors. Evidence in children with ASD shows decreased GM density in STS (Boddaert, Chabane et al. 2004). In summary, our results that greater right TPO GM is associated with less anxiety-like behavior, fit with a bigger literature indicating that a larger TPO allows for enhanced processing of socioemotional stimuli. Interestingly, right AMYG and right TPO correlated with physiology or behavioral measures and sMRI analyses revealed that both the TPO and AMYG were larger in the right than the left hemisphere, providing evidence that this structural laterality may have functional significance.

As part of the multiple regression analysis, Lupron-treatment and rank also predicted a significant amount of the variance for HPA axis negative feedback and social behaviors.

There were several limitations of this study. One was the narrow developmental time frame and the limited number of measurements, only taken at two early time points, one at pre-puberty and one at peri-puberty. This limits the conclusions, as we cannot extrapolate the effects of age, rank, and Lupron-treatment on brain structural changes through adulthood. Another limitation is that, based on the atlas used, we could only assess the TPO GM, and not effects on WM. In addition, it would be important to do follow-up studies in sub-regions and sub-nuclei within the ROIs we studied, such as medial PFC, dorsolateral PFC, and orbitofrontal cortical areas, as well as basolateral, medial and central AMYG sub-nuclei. In this study, these sub-regions were grouped together into the area we termed “PFC” or “AMYG”. Specific regions of these larger brain areas could play distinct roles in brain function and behavior that we were unable to examine. Finally, it is important to note that while several brain regions were found to significantly predict either behavioral or stress physiology measures, none of these predicted

greater than 43% of the variance in the model. This indicates that there are other variables that explain the rest of the variance that were not identified in this study.

In summary, the findings from this study suggest that E2 plays an important role in the development of brain regions including PFC and AMYG, as hypoestrogenism due to Lupron treatment was associated with smaller AMG and PFC WM and GM volumes. Future studies are necessary to elucidate additional brain regions affected by Lupron-treatment, as this is a drug currently prescribed to treat precocious puberty in children. In addition to the effects of hypoestrogenism on several brain areas, we found effects of social rank on PFC GM, indicating that this region is important to successfully navigate the complexities of the social hierarchy. Lastly, we found that these structural brain changes had important functional correlates to social and emotional behaviors, as well as HPA axis basal and stress function, indicating that the development of PFC, AMYG, and TPO during the pubertal transition have behaviorally and neuroendocrine relevant consequences.

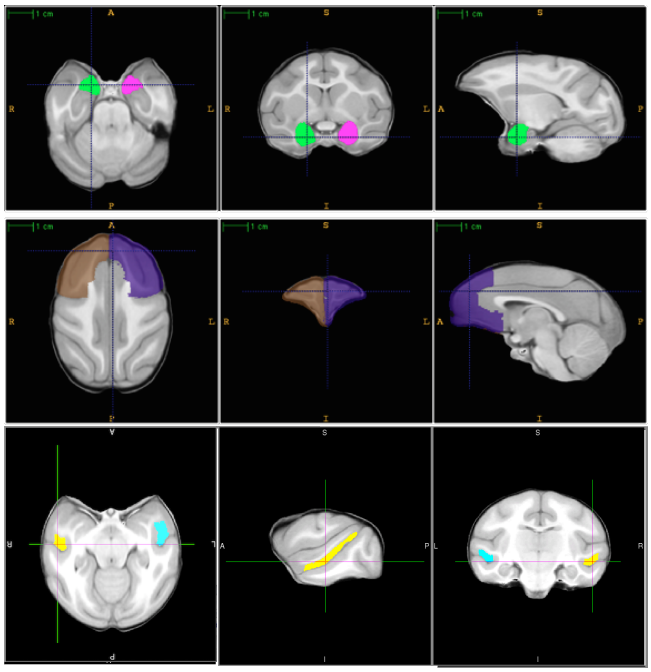
Tables

		Overall Model				Predictor Variables					
	Dependent Variable	n	*Adj. R^2*	*F*	*Sig.*	Retained Predictor Variables	B	Std. Error	β	*t*	*Sig.*
Age 1; PRE-Pub	ΔCortisol Separation	No variables were entered - non significant									
	Baseline Cortisol		0.228	6.907	0.017	Left PFC GM Age 1	-2590	985.45	-0.516	-2.628	0.017
	Post Dex Cortisol	No variables were entered - non significant									
	ΔCortisol Dex		0.195	5.603	0.029	Left PFC GM Age 1	**2139.79**	904.009	0.487	2.367	0.029
	Affiliation Duration	No variables were entered - non significant									
	Submission Duration	No variables were entered - non significant									
	Play Wrestle Duration	No variables were entered - non significant									
	Anxiety Behavior Frequency	No variables were entered - non significant									
	Sit Alone Duration	No variables were entered - non significant									
	Freeze Duration	No variables were entered - non significant									
Age 2; PERI-Pub	ΔCortisol Separation Stress		0.163	4.896	0.039	Right Amygdala Age 2	9182.65	4150	0.453	2.213	0.039
	Baseline Cortisol	No variables were entered - non significant									
	Post Dex Cortisol	No variables were entered - non significant									
	ΔCortisol Dex		0.281	8.829	0.008	Cohort	**-13.151**	4.426	-0.563	-2.971	0.008
	Affiliation Duration	No variables were entered - non significant									
	Submission Duration		0.146	4.411	0.049	Cohort	2.738	1.303	0.434	2.1	0.049
	Play Wrestle Duration		0.421	4.815	0.041	Total ICV Age 2	0.00013	5.8E-05	0.45	2.194	0.041
	Anxiety Behavior Frequency		0.232	7.034	0.016	Right TPO Age 2	-7349.5	2771.09	-0.52	-2.652	0.016
	Sit Alone Duration		0.22	6.642	0.018	Relative Rank Age 2	-14.647	5.683	-0.509	-2.577	0.018
	Freeze Duration		0.151	4.544	0.046	Right TPO Age 2	-1227.4	575.822	-0.439	-2.132	0.046

Table 1. Multiple Regression Analysis. Candidate predictor variables were age 1 and age 2 volumes of total WM, total GM, and total ICV, as well as right and left of PFC GM, PFC WM, AMYG, and TPO GM, and Rank and Lupron Treatment. Each predictor value was entered into the stepwise model and those variables that significantly contributed to it ($p<0.05$) were retained. If more than one model was identified at a significance level of $p<0.05$, the model that accounted for the most variance was selected, provided the condition index did not exceed 30 (to exclude multicollinear models).

Figures

A.



B.

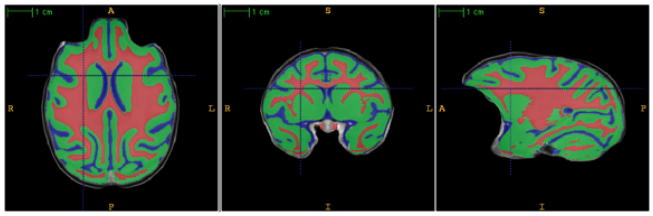


Figure 1. Representative images of the structural MRI atlas, ROIs and automatic tissue segmentation and parcellation processes using the AutoSeg pipeline. (A) AMYG –top row-, PFC –middle row- and TPO –bottom row- ROIs used to generate the automatic cortical and subcortical parcellations in AutoSeg (Styner, Knickmeyer et al. 2007, Howell, Grand et al. 2014); L: left, R: right; A: anterior, P: posterior; S: superior, I: inferior. (**B**) Results of automatic tissue segmentation for GM (green), WM (red), and CSF (blue) using AutoSeg. L: left, R: right; A: anterior, P: posterior; S: superior, I: inferior. Images provided by J. Godfrey.

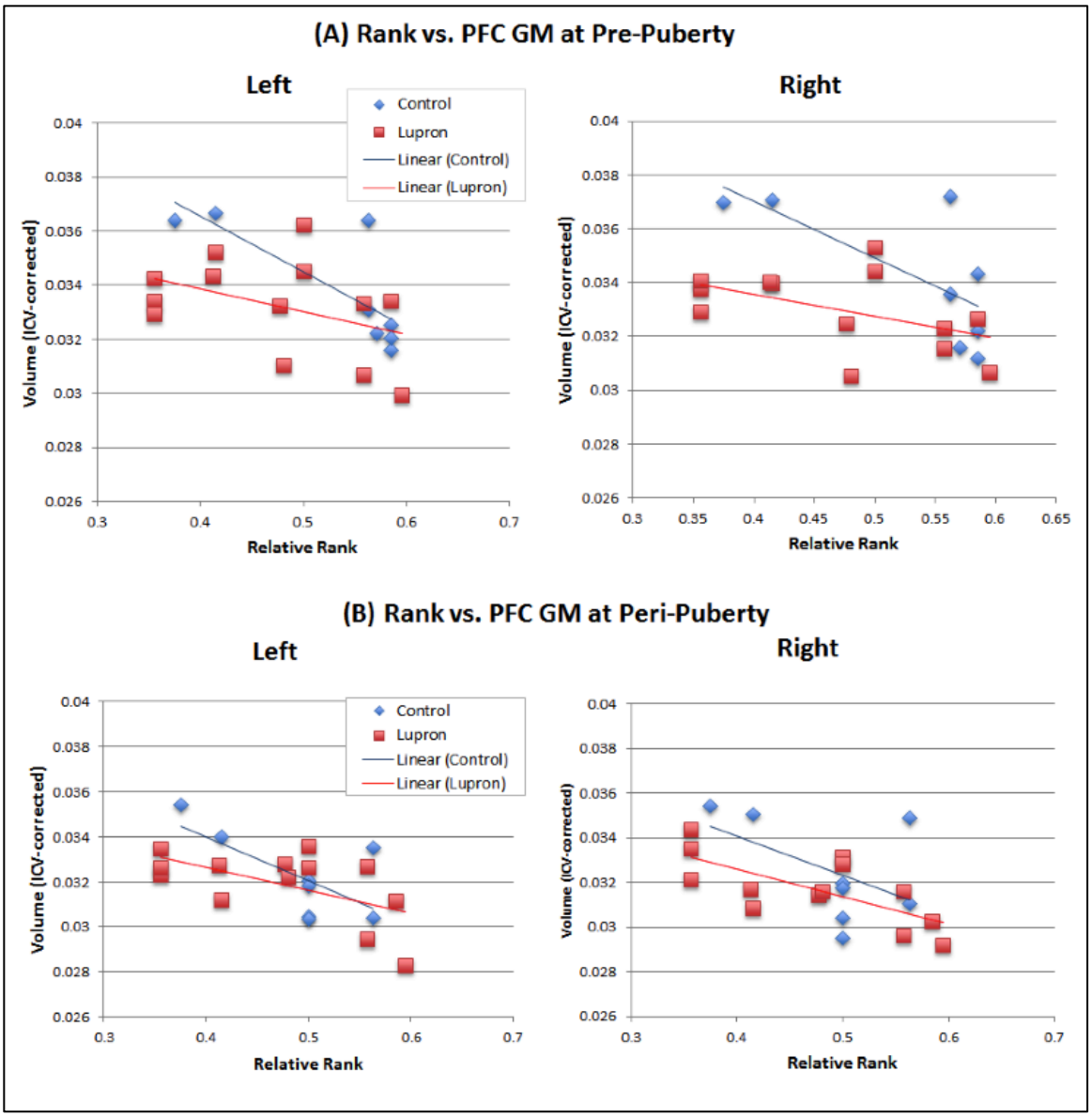


Figure 2. Relationship between rank and PFC GM at pre-puberty (A) and peri-puberty (B). A significant negative correlation between social rank and left PFC GM volume at pre-puberty (r=-0.474, p=0.03) was detected, with bigger PFC GM volumes in higher-ranked relative to lower-ranked subjects. A significant negative correlation was also found between rank and both right and left PFC GM volume at peri-puberty (right: r=-0.544, p=0.011; left: r=-0.574, p=0.007), with bigger PFC GM volumes in higher- than lower-ranked animals.

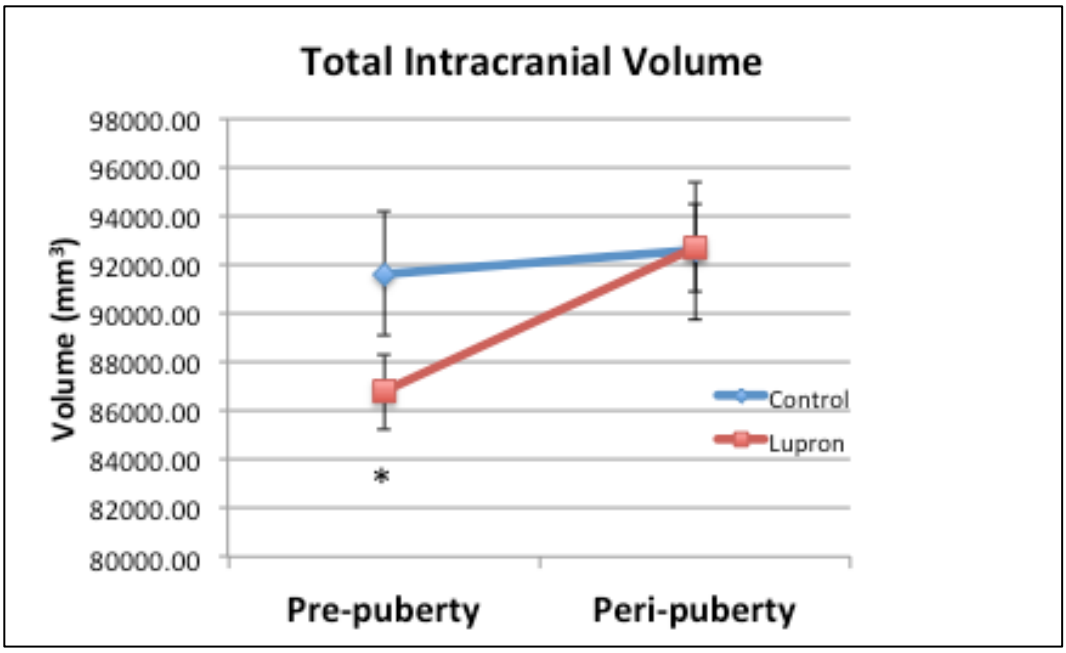


Figure 3. Developmental effects of Lupron treatment on total ICV. There was a main effect of age on ICV ($F_{1,19}$=21.111, p=0.000198, $\eta^2_{partial}$=0.526), which increased from pre- to peri-puberty. A significant age x Lupron-treatment interaction effect was also identified ($F_{1,19}$=11.007, p=0.00362, $\eta^2_{partial}$=0.367), indicating that the effect was driven by smaller ICV in Lupron-treated subjects at pre-puberty ($p=3.803 \times 10^{-6}$) relative to controls.

*: $p<0.05$, significantly different from Lupron Peri-puberty.

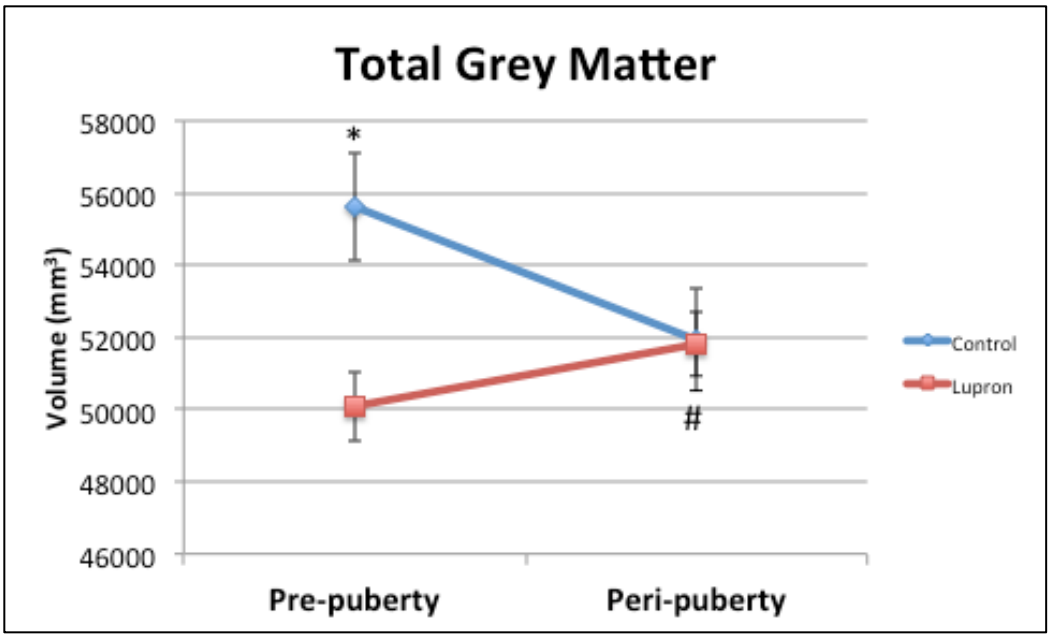


Figure 4. Developmental effects of Lupron treatment on total GM volume. There was a main effect of age, where total GM decreased from pre- to peri-puberty ($F_{1,19}$=4.698, p=0.0431, $\eta^2_{partial}$=0.198). A significant age x Lupron treatment interaction was also identified ($F_{1,19}$=35.975, p=0.00000901, $\eta^2_{partial}$=0.654). *Post hoc* analyses indicated that the effect of age was driven by differences in volume at pre-puberty, where control subjects had significantly larger total GM volumes (p=0.00417).

*: p<0.05, significantly different from Lupron Pre-puberty

#: p<0.05, Lupron Peri-puberty significantly different from Lupron Pre-puberty and Control Peri-puberty significantly different from Control Pre-puberty

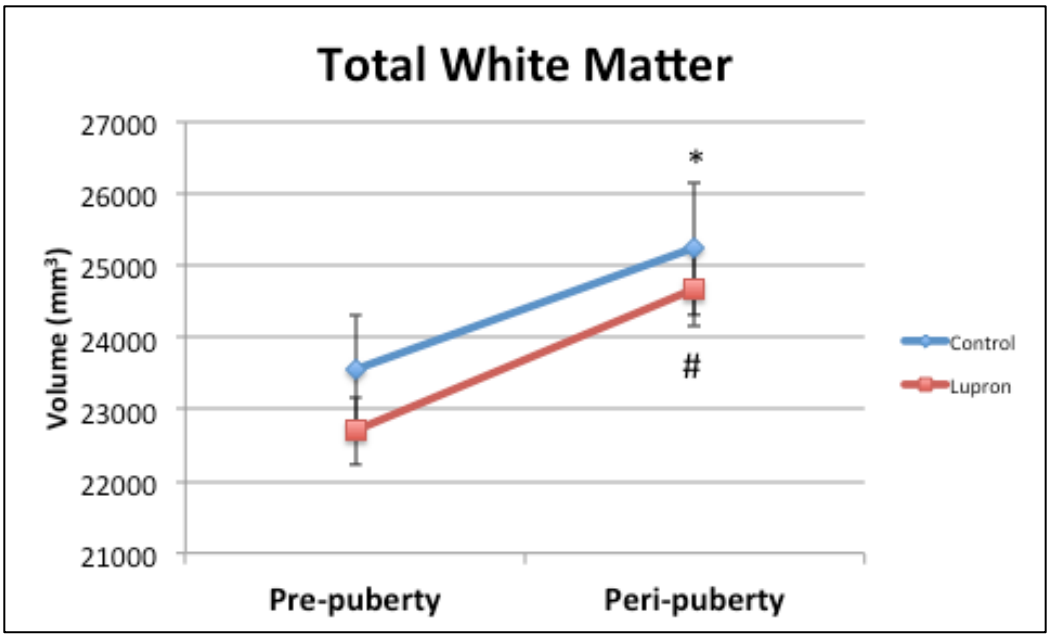


Figure 5. Developmental effects of Lupron treatment on total WM volume. A main effect of age was identified, where total WM volume increased with age ($F_{1,19}$=136.727, $p=4.0084x10^{-10}$, $\eta^2_{partial}$=0.878).

*: $p<0.05$, significantly different from Control Pre-Puberty

#: $p<0.05$, significantly different from Lupron Pre-Puberty

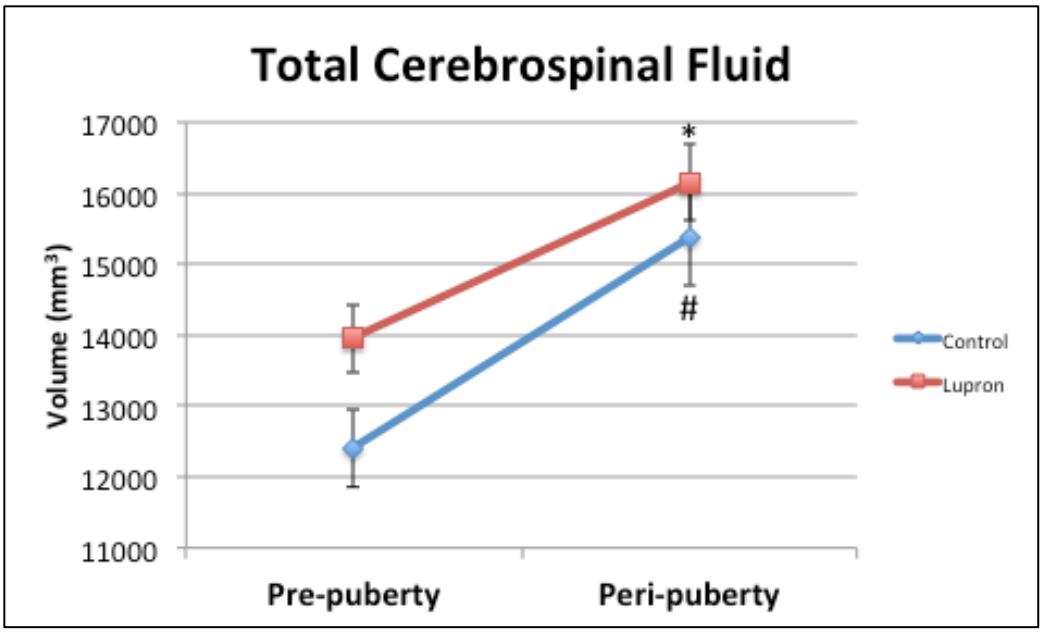


Figure 6. Developmental effects of Lupron treatment on total CSF volume. A main effect of age was identified, where CSF volume increased from pre- to peri-puberty ($F_{1,19}$=31.694, p=00.00001987, $\eta^2_{partial}$=0.198).

*: p<0.05, significantly different from Lupron Pre-puberty

#: p<0.05, significantly different from Control Pre-puberty

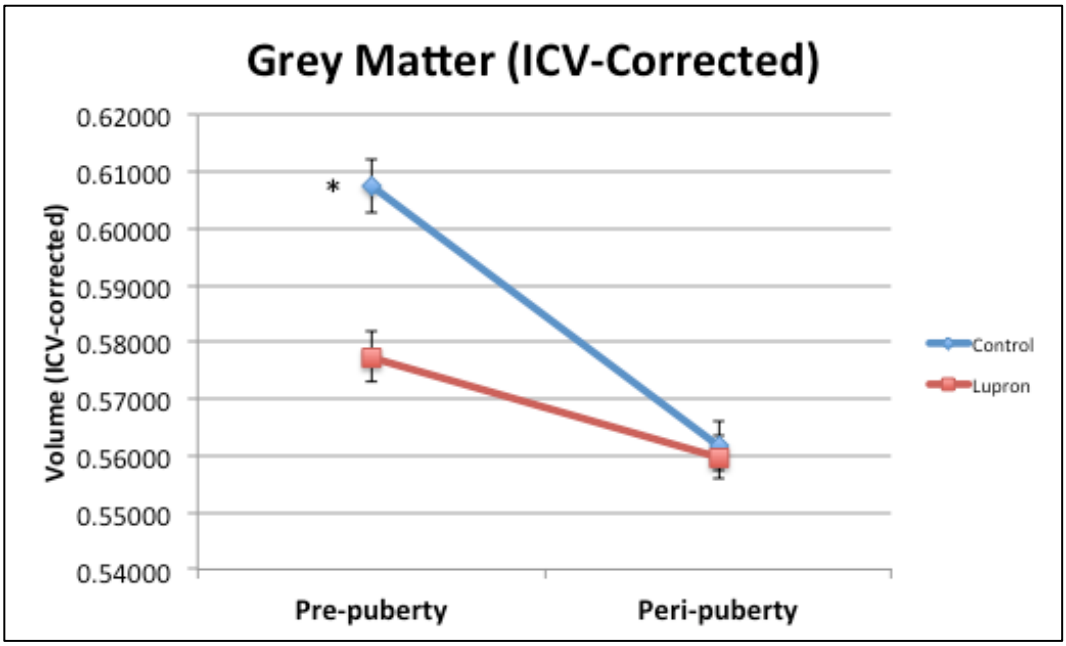


Figure 7. Developmental effects of Lupron treatment on GM volume (as a proportion of total ICV). Main effects of age ($F_{1,19}$=77.647, p=3.875x10^{-8}, $\eta^2_{partial}$=0.803) and Lupron ($F_{1,19}$=9.435, p=0.00628, $\eta^2_{partial}$=0.332) were identified for total GM volume, which decreased with age, and was bigger in control than Lupron-treated subjects. An age by Lupron interaction effect was also found ($F_{1,19}$=15.259, p=0.000949, $\eta^2_{partial}$=0.445), with *post hoc* tests indicating that it was driven by smaller GM volumes in Lupron-treated animals than controls at pre-puberty (p=0.000241), but not at peri-puberty.

*: p<0.05, significantly different from Lupron Pre-puberty.

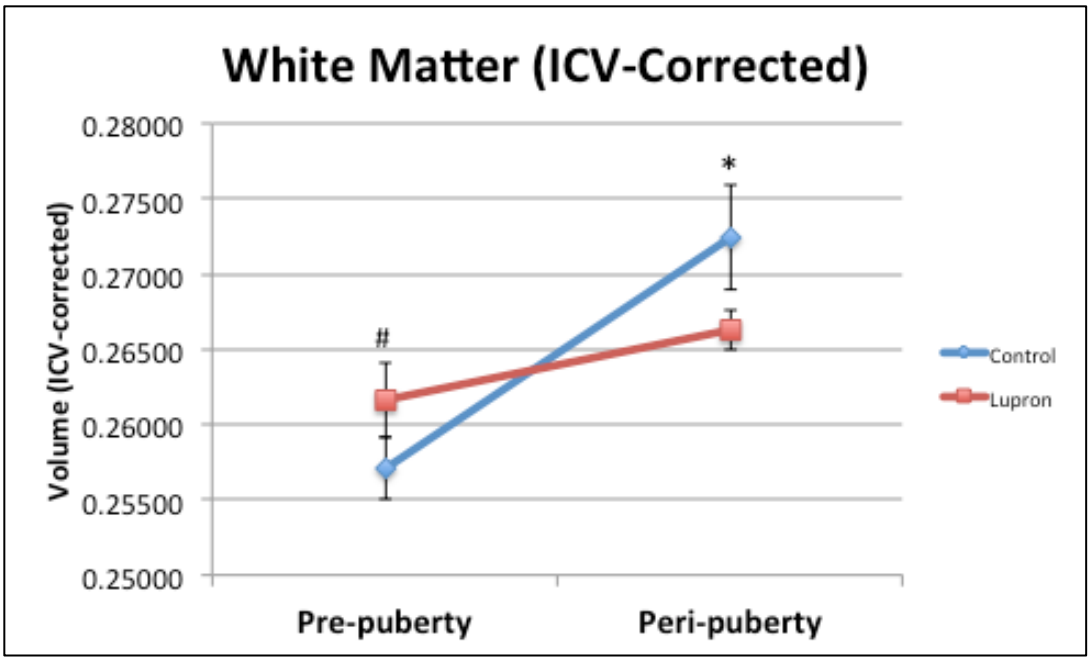


Figure 8. Developmental effects of Lupron treatment on WM volume (as a proportion of total ICV). A main effect of age ($F_{1,19}$=32.284, p=0.0000178, $\eta^2_{partial}$=0.630) was found, with total WM volume increasing from pre- to peri-puberty. A significant age by Lupron interaction effect ($F_{1,19}$=9.246, p=0.00673, $\eta^2_{partial}$=0.327) was identified. *Post hoc* analyses indicated that total WM was significantly greater at peri-puberty than at pre-puberty for both control (p=0.0000240) and Lupron-treated subjects (p=0.046). There was no significant difference in total WM volume between control or Lupron-treated subjects at pre- or peri-puberty.

*: p<0.05, Control Pre-puberty significantly different from Control Peri-puberty

#: p<0.05, Lupron Pre-puberty significantly different from Lupron Peri-puberty

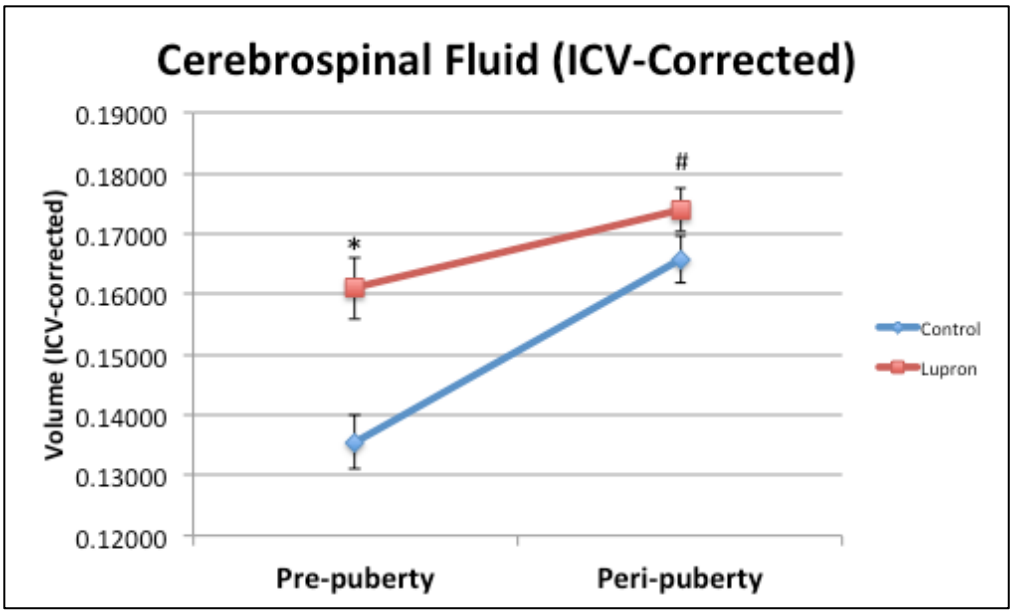


Figure 9. Developmental effects of Lupron on CSF volume (as a proportion of total ICV).

Main effects of age ($F_{1,19}$=23.927, p=0.000101, $\eta^2_{partial}$=0.557) and Lupron treatment ($F_{1,19}$=12.380, p=0.00230, $\eta^2_{partial}$=0.395) were found for total CSF volume, where there was an increase in CSF volume with age, and CSF volume was greater in Lupron-treated subjects compared to controls. Although no significant age by Lupron interaction effect was found, visual inspection of the graph suggests that there was a bigger volume increase in control than Lupron subjects.

*: $p<0.05$, Lupron significantly different from Control for combined ages

#: $p<0.05$, significantly different from Pre-puberty for combined Lupron-treated and Control groups

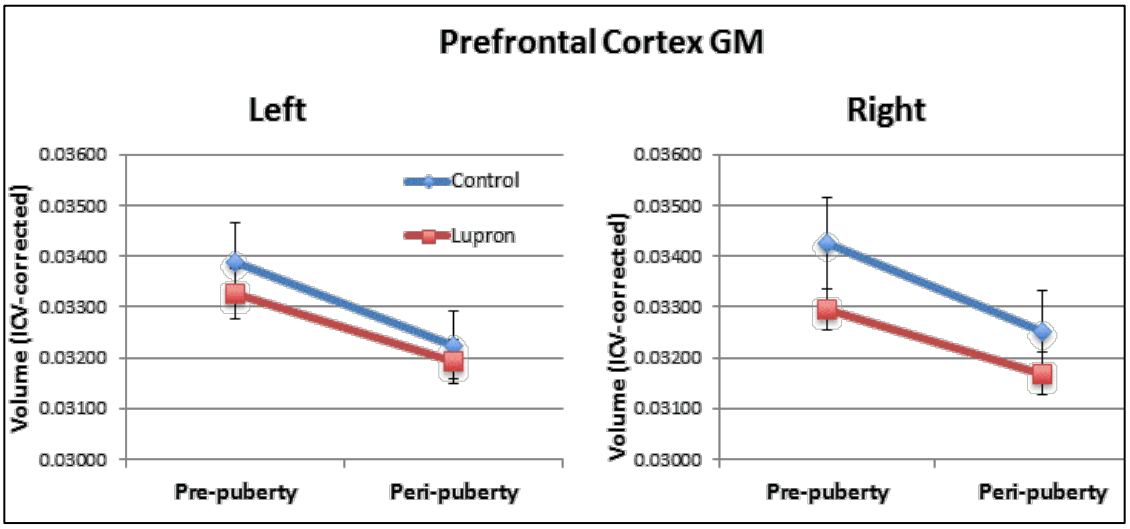


Figure 10. Developmental effects of Lupron treatment on PFC GM volume. A significant main effect of Lupron treatment was identified, with bigger PFC GM volumes in control relative to Lupron-treated subjects ($F_{1,17}$=13.854, p=0.002, $\eta^2_{partial}$=0.449).

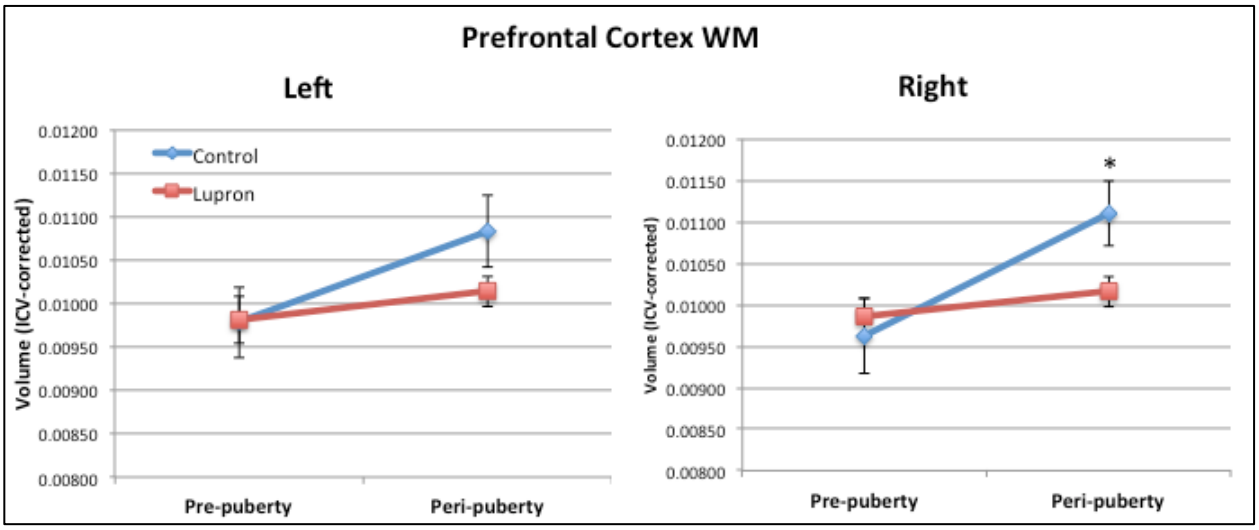


Figure 11. Developmental effects of Lupron treatment on PFC WM volume. A main effect of age was identified ($F_{1,19}$=36.698, p=7.942x10^{-6}, $\eta^2_{partial}$=0.659), with WM volume increasing from pre- to peri-puberty. Interactions effects were also found for age by Lupron treatment ($F_{1,19}$=13.186, p=0.00178, $\eta^2_{partial}$=0.410) and age by Lupron by hemisphere ($F_{1,19}$=5.089, p=0.0361, $\eta^2_{partial}$=0.211). *Post hoc* analyses revealed that control subjects had greater PFC WM volumes relative to Lupron-treated subjects at peri-puberty (p=0.045), but there were no significant differences between the groups at pre-puberty. PFC WM was significantly greater at peri-puberty compared to pre-puberty in control subjects (p=6.440x10^{-6}), but there was no significant difference in PFC WM volume between both ages for Lupron-treated subjects. In addition, PFC WM volume was significantly greater in the right hemisphere of control subjects relative to Lupron-treated subjects at peri-puberty (p=0.023), but not in the left hemisphere.

*: p<0.05, significantly different from Lupron Peri-puberty, Right hemisphere.

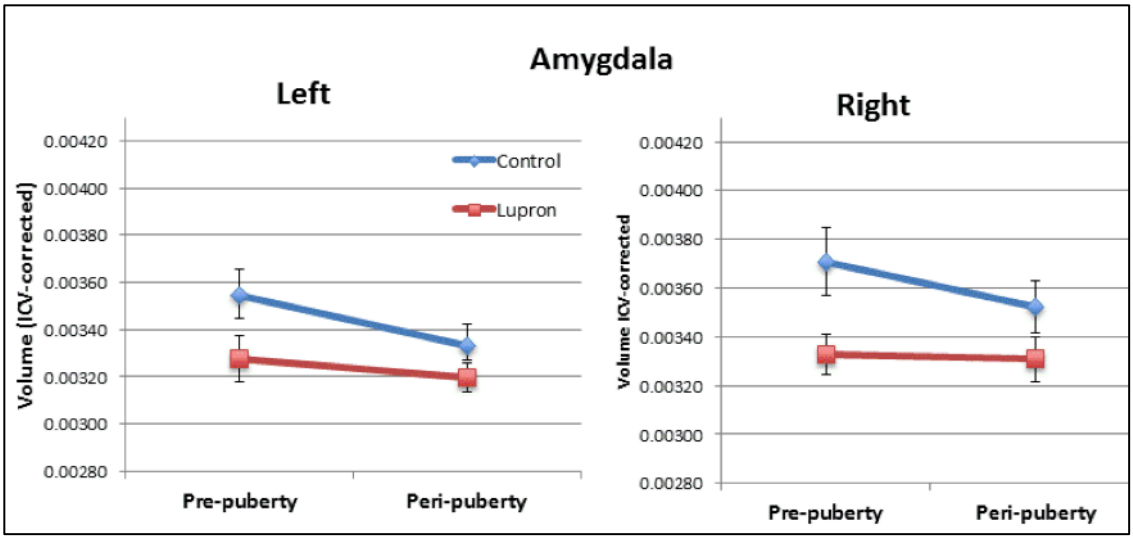


Figure 12. Developmental effects of Lupron treatment on AMYG volume. Main effects of Lupron treatment ($F_{1,19}$=5.418, p=0.031, $\eta^2_{partial}$=0.222) and hemisphere ($F_{1,19}$=7.526, p=0.013, $\eta^2_{partial}$=0.284) were found for amygdala volume, with greater volumes in control relative to Lupron-treated subjects, and in the right hemisphere relative to the left.

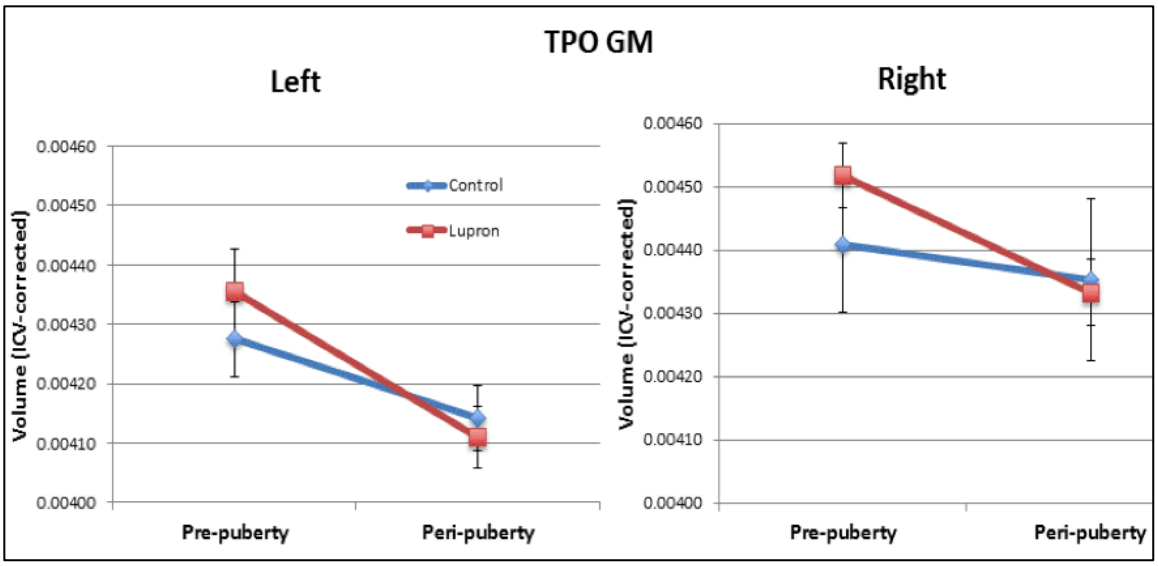


Figure 13. Developmental effects of Lupron treatment on TPO GM volume. Significant main effects of age ($F_{1,19}$=14.321, p=0.001, $\eta^2_{partial}$=0.430) and hemisphere ($F_{1,19}$=16.059, p=0.001, $\eta^2_{partial}$=0.458) were identified, where TPO GM volume decreased with age, and was bigger in the right relative to the left hemisphere.

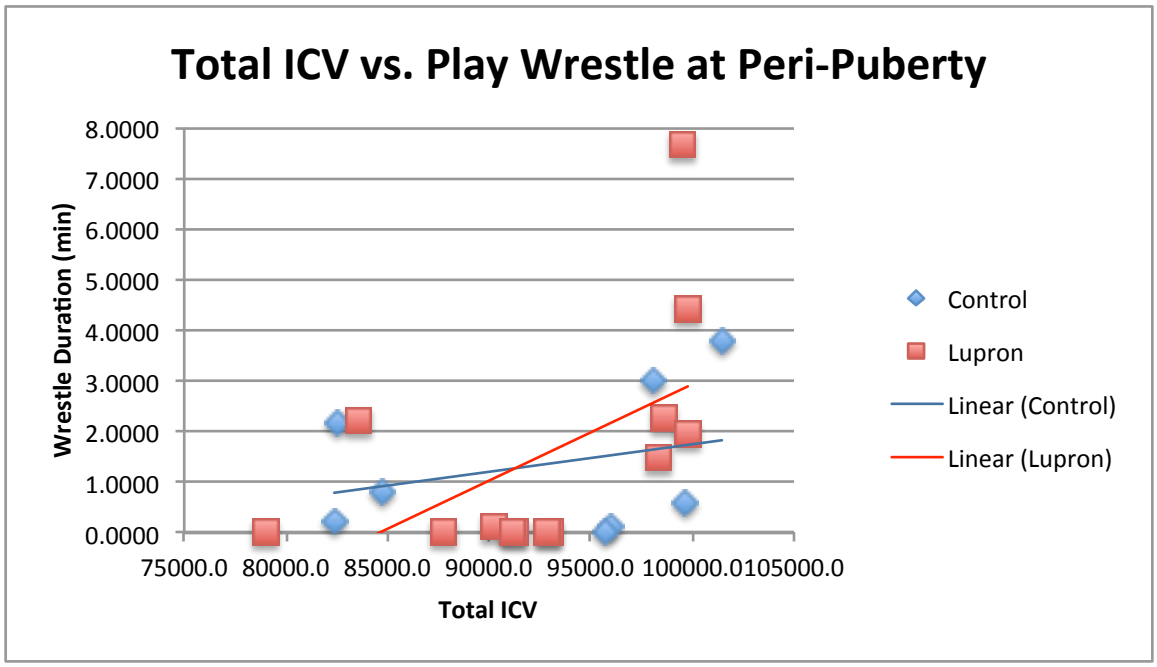


Figure 14. Relationship between total ICV and play wrestle behavior at peri-puberty. Total ICV predicted play wrestle duration at peri-puberty, where greater ICV predicted more wrestle behavior ($p=0.041$, adj. $R^2=0.421$).

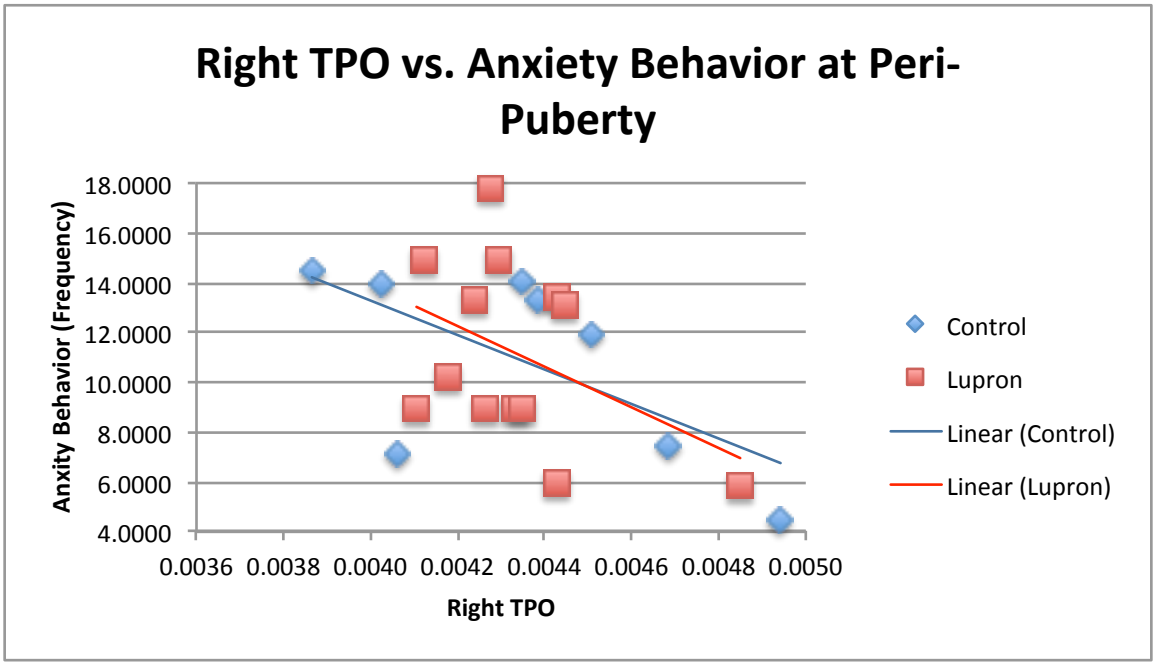


Figure 15. Relationship between right TPO and anxiety behavior at peri-puberty. Right TPO volume predicted anxiety behavior at peri-puberty, where greater ICV predicted less anxiety behavior ($p=0.016$, adj. $R^2=0.232$).

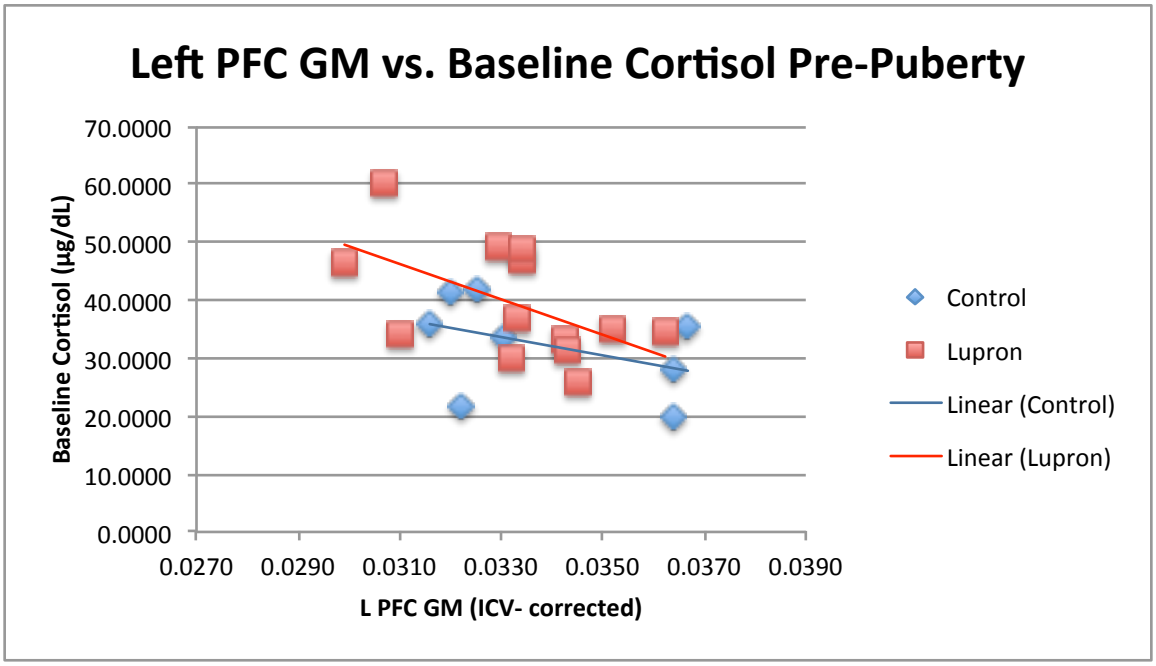


Figure 16. Relationship between left PFC GM and baseline cortisol at pre-puberty. Left PFC GM volume predicted baseline cortisol levels at pre-puberty, where greater PFC volume predicted lower baseline cortisol ($p=0.017$, adj. $R^2=0.228$).

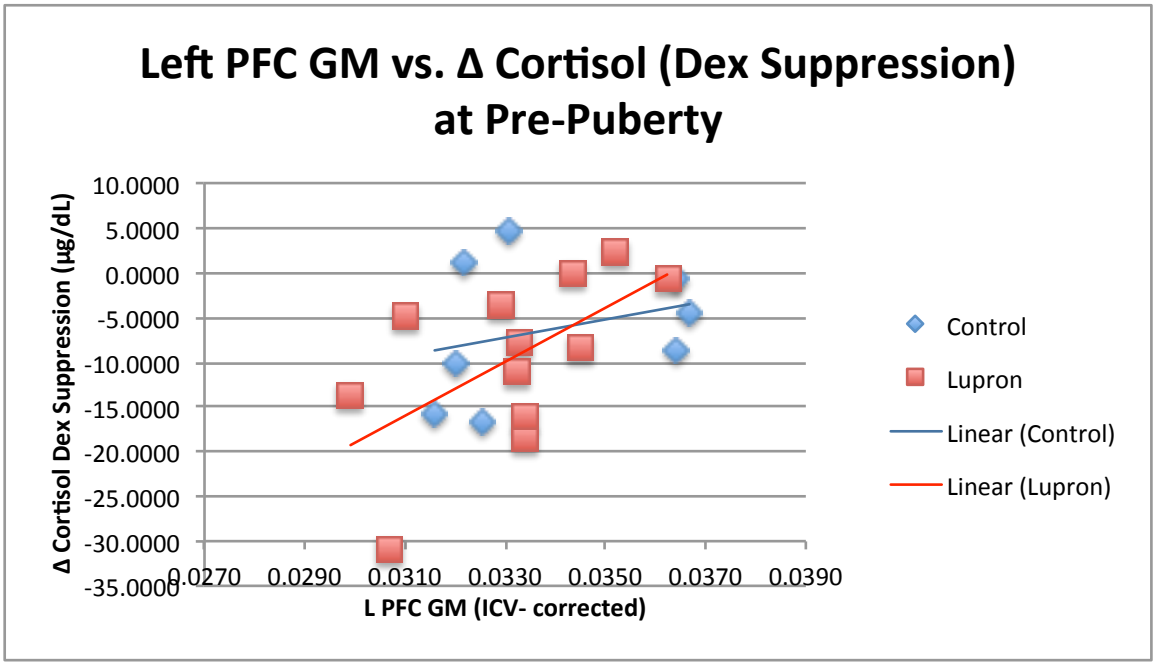


Figure 17. Relationship between left PFC GM and change in cortisol following Dex administration at pre-puberty. Left PFC GM volume predicted the change in cortisol following Dex administration at peri-puberty, where greater PFC GM volume predicted a less negative change in cortisol levels, indicating less suppression of cortisol after Dex administration ($p=0.029$, adj. $R^2=0.195$).

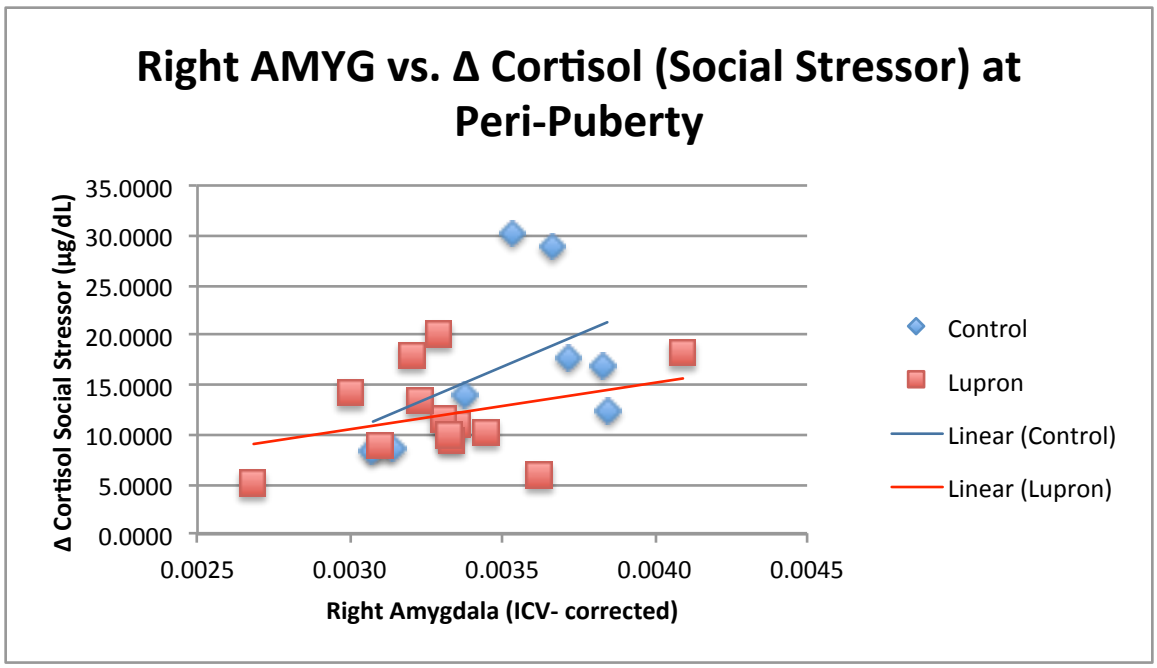


Figure 18. Relationship between right AMYG and change in cortisol following acute stress at peri-puberty. Right AMG volume predicted the change in cortisol from baseline following separation stress, where greater AMYG volume predicted a greater increase in cortisol ($p=0.039$, adj. $R^2=0.163$).

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