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Psychosocial stress modifies the effects of estradiol on female rhesus monkey brain and behavior

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Psychosocial stress modifies the effects of estradiol on female rhesus monkey brain and behavior

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An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Neuroscience

2014

Abstract

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By Katherine Reding

Exposure to stressors can be a precipitating factor in the development of psychopathology. Women are more likely to develop stress-related disorders than men; however, the mechanisms of this increased susceptibility are not understood. One hypothesis for this gender bias in disease prevalence is that exposure to chronic stress alters the beneficial effects of the ovarian hormone estradiol (E2) on behavior, thus increasing incidence of behavioral disorders. Few studies have examined the effects of chronic stressor exposure on E2's actions in the female brain. To determine whether stress alters E2's effect on both behavior and brain structure and function, I used the well-defined and highly translational female rhesus monkey model of social subordination to examine the consequences of chronic stress on the action of E2 on brain and behavior. I tested the hypothesis that E2 would dose-dependently increase socio-sexual behavior in dominant but not subordinate females. In vivo neuroimaging techniques including structural magnetic resonance imaging (MRI) and resting-state functional MRI were used to identify changes in structure and functional connectivity (FC) of cortico-limbic brain region in ovariectomized females both with and without short term E2 replacement. Evidence from these studies suggests that social subordination attenuates E2's activational effects on sociosexual behavior, which are not overcome with higher dose of E2. The neuroimaging data demonstrate dichotomous effects of E2 on brain structure, such that cingulate gray matter volumes decreased in subordinate and increased in dominant females. Additionally, subordinate females showed decreased negative FC between the amygdala and ventral pallidum and increased positive FC between the anterior cingulate cortex and the supplemental motor area, posterior cingulate cortex, and the superior temporal sulcus. Furthermore, FC between the amygdala and the medial prefrontal cortex increased between in subordinate females and decreased during the E2 condition, although no interaction effects were found. Together, these data represent the first studies identifying the effects of chronic stress, imposed by social subordination, and E2 on the adult female primate brain and behavior. The experiments serve to fill critical gaps in our knowledge of how adaptation to psychosocial stress can increase susceptibility to behavioral disorders in women.

Psychosocial stress modifies the effects of estradiol on female rhesus monkeys brain and behavior

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Acknowledgements

Dr. Mark Wilson and Dr. Mar Sanchez have been a source of unvarying support and guidance throughout my graduate career. I am ever grateful for their support, both in navigating me through the past five years of my graduate career and encouraging me to do so with rigor. Dr. Helen Mayberg, Dr. James Rilling, Dr. Donna Toufexis, and Dr. Kim Wallen have all helped me to be the scientist that I am today, and their guidance has steadily improved and broadened my research horizons. I also want to thank Dr. Damien Fair and Dr. Martin Styner and their research teams for the technical neuroimaging training they have provided me, as well as their constant availability to answer even my smallest questions.

The non-human primate studies included in this dissertation were conducted with the expert technical assistance of Jennifer Whitley, Natalie Brutto, Angela Tripp, Shannon Bounar, Jonathon Lowe, Casie Lyon, and Erin O'Sheil. In addition, for their hard work, support (both moral and technical), and teamwork needed to succeed both non-human primate studies and graduate life, I thank Dr. Vasiliki Michopolous, Dr. Brittany Howell, Dr. Carla Moore, Dr. Christa Payne, Jodi Godfrey, Jordan Kohn, Amanda Mummert, and Dora Guzman. These studies would not have been possible without the dedication of the animal husbandry and veterinarian staff at the Yerkes National Primate Research Center. A special thanks to Dr. Sarah Pruett and the Biomarkers Core at Yerkes National Primate Research for both her mentorship and assistance in conducting the hormonal assays included in this dissertation.

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Chapter 1: Introduction

Exposure to stressors can be a precipitating factor in the development of psychopathology. The accumulation of stressful events across the lifespan and exposure to chronic psychosocial stressors can render an individual more susceptible to the development of mood and anxiety disorders (Hammen, 2005b). Women are two to three times as likely to develop stress-related psychopathology disorders than men and this gender difference emerges around puberty and is maintained across the lifespan (Seeman, 1997; Kessler, 2003). Importantly, history of exposure to stressful life events is the best predictor for development of depression among women (Kendler et al., 1993) as well as other forms of anxiety disorders (Hunter and McEwen, 2013). The biological mechanisms of this susceptibility, however, are far from being understood. Evidence suggests that the ovarian hormone estradiol (E2) may have positive effects on mood, cognition, and neurobiology (McEwen and Alves, 1999). One hypothesis for this gender bias in disease prevalence is that exposure to chronic stress inhibits the beneficial effects of E2 on behavior (White and Uphouse, 2004; Walf and Frye, 2005a), thus increasing incidence of behavioral disorders. The goal of this dissertation was to examine the hypothesis that stressor exposure alters the neurobehavioral effects of E2. The studies provide evidence that adverse social experience attenuates the E2's effects on both brain (structure and function) and prosocial behaviors.

This introduction provides a comprehensive review of the literature that overall supports the hypothesis that chronic stressor exposure modifies the effect of E2 on brain structure and function, thus affecting the behavioral health of women. The chapter includes a review of the neurobehavioral actions and distribution of estrogen and stress hormone

receptors, including GC and corticotropin releasing factor (CRF) receptors, in cortico-limbic regions of the brain. Furthermore, neurobiological and behavioral effects of E2 are reviewed, with specific attention focused on how chronic stress may modify these activational effects E2. Data are derived from a variety of clinical and preclinical models, including intact and ovariectomized rodents and non-human primates, as well as pre and postmenopausal women, with and without E2 replacement therapy (ERT). Behavioral data suggest that the activational (e.g. sexual and prosocial) effects of E2 may be inhibited by exposure to chronic stressors while neurobiological analyses, including structural and functional neuroimaging data, suggest that chronic stress modifies, E2's effects on neuroplasticity in cortico-limbic circuits, reflecting neural adaptations to these adverse social experiences. My overriding hypothesis is that chronic social stress attenuates the neurobiological effects of E2 on brain structure and function, resulting in alterations within cortico-limbic circuits and differences in socioemotional behavior.

1.1 Stress and gonadal hormone receptors in the brain

Even before steroid hormone receptors were identified, data pointed to the direct role of both GCs and E2 on central nervous system tissues (Eisenfeld and Axelrod, 1965; McEwen et al., 1969). Initially, these receptors were thought to act only in the nucleus of the cell (McEwen et al., 1975). Over the past 50 years, data have shown that E2 and GCs can act at both the genomic level to activate or repress gene transcription or at the non-genomic level to alter electrochemical properties of both neurons and glia, and that these actions can take place either via binding to membrane, cytosolic, or nuclear receptors

(McEwen, 1991). GCs and E2 are the main outcome hormones and key regulators of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary gonadal (HPG) axes (discussed in section 1.2), respectively, but they also exert effects on extrahypothalamic, corticolimbic, brain regions that both modulate these axes and affect behavior and neuronal morphology. However, it is apparent that CGs are not the sole mediators of the physiological stress response. The identification of CRF (Vale et al., 1981) and mapping its expression throughout the brain has highlighted the ubiquitous role of this stress neuropeptide not only modulating both the HPA and HPG axes, but influencing behavior (Owens and Nemeroff, 1991).

1.1.1. Estrogen Receptors

Estrogens, including E2, regulate gene transcription by binding to cytoplasmic estrogen receptors (ERs) to form receptor-ligand complexes which then can bind to DNA via estrogen response elements (EREs) or activating protein (AP1) elements to activate or repress gene expression (Lee and McEwen, 2001). In 1996 the ER-beta (ß) E2 receptor was discovered (Kuiper et al., 1996) and found to have a similar binding affinity for E2 (Kd=.4nM) compared to the originally discovered ER-alpha (α) (Kd=.1nM) (Kuiper et al., 1997). The ability of E2 to increase or decrease gene transcription depends on multiple factors, including the association with promoter elements, concentration of ligand, and ratio of ERα to ERß in the same cell. Both ER subtypes are generally thought to increase gene transcription when bound to EREs, but when bound to AP1 elements ERα increases while ERß inhibits gene transcription (Paech et al., 1997). Furthermore, when E2 levels are high, both receptors increase gene transcription at EREs, while at low

E2 concentrations, ERß can act as an inhibitor of ERα, suggesting that a cell's response to E2 depends on the concentration of E2 and ratios of ERs (Hall and McDonnell, 1999). More recently, additional isoforms of ERß have been discovered, some of which regulate transcription independent of E2 and bind with ERα receptors to inhibit gene transcription (Moore et al., 1998; Poola et al., 2005). Overall, the effects of ERα appear to be modified by the effects of ERß.

E2 can also bind to non-nuclear receptors to cause fast, non-genomic, effects or genomic effects mediated through second messenger systems either on the cell membrane or in the cytosol (Bjornstrom and Sjoberg, 2005). These effects are most likely localized in the same neurons that express ERs, as membrane receptors are derived from ERα and ERß mRNA with subsequent post-translational modifications to localize the receptors to the membrane (Razandi et al., 1999). Often these non-nuclear ERs interact directly or indirectly to G-protein coupled receptors (GPCRs) (Kelly and Wagner, 1999). However, E2 can also act at GPCRs independent of ERs, such as at GPR30 located in the endoplasmic reticulum (Revankar et al., 2005). Through these mechanisms, E2 can induce rapid changes in cell signaling by altering calcium and potassium channels (Kelly and Levin, 2001) or can regulate gene transcription through cAMP response elements (CREs) or serum response elements (SREs) (McEwen, 1991).

ERα and ERß are expressed throughout the hypothalamus, amygdala, hippocampus, and cortex in rats (Shughrue et al., 1997; Shughrue et al., 1998a; Shughrue and Merchenthaler, 2000), rhesus monkeys (Pau et al., 1998; Blurton-Jones et al., 1999; Perez

et al., 2004; Bao et al., 2006), pigtail macaques (Gundlah et al., 2000), cynomolgus monkeys (Register et al., 1998; Osterlund et al., 2000b), and humans (Register et al., 1998; Osterlund et al., 2000a; Osterlund et al., 2000b). In the amygdala, rodents and humans show expression of both ERα and ERß within the medial amygdala (MeA), while rhesus monkeys show either no ER binding (Perez et al., 2004) or only ERα (Blurton-Jones et al., 1999). Additionally, in the basolateral nucleus of the amygdala (BLA), rats show binding of both ERα and ERß (Shughrue et al., 1997; Shughrue et al., 1998b) while data from humans and cynomolgus monkeys indicate ERα is more prominently expressed compared to ERß (Osterlund et al., 2000b; Osterlund and Hurd, 2001). Data from rhesus monkeys suggests no ER expression in the BLA (Perez et al., 2004), which is difficult to understand given the data from the closely related cynomolgus macaque. Furthermore, the bed nucleus of the stria terminalis (BNST), which is considered the extended amygdala, expresses both ERα and ERß in rodents, while only ERα has been see in macaque monkeys (Blurton-Jones et al., 1999; Gundlah et al., 2000). However, no ER expression is reported in the BNST of humans (Osterlund et al., 2000a; Osterlund et al., 2000b). These species differences need further examination, as they could be due in part to different methodologies used to assay ERs, which included mRNA in situ hybridization using both human (Register et al., 1998) and monkey ER cDNA (Pau et al., 1998; Gundlah et al., 2000) in addition to immunohistochemistry (Blurton-Jones et al., 1999; Perez et al., 2004).

Data also show a lack of a consistent expression pattern in the neocortex across species, specifically in the prefrontal cortex (PFC). As detailed below, all studies that examined

cortical ERs showed expression of ERα and ERß (Shughrue et al., 1997; Shughrue et al., 1998a; Shughrue and Merchenthaler, 2000) (Pau et al., 1998; Blurton-Jones et al., 1999; Perez et al., 2004; Bao et al., 2006) (Register et al., 1998; Osterlund et al., 2000b), except one that only showed cortical expression or ERß (Shughrue et al., 1998a). In humans, ER expression has been described in the temporal cortex, with ERα in layer V and ERß localized to layer V/VI (Osterlund and Hurd, 2001), as well as ERα expression throughout all layers (I-VI) in the dorsolateral (dl)PFC (Montague et al., 2008). In rhesus monkeys, data suggest the frontal cortex expressed ERß only, while the PFC expresses both ERs (Pau et al., 1998). Finally, in rodents the cingulate and prelimbic PFC show expression of ERα (Montague et al., 2008). While the data across species show some differences in local ER expression in cortical areas, it is clear that E2 can target the PFC given the localization of ERs in this brain region.

1.1.2 Glucocorticoid receptors

Two types of GC receptors are present in brain tissue, the type-I mineralocorticoid receptor (MR) and the type-II GC receptor (GR), with distinct receptor expression and binding affinity to GCs, which are produced by the adrenal gland, primarily corticosterone in rodents and cortisol in primates (Dekloet et al., 1987). MRs have 5 to 10 fold higher affinity for GC, compared to GRs (Reul and Dekloet, 1985). This difference in affinity causes MRs to be saturated even at very low concentrations of GCs, while GR availability can fluctuate due to varying GC levels across the circadian rhythm and stress-induced changes in GC secretion (Dekloet et al., 1987). When GCs binds to either MR or GR, they form a ligand receptor complex that associates with either positive or negative

GC response elements (GREs) that act as transcription factor to regulate gene expression, activating or repressing it (De Kloet et al., 1998). GCs, similar to E2, can also act through rapid, non-genomic, mechanisms, and elicit effects through binding directly or indirectly with GPCRs (Tasker et al., 2006).

Distribution of MRs and GRs in the brain varies across species. In rodents, MRs and GRs are located in the septum, hippocampus, and nucleus tractus solitarii (NTS) while only GRs are located in the cerebral cortex, amygdala, basal ganglia, and hypothalamus – including the ventromedial hypothalamus (VMH), arcuate, and posterior ventral regions (Reul and Dekloet, 1986; Fuxe et al., 1987). Whereas rodents show high GR and MR expression in the hippocampus and limited GR expression in the neocortex, rhesus monkeys show weak labeling of GRs in the hippocampus and increased GR and MR expression in the PFC suggesting significant species differences in the effects of GCs (Sánchez et al., 2000). Rhesus monkeys also show low expression of GRs in the central amygdala (CeA) and high expression of GRMRs in the hypothalamus, pituitary, entorhinal cortex, and cerebellum (Sánchez et al., 2000). In humans, GR and MR expression are similar to each other in most brain areas tested including the amygdala, with higher abundance in the cerebral cortex (Cao-Lei et al., 2013). In the hippocampus, similar to rhesus monkeys, MRs were more abundant than GRs, while in the pituitary GRs were more abundant than MRs (Cao-Lei et al., 2013).

The classic role of GC and MRs and GRs is on basal functioning and negative feedback inhibition of the HPA axis (De Kloet, 2004), and life history of exposure to stressors can

alter response to GCs (Joels et al., 2009). Although the mechanism are not completely understood, the balance of both genomic and non-genomic responses of GRs, MRs, and the myriad other players in the stress-response (e.g. CRF, epinephrine, norepinephrine) within corticolimbic regions including the hippocampus, amygdala, and PFC, play a key role in the response to stress, either adaptive or maladaptive (Groeneweg et al., 2011). The overlapping distribution of ERs within these same regions, and potential for interactions among receptors provide a mechanism that would support the hypothesis that stress signals can disrupt E2 signaling and action.

1.1.3 Corticotropin releasing factor receptors

As mentioned above, CRF is a critical stress neuropeptide expressed throughout the brain. There are two of CRF receptors subtypes expressed in brain tissue, type-1 (CRFR1) and type-2 (CRFR2). These are G-protein coupled membrane receptors whose effects are mediated through their association with GPCRs and the cAMP and protein kinase A pathways (Perrin and Vale, 1999), although genomic effects have also been reported through activation of the mitogen-activated protein kinase (MAPK), as well as extracellular signal-related kinase (ERK) pathways (Refojo et al., 2005). CRFR1 receptors have a 10-fold higher affinity for CRF compared to CRFR2 receptors and, in addition to CRF, they also bind other CRF-like neuropeptides in the Urocortin family, including Urocortin 1, while Urocortin 2 and Urocortin 3 have higher affinity for CRFR2 (Bale and Vale, 2004).

In rodents, CRF1 receptors have a higher distribution within the brain compared to CRF2 (Bale and Vale, 2004), although the distribution vary by species. CRFR1 receptors are widely distributed throughout the brain in rodents, and include expression in cortex (frontal, cingulate, and frontoparietal cortices), hippocampus, amygdala (MeA, BLA), BNST, caudate, nucleus accumbens, hypothalamus -including the paraventricular nucleus (PVN), dorsal medial hypothalamus, and the arcuate nucleus (Aguilera et al., 2004). Regional overlap with CRFR2 receptors is seen in the BNST, MeA, and hippocampus (Aguilera et al., 2004), while no CRFR2 receptors were seen in the cortex or pituitary (Lovenberg et al., 1995). In rhesus monkeys, the distribution of CRFR1 and CRFR2 is show higher CRFR1 mRNA in the pituitary, amygdala, cerebellum, and cortex (frontal, cingulate, insula) compared to CRFR2 (Sanchez et al., 1999). In the hippocampus, CRFR1 receptor binding was seen in the dentate gyrus, while CRF2 showed receptor binding in the subiculum and CA1, and both were found in the entorhinal cortex, and CA4 region (Sanchez et al., 1999). Within the amygdala, CRFR1 predominated in the basal accessory, lateral, and medial regions, with less expression in the CeA, while CRFR2 was most predominant in the CeA compared to the basal accessory and medial nuclei which showed only moderate expression (Sanchez et al., 1999). CRFR2, but not CRFR1, was located in the BNST and the inverse was seen in the NTS (Sanchez et al., 1999). In the human, CFRR1 and CRFR2 were also both expressed in the pituitary, hippocampus, amygdala, and thalamus (Hiroi et al., 2001). Data show CRF receptors in the cortex as well, although differences in total cortical distribution of CRFR1 versus CRFR2 have not been examined (Nemeroff et al., 1988; Vita et al., 1993; Hucks et al., 1997; Merali et al., 2004). Because the distribution of CRFR1 is located throughout

similar cortico-limbic regions where ERs and GRs are expressed, and CRF is an important stress neuropeptide implicated in a number of stress-related disorders (Ehlert et al., 2001), it could be an important biological signal implicated in the disruption of the neurobehavioral effects of E2 caused by stress, in addition to the role of GCs.

1.1.4 Summary

Distribution of the ERs, GR/MR, and CRF type 1 receptors overlap in corticolimbic regions that are important for the regulation of a socioemotional behaviors. However, to date, the co-localization of these receptors within these regions has not been thoroughly examined (Handa and Weiser, 2014). Variability in the distribution of these receptors, combined with their potential influence on one another could produce myriad combinations of effects within these regions. Furthermore, as MR versus GRs and ERα versus ERß have different, and at times opposing effects, the ratio of receptor co-expression can also impact interactions between stress-hormones and E2. For example E2 acts to increase ERα expression in the VMH (Malikov and Madeira, 2013) while decreasing ERß expression in the posterior hypothalamus and the CeA (Yamaguchi and Yuri, 2014), mediated by epigenetic regulation (Imamura, 2011). To give yet another example of the complex interactions between all these systems, dexamethasone, a synthetic GC, can increase expression of ERß (Suzuki and Handa, 2004), and ERß can up regulate CRF expression (Miller et al., 2004). Therefore, altogether, the levels of expression and actions of these receptors are not only regulated in a complex way, but they are a dynamic process, providing a strong rationale for investigating the interactions

between stress and E2 within corticolimbic regions, including the hippocampus, amygdala, and the PFC.

1.2 Estradiol and stress effects on neuroendocrine regulation

Chronic stress is associated with repeated activation of stress-response systems, which lead to up regulation of a number of stress neuropeptides, including CRF and vasopressin (AVP), as well as a chronic proinflammatory condition (Ulrich-Lai and Herman, 2009; Hansel et al., 2010). Therefore high peripheral concentrations of glucocorticoids are most often used as surrogate markers of acute or sustained stress. However, the long-term effects of repeated/sustained stress can be complex, and sometimes down regulated HPA axis activity has been reported (Fries et al., 2005). Thus, in the literature there is evidence that stress-induced psychopathology is associated with both elevated basal cortisol (e.g. major depressive disorder) or reduced basal cortisol levels (e.g. post traumatic stress disorder, PTSD) (Ehlert et al., 2001). In attempting to understand how chronic stressor exposure may modify E2 actions on behavior, it is critical to appreciate that E2 modifies HPA axis function and, similarly, that GCs and CRF can alter the HPG axis in return. Given that females have a higher prevalence of stress-induced disorders than males, the cross talk between these two systems requires examination as a possible mediator of sexually dimorphic disease risk. As described below, the effect of E2 on the HPA axis has been well described, and appears to be mediated differentially by ER subtypes (e.g., (Handa and Weiser, 2014)). While an attenuation of the HPG axis activity is a well accepted consequence of chronic stressor exposure, particularly in females (Berga and

Naftolin, 2012), the underlying mechanisms are not well understood (Kaplan and Manuck, 2004).

1.2.1 Stress signals modify the hypothalamic-pituitary-gonadal axis

The HPG axis is three-tiered endocrine pathway that regulates the production of gonadal hormones and fertility. The hypothalamus releases gonadotropin-releasing hormone (GnRH) into the median eminence to act on the anterior pituitary to release both luteinizing hormone (LH) and follicle stimulating hormone (FSH) into peripheral circulation which stimulates follicular growth and ovarian steroid (e.g. E2) synthesis and release (Vadakkadath Meethal and Atwood, 2005). GnRH neurons are dispersed throughout the hypothalamus, with the highest concentration in the rostral preoptic area (rPOA), and they receive inhibitory and excitatory input via diverse neurochemical messengers (e.g. kisspeptin, serotonin, and norepinephrine) (Campbell, 2007; Terasawa et al., 2010).

Ovarian hormonal and ovulatory cycles are markedly different across species. Intact female rodents typically have a 4 - 5 day estrous cycle, with low levels of E2 and progesterone (P4) during diestrous 1, rising medium levels of E2 and P4 during diestrous 2, and peak levels of both E2 (morning) and P4 (afternoon) in proestrous, and a sharp declining in E2 and P4 during estrous (Becker et al., 2005). Rhesus and cynomologous monkey show roughly a 28 - 32 day ovarian cycle (Weinbauer et al., 2008), similar to that seen in humans (Thorneycroft et al., 1971). Rhesus monkeys, but not humans or cynomologous monkeys, show a season distribution of ovulatory cycles, limited to the

fall and winter months from mid-September to mid-April (Walker et al., 1984). Overall, the primate ovarian cycle can also be divided into five stages; early follicular, mid/late follicular, ovulation, early luteal, and mid/late luteal. Both E2 and P4 are at their nadir during the early follicular phase, while E2 levels rise during the mid/late follicular period unopposed by P4, and reach their peak at ovulation, causing a surge in FSH and LH release that, in turn, causes follicle rupture and egg release (Buffet and Bouchard, 2001). After ovulation, E2 levels drop precipitously to early follicular levels, but then both E2 and P4 are produced by the corpus luteum and begin to rise during the early luteal stage (Buffet and Bouchard, 2001). During the mid/late luteal phase, P4 reaches its peak while E2 reaches its second peak, although E2 levels remain lower than during the ovulatory peak (Buffet and Bouchard, 2001).

Stress attenuates HPG activity by suppressing hypothalamic GnRH, and/or pituitary LH/FSH pulsatile release, as well as E2 synthesis in the ovary (Rivier and Rivest, 1991; Sapolsky et al., 2000). The mechanisms of this attenuation, and the specific effects of either CRF or GSs are complex and are not fully understood (Berga and Loucks, 2007). Exogenous treatment with CRF inhibits GnRH pulse frequency in rats (Petraglia et al., 1987). In intact ewes CRF inhibits the preovulatory LH surge (Polkowska and Przekop, 1997), while in ovariectomized ewes GCs also decreases LH pulse amplitude from the pituitary secondary to reduced sensitivity to hypothalamic GnRH (Breen and Karsch, 2004). However, additional studies suggest that GC suppression of LH at the level of the pituitary is dependent on E2 (Pierce et al., 2009). In rhesus monkeys, CRF inhibits LH (Olster and Ferin, 1987), while the synthetic GC, dexamethasone, is unable to decrease

ovarian functioning following 24 days of adrenal suppression (Lovejoy and Wallen, 1990). In ovariectomized rhesus monkeys, psychosocial stress imposed by social subordination potentiates E2 negative feedback inhibition of LH secretion (Michopoulos et al., 2009), although it has no effect on positive feedback (Michopoulos & Wilson, unpublished data). Together, these data indicate that stressor exposure, possibly via CRF, may act both centrally on GnRH neurons and at the pituitary to suppress ovarian function.

One of the main adverse consequences of chronic stress on ovarian function, both in non-human primates and humans, is infertility, expressed as luteal phase insufficiency and anovulation (Berga et al., 1997; Kaplan and Manuck, 2004; Berga and Loucks, 2007). Indeed, stress-induced hypothalamic anovulation is a significant contributor to infertility in women (Berga and Loucks, 2005). In macaque monkeys, exposure to social subordination, interpreted as a chronic psychosocial stressor, also has a graded effect on ovarian cycle parameters, with lower social rank (higher stress) associated with increased cycle length as well as a higher incidence of anovulation (Pope et al., 1986; Kaplan et al., 2010). As a consequence, most low ranking females show reduced circulating ovarian hormones including E2 and P4 but maintain regular ovarian cycling in both cynomolgus (Kaplan et al., 2010) and rhesus monkeys (Pope et al., 1986), while approximately 23% of subordinate cynomolgus females show luteal insufficiency and another 23% are anovulatory (Kaplan and Manuck, 2008). Furthermore, cynomolgus monkeys show a range of sensitivity to ovarian cycle disruption following stressor exposure (Bethea et al., 2008), suggesting genetic and or experiential factors may be important.

1.2.2 Estradiol modifies hypothalamic-pituitary-adrenal axis activity

Following exposure to an acute stressor, either real or perceived, the HPA axis gets activated, resulting in the secretion of GCs from the adrenal cortex, which acts on both peripheral and central tissues. The HPA axis stress response is initiated by activation of the release of CRF from hypothalamic paraventricular nucleus (PVN), as well as additional secretagogues such as arginine vasopressin (AVP), into the hypothalamo-hypophysial portal vasculature (Dallman et al., 1987). CRF then induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the bloodstream which acts on the adrenal cortex to activate GC synthesis and release (Dallman et al., 1987). In parallel, stressors also activate the sympathetic adrenal medullary (SAM) axis, to stimulate the release of both epinephrine and norepinephrine from the adrenal medulla (Ulrich-Lai and Herman, 2009). Both basal and stress-induced activation of the HPA axis is regulated, in part, but the many inputs that converge on the PVN. Corticolimbic regions including the PFC, amygdala, and hippocampus send second-order connections through the BNST and hypothalamic nuclei to the PVN to regulate HPA axis activity (Herman, 2005). These regulatory pathways converge on the PVN, to either increase or inhibit release of CRF. Circadian fluctuations in basal HPA axis function are controlled by a "biological clock" located in the suprachiasmatic nucleus of the hypothalamus, and follow a circadian rhythms regulated by light/dark, sleep/wake and eating patterns (Casicio et al., 1987). Other factors that regulate HPA activity include internal shifts in homeostasis, such as changes in blood oxygenation and pH (McEwen and Gianaros,

2011b), which activate PVN via brain stem inputs from the NTS (Swanson and Sawchenko, 1983).

GCs are highly catabolic hormones that have multiple actions during the stress response including mobilization of energy resources, increase of cardiovascular tone and vasoconstriction, reduction of inflammation and reproductive function, and, through their negative feedback actions on the HPA axis, suppressing the release of CRF and pituitary ACTH to shutdown the HPA axis activation (Sapolsky et al., 2000). To avoid the deleterious effects of chronic exposure to elevated levels of GCs and maintain homeostasis following exposure to a stressor the HPA-axis is shutdown, in part, by GC negative feedback, mediated mainly by GRs although MRs are also involved (Dallman et al., 1994). In addition to the main negative feedback of GCs via binding to GRs at the level of the anterior pituitary and PVN, GC negative feedback also involves inhibitory monosynaptic input from surrounding hypothalamic nuclei and the posterior BNST and inhibitory polysynaptic input from cortico-limbic regions including the rodent PFC (specifically the prelimbic area), lateral septum, and the hippocampal subiculum (Herman et al., 2003; Herman et al., 2005). Within the anterior pituitary, activation of GRs inhibits ACTH release by acting on negative GREs to reduced production of ACTH precursor pro-opiomelanocortin (POMC), which is typically stimulated by CRF (Dostert and Heinzel, 2004). Data also that show rapid GC non-genomic actions on GRs in the PVN can activate local release of endocannabinoids, which lead to autoinhibition through binding to their receptors (Evanson et al., 2010).

Evolutionarily, the response to an acute stressor is adaptive, and allows the individual to mobilize metabolic resources and prepare the immune system to react appropriately to the threat (McEwen and Gianaros, 2011a). The sustained activation of stress response systems caused by chronic stress can also be considered adaptive if it increases chances of survival during hostile conditions (Dallman, 1993, 2003). Thus, the duration of exposure to the stressors, as well as the nature and severity of the stressor, are all factors that influence the nature of the initial stress response by the organism, as well as the way it adapts to chronic stress (Miller et al., 2007). For example, individuals with stress-related disorders, show two types of HPA axis adaptation to chronic stress, either a loss of negative-feedback resulting in hypercortisolemia (e.g. major depressive disorder) or a hypersensitivity to GC negative feedback of the HPA-axis resulting in hypocortisolemia (e.g. post traumatic stress disorder, PTSD) (Ehlert et al., 2001). In both examples, however, there is evidence of CRF hypersecretion (Ehlert et al., 2001) associated with negative effects on physiology, emotional regulation, and mood (Chrousos, 2000; Raison and Miller, 2003; Gillespie and Nemeroff, 2005). Thus, the type, duration, and severity of the stressor are likely important determinants of the resulting stress response and long-term consequences.

E2 has direct effects on the HPA axis, and can potentiate peak GC secretion in response to an acute stressor as well as diminish GC negative feedback, at least based on evidence from studies in ovariectomized rodents with E2 replacement (Burgess and Handa, 1992; Carey et al., 1995). Data from intact rodents also show increased basal and stress-induced GC and ACTH secretion during proestrous, when both E2 and P4 are elevated (Carey et

al., 1995). Studies in rhesus monkeys have shown that E2 also leads to both reduced GC negative feedback following administration a synthetic GC, in a dexamethasone suppression test (DST) as well as increases adrenal GC production following a combined DST/CRH challenge (Wilson et al., 2005). Importantly, ovariectomized female rhesus monkeys exposed to chronic social subordination, a potent psychosocial stressor, showed a significant and earlier escape from GC negative feedback inhibition as well as increased GC response to CRH following E2 (Wilson et al., 2005). Therefore, these data suggest that E2 may in fact act to exacerbate the effects of chronic stress on the HPA axis.

E2 effects on the HPA axis, however, appear to be receptor subtype specific, with ERα exacerbating and ERß attenuating HPA response to stressors. In rodents ERα, but not ERß, reduces GCs negative feedback to restraint stress (Weiser and Handa, 2009). ERß, however, acts to blunt the diurnal peak corticosterone response as well as GC response to an acute stressor (Weiser and Handa, 2009). Infusion of E2 or propyl pyrazol triol (PPT), an ERα agonist, into the PVN potentiates GC response to 30 minutes of restraint stress, while both diarylprpionnitrile (DPN), an ERß agonist, and ICI 182,780, an non-selective ER antagonist decrease GC secretion relative to untreated females (Liu et al., 2012). It is important to remember that E2 interacts with both ER subtypes in females. These data suggest that individual differences and alterations in HPA axis function following E2, such as after prolonged exposure to chronic stress, may be mediated by alterations by expression of ERα/ERß, although data on this question are not available.

The effects of E2 on the human female stress response are less clear, as studies specifically testing effects of ERα and ERß agonists, such as PPT and DPN (respectively), are unavailable in women, and other selective ER modulators (SERMs) produce a mixture of agonist and antagonist ER effects (Paech et al., 1997; Wilson et al., 2003). Acute stress assessments with the Trier Social Stress Test (TSST), which includes 15 minutes of public speaking and mental arithmetic, showed that low concentrations of E2 unopposed by P4 during the early follicular phase were associated with blunted peak cortisol compared to the mid-luteal phase when both E2 and P4 are high (Kirschbaum et al., 1999). Data from DST suggests that GC negative feedback is also increased during this early follicular phase (Altemus et al., 1997). Interpreting these results between low (early follicular) and high (late-follicular) E2 levels, one would conclude that increasing concentrations of E2 reduce GC negative feedback, and elevate cortisol response to an acute stress similarly to the evidence reported from studies in animals. However, this view ignores the role of circulating P4 and its interaction with E2, which has been shown to both enhance E2's potentiation of basal HPA activity and peak corticosterone response to a stressor in rodents (Carey et al., 1995; Wilson et al., 2005) and to reduce E2's attenuating effects on HPA negative feedback in rhesus monkeys.

Alternatively, studies in post-menopausal women, where both endogenous E2 and P4 are negligible, indicate that two weeks of E2 replacement therapy (ERT, no P4) have no effect on basal cortisol, the cortisol response to the TSST, or GC negative feedback (Kudielka et al., 1999). These data did show that E2 blunts cortisol release, but not ACTH, following a DST/CRH challenge, suggesting a E2 may act directly at the adrenal

to diminish cortisol synthesis (Kudielka et al., 1999). Thus, findings from studies in women are mixed, with some suggesting E2 facilitates stress hormone reactivity, similar to that observed in animal models, while other studies suggest a limited effect of E2 on HPA regulation.

1.2.3 Summary

In general, the data suggest a potentiating effect of E2 on HPA reactivity to both acute and chronic stressors while stress hormones suppress HPG function. These intricate connections between the two axes provides a challenge when attempting to elucidate the effects of either one individually (Viau, 2002). While stressor exposure can suppress the reproductive axis that, for females, produces a chronic condition of hypoestrogenism, it does not do so in all cases. That is, some proportion of females exposed to chronic stressors still shows normal ovulatory function and E2 secretion (Kaplan and Manuck, 2004). Thus, the question directly relevant to this dissertation, is how stressor exposure disrupts the activational effects of E2 on the brain and behavior and whether this is the result of stress hormones disrupting ER signaling or downstream effects of ER activation.

1.3 Animal models of stress

Animal models of both acute and chronic stress can be generated through social or physical environmental manipulations and are primarily used to understand mechanisms of stress-related disorders in people, such as anxiety, depression, or addiction. These models can be evaluated on the basis of their face, construct, and predictive validity

described as follows: (1) face validity holds that animal models of human disorder should include symptoms typically seen in clinical populations, (2) construct validity concerns developing the model using methods similar to those seen to induce the disease in humans, and (3) predictive ability assesses the efficacy of treatment in response to pharmaceutical compounds, although this final validity is circular in that many of the animal models available are based on this final criteria (Nestler and Hyman, 2010). Additional sets of criteria have also been proposed, and include homological (species/strain), pathogenic (developmental), and mechanistic (physiology) validity (Belzung and Lemoine, 2011). However, the majority of animal models of stress have been validated and tested on male rodents (rats and mice), although sex and species differences often produce contrasting physiological or behavioral outcomes (Palanza, 2001). In this section, I will discuss several well-established stress paradigms that are used to study the behavioral, physiological, and neurobiological consequences of exposure to stress, and assess their face and construct validity for use as female models of stress-related disorders such as anxiety and depression.

1.3.1 Rodent models of stress

Rodent models of stress-induced anxiety and depressive-like behavior can be grouped into two main classes, conditioned or unconditioned responses (Bourin et al., 2007). Conditioned responses, including classical fear-conditioning often show sex-differences in acquisition (Maren et al., 1994)}, and may be attenuated in females following acute stress (Maeng and Shors, 2013). Therefore deficits in these paradigms may be due to reduced fear-learning as opposed to reduced fear-like or anxiety behavior, which will be

discussed in Section 1.4.2 "*Non-reproductive behavior*". Unconditioned stressor paradigms include both social and environmental stressors that naturally elicit anxiety-like or fear-like behavior that are often sex and species dependent. Two of the most commonly used behavioral tasks to examine the effects of environmental stress on fear and anxiety are the open field test and elevated plus maze, which measure a combination of exploratory and avoidant behavior in a novel environment (Lister, 1990). These paradigms show sex-differences in behavior where females appear less anxious than males (Palanza, 2001). Female behavior within these paradigms, at least in Wistar rats, is often driven by increased locomotion behavior, while male behavior is predominantly driven by anxiety-like behavior, such as decreased exploration in the open arm of the elevated plus maze (Fernandes et al., 1999). Increased locomotor activity in females is then interpreted as decreased anxiety-like behavior (Morgan et al., 2004). This suggests both low face and construct validity for female rodents.

Physical stressors include exposure to sensory stimuli that are threatening, including predator odors or ultrasonic vocalizations and stimuli that restrict movement or are uncomfortable or painful, including restraint, forced swim test, and foot or tail shock (Bourin et al., 2007). Overall, acute physical stressors have low face and construct validity. In the forced swim task, depressive behaviors are measured as a reduction in escape behavior (e.g. swimming, struggling) or immobilization, and females show reduced depressive behavior compared to males (Palanza, 2001). Criticism of the paradigm suggests that this 'depressive' behavior may actually be adaptive in nature (Palanza, 2001), and this difficulty of interpreting these results suggest both low face and

construct validity in females. Acute restraint stress, where rodents are immobilized in plastic tubes for 5-30 minutes is another common stress paradigm. In females, this paradigm appears to increase markers of anxiety-like and depressive behaviors (Walf and Frye, 2005a). However these metrics were evaluated using the open field test, elevated plus maze, and forced swim tests previous mentioned, and the face validity of the stressor paradigm is dependent on the face validity of the behavioral tests used to assess the consequences of the stressor.

Although females appear less susceptible to acute stress in a range of paradigms, they often show increased depressive and anxiety-like behaviors following chronic presentations of these same stressors than males (Palanza, 2001). In females, exposure to chronic restraint decreases exploration during an open field test and heightened acoustic startle reflex compared to males, but these apparent sex-differences are species dependent as neither male nor females Long-Evans rats respond to restraint stress (Faraday, 2002). Additionally female Sprague Dawley rats show greater neural activation, measured by c-Fos, and GR expression in the PVN than males following both acute and chronic restraint stress (Zavala et al., 2011). Chronic mild stress, or chronic variable stress (CVS), are paradigms that include repeated presentations of physical stressors including restraint inside of a constrictive tube, unpredictable shock, forced running, forced swim, or a combination of all of these factors presented in a randomized order (Willner, 2005). Following CVS, females show decreased open field exploration and sucrose intake, interpreted as anhedonia, as well as increased basal corticosterone (Dalla et al., 2005). Furthermore CVS also increases c-Fos expression in the PVN as well as in cortico-limbic

brain regions in female, but not male rats (Carvalho-Netto et al., 2011). The symptomatology produced by these stress models suggests they have face validity, but the artificial nature of the stressors questions their construct validity.

Chronic social stress in rodents is commonly assessed in either a visible burrow system or exposure to social defeat through a resident-intruder task. The visible burrow system (VBS) is a social subordination rat model of chronic stress, induced by the formation of a male social dominance hierarchy in mixed male and female groups. This model has not been tested in females rodents as they do not form similar social hierarchies and actually prefer to live in groups, although these preferences for group living do vary by species (Palanza et al., 2001). Alternative social instability models have been proposed, and females respond to alternating exposure between crowded cages and social isolation with elevated basal levels of corticosterone (Haller et al., 1999; Herzog et al., 2009). Social defeat is another paradigm similar to social subordination in rats that models chronic stressor exposure. For this model, the target animal is introduced as 'intruder' into the cage of a same-sex resident that is more aggressive (Razzoli et al., 2009; Holly et al., 2012). Following repeated episodes of defeat, the intruder typically exhibits a range of depression like symptoms including anhedonia, and decreased social behavior. This model is considered to have both construct and face validity (Nestler and Hyman, 2010) and has been shown to be effective in both males and females (Holly et al., 2012).

The extensive application of rodent models allow for the systematic investigation of the behavioral, neurobiological, and physiological consequences associated with chronic

stress that are simply not possible in humans (Armario and Nadal, 2013). These stressor paradigms are commonly used as models of anxiety and depression, to better understand the etiology and mechanisms of psychopathology in humans (Nestler and Hyman, 2010). Limitations of these models include their low construct validity in females, such that many of the stressors are not comparable to the typical chronic psychosocial stressors experienced by women.

1.3.2 Macaque model of stress

Nonhuman primates provide an more translational alternative approach to modeling the adverse effects of both acute and chronic stress in humans (Abbott et al., 2003). Their behavioral, physiological, neurobiological, and phylogenetic similarities with humans provide increased homological validity in comparison to rodents (Belzung and Lemoine, 2011). Often, both acute and chronic stressors attempt to approximate anxiogenic conditions animals may exhibit in the wild (Barros and Tomaz, 2002). These models can also be assessed by their face, construct and predictive validity as described previously (Nestler and Hyman, 2010), and overall provide a more ethologically relevant model to explore stress-related pathology in women. In addition to studies of wild populations of baboons (Sapolsky et al., 1997), a number of captive species of nonhuman primates have been used to study the consequence of social stress on a number of health related outcomes, including marmosets (Abbott et al., 2003; French et al., 2007) , squirrel monkeys (Levine et al., 1997), and rhesus and cynomolgus macaques (Shively and Clarkson, 2007) (Shively et al., 1997; Michopoulos et al., 2012a). Given the higher availability of captive populations, macaque models are most often used.

Acute stress paradigms are used to identify stress-induced changes in physiology and behavior. Social isolation paradigms have high construct validity and are considered a psychogenic ethological stressor (Barros and Tomaz, 2002). Short durations of separation produce increases in circulating stress hormones (Ayala et al., 2004; Arce et al., 2010) and anxiety-like behavior (e.g. distress calls) (Barros and Tomaz, 2002) suggesting construct validity. Intruder paradigms are also used in macaques, and placement of unfamiliar animals in adjacent cages separated only by Plexiglas dividers significantly increase stress hormones, anxiety-like and agonistic behavior in males (Habib et al., 2000).

More complex acute stress paradigms used in the literature include the human intruder (HI) and Approach-Avoidance (AA) tests. The HI test elicits both anxiety-like and fear behaviors, and agonistic behaviors in response to the presence of an unfamiliar human (Kalin and Shelton, 1989, 2003). The AA test measures the behavioral response of a monkey to the simultaneous presentation of a food reward in the presence of a threatening object (i.e. rubber snake), although this task often combines both conditioned and unconditioned stimuli (Machado et al., 2009). Both the HI and AA tests attempt to simulate naturally aversive stimuli for macaques and were developed specifically to translate to human task, although predominantly in children, suggesting that these tests also have high construct validity. Although the majority of the data using these paradigms has been collected separately in males (Kalin et al., 2004; Kalin et al., 2005; Machado et

al., 2009) and in females (Arce et al., 2010; Howell et al., 2013), the data do not suggest sex-differences in behavioral or physiological response.

Non-human primate chronic stress paradigms utilize psychogenic stress, sometime accompanied by physical or metabolic stressors. The chronic variable stress model is a well-established stress model that includes both social stressors, including living in single-housed conditions with repeated relocation to new housing environment, and metabolic stressors, including calorie restriction and treadmill running (Cameron, 1997; Bethea et al., 2008). Following exposure to this paradigm, females show attenuation of HPG function (Kaplan and Manuck, 2004) providing face validity to the human condition. The chronic variable stress paradigm does not uniformly produce stress-related dysfunction, and the model has also been used to study stress vulnerability in females (Bethea et al., 2008). The inclusion of diet restriction and treadmill running with the social stressor in this paradigm has high construct validity to that of women, as dieting and excessive exercise are frequent characteristics of women with stress-induced amenorrhea (Berga et al., 2003), and suggests strong construct validity in females.

In contrast to the models presented above, chronic psychosocial stress in the form of social subordination stress in female macaques has both face and construct validity, given it is the consequence of the naturally occurring dominance hierarchies (Shively and Kaplan, 1984; Sapolsky, 1995; Sapolsky, 2005; Jarrell et al., 2008). The neurobehavioral consequences of differences in social rank make social subordination in macaque females a potent psychosocial stressor (Sapolsky, 2005). In female rhesus macaques, social

groups have a matrilineal structure in which social rank is determined by birth (de Waal and Luttrell, 1985), and familial, or kin support acts as a natural buffer to rank-related stress (Kikusui et al., 2006). Dominance hierarchies maintain group stability (Bernstein, 1970), however, lower-ranking females receive disproportionally greater amounts of aggression from higher-ranking females and engage in higher levels of submissive behavior (Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984). In captive populations, dominance hierarchies can also be established experimentally by removing a female from her natal group, and introducing her to another group (Shively et al., 1997; Jarrell et al., 2008). Whether members of their natal groups or these experimentally formed groups, subordinate females living in long-term stable groups are continually exposed to unpredictable harassment from more dominant animals, somehow similar to the intermittent social defeat in rodent models (Miczek et al., 2008) and provide a powerful model to explore the consequences of chronic stress exposure in women (Michopoulos et al., 2012a).

1.3.3 Summary

A wide variety of animal models exist to test the effects of stress on both brain and behavior, each with it's own advantages and limitations. Rodent models present a high throughput, cost-effective method of measuring particular aspects of an organism's stress response t, a number of interventions and of studying the cellular and molecular underlying mechanisms. Consequently, these models have provided the majority of our understanding of the physiology of stress-related changes in brain and behavior. However, these rodent models often do not produce relevant phenotypes to assess stress

response in women. In contrast, nonhuman primate models of social subordination stress, specifically studies of female macaques, present an opportunity to examine how chronic stress resulting from this adverse social experience impacts neurobehavioral outcomes that are translatable to women.

1.4 Chronic stress modifies E2's activational effects on behavior

The activational effects of E2 on the expression of sociosexual behavior are well established (Beach, 1976; Wallen and Goy, 1977; Wallen, 1990; Zehr et al., 1998). Briefly, the original hypothesis (Phoenix et al., 1959) suggested that in adulthood, hormones acted upon "tissues mediating reproductive behavior" to induce sexual behavior (Wallen, 2009). In general, the activational effects of E2 are those that produce changes in behavior different from a hypoestrogenic condition, e.g., increases in reproductive and social (e.g. affiliative and aggressive) behaviors that may be parallel to decreases in emotional reactivity (e.g. anxiety and fear). Importantly, chronic stress resulting from some adverse experience or exposure to novel environments (Morgan and Pfaff, 2001) may attenuate these effects on behavior (White and Uphouse, 2004; Uphouse et al., 2005; Pierce et al., 2008), suggesting activation of stress hormone reactivity is an important determinant of E2 action. The activational effects of E2 on adult rodents, sheep, and non-human primates are reviewed below, with a focus on the their modulation by chronic stress.

The activational hypothesis, though formulated based on research on animal models (Beach, 1976; Wallen and Goy, 1977; Wallen, 1990; Zehr et al., 1998; Schulz et al., 2009), is clearly applicable to sex-specific activational effect of hormones seen in humans. However, interventional studies manipulating exposure to hormones in adulthood is ethically and technically challenging in humans. The majority of data on the activational effects on women are based on examining the effects of E2 across the menstrual cycle or the effects of ERT in postmenopausal women. Additionally, manipulations of exposure to chronic stress are also difficult in human populations, and therefore direct assessments of the interactions of E2 with stress history are limited and requires more invasive and experimentally-controlled studies in animal models.

1.4.1 Reproductive behavior

Ovarian hormones influence three major constructs of sexual behavior in females, including attractivity, receptivity to male sexual behavior, and proceptivity sexual solicitation towards males (Beach, 1976). Attractivity refers to a female's potential to act as a rewarding stimulus to a male. Proceptivity refers to behaviors emitted by the female towards the male to initiate copulation while receptivity reflects a female's willingness to allow a male to mount (Beach, 1976). In general, the occurrence of reproductive behavior in females is tightly coupled to increases in E2 and P4 during the periovulatory period, with the exception of primates, both human and non-human (Wallen, 1990). However, disruption of the activating effects of E2 on female sexual behavior by exposure to chronic stressors in adulthood may be present regardless of these species differences.

Sexual attractivity is based on alterations of physical characteristics such as scent, or sexual swelling, that can stimulate male sexual behavior (Everitt et al., 1987; Girolami and Bielert, 1987; Tilbrook and Lindsay, 1987). In rodents, the stimulus value of a female may be measured operationally in a second-order conditioning paradigm, where a male must press a lever in order to gain access to a female (Everitt et al., 1987). Similar studies conducted on naïve virgin males show that E2 primed females elicit a greater motivational response from males, suggesting an inherent attractiveness in hormonally primed versus unprimed females (Lopez et al., 1999). In studies of rhesus monkey sexual behavior, attractivity translates to male approach and initiation of proximity to females as well as mount behavior (Wallen and Tannenbaum, 1997). However, proceptive female behaviors, which include approaching and initiating proximity to a male, may also increase a female's attractiveness in rhesus monkeys and both are potentiated by E2 (Carpenter, 1942; Johnson and Phoenix, 1976; Cochran, 1979; Wallen et al., 1984).

In rodents E2 is able to induce both proceptive and receptive reproductive behaviors independent of progesterone (P4), and these effects are dependent on ERα expression in the VMH including both the core and the surrounding shell (Musatov et al., 2006; Blaustein, 2008). Specifically, that presence of E2 is required for the expression of the lordosis reflex arc, possibly by potentiating the excitatory effect of the lordosis-triggering stimulus (Kow and Pfaff, 1998). In ewes, increasing levels of E2 during estrous increase both proceptive and receptive reproductive behavior (Tilbrook et al., 1990), mediated in part by the medial basal hypothalamic (MBH) nucleus (Blache et al., 1991).

E2 can also stimulate the occurrence of receptivity and proceptivity in rhesus monkeys (Wallen et al., 1984), however, receptivity can be seen independent of E2 (Wallen, 1990). Additionally, data in rhesus monkeys comparing sexual behavior in pairs versus multi-male/multi-female groups, suggest a decoupling between E2 and proceptive behavior dependent on social context (Wallen, 2001). Decreased hormonal effect on sexual behavior, e.g. reduced correlation between E2 and sexual behavior, are more observable in pairs-test (Wallen and Winston, 1984) especially when housed in smaller enclosures (Wallen, 1982), both conditions which facilitate proximity and access. Multi- or single-male/multi-female groups show greater association between E2 and sexual behavior (Wilson et al., 1982; Wallen and Winston, 1984). It has been suggested that this greater coupling in large social groups may also be a result of increased social risks of mating and females competing for mate. Thus, the initiation of sexual behavior would only occur as follicular phase levels of E2 had increased sufficiently to increase sexual motivation to override risk avoidance (Wallen, 2001). Furthermore data suggests that proceptive sexual behaviors, as opposed to receptive behavior, show the most inducement by E2 (Wallen and Tannenbaum, 1997; Zehr et al., 1998; Dixson, 2001), although these effects are not seen in all primate species (Lipschitz, 1997).

If chronic stress disrupts the action of E2 on female sexual behavior, it could do though any of the three constructions that encompass these behaviors. Data on the effects of chronic stress on attractivity are limited. However, studies in sheep show female attractiveness, measured by a ram's approach to a tethered ewe within an enclosed T-maze is attenuated by prior exposure of the a chronic social stressor despite no effect on

estrus or fertility (Pierce et al., 2008). This reduction in attractivity, however, is like also mediated by the parallel reduction in proceptivity but not receptive behavior (Pierce et al., 2008). Thus, even in the presence of a normal estrous cycle, stressor exposure does attenuate certain aspect of sexual behavior in ewes. Infusion of cortisol to levels similar to those seen following the psychosocial stressor, were unable to produce these same effects, but instead, significantly reduced ewe receptive behaviors (Papargiris et al., 2011a). The neural mediators of these differences, however, are unknown although some data suggests that limbic CRF secretion, including the CeA and BNST, may be a possible mechanism of action for stress-induced attenuation of attractiveness and proceptivity (Dobson et al., 2003).

Very little is known about the effects of chronic stress on E2's activational effects in non-human primates. Data suggest that, in social subordination models, more subordinate females require higher levels of E2 to initiate proceptive sexual behavior compared to dominant females (Wallen, 1990). However, this attenuation in the of induction of proceptivity at lower doses of E2 in subordinate females could be attributed to increased behavioral inhibition due to a perceived risk of aggression in competing for mates from higher ranking females. However, this does not negate the hypothesis that chronic stress diminishes the prosocial effects of E2.

Most support for the hypothesis of a stress-induced reduction in the activational effects of E2 on female sexual behavior comes from the literature on the attenuating effect of stress in rodents, as well as the effects of stressor exposure on other activational effects of E2

(discussed in section 1.4.2). The data show that stress can alter the efficacy of E2 through a number of different mechanisms. In E2 and E2/P4 primed ovariectomized rats repeated short durations (5 minutes) of restraint result in transient decreases in reproductive behavior, including time spent affiliating with males and frequency of lordosis behavior (White and Uphouse, 2004; Uphouse et al., 2005). However these effects are reversed with increasing doses of E2 (Uphouse et al., 2007), similar to the to social rank differences in E2 responsivity mentioned above (Wallen, 1990). In addition, infusion of serotonin receptor 5HT-2 agonist or 5HT-1 antagonists into the MBH (Uphouse et al., 2007), suggesting that the effects may be mediated by serotonin signaling. In support of this hypothesis, treatment with a 5HT-2 receptor antagonist acted synergistically with stressor exposure to further reduce lordosis in proestrous females more than the effect of each intervention alone (Uphouse et al., 2003).

Other rodent data also suggest a role for the amygdala in stress-induced attenuation of E2's reproductive effects. Constitutive increase of CRF in the CeA decreases proceptive behavior in E2 plus P4 primed females although no effects were seen in lordosis or male behavior (Keen-Rhinehart et al., 2009). Furthermore, other data from intact females suggest a dose effect of stressor exposure, as showed that longer durations of restraint were able to inhibit lordosis behavior on the afternoon of proestrous (Donadio et al., 2007).

Together, these data from rats, ewes and monkeys suggest that, indeed, chronic exposure to stressors may decrease the induction of female sexual behavior by E2. While the data

are somewhat limited, the most consistent effect appears to be on the reduction in female sexual motivation or proceptivity, as receptive behavior was inconsistently affected by the stress paradigms employed. However, it is important to appreciate that stress does not likely abolish the behavioral effects of E2 but rather may shift the activation of difference neural circuits activated by E2 so other non-reproductive behaviors predominate.

Analyses of E2's activational effects or their inhibition by chronic stress are limited in human studies. Analyses of these effects are not straight-forward, given the apparent decoupling of the capacity of sexual behavior in women from hormonal influence, analogous to what occurs under certain circumstances in rhesus monkeys (Wallen, 1990). Physiologically, E2 increases vaginal lubrication and blood-flow to the clitoris, as well as maintain vaginal pH and is therefore thought to facilitate reproductive behavior, which is often diminished in women with sexual dysfunction (Berman, 2005). Behavioral data also suggest that E2 increases women's proceptivity, identified by increased sexual desire, either mentally through erotic fantasy or physically through increased initiation of intercourse (Bullivant et al., 2004; Dawson et al., 2012). Contrary to this E2 induced increase in physical and psychological arousal, some studies show no difference in sexually initiated behavior in heterosexual couples across the menstrual cycle (Grebe et al., 2013). However, increased female initiated sexual behavior during mid-cycle can be seen in single, but not partnered women (Caruso et al., 2014).

Recent updates to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) indicate that the clinical definition of female sexual dysfunction is still being developed (Sungur and Gunduz, 2014). Although the role of E2 on human sexual behavior is not considered in these definitions, attenuation of physiological and psychological desire and arousal are also commonly implicated in sexual dysfunction in women (Laumann et al., 1999). Epidemiological data suggests that sexual dysfunction is more prevalent in women (43%) than in men (31%) (Laumann et al., 1999). In a metanalysis regarding females sexual dysfunction, about 12-25% of cases are associated with 'personal distress' (Palacios et al., 2009). Furthermore, acute and chronic stress have been shown to reduce both psychological and physiological arousal, measured using vaginal photoplethysmography to detect vaginal pulse amplitude, or increased vaginal vasocongestion (Ter Kuile et al., 2007). Additional data on the effects of anxiety, however, indicate that both low and high levels of anxiety reduce physiological arousal (Bradford and Meston, 2006). However, the difficulty interpreting the possible stress-induced attenuation of sexual function in people is that it is not clear whether the deficits are due to stress-induced reduction on ovarian hormone secretion or to an attenuation of E2 efficacy on neural substrates. The use of animal models and the ability to systematically control E2 and stressor exposure provide a distinct advantage to address this question.

1.4.2 Non-reproductive behavior

E2 has a significant effect on arousal (Morgan and Pfaff, 2001; Pfaff, 2005), increasing locomotor activity, sensory perception, and emotional reactivity (Morgan et al., 2004).

One hypothesis is that E2's activating effects on behavior stem from this general increased arousal, such that the specific behaviors that emerge, e.g., expression of fear and anxiety (Morgan et al., 2004) or affiliation and aggression (Walker et al., 1983), may depend on the context or environment the individual is in. Often inconsistencies in the literature can be explained by differences in experimental paradigm, or interpretation of locomotion as anxiety (Morgan et al., 2004).

An important distinction to make while discussing the anxiolytic effects of E2 and their modulation by stress, is that anxiety can be tested with or without prior exposure to a stressor. Behavioral paradigms that measure anxiety- or depressive-like behaviors in rodents, such as the open field test or the forced swim paradigm can measure an animals' adaptive behaviors used to cope with a threatening environment, and E2 appears to increase coping (e.g. increased exploratory behaviors) without a history of exposure to chronic stress. However, chronic stress can alter these adaptive socioemotional behaviors and may in parallel modify E2's effects on these behaviors.. For example, a history of stressor exposure can attenuate E2's anxiolytic effect measured in the open field and elevated plus maze, and unlike sexual behavior, higher doses of E2 do not reverse the effects of prior stressor exposure (Walf and Frye, 2005a). Being aware of such experimental differences can provide a better insight into the effect of stress in modulating E2's effects on anxiety-like and depressive behavior.

Often when E2, or E2 benzoate (EB), are administered to previously unstressed females, the effects on rodent anxiety and fear-like behavior are mixed, with some studies showing

increased anxiety, while others show decreased (Morgan et al., 2004). E2's putative anxiogenic or anxiolytic effects on behavior without a history of chronic stress exposure are influenced by E2 specific interaction with ER subtypes. Administration of SERMs that are agonists for ERß, including DPN and coumestrol, are anxiolytic in unconditioned acute stressor paradigms in rodents (e.g. elevated plus maze) without prior exposure to stress, while administration selective ERα agonists, PPT and 17α estradiol, are either not effective (Walf and Frye, 2005b), or anxiogenic (Lund et al., 2005). Female ERß knockout rodent models also show higher levels of anxiety in unconditioned acute stressor paradigms than wild-type rodents, along with increased plasma corticosteroid secretion (Walf et al., 2009). ERß knockout mice also show decreased serotonin monoamines in the hippocampus, BNST, and MPOA, and decreased dopamine monoamines in the caudate and putamen (Imwalle et al., 2005). These data suggest that activation of ERß produces anxiolytic effects may be mediated by its genomic effects within these brain regions.

ERα and ERß, agonists have similar effect on the GC stress-response following chronic stress (Serova et al., 2010), however tests of there individual effects on anxiety-like behavior paradigms following chronic stress have not been assessed. In rhesus monkeys, E2 reduces anxiety-like behavior in females with high baseline rates of anxiety; however, socially subordinate females with the short promoter length variant in the gene encoding serotonin transporter were unresponsive to the anxiolytic effects of E2 (Michopoulos et al., 2011), suggesting differences in E2's anxiolytic effects following chronic stressor exposure.

Several fear-conditioning paradigms including classic fear learning, contextual fear learning, and fear potentiated startle have been used to examine E2 affects on fear and anxiety in rodents. These paradigms are often implemented in naïve rodents, without history of exposure to chronic stress. Broadly speaking these paradigms involve behavioral measures regulated by hippocampal, amygdala, and PFC circuitry (Davis et al., 1997; Markus and Zecevic, 1997; Lebron-Milad and Milad, 2012). Both contextual and classical fear conditioning pair an aversive unconditioned stimulus (e.g., foot shock) with a conditioned stimulus, e.g., a light or a tone, while in contextual conditioning, chamber in which the shock is delivered serves as the conditioned stimulus. Fear potentiated startle paradigms are used to assess alterations in an unconditioned stress response, such as the acoustic startle reflex. Experiments can manipulate the anticipation of a stressor by presenting a previously (fear) conditioned stimulus. However, unconditioned or physiological stressors can also cause potentiated startle by increasing the animal's anxiety (e.g. testing in a brightly lit environment for rodents, or in a dark chamber for macaques), or by mimicking elevated stress levels by CeA over expression of CRF (Keen-Rhinehart et al., 2009). Whether exposure to chronic stress affects fear learning has not been robustly tested in females, while males show facilitation of fear-learning following both corticosterone administration and stress (Farrell et al., 2013). Some data suggests that in female rodents, a history of chronic stress may actually inhibit fear conditioning (Maeng and Shors, 2013).

E2 may exert some of its putative anxiolytic effects by reducing contextual fear acquisition or potentiating classical fear extinction, however, these too may be attenuated by exposure to chronic stress. Contextual fear conditioning shows marked sex-differences (Maren et al., 1994), thought to be explained by the effects of higher levels of E2 in the hippocampus. In ovariectomized females, E2 attenuates contextual fear conditioning concomitant with reduced long term potentiation (LTP) in the hippocampus (Gupta et al., 2001). In intact females, hippocampus-dependent contextual fear-learning, but not cue-conditioned fear learning, is attenuated during proestrous, when E2 and P4 levels are at their peak (Markus and Zecevic, 1997). These findings, however, are not consistent across studies, as E2 has also been shown to enhance (Jasnow et al., 2006) or have no effect on contextual fear conditioning (Chang et al., 2009). One explanation of this discrepancy is that E2's effects are dose dependent, as low doses increase while higher doses, equal to or greater than those seen in proestrous, decrease fear conditioning (Barha et al., 2010). Another hypothesis is that, similar to anxiety-like behavior, contextual fear-conditioning is also dependent on ER subtype. This is supported by data from ERß knockout mice that show both decreased contextual fear learning and CA1 hippocampal long term potentiation (LTP) compared to controls (Day et al., 2005). However, a third hypothesis is that history of exposure to stress may alter the effects of E2 in contextual fear learning. Although this hypothesis has not been examined, data show that chronic stress and concurrent E2 replacement facilitate both contextual freezing compared to stress alone (Hoffman et al., 2010) and hippocampal-dependent spatial learning during the Morris water maze task compared to stress or E2 alone (McLaughlin et al., 2010).

Chronic stress has been shown to modify E2's effect on classical (cued) fear conditioning. In contrast to contextual fear conditioning, classical eye-blink conditioning is enhanced at proestrous (Shors et al., 1998) and auditory cued fear conditioning is enhanced following EB treatment in ovariectomized rats (Morgan and Pfaff, 2001). Exposure to an acute stressor is then able to attenuate E2's effects, and impairs eye-blink conditioning when females are concurrently exposed to higher endogenous levels of E2 (Shors et al., 1998; Maeng et al., 2010; Maeng and Shors, 2013). Exposure to chronic stress has also been shown to impair fear conditioning recall, but not learning, in females (Baran et al., 2009), although estrous cycle phase was not addressed.

Fear extinction learning is also enhanced during proestrous and administration of non-selective E2 antagonists during extinction learning decreases extinction recall (Milad et al., 2009). These effects are also dependent on ER subtype, as ERß, but not E2 or an ERα agonists, enhanced extinction recall when administered prior to extinction learning (Zeidan et al., 2011). Although E2 appears to facilitate extinction learning and recall to a single-cue, it has also been shown to impair fear inhibition or 'safety-signal learning' following cued fear conditioning (Toufexis et al., 2007). This paradigm pairs two previously conditioned stimuli, one associated with an aversive stimulus and the other not associated with a stimuli, and thus a safety cue (Myers and Davis, 2004). ERα and ERß agonists both disrupt discrimination learning between the two cues and reduce fear inhibition during presentation of the 'safety-signal' producing a generalization of fear learning (Toufexis et al., 2007). Together these data suggest that although E2 can

increase extinction recall, it may do so only when the threat is removed, and inhibits the use of a 'safety-signal' to identify a non-threatening environment.

Concurrent with an increase in female sexual behaviors, rising concentrations of E2 also generally increase affiliation and aggression. Studies of socially-housed rhesus monkeys, suggest that affiliation toward males and aggression toward females increases with peak estrous behavior (Carpenter, 1942) and in conjunction with peak ovarian E2 (Wallen and Tannenbaum, 1997). The specific behavioral effects of increased E2 can depend on social context, such that E2 increases affiliation in female-only groups (Michopoulos et al., 2011) but decreases female-female and increase female-male affiliation when males are present (Wallen and Tannenbaum, 1997). In parallel, aggressive behaviors in rhesus monkeys have also been shown to increase when E2 is elevated (Wallen and Tannenbaum, 1997; Michopoulos et al., 2011) although these alterations in aggression may in fact be secondary to increased affiliative behavior (e.g. increased proximity) or competition for mates (Walker et al., 1983).

Some aggressive behaviors, however, can be isolated to specific ER subtypes. In rodents, intact ERα knockout mice show increased female aggression in a resident-intruder task (Ogawa et al., 1998), while ERß knockout mice and wild-type mice showed no aggression (Ogawa et al., 1999), suggesting that ERα acts to reduce aggression. However, administration of an ERß agonist, WAY-200070, to intact female mice increased both aggressive behavior as well as affiliative behavior suggesting that ERß increases overall

social behavior which increases the likelihood of aggressive interactions (Allen et al., 2010).

More broadly, E2 can augment social recognition which has been suggested as an process strongly influencing both aggressive and affiliation behavior (Shelley et al., 2006). Studies of social recognition in mice suggest that both ERα, and to a lesser extent, ERß increase social recognition, possibly through the up regulation of the prosocial neuropeptide oxytocin (OT) in the hypothalamus and it's receptor in the amygdala (Choleris et al., 2003; Choleris et al., 2006). In mice, knockdown of ERα in the medial amygdala abolished social recognition although it did not subsequently increase aggression (Spiteri et al., 2010).

Literature on the effects of E2 on behavior in women suggests that E2 can improve positive mood and affect. These data appear inconsistent with reports that women experience increased prevalence of mood and anxiety disorders throughout their life span (Lasiuk and Hegadoren, 2007) and at times of hormonal transitions either as E2 begins to increase during puberty (Angold et al., 1999) or as ovarian E2 is reduced post partum (Ahokas et al., 2001) and during menopause (Soares et al., 2003). An additional and important consideration is that history of stressful life events is the best predictor for development of stress-induced disorders among adult women (Kendler et al., 1993). Rodent research, reviewed above, suggests that a consequence of chronic stress is a reduction in the activational effects of E2 on behavior in certain contexts. Therefore,

examining the effect of E2 without consideration of a women's stress history may limit interpretability of the data.

Examining the effects of E2 across the menstrual cycle and loss following menopause present two natural experiments on the effects of E2 on women's mood and affect. Correlating changes in mood across the menstrual cycle with E2, however, is difficult, and measures of E2 can be elevated either in isolation of P4 (late follicular phase) or concomitant with elevated measures of P4 (mid-luteal). Nonetheless, data suggest that women show no systematic variation in mood across the menstrual cycle, despite prevailing dogma (Romans et al., 2012). On the other hand, women with premenstrual dysphoric disorder (PMDD), classified as a clinical mood disorder (Epperson, 2013), show increased negative affect when E2 and P4 levels are declining prior to menses, but alternatively show a negative response to both E2 and P4 replacement following leuprolide ovarian suppression compared to healthy controls (Schmidt et al., 1998). Although the role of chronic stress in the development of PMDD is not known, prior history of stress-related psychopathology is a risk factor for developing PMDD (Soares and Zitek, 2008). These data suggest that indeed some women with stress-induced disorders may show a different response to E2 and P4 than disease free women.

Data on postmenopausal women, also offers an opportunity to examine the effect of E2 on mood and affect by prospective and cross-sectional studies of hormonal replacement therapy, including ERT, and measures of depression and anxiety. Risk of first-onset episodes of depression is increased during the menopausal transition, but these effects are

not seen in all women (Cohen et al., 2006). Early studies using ERT show that conjugated equine estrogen (CEE) increases positive affect when administered alone, compared to combination therapy with medroxyprogesterone acetate (MPA) (Sherwin, 1991). This observation is supported by a meta-analysis of various combinations of hormonal replacement therapies (e.g. E2, CEE, CEE+MPA) and administration methods, which suggest that E2 and CEE act to reduce depressive symptoms, and that concomitant administration of MPA or synthetic progestins attenuates these positive effects (Zweifel and O'Brien, 1997). Furthermore, acute treatment with E2 can decrease depressive symptoms in menopausal females, and E2 withdrawal can increase negative mood in premenopausal healthy females (Schmidt and Rubinow, 2009). Some studies, suggest that E2's effect on availability and function of monoamines elevate mood and reduce clinical symptoms of depression (Soares et al., 2003). However, other studies suggest that this elevation in mood may be the result of a reduction of vasomotor symptoms (hot flushes) associated with ERT (Holte, 1998) and not general improvement in positive affect. Furthermore, this ameliorating effect is not universal, and some respond with decreased mood independent of type of hormonal replacement (Toffol et al., 2013). Stress history may be a critical factor that explains the variance in response to E2.

Measures of fear and anxiety behavior using startle paradigms have also been tested in both clinical and healthy control populations of premenopausal women, and the results depend on what phases of the menstrual cycle are compared. For example, data suggest that when examining behavior based on serum levels of E2 (low versus high), independent of P4, high E2 facilitates extinction recall (Milad et al., 2010; Zeidan et al.,

2011; Glover et al., 2012; Lebron-Milad and Milad, 2012), and, in contrast to data from rodent, increases safety-signal learning (Glover et al., 2013). When looking at menstrual cycle phases in the context of both E2 and P4, extinction recall is impaired in the late-follicular phase (high E2 unopposed by P4) compared to the early follicular phase (low E2 unopposed by P4) (Milad et al., 2006). Similarly, safety-signal learning is impaired in the follicular phase compared to the mid-luteal phase, and females show reductions in fear discrimination between stimuli (Glover et al., 2013), which suggest that E2 unopposed by P4, impairs safety-signal learning and recall. Overall, these alterations in fear and anxiety in women suggests a similar alteration of reproductive strategies to rodents, in that stressors or indicators of a harsh environment, either real or imagined, reduce E2's activational effects.

1.4.3 Summary

In general, E2's increases sexual behavior, mediated by ERα, while also increasing social behavior and decreasing fear and anxiety-like behavior, perhaps mediated by ERß within the hippocampus, amygdala, or PFC. Ultimately, these behaviors serve to increase opportunities for mating, as proceptive behavior is elevated along with locomotor, exploratory, and prosocial behavior, while risk-avoidance and contextual conditioning is reduced. Exposure to chronic stress appears to attenuate reproductive and anxiolytic effects of E2, at least in rodents and ewes, and serves perhaps to modify reproductive strategies during challenging, hostile or harsh conditions. Importantly, the attenuation of both reproductive and non-reproductive behavior in rhesus monkeys has not been examined within the context of mixed male and female social groups.

Overall, as in rodents, E2 appears to increase reproductive behaviors and seems to be both anxiolytic and anxiogenic in humans. Direct hormonal manipulation and use of ER subtype specific agonists and antagonist may improve our understanding of the effects of E2 on the latter. However, from the review above it is important to note that another critical variable we need to consider is the individual's history of chronic stress, as it may alter the expression of ERs in different brain regions to alter behavior, and directly affect HPG function via GCs or CRF over activity. Given that chronic and acute stress attenuate E2's activational reproductive effects in animal models, a woman's current or cumulative stress history may better explain the variance in females response to E2 in the studies summarized above. Additionally, as chronic exposure to stressors may reduce fertility (Berga and Loucks, 2007), alterations in reproductive and non-reproductive behavior described above may be due in part to a reduction of circulating E2. Studies in postmenopausal women, although allowing for exogenous hormonal control, present additional challenges, as the effects of E2 may be affected by age-dependent differences in ER availability (Sherwin and Henry, 2008). This highlights the importance of studies controlling for circulating E2 to differentiate between effects of reduced E2 versus attenuated actions of E2 by chronic stress. Rhesus monkey exposed to social subordination stress present an ethologically relevant model to address these issues, and behavioral, physiological, and neurobiological homologies make these findings highly translational to both stress-induced pathology in women.

1.5 Estradiol effects on brain structure and function: Interactions with chronic stress

Data from animal models including rodents (Watanabe et al., 1992; Magarinos and McEwen, 1995a; Martinez et al., 2002; Radley et al., 2013), tree shrews (Magarinos et al., 1996), vervet monkeys (Uno et al., 1989), and cynomolgus monkeys (Willard et al., 2009) indicates that a history of chronic stress leads to both morphological and functional changes in the hippocampus, amygdala, and PFC, although the data rely on a myriad array of techniques, animal models, and stress paradigms. The majority of these data have been limited to males and indicate that chronic stress decreases dendritic arborization and spine density in the hippocampus and PFC, while increasing amygdala dendritic arborization, specifically in the BLA (Leuner and Shors, 2013). In females, in the absence of stress E2 has neurotrophic and neuroprotective effects in the amygdala, hippocampus and PFC and increases both cell survival and growth (Lee and McEwen, 2001). These effects are thought to be mediated in part by E2's genomic regulation of brain derived neurotrophic factor (BDNF) (Sohrabji et al., 1995) as well as activation of MAPK (Toran-Allerand et al., 1999). Importantly, chronic stress and E2 interact to cause morphological and functional changes to the hippocampus, PFC, and amygdala due in part to the activation of both stress hormone (GCs, CRF) and E2 receptors (Sousa et al., 1989; Morimoto et al., 1996; Shughrue et al., 1997). However, it is unclear from the studies in the literature whether the effects of E2 are protective or detrimental under situations of chronic stress. Further examination of the data is needed to assess the interaction of stress and E2 on the female brain, and the use of a rhesus money model allows for both an ethologically relevant model of stress for women, and a greater phylogenetic similarity of neuroanatomy and neuroendocrinology to identify these effects.

1.5.1 Hippocampus

The hippocampus regulates HPA axis function by maintaining basal tone as well as GC negative-feedback on the PVN via polysynaptic connections originating in the ventral subiculum and CA1 region and terminating on GABAergic neurons in the BNST or hypothalamus (Herman et al., 2003; Herman, 2005). Chronic exposure to stressors, or infusion of GCs causes both structural and functional alterations in CA1 and CA3 hippocampal regions and this is associated with impaired HPA axis function and cognitive alterations (McEwen, 1999; Joels, 2008). In male rodents, chronic restraint results in the atrophy of apical dendrites in CA3 neurons, without detrimental morphological effects to the CA1 or dentate gyrus (Woolley et al., 1990b; Magarinos and McEwen, 1995a). Physiological alterations within CA1 appear to be mainly related to changes in LTP and neuronal signaling (Joels, 2008). Similar to males, chronic restraint in ovariectomized females results in dendritic retraction in CA3 neurons independent of E2 treatment (McLaughlin et al., 2005). Follow-up studies have suggested that both E2 and cholesterol replacement were able to reverse these effects (Takuma et al., 2007; McLaughlin et al., 2010). Together, these data support stress-induced retraction of CA3 neuronal dendritic fields in both males females, and that the neuroprotective role of E2 is no greater than placebo.

There are some data that support sex-specific and E2-dependent effects on morphological alterations in CA1 neurons following both acute (Shors et al., 2001) and chronic stress (Conrad et al., 2012). In general in the absence of stress, E2's neurotropic effects on the

hippocampus are mainly seen in CA1, and not within CA3 neurons or the dentate gyrus (Woolley et al., 1990a; Woolley and McEwen, 1992). For example, ovariectomy leads to a loss of CA1 spines, which can be rescued with E2 replacement (Gould et al., 1990), and increases in spine density in CA1 are seen during proestrous (Woolley et al., 1990a; Woolley and McEwen, 1992). These effects are thought to include non-genomic changes in neural function as well as genomic changes that reduce neuronal atrophy (McEwen, 1999). History of chronic stress, however, modifies E2's effects on neuronal morphology within the CA1 region. E2 increased CA1 dendritic arborization when subjects were exposed to chronic restraint compared to both a placebo treatment and a no stress condition, although this interaction also resulted in decreased CA1 spine density compared to E2 or stress alone (Conrad et al., 2012). Data from rhesus monkeys also suggests that E2 increases synaptic protein expression within the stratum oriens and stratum radiatum in CA1 (Choi et al., 2003). However, naturally cycling cynomolgus female monkeys with spontaneous expression of a depressed phenotypes (e.g. frequent slumped shoulder posture), show decreased anterior hippocampus volumes (Willard et al., 2009) due primarily to decreased neuronal volume within the CA1 and dentate gyrus compared to non-depressed monkeys (Willard et al., 2013). The overall impact of chronic stress and E2 on total hippocampal volume, however, has not been examined.

Hippocampal atrophy is also seen in stress-related disorders in both men and women (McEwen, 1999; Campbell et al., 2004). Using structural magnetic resonance imaging (sMRI) to measure hippocampal volumes in vivo, longer lifetime duration of depression was associated with smaller volume in women (Sheline et al., 1999). Postmortem

analysis of individuals diagnosed with major depressive disorder indicate that this reduction in volume is associated with decreases in somata volume of pyramidal neurons within the CA1-3 regions and dentate gyrus, in combination with increased density of neurons and glia compared to controls (Stockmeier et al., 2004). No differences were seen between individual CA regions, and all showed a similar pattern of atrophy (Stockmeier et al., 2004).

Altogether these data suggest that chronic stress exposure causes hippocampal atrophy in males, and changes appear mainly due to the retraction of CA3 neurons. While in females chronic stress in combination with E2 may act to increase dendritic arborization or decrease spine density in CA1 neurons in addition to CA3 regions. Data from female monkeys, suggests that chronic stress-induced depressive behavior is associated with decreased CA1 neuronal dendritic field and subsequent volume, although the effects of E2 or chronic stress were not examined. Further investigation is needed to investigate the interaction of stress and E2 on the hippocampus, and identifying alterations in gross volume in rhesus monkeys following E2 replacement and social subordination can help elucidate these effects in an ethologically relevant model of stress for women.

1.5.2 Prefrontal Cortex

The PFC plays a significant role in gating amygdala responses to threat or salient emotional stimuli, therefore regulating behavioral, neuroendocrine, and autonomic responses to stressors, and modulating fear learning and extinction processes (Quirk and

Beer, 2006; Quirk and Gehlert, 2003; Sotres-Bayon and Quirk, 2010). The PFC is also a key inhibitor of the HPA axis through its connections to the hypothalamus, BNST, and NTS (Herman et al., 2005). In male rodents, chronic restraint stress decreases dendritic arborization (Radley et al., 2013; Shansky and Morrison, 2009) and excitability of PFC pyramidal neurons (Jackson and Moghaddam, 2006). This reduction is associated with increased anxiety-like behavior in males (Faraday, 2002).

Females, however, show a different PFC response to chronic stress, which appears E2-dependent. Notably, chronic restraint stress in ovariectomized female rats with E2 replacement results in increased apical dendritic length (Garrett and Wellman, 2009) and spine density in PFC neurons projecting to the BLA (Shansky et al., 2010). These data suggest that chronic stress and E2 act to increase dendritic fields and synaptic density in the amygdala – medial (m) PFC pathway of female but not male rodents. In the PFC E2 has neurotropic effect in the absence of stress, as ovariectomy without E2 reduces spine density of pyramidal neurons (Wallace et al., 2006), while E2 treatment after ovariectomy increases spine density in the medial PFC (Shansky et al., 2010). Similar effects, however, have not been detected across the estrous cycle (Markham and Juraska, 2002). In rhesus monkeys, spine density has also been shown to increase in the dorsolateral (dl)PFC decreased following E2 replacement after ovariectomy (Tang et al., 2004). Chronic stress and E2 appear to act synergistically to cause greater dendritic growth in the PFC compared to E2 alone (Garrett and Wellman, 2009). Interestingly, in normally cycling women E2 concentrations are negatively correlated with PFC volume,

specifically the ACC, (De Bondt et al., 2013), suggesting, that in opposition to the rodent literature, E2 may reduce dendritic complexity in women.

In humans, reductions in PFC volume have also been linked with stress-related disorders such as PTSD (Eckart et al., 2011; Karl et al., 2006) and depression (Kempton et al., 2011). For example, reductions in the volume of the subgenual cingulate cortex are characteristic alterations in individuals with familial major depressive disorder (Drevets et al., 2008). These decreases, however, are not universal (Hamani et al., 2011) and some data suggest no difference in subgenual volume in bipolar or unipolar depression (Brambilla et al., 2002). Postmortem studies of individuals diagnosed with major depression or bipolar disorder suggest that subgenual volume loss may be a result of a reduced number of glia while reduction in volumes of other PFC subdivisions, including the dlPFC and the orbitofrontal (OFC), are associated with reduced cell volumes of both neurons and glia (Rajkowska, 2000).

The rodent literature suggests that chronic stress modifies E2 effects to increase measures of cortical volumes (e.g. spine density and dendritic arborization (Kassem et al., 2013)). However, human data suggest that both E2 and stress-related disorders show a marked decrease in PFC volumes. These contradictory findings could be due to species-specific differences, including those reviewed above in either distribution of ER and GR receptors, Given that women are more susceptible to mood and anxiety disorders than men, and that chronic stress can increase this risk, understanding how a history of chronic stress can modify E2's effects may provide greater insight into the etiology of stress-

related disorders. Further examination is needed to assess this interaction, and again, the use of an ethologically relevant rhesus money model of chronic stress allows for both greater translational potential and phylogenetic similarity of neuroanatomy to identify these effects in a model of chronic stress in women.

1.5.3 Amygdala

Activation of the amygdala is thought to stimulate the HPA axis through indirect connections to the PVN through the BNST, and other regions, including NTS, and additional hypothalamic nuclei (Herman et al., 2005). The amygdala itself is a complex structure and can be divided into many subnuclei, however, three main divisions are thought to be involved in the stress-response including the MeA, CeA, and BLA (Herman et al., 2003). Integration of PFC and hippocampal information through the BLA, and its subsequent activation of CeA output projections is thought to be the primary circuit involved in the regulation of anxiety and fear learning behavior (Davis and Whalen, 2001). In males, chronic stress increases anxiety-like behavior in parallel to increased dendritic arborization in the BNST and BLA (Vyas et al., 2003; Vyas et al., 2006). In the BLA these changes in anxiety-like behavior and neuronal arborization persist even after termination of the chronic stressor (Vyas et al., 2004). Alternatively, spine density in the MeA is reduced following chronic stress (Bennur et al., 2007), while no effects are seen in the CeA, at least in males (Vyas et al., 2003).

The amygdala is responsive to E2, and dendritic spines in medial amygdala (MeA) neurons, a sexually dimorphic region in rodents, show cyclical alterations in spine density

across the estrus cycle (Rasia-Filho et al., 2004). However the highest spine density (Rasia-Filho et al., 2004) and somatic volume (Hermel et al., 2006) was seen during diestrous, just prior to proestrus when E2 and P4 are at their peak. This is a curious finding, as other studies show that ovariectomy followed by E2 replacement increases dendritic spine density in the posterodorsal MeA and that was further potentiated by P4 replacement (de Castilhos et al., 2010). In the BLA, though, females showed no difference in dendritic arborization or spine density across the estrous cycle, although there was a trend for decreased spine density during proestrous (Rubinow et al., 2009). Similarly, amygdala volume in human healthy women without psychopathology is reduced in the late follicular phase when E2 levels are at their peak (Ossewaarde et al., 2013). To the best of my knowledge, there are no studies in the literature on the effects of stress on the E2 actions on amygdala morphology (Farrell et al., 2013).

Data on amygdala volume in patients with anxiety or depressive disorders are mixed. Decreased amygdala volumes are evident in individuals with PTSD (Karl et al., 2006b; Rogers et al., 2009), while data on major depressive disorder suggest no effects on amygdala volume (Hajek et al., 2009). A review of the literature also suggests equivocal findings in the amygdala (Tottenham and Sheridan). Differences in etiology of depression and gender may account for variability in the effects on amygdala volume across patients. A recent study suggests that in females, but not males, amygdala volumes depended on family history of depression, with increased volume see in those without prior history (Saleh et al., 2012). Life history of stressor exposure or other factors that may precipitate onset of depression, however, were not assessed (Saleh et al., 2012).

1.5.4 Summary

There is limited data on how exposure to chronic stressors modifies the effects of E2 on adult brain volumes and underlying cellular morphology, particularly of PFC-amygdala-hippocampal regions. Behavioral data reviewed previously indicates that chronic stress can attenuate E2's activational effects on both reproductive and non-reproductive behaviors. These alterations in socioemotional behavior may be the result of underlying changes in brain structure. To date, the information we do have suggests that stress and E2 can both potentiate and attenuate CA1 neuronal morphology and result in synergistic increases in PFC dendritic complexity. Less is known about the interaction of stress and E2 on the amygdala, but its involvement both in mediating sexual behaviors (MeA) and anxiety and fear behaviors (CeA, BLA) suggests that the amygdala may show modulation of E2's effects. To better understand how exposure to chronic stress modifies the effects of E2 on brain morphology, and the possible protective or detrimental interactions between them, prospective studies that can manipulate E2 concentrations in the context of different levels of stressor exposure are needed. Although the majority of the information available comes from rodent models of chronic stress, differences in organization of the PFC in rodent and primate neuroanatomy (Preuss, 1995) suggest the use of a non-human primate model is critical to examine the effects of stress and E2 on structural changes in volumes in the hippocampus, amygdala, and PFC.

1.6 Estradiol effects on brain functional connectivity: Interactions with chronic stress

As outlined above, E2 and chronic stress have numerous independent influences on behavior and brain structure, and these data suggest subsequent alterations in brain function. It is possible that the two are dissociated, with structural differences in brain volume and functional connectivity between these structures being unrelated. However, both approaches are informative and together may provide a more complete understanding normal and disrupted brain function. Importantly, there are no studies elucidating these effects in animal models, and effects in humans are mainly observational without randomized controlled studies. Studying both structure and function in a translationally relevant animal model, such as the rhesus monkey model of subordination stress, will help us better understand the association between chronic stress and disease, as well as the increased vulnerability to develop mood and anxiety disorders in females.

Data from electrophysiological studies following chronic stress in males, suggests that stress-induced reductions in dendritic morphology in the PFC (Radley and Morrison, 2005) may be associated with decreased coherence of functional activity with both the amygdala and hippocampus (Lee et al., 2011). In males, data specific to the amygdala – PFC pathways suggests that stress increases both mPFC (Jackson and Moghaddam, 2006) and amygdala excitability (Rosenkranz et al., 2010), while also reducing amygdala – mPFC long-term potentiation (Joels et al., 2007; Maroun and Richter-Levin, 2003) and synchronization of electrophysiological activity between these regions, suggestive of impaired top-down inhibitory control of the amygdala by the mPFC (Lee et al., 2011). There have been no similar experiments in electrophysiology in females following

chronic stress and E2 treatment. As described previously, plasticity of the amygdala - PFC pathway in females following chronic stress is associated with increased, and not decreased, synaptic complexity of PFC neurons (Shansky et al., 2009). PFC neurons projecting to the BLA are thought to be excitatory, but ultimately exert inhibitory effects on BLA output, likely mediated by inhibitory interneurons (Rosenkranz and Grace, 2002). Intracellular recordings in slice preparation showed reduced excitatory postsynaptic potentials (EPSPs) of pyramidal BLA neurons (projecting out of amygdala) in bath applications of E2 versus saline (Womble et al., 2002). One hypothesis linking these effects together is that following chronic stress, E2 increases excitatory input to the BLA to suppress its output, and may show increased coherence in functional activity.

One method of examining neural connectivity in vivo is by examining low frequency oscillations (<.1Hz) in blood-oxygen level dependent signal (BOLD) in resting-state (rs)-fMRI analysis (van den Heuvel and Hulshoff Pol, 2010). Correlations in the resting BOLD signal recapitulate functional brain networks (e.g. motor network) and are thought to be proximate measures of functional connectivity (FC) in the brain (Biswal et al., 1995). A second method of examining neural connectivity, measures the correlation of low frequency BOLD signal during task-based fMRI (Hampson et al., 2002). This method is sensitive to the task being preformed and correlations in BOLD signal reflect functional coupling during the task, and not intrinsic FC detected during resting-state (Buckner et al., 2013).

In humans, increased PFC – amygdala FC is associated with enhanced top-down deactivation of amygdala activity (Hare et al., 2008). Increased structural connectivity (Kim and Whalen, 2009) and FC (Kim et al., 2011a) in this pathway is also associated with lower levels of trait anxiety in healthy individuals. Conversely, individuals with stress-induced psychopathology show a marked decrease in amygdala - PFC FC (Hahn et al., 2011; Prater et al., 2013; Stevens et al., 2013). One possibility is that chronic stress reduces the FC within this pathway, resulting in disruption of socioemotional processing. Given the sex-differences apparent in the rodent literature and the effects of stress and E2 on both structure and function, the increased incidence of stress-related psychopathology in women may be in part due to how exposure to chronic stress alters the effects of E2 on the amygdala – PFC pathway.

Considered together, the rodent model suggests increased FC within this pathway, which in light of the human data further suggest enhanced top-down modulation of the amygdala. This would then suggest that E2 facilitates this top-down modulation. Therefore the gap between E2's putative facilitation effects on amygdala – PFC FC and the significant decreases in FC in mood and anxiety disorders needs further attention. This is especially true given the increased vulnerability of women to developing mood and anxiety disorders. Exposure to chronic stress may in fact reduce E2's facilitation of FC.

1.7 Aims

The data presented in this introduction serve to support my thesis that chronic stress modifies the effects of E2 on both brain and behavior, and provides a possible explanation for the incidence of mood and anxiety disorders in women. Neurobiological data show that both E2 and stressor exposure, whether mediated by CGs or CRF, can have long-lasting genomic effects on brain structure even in adulthood. These brain structures include cortico-limbic regions which and modulate both reproductive and non-reproductive behaviors. Importantly, although chronic stress can attenuate the behavioral effects of E2, less is known about the role of E2 and brain structure and functional connectivity and how these are modulated by exposure to chronic stressors.

To better understand the role of both chronic stress and E2 on the female brain and behavior, I will examine three independent hypotheses in a ethologically relevant rhesus monkey model of chronic stress. In Aim 1, I will examine the hypothesis that social subordination attenuates the activational effects on behavior and that higher doses of E2 will rescue these effects. In Aim 2, I will examine the hypothesis that social subordination attenuates E2 plasticity on brain structure in the hippocampus, amygdala, and PFC. In Aim 3, I will test whether social subordination attenuates the effects of E2 on the PFC – amygdala functional connectivity.

These studies presented in this dissertation therefore aim to resolve several gaps in the literature of stress-related attenuation of E2's activational effects. Initially, to provide data on the modification of E2's effects on rhesus monkey behavior following a history of chronic stress, I will attempt to replicate findings from the rodent literature that

suggest that higher doses of E2 are need to overcome the attenuating effects of stress. Once the effects of both chronic stress and E2 on behavior have been established in the rhesus monkey model of chronic subordination stress, I will attempt to understand the neurobiological underpinnings of these effects using in vivo neuroimaging techniques. These studies will make a significant contribution to the field, as there are limited data supporting neurobiological effects of E2 and their modification by history of chronic stress exposure. Understanding the structural and functional alterations in the brain, specifically within cortico-limbic circuits, in the social subordination model of female rhesus monkeys will provide novel data on the possible interaction of E2 and chronic stress that may make the female brain more vulnerable to stress-induced psychopathology.

Chapter 2: Social status modifies estradiol activation of sociosexual behavior in female rhesus monkeys

2.1 Abstract

Estrogen (E2) has activational effects on sexual motivation and mitigating effects on anxiety-like behaviors that can be attenuated with chronic exposure to psychosocial stress. Some studies suggest that this attenuation can be overcome by higher doses of E2, while others show that chronic psychosocial stress may alter the mechanisms of E2 function, thus reducing any positive benefit from higher doses of E2. To determine the interaction between psychosocial stress and E2 dose on behavior, we examined the scope of attenuation across a suite of socioemotional behaviors, including reproduction, affiliation, aggression, submission, and anxiety-like behaviors on 36 ovariectomized female rhesus monkeys. Females were exposed to graded psychosocial stress, established by an intrinsic female dominance hierarchy, where subordinate animals receive high amounts of harassment. Our data show that E2 dose-dependently increased sexual motivation and male-affiliation in dominant (e.g. low-stress) females, while subordinate females showed no positive effects of E2, even at higher doses. In addition, contact aggression was attenuated in dominant females, while non-contact aggression was attenuated in both dominant and middle-ranking females. These results suggest that the stress-induced attenuation of E2's activational effects on sexual behavior and affiliation with males may not be overcome with higher doses of E2. Furthermore, the observed behavioral consequences of psychosocial stress and E2 dose may be dependent on the behaviors of all the females in the social-group, and better resolution on these effects depends on isolating treatment to individuals within the group to minimize alterations in social-group interactions.

2.2 Introduction

While the activational effects of estradiol (E2) on the expression of sociosexual behavior are well established (Beach, 1976; Wallen and Goy, 1977; Wallen, 1990; Zehr et al., 1998) and the observed magnitude of these behaviors is disrupted by exposure to chronic and acute stress (White and Uphouse, 2004; Pierce et al., 2008), the mechanisms underlying the synergistic effects of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis and the limbic-hypothalamic-pituitary-gonadal (LHPG) axis on behavior are unknown. In response to a stressor, the LHPA axis mounts a systemic response to increase adrenal glucocorticoid secretion, and under conditions of chronic stress, a sustained LHPA axis response can lead to subsequent hypo- or hypercortisolemia and a dysregulation of glucocorticoid negative feedback (Chrousos and Gold, 1992; Chrousos, 2009). Consequently, chronic exposure to stress or stress hormones can potentiate E2 negative-feedback inhibition of LHPG axis function (Ronnekleiv et al., 2010) and result in reduced hypothalamic release of gonadotropin releasing hormone (GnRH) (Oakley et al., 2009), pituitary release of luteinizing hormone (LH) (Chrousos et al., 1998; Berga and Loucks, 2007; Michopoulos et al., 2009), or impair normal ovarian function and subsequent ovulation (Adams et al., 1985). The mechanism for this inhibition, however, is unclear, as administration of exogenous glucocorticoids do not consistently interfere with reproductive physiology in women (Saketos et al., 1993; Samuels et al., 1994) or monkeys (Lovejoy and Wallen, 1990).

In addition to the potentiation of E2 negative-feedback of the LHPG axis, stress can concomitantly attenuate the expression of E2's activational effects on female sexually motivated behavior in ovariectomized rats (White and Uphouse, 2004), ewes (Pierce et al., 2008), and intact rhesus monkeys (Wallen, 1990). In ovariectomized female rats receiving E2 and progesterone (P4) replacement, over-expression of corticotropin-releasing factor (CRF) in the central nucleus of the amygdala inhibits expression of sexual behaviors (Keen-Rhinehart et al., 2009). However, supraphysiological concentrations of E2 can rescue lordosis behavior in rodents following acute exposure to restraint stress (White and Uphouse, 2004; Uphouse et al., 2005), suggesting a balance between the LHPA and LHPG axes. Nevertheless, E2 reinstatement of sexual behavior following stress has only been shown following acute physical restraint, and may not generalize to sexual behavior following chronic exposure to stress. Support for the latter comes from behavioral observations of socially subordinate intact rhesus monkeys, whereby social subordination is thought to act as a chronic stressor, that increased levels of E2 are needed to induce sexual behavior (Wallen, 1990). Subordinate females expressed sexual behavior mainly during peak periovulatory E2 concentrations, whereas more dominant females expressed both proceptive and receptive behaviors significantly earlier in the cycle when E2 levels were lower, suggesting higher sensitivity to E2's activational effects. Despite the implications of these data, little is know about the interaction between graded exposure to chronic social subordination stress and increasing doses of E2 on the expression of female sociosexual behavior.

In conjunction with sexual motivation, data from human clinical research and animal models demonstrate a role for E2 in altering anxious, affiliative, and aggressive behavior in females, although the directionality of the effect may be context dependent. In human clinical studies, major hormonal shifts in LHPG activity precipitate onset of mood and anxiety disorders (Halbreich and Kahn, 2001), and E2 replacement therapy (ERT) is anxiolytic and anti-depressive during peri-menopause (Cagnacci et al., 1997; Schmidt et al., 2000) and post-partum (Moses-Kolko et al., 2009). Rodent studies, however, suggest that the effects of E2 are often inconsistent, as E2 can act to increase or decrease anxiety depending on its interaction with E2 receptors (ERs) (Lund et al., 2005; Walf and Frye, 2005b), and it can be anxiolytic in familiar environments such as a female's home cage while anxiogenic in novel environments (Morgan and Pfaff, 2001). Data on the effects of E2 on either affiliation or aggression are more limited. The majority of rodent research focuses on E2's role in augmenting maternal bonding and increasing post-partum maternal aggression, and not on its influence on prosocial or agonistic behavior (Bos et al., 2012). Studies observing gonadally intact rhesus monkeys suggest that affiliation toward males and aggression toward females increases with peak estrus behavior (Carpenter, 1942) and in conjunction with peak ovarian E2 (Walker et al., 1983; Wallen et al., 1984). Ovariectomized rhesus monkeys given E2 replacement, without access to male partners, also show an amplification of aggression toward females along with an attenuation of anxiety-like behaviors (Pope et al., 1987; Michopoulos et al., 2011). Increased female-female aggression, however, was seen in conjunction with increased affiliative behavior between females, suggesting that higher rates of proximity and interaction may be linked to higher rates of aggression or vice versa. Taken together,

these data suggest that chronic stress can moderate E2's effects on anxious, aggressive, and affiliative behavior, similar to that seen in sexual behavior.

The present study tested the hypothesis that stress from chronic subordination diminishes the behavioral efficacy of E2 on a range of socio-emotional behaviors in female rhesus monkeys. We hypothesize that females exposed to chronic stress develop a physiological state that blunts E2's dose dependent beneficial effect on attenuating anxiety and increasing motivation to engage in sexual behavior, prosocial male-directed behaviors, and aggressive female-directed behaviors. Furthermore, we hypothesize that socially subordinate females will require higher levels of E2 in order to stimulate these behaviors.

2.3 Material and Methods

2.3.1 Subjects

Subjects were 50 ovariectomized adult female rhesus monkeys (*Macaca mulatta*) socially housed and maintained at the Yerkes National Primate Research Center (YNPRC). Ten groups of five females, each with one resident adult male, were housed in run-type enclosures that measured 20 X 15 X 8 feet each and included both indoor and outdoor areas. Animals were between the ages of 11-14, and were fed a standard commercial low-fat high-fiber diet (Ralston Purina Company, St. Louis MO) *ad libitum* supplemented daily with seasonal fruits and vegetables. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) 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 groups were comprised of previously multiparous adult females between 7 and 10 years of age, selected from larger breeding group as described previously (Jarrell et al., 2008). Females were ovariectomized six months prior to small group formation and then placed in one of ten groups of five unfamiliar female conspecifics, and remained in these groups for at least four years prior to the onset of the present study. An individual resident male was introduced into each group approximately three years following group formation. Since social group formation, females participated in several protocols in which they received replacement therapy with physiological concentrations of gonadal steroids (2001; Michopoulos et al., 2009; Michopoulos et al., 2011). All females had at least three months of E2-washout between the completion of any previous study and the beginning of the current study. During the course of data collection, ten females were unable to complete all treatment conditions due to intermittent health concerns, and were therefore excluded from the analysis, reducing the sample size to 40 rhesus monkey females. Four additional females were excluded based on changes in social rank bringing the final sample size to 36.

2.3.2 Social Subordination Stress

The use of dominance social hierarchy to model adverse health outcomes associated with chronic psychosocial stress is well-established in the literature (Adams et al., 1985; Kaplan et al., 1996; Paiardini et al., 2009; Michopoulos et al., 2011; Michopoulos et al.,

2012a). Rhesus monkey social groups are organized by a linear dominance hierarchy that maintains group stability (Bernstein, 1970) at the expense of lower-ranking females, which receive disproportionally greater amounts of aggression from higher-ranking females and engage in higher levels of submissive behavior (Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984). As a consequence, subordinate females are exposed to unstable and stressful environments, which can result in LHPA-axis dysregulation (Michopoulos et al., 2012b) evinced through increased adrenal gland size (Kaplan et al., 1984) and hypercortisolemia operationally defined as elevated cortisol response to social challenge (Cohen et al., 1997) and impaired physiological responses to dexamethasone suppression (Shively et al., 1997; Jarrell et al., 2008; Wilson et al., 2008; Kaplan et al., 2010) or ACTH challenge (Shively, 1998).

In the wild, social groups have a matrilineal structure in which social rank is determined by birth (de Waal and Luttrell, 1985), and familial, or kin support acts as a natural buffer to rank-related stress (Kikusui et al., 2006). In the present study, all females were taken from middle ranking social groups within larger compounds, and placed in small non-kin groups to maximize rank-related social stress (Jarrell et al., 2008). New social status rankings were determined based on observations of dyadic agonistic interactions (e.g. submissive behaviors) between females within a group. Females were then classified into one of three rank categories; Alpha – the highest ranking female (N=8), Middle – the second and third ranking female (N=16), and Subordinate – the fourth and the fifth ranking female (N=12). This division reflects the uniqueness of the alpha female, who receives little to no aggression and does not express submissive behaviors during dyadic

interaction (Table 2), and therefore is not exposed to the chronic stress of social subordination (Michopoulos et al., 2011). A total of 4 females changed ranks midway through the 12-month study, such that they could no longer be classified as one of the three ranking categories, and were therefore excluded from the current analysis (N=36, Table 2.1).

2.3.3 Experimental Design

Rhesus monkeys exhibit a seasonal breeding cycle with ovulatory cycles and fertility occurring from August to April followed by anovulation, secondary to reduced gonadotropin secretion during the May to July nonbreeding season (Walker et al., 1984). Therefore, all animals were studied during months defined as the breeding season for gonadally intact females, notably from January to April, 2010 followed by August to December, 2010. As data collection was spread across two consecutive breeding seasons, all doses were counterbalanced across time. The conditions consisted of one placebo treatment (0μg/kg/day) and three E2 replacement treatments at low (2μg/kg/day), medium (4μg/kg/day), and high (8μg/kg/day) doses delivered using 21-day sustained release pellets (Innovative Research of America) containing either 17β-estradiol or cholesterol (placebo). Pellets were implanted subcutaneously between the scapula following anesthesia with ketamine (10mg/kg intramuscular injection), antiseptic preparation of the site, and sterile procedures as described previously (Mook et al., 2005). Each pellet was designed to continuously release E2 at one of the four treatment doses over the course of three weeks, and was followed by a minimum of a three-week washout period of no treatment. Members of each small group received identical treatments, and

order of treatment conditions was counterbalanced across groups. Pellets were implanted three days prior to the initiation of behavioral observations.

2.3.4 Behavioral Outcome Measures

Behavioral group observations, consisting of simultaneous focal group observations on the members of each small group, were conducted live for 30 min, at 14:00 hr on three separate days during both week one and week three of each treatment. Thus, a total of six observations were obtained ranging from day 3 to day 20 of each treatment. Observers were blind to dose of E2, however, due to swelling and pigment changes in face, genitals, and buttocks following E2, observers were not blind to control versus E2 treatment. Data were recorded in an actor – behavior – recipient format using the "HandObs" program developed by the Center for Behavioral Neuroscience (Graves and Wallen, 2006). Inter-observer reliability exceeded 90% in each of three live reliability trials. Behavior was coded using a well-established ethogram (Pope et al., 1987; Jarrell et al., 2008) and was analyzed both individually and in the behavioral categories of (1) proceptive sexual behavior directed toward males (e.g. female hindquarter presentation, handslap, standup, threataway, or crouch behavior), (2) receptive sexual behavior toward males (e.g. received hiptouch or mount from male), (3) affiliative behavior toward males (initiates proximity or grooming), (4) affiliative behavior toward females (initiates proximity or grooming), (5) anxiety-like behavior (yawns, body-shakes, pacing, or self-directed scratching and bodily exploration), (6) agonistic behavior toward females (attacks, chases, or threats with and without contact), and (7) submissive behavior toward females

(withdraws or grimaces). Additionally, duration (seconds) of affiliative behavior directed and received from both males and females was calculated.

2.3.5 Hormonal Assay

To confirm E2 concentrations, 3mL of serum were collected from the saphenous vein located on the back of the leg directly following observations. Animals were trained to present their hind legs for conscious venipuncture, which allows for the collection of blood in awake unanesthetized monkeys. Serum was collected at five time points, two during week one, one during week two (no behavioral observations), and two during week three. All assays were done in the Biomarkers Core Laboratory at the YNPRC using a modification of a previously validated commercial assay (Siemens/ Diagnostic Products Corporation, Los Angeles, CA (Pazol et al., 2004). The assay had a sensitivity of 5 pg/mL and intraassay and interassay coefficients of variation (CVs) of 7.95% and 11.3% respectively.

2.3.6 Statistical Assessment

Data were analyzed using repeated measures analysis of variance (rmANOVA) with rank categorization (Alpha, Middle, Subordinate) and genotype (serotonin transporter, long and short variant: see Jarrell et al., 2008) as between subject factors, and dose (0, 2, 4, 8 μg/kg/day), week (1^{st}, 3^{rd}), and observation (1, 2, 3) as with-in subjects factors. Main and interaction effects of genotype and observation are not reported. All analyses were conducted using IBM SPSS 19, a statistical software package. Variance was not normally distributed across all variables, and data were log transformed using the formula

$Log_{10}(X+1)$ to account for values of zero. Variance and sphericity were reduced with this transformation, but not all values were normally distributed following the transformation. These data are reported with a Greenhouse-Geisser correction. All results where $p \leq .05$ were considered significant, and post-hoc tests were conducted, if necessary, using a Bonferroni correction for multiple comparisons. All non-significant main and interaction effects with a $p<.10$ were reported with an effect size (Cohen's d), and a $d>.50$ was considered a non-significant trend. Results are summarized as mean ± standard error of the mean (SEM) of untransformed data.

2.4 Results

2.4.1 Hormonal Assays

Serum samples collected during E2 treatment (Figure 2.1) show a significant main effect of dose ($F_{2,50}$ =35.67, $p<0.001$; low dose: 36.8±4.8pg/mL, medium dose: 80.3±7.4pg/mL, high dose: 128.2±11.5 pg/mL) and sample time point ($F_{2,50}$ =41.60, $p<0.001$) on serum E2, but no other main effects or interaction effects were significant. Serum samples collected during placebo treatment were not assayed for E2, because serum concentrations in ovariectomized females are below assay detectability (Zehr et al., 1998). As serum levels of E2 were significantly different across time, we further investigated the data to assess the differences in serum E2 between observational weeks 1 and 3 in a second rmANOVA model using rank as a between subject variable, and dose (2,4,8 μg/kg/day), week (1,3), and sample number (1,2) as with-in subject variables. Results showed a significant main effect of dose ($F_{2,54}$ =39.75, $p<0.001$;) and week ($F_{2,54}$

=93.25, p<0.001) on E2, but no effect of rank ($F_{2,27}$ =0.54, p=0.588). Additionally, there was a non-significant trend for a dose by week interaction ($F_{2,54}$ =3.36, p=0.060, d=0.51). Post hoc tests were significant for all dose x week interactions, such that the 8µg/kg/day dose resulted in a higher serum E2 concentration than the 4ug/kg/day dose which was greater than the 2µg/kg/day dose and week one was greater than week three (Table 2.2).

2.4.2 Sexual and Social Behavior

Rank had a significant effect on proceptive ($F_{2,30}$ =4.11, p=0.026) and receptive sexual behaviors ($F_{2,30}$ =12.57, p<0.001), as well as on female initiation of affiliation towards males ($F_{2,30}$ =5.47, p=0.009) and affiliation toward females ($F_{2,30}$ =4.41, p=0.021). Post hoc analysis determined that alpha females had a higher frequency (per 30 minutes) of proceptive sexual behavior (1.03 ± .19) than subordinate females (.21 ± .15, p=0.024) and a higher frequency of receptive sexual behavior (1.89 ± .30) compared to both middle-ranking (.28 ± .21, p<0.001) and subordinate females (.30 ± .24, p<0.001). Alpha females also showed a higher frequency of initiating male-directed affiliation (1.78 ± .43) compared to subordinate females (.40 ± .34, p=0.007), while female-directed affiliation was highest in middle-ranking females (3.0 ± .44) and greater than subordinate females (1.34 ± .50, p=0.024).

There was a main effect of E2 dose and an interaction between dose and rank for both proceptive sexual behavior (dose: $F_{3,90}$=3.41, p=0.021; interaction: $F_{6,90}$=2.30, p=0.041; Figure 2.2a) and receptive sexual behavior (dose: $F_{3,90}$=4.84, p=0.008; interaction; $F_{6,90}$=2.45, p=0.031; Figure 2.2b), but not male-directed affiliation (dose: $F_{3,90}$=1.74,

p=0.182; interaction: $F_{6,90}$=.77, p=0.592; Figure 3) or female-directed affiliation (dose: $F_{3,90}$ =0.17, p=0.914; interaction: $F_{6,90}$=0.28, p=0.945, Table 2.3). In comparison to the lowest dose, proceptive sexual behavior was maximally enhanced at a medium dose of 4μg/kg/day (p=0.046), while receptive sexual behavior was enhanced at both a medium dose (4μg/kg/day, p=0.026) and a high dose (8μg/kg/day, p=0.011) of E2. Post hoc analysis of the interaction between dose and rank revealed that at the medium dose, alpha females showed significantly more proceptive sexual behavior than middle-ranking females (p=0.008) and subordinate females (p=0.001) and significantly more receptive behavior than middle-ranking females (p=0.001) and subordinate females (p=0.001). There were no significant differences between middle-ranking and subordinate females for either proceptive (p=0.612) or receptive (p>0.999) behavior. At the highest dose, alpha females showed greater frequency of receptive behavior only when compared to subordinate females (p=0.048). Within ranks, alpha females showed a higher frequency of proceptive behavior following the medium dose compared to both the placebo (p=0.019) and low dose (p=0.024), as well as a higher frequency of receptive sexual behavior following both the medium (p=0.002) and the high (p=0.034) dose as compared to the low dose. There was no significant difference between the medium and high dose in proceptive sexual behavior (p=0.058) or receptive sexual behavior (p=0.585). Middle ranking and subordinate females did not show any significant alterations in proceptive or receptive sexual behavior as a result of E2 dose.

There was no main effect of observation week when looking at proceptive ($F_{1,30}$=1.06, p=0.313) and receptive ($F_{1,30}$=3.01, p=0.093, d=.39) sexual behavior. However,

proceptive behavior did show a dose by week interaction effect ($F_{3,90}$=2.96, p=0.036) while receptive behavior did not ($F_{3,90}$=1.53, p=0.222; Table 2.2). Post hoc analysis showed that the only significant difference in behavior between week one and week three of treatment was seen in the placebo dose. There was no change in behavior as a result of treatment week for the 2, 4, or 8 μg/kg/day doses. The behavioral data shows that the difference in the placebo dose was due to reduced sexual activity during week three as compared to week one (p=0.012).

Since dose did not have an effect on either male or female affiliation, data were collapsed across all non-placebo doses. Results showed that there was a main effect of E2 treatment on male-directed affiliation ($F_{1,30}$ =9.45, p=0.004, Figure 2.3), but not female-directed affiliation ($F_{1,30}$ =0.85, p=0.363). Affiliation directed toward males and females both showed a main effect of Rank (male: $F_{2,?0}$ =5.50, p=0.009; female: $F_{2,30}$ =4.09, p=0.027) but no interaction effect of Treatment and Rank (male: $F_{1,30}$ =0.04, p=0.840; female: $F_{1,30}$ =0.01, p=0.915). Post Hoc analysis showed that following E2, females initiated significantly more affiliative behaviors toward males (Placebo: 0.75±0.17, E2: 1.15±0.24, p=0.031), and alpha females increased male-directed affiliation (Placebo: 1.25±0.34, E2: 2.77±0.63, p=0.020), while middle-ranking (Placebo: 0.77±0.24, E2: 1.04±0.34, p=0.075) and subordinate females (Placebo: 0.24±0.27, E2: 0.45±0.39, p=0.365) did not.
Further analysis was done to identify E2's effects on time spent in affiliation, as opposed to frequency of initiating affiliation. Total affiliation time was analyzed using rmANOVA with sex of partner as a with-in subject measure. Sex of partner significantly affected duration of affiliation ($F_{1,30}$=10.80, p=0.003), being longer with female (4.06±0.42s) than

male partners (2.11±.61s), but durations were unaffected by E2 dose ($F_{3,90}$=1.17, p=0.325) or rank ($F_{2,30}$=1.54, p=0.231). An interaction with sex-of-partner and rank ($F_{2,30}$=7.19, p=0.003) emerged, such that middle-ranking females (female: 5.40±0.54s; male: 2.96±0.78s, p<0.001) and subordinate females (female: 4.0±0.56s; male: 0.81±0.82s, p=0.001) were found to spend more time with females compared to males, while alpha females distributed affiliation time equally between males and females (female: 3.0±0.79s; male: 3.04±1.15s, p=0.234).

2.4.3 Agonistic Behavior

There was a main effect of rank on aggression directed toward other females ($F_{2,30}$=5.02, p=0.013), aggression received from other female ($F_{2,30}$=15.35, p<0.001), and submission toward females ($F_{2,30}$=18.50, p<0.001; Table 2.3). However, there was no main effect of dose, or an interaction between dose and rank on directed aggression (dose: $F_{3,30}$=0.97, p=0.399; interaction: $F_{6,90}$=0.77, p=0.596) or aggression received (dose: $F_{3,30}$=1.51, p=0.225; interaction: $F_{6,90}$=0.92, p=0.484). There was a non-significant trend for a main effect of dose on submission ($F_{3,30}$=2.42, p=0.071, d=.59) but not a dose by rank interaction ($F_{6,90}$=1.23, p=0.299). Additionally, there were no significant main effect of observation week on the frequency (per 30 minutes) of aggression directed toward other females (week one: 2.24±0.56; week three: 2.56±0.59, $F_{1,30}$=3.21, p=0.084, d=.41), aggression received from other female (week one: 1.77±0.51, week three: 2.18±0.55, $F_{1,30}$=2.72, p=0.110), and submission toward females (week one: 4.17±0.74, week three: 5.21±1.01, $F_{1,30}$=18.50, p=.056, d=.49).

As the aggression category included both contact and non-contact aggressive behaviors that may reflect different levels of motivation, each was analyzed independently using the same rmANOVA model. There was a significant main effect of dose and a main effect of rank, but no interaction effect between dose and rank for both contact aggression (dose, $F_{3,90}$=3.80, p=0.022; rank, $F_{2,30}$=11.89, p<0.001; interaction, $F_{6,90}$=1.48, p=0.212) and non-contact aggression (dose, $F_{3,90}$=7.40, p=0.001; rank, $F_{2,30}$=4.57, p=0.019; interaction, $F_{6,90}$=1.46, p=0.215). To compare the frequencies of these two aggression types, another rmANOVA model was run with type of aggression (contact versus non-contact) as a within subjects factor. There was a main effect of aggression type ($F_{1,30}$ =32.38, p<0.001), rank ($F_{2,30}$=6.36, p=0.005), and dose ($F_{3,90}$=7.72, p<0.001), and an interaction between dose and aggression type ($F_{3,90}$=5.92, p=0.001, Figure 2.4). Post hoc analysis illustrated that frequency of non-contact threat (2.73±0.67) during 30 minute observations was significantly greater than that of contact threat (0.16±0.03, p<0.001), and that E2 uniquely attenuated the expression of contact and non-contact aggression. Contact aggression was significantly attenuated in alpha females with a medium dose of 4μg/kg/day, when compared with either the placebo (0μg/kg/day, p=0.039) or the high dose (8μg/kg/day, p=0.007). However, E2 attenuated non-contact aggression in both alpha and middle-ranking females. Alpha females showed reduced non-contact aggression with either a dose of 2μg/kg/day (p=0.017) and 4μg/kg/day (p=0.039), while E2 attenuated non-contact aggression in middle-ranking females at all doses (2μg/kg/day, p=0.002; 4μg/kg/day, p=0.050; 8μg/kg/day, p=0.004).

2.4.4 Sociosexual versus Aggressive Behavior

In order to better compare expression of sexual motivation, male-directed affiliation, and female-aggression across all doses and rank categories, we added the frequency of sexual behaviors and male-directed affiliation for each female across all six observations (180 minutes) during each of the four E2 treatment conditions to create a new 'sociosexual' behavior variable for each female at each dose. In parallel, we added contact and non-contact aggressive behaviors given across all six observations to create an 'aggressive' behavior variable. These two behavioral categories were analyzed using a new rmANOVA, including both rank categorization (Alpha, Middle, Subordinate) and genotype (not reported) as between subject factors, and dose (0, 2, 4, 8 μg/kg/day) and behavior (sociosexual, aggressive) as within subject factors to statistically test if sociosexual and aggressive behavior were similarly influenced by social rank and E2 dose. We found that, as expected, there was a main effect of dose ($F_{3,90}$=3.50, p=0.033), a main effect of rank ($F_{2,30}$=13.59, p<0.001) on both behaviors combined, but no main effect of behavior sub-type ($F_{1,30}$=1.90, p=0.178; Figure 2.5). Additionally, the data show a significant interaction between dose and behavior type ($F_{3,90}$=4.56, p=0.005) and a non-significant trend for an interaction between dose, behavior, and rank ($F_{6,90}$=1.99, p=0.075, d=0.70), indicating no significant difference in the occurrence of sociosexual and aggressive behavior and that E2 doses do not uniformly alter the occurrence of sociosexual and aggressive behaviors. Post hoc analysis show that at a dose of 4μg/kg/day, total sociosexual behavior observed (22.79±4.95) is greater than aggressive behavior (12.10±3.21; p=0.016) while the occurrence of these behaviors was not significantly different at other doses (p's>0.05). Furthermore, this difference is driven by

the alpha female, who alone shows significantly greater sociosexual behavior compared to aggressive behavior following only the medium 4μg/kg/day dose ($p=0.002$).

2.4.5 Anxiety-Like Behavior

Grouping all anxiety-like behaviors together, there were no rank, dose, or interaction effect on anxiety-like behavior (rank: $F_{2,30}=2.17$, $p=0.132$; dose: $F_{3,90}=0.05$, $p=0.986$; interaction: $F_{6,90}=1.65$, $p=0.142$; Table 2.2). There was no effect of week on frequency of anxiety behavior ($F_{6,90}=0.05$, $p=0.828$). Only when looking at individual anxious behaviors collapsed across all E2 doses, did E2 significantly increased self-scratch (Placebo: 2.84±0.49, E2: 2.90±0.31, $F_{1,30}=5.33$, $p=0.028$) and self-explore behavior (Placebo: 0.71±0.14, E2: 0.80±0.12, $F_{1,30}=7.40$, $p=0.011$). Similarly, across all doses, an interaction effect emerged between treatment and rank on yawning behaviors ($F_{2,30}=3.77$, $p=0.035$). Post hoc analysis showed that E2 increased yawning in middle-ranking (Placebo: 0.38±0.19, E2: 0.52±0.14, $p=0.011$) females. We found no main effect of rank on any individual anxiety-like behaviors (all $p<0.10$).

2.5 Discussion

The current findings demonstrate that exogenous administration of E2 increases sexual and male-directed social behavior in female rhesus monkeys, thus adding support for the activational effects of E2 on rhesus monkey behavior as suggested by the correlation of endogenous E2 with increased sexual behavior (Wilson et al., 1982; Wallen et al., 1984;

Michael and Zumpe, 1993). Importantly, social subordination attenuated E2's activational effects on sociosexual behavior, and higher doses of E2 were not sufficient to increase the frequency or duration of these behaviors in subordinate females. E2's main effect on behavior was seen in addition to the influence of rank on sexual and male-affiliative behaviors, suggesting a synergistic effect of both treatment and social status on sociosexual behavior. These findings are novel in light of the current literature which suggests that concentrations of endogenous (Wilson et al., 1982; Wallen et al., 1984; Michael and Zumpe, 1993) or exogenous (Zehr et al., 1998) E2, higher than those observed in the current study, continue to increase sexual behavior in rhesus monkeys. The current study additionally provides data on the effects of E2 dose and chronic social subordination on non-mating behaviors, showing that E2 replacement attenuated female agonistic behaviors in dominant and middle-ranking females. In contrast, E2 had no effect on either female-directed affiliation or self-directed anxiety-like behavior, suggesting that E2's putative effects on alleviating anxiety-like behavior are not seen in rhesus social group interactions. These findings further suggest that the dose-dependent increase in sexually motivated behaviors by E2 is attenuated in lower ranking female rhesus monkeys, despite the alleviation of aggressive behaviors by alpha and middle-ranking females and no detectable change in anxiety-like behavior.

Why the largest dose employed, which produced blood E2 levels substantially below the preovulatory peak seen in intact cycling rhesus monkeys, did not increase sexual activity above and beyond the medium dose is puzzling. An inverted U-shaped dose response curve was seen for sexual behaviors in the alpha females, with the medium dose of E2

creating the greatest increases in behavior and the low and high doses being inadequate to do so. We hypothesize that this dose-response curve is a product of the pellets used for E2 administration, and may be related to the subsequent fluctuation of E2. Females received subcutaneous E2 pellet implants following a minimum three-week period of E2 washout, and the resulting spikes in circulating E2 may not mimic the gradual increases observed during a natural ovarian cycle (Wallen et al., 1984). Additionally, studies looking at the exogenous administration of E2 mainly used Silastic capsules, which allow for a chronic release of E2 so long as it remains implanted subcutaneously (Zehr et al., 1998). The administration of E2 via subcutaneous pellets with their rapid release profile may have diminished E2's efficacy to induce behavioral changes. Future studies may benefit from using alternative methods producing a more consistent release profile. However, despite the significant decline in E2 serum concentrations between observation week one and week three across all doses, there were no significant differences in either proceptive or receptive sexual behavior or male-directed affiliation. Taken together, we see that E2 has a significantly different effect on sociosexual behavior following the medium dose of E2 that is absent in middle and subordinate ranking females. We suggest that the lack of a U-shaped curve in lower ranking female behavior may result from exposure to chronic subordination stress dampening the physiological and behavioral responses to E2.

Our data support the hypothesis that exposure to social subordination, a model of psychosocial stress that induces stress-related changes in physiology (Sapolsky, 1995), alters the activational effect of E2 on neural systems that mediate sociosexual behavior in

rhesus monkeys. The effects of stress on LHPG axis function have been shown at the hypothalamic-pituitary level, whereby chronic stress amplifies LHPG axis sensitivity to E2 negative-feedback, resulting in the suppression of pituitary LH secretion (Michopoulos et al., 2009). The mechanism by which this interaction takes place is still unknown. In rhesus monkeys, glucocorticoids are one possible mediator, as chronic administration (2-3 months) of hydrocortisone acetate was able to elevate circulating cortisol and reduce serum concentrations of LH and FSH in gonadectomized males (Dubey and Plant, 1985). Additionally, in intact female cynomolgus monkeys, serum concentrations of E2, FSH, and LH were significantly reduced following administration of the adrenal androgen dehydroepiandrosterone sulfate (DHEAS) (Kowalski and Chatterton, 1992a), but not adrenocorticotropin-(1-24) (ACTH) (Kowalski and Chatterton, 1992b) over the course of one menstrual cycle. Furthermore, following the administration of metyrapone, an adrenal steroid synthesis inhibitor, subsequent elevations in CRH and ACTH were insufficient to decrease LH (Van Vugt et al., 1997). However, the majority of studies suggest that glucocorticoid suppression of the LHPG axis may be specific to chronic administration, and CRH is the mediator of short-term suppression of the LHPG axis (Tilbrook et al., 2000).

Furthermore, the mechanism by which chronic stress alters the effects of E2 on sexual behavior remains unclear. The current study suggests that exposure to chronic stress desensitizes brain systems that mediate the sociosexual effects of E2 on behavior, independent of LHPG and ovarian function, and further suggests that this desensitization is not overcome by increased administration of E2. So far, two mechanisms have recently

garnered support from research on the effects of corticosterone on adrenalectomized female rats, although it is important to keep in mind that species differences exist in LHPA function (Rivier and Rivest, 1991; Tilbrook et al., 2000). In one study, adrenalectomized gonadally intact female rats showed a dose-response increase in ERs (specifically the β-subtype) mRNA expression in the hypothalamic paraventricular nucleus after implantation subcutaneous corticosterone pellets (Isgor et al., 2003). These data suggest a mechanism by which chronic activation of the LHPA axis and rising levels of CRF and corticosterone alter the expression of ERs to then affect behavior. In another study, ovariectomized and adrenalectomized female rats given E2 were found to have no change in the expression of ERs in the pituitary following dexamethasone injection, however there was an attenuation of the downstream effects of ERs on progesterone receptor expression and pituitary weight (Terakawa et al., 1985). Thus, a second mechanism might utilize the disruption of ER function as a transcription factor to reduce or alter the expression of genes and proteins normally regulated by E2 that in turn regulate socio-sexual behavior.

The current study additionally demonstrates that administration of E2 decreases both non-contact and contact aggression in parallel to the potentiation of sexually motivated behavior. However, we suggest that these data represent a byproduct of E2's activational effects on a female's motivation to engage in socio-sexual behavior combined with ready access to a male, and not a direct effect of E2 on aggressive behavior. Greater access and proximity to males increases rates of sexual behavior uncoupled from hormonal state (Wallen, 1990) and may serve to decrease aggression in females (Walker et al., 1983).

Male-female pairs of rhesus monkeys observed in large compounds showed tighter coupling between the female's ovarian cycle and fluctuations in sexual activity than did pairs of rhesus monkeys observed in small cages (Wallen, 1982). Multi-female groups, when given restricted access to a single male, also showed higher correlations between sexual behavior and hormonal condition than when observed in male-female pairs (Wallen and Winston, 1984). The decreased coupling of sexual behavior and E2, as a result of small group enclosures and unrestricted access to males in the current study therefore provides greater opportunities for sexual behavior. This, however, may only be true of alpha females, and social subordination stress may elevate threshold levels of E2 needed to elicit sexual behavior (Wallen, 1990). Regardless, the increase in reproductively motivated behavior in alpha females, and the environmental opportunity to access males may be the cause of the decreased aggressive behavior seen following the medium dose of E2. In support of this hypothesis, ovariectomized females pair-housed with a male do not show any increase in aggression following treatment with E2 (Zumpe and Michael, 1970), and intact multi-female groups with unrestricted access to a single-male showed a corresponding decrease in aggressive behavior (Walker et al., 1983). In contrast, intact multi-female groups with restricted access to males showed an increases in less severe non-contact aggression with higher levels of E2 during peri-ovulation (Walker et al., 1983), as did E2 treated ovariectomized females with no access to males (Wallen and Tannenbaum, 1997; Michopoulos et al., 2011). The trend for aggression to increase in alpha females following the highest dose of E2, without a corresponding increase in proceptive sexual behavior, requires further investigation, and suggests that

the decreased aggression may be due to increased motivation for sexual behavior and not increased receipt of sexual male sexual behavior.

E2 has also been implicated in the reduction of anxiety-like behavior in animals and in elevating positive emotion and affect in human beings (Halbreich and Kahn, 2001; Toufexis et al., 2006). Hence, it is somewhat surprising that we did not observe very many changes in anxiety-like behavior related to E2 in this study. In fact, E2 tended to increase self-directed anxiety behavior in our subjects irrespective of rank. This is in contrast to what has been observed in female rodents. For example, proestrous females, in which E2 levels are peaking, show reduced periods of immobility in the Porsolt forced swim tests, increased latencies in burying an electrified prod, and more time spent in the open arms of the elevated plus maze than do diestrous females or males (Marcondes et al., 2001). In other studies looking at the effect of long periods of anovulation in female macaques, it was found that these hypoestrogenic females exhibited behavior analogous to that seen in women with clinical depression (Shively et al., 2002). The discrepancies between these reports and the present study may be related to the type of anxiety-like behavior being scored and stress level present when the behavioral observations were undertaken. For example, rodent studies concentrate on changes in motor activity (i.e. freezing) and exploratory behavior, while in this present study we looked at self-directed anxiety-like behaviors. In addition, our behavioral observations were not undertaken during experimental conditions that have been shown to enhance stress reactivity for primates wherein changes in anxiety-like behavior due to E2 may be easier to determine. Thus, we are currently examining the interaction of E2 and social rank on

approach/avoidance behavior, a paradigm that has been shown to measure anxiety as indexed by a more complete range of behaviors, including exploratory behavior, in macaques (Coleman et al., 2011).

These data show that social subordination inhibits the expression of E2-mediated increases in sexual and male-affiliative behaviors, and that these changes may not be overcome by higher doses of E2. Furthermore, female-female aggression was seen to decrease following E2 treatment, suggesting that E2's influence on aggressive behavior is influenced by social and environmental factor more so than on hormonally mediated changes in neural systems underlying aggression. Finally, the current study does not support the hypothesis that E2 alters the expression of anxiety-like behavior, as measured in our study. Future studies using paradigms that enhance stress reactivity are necessary to elucidating the complex interaction between stress, E2 and anxiety-like behavior.

Table 2.1. Subject Group Composition. The alpha females (N=8), Middle-Ranking females (N=16), and Subordinate females (N=12) included in the analysis were members of 9 social groups. All excluded females are denoted by (--). An asterisks (*) denotes female that were excluded based on alterations in group social status, while the remaining females were excluded because they did not receive all 4 doses of Estradiol (E2; 0, 2, 4, 8 µg/kg/day) due to health concerns during the course of their treatment.

RANK (Category)	*Group 1*	*Group 2*	*Group 3*	*Group 4*	*Group 5*	*Group 6*	*Group 7*	*Group 8*	*Group 9*	*Group 10*	*Total*
1 (Alpha)	*Included*	*Included*	*Included*	*--*	*Included*	*-- **	*Included*	*Included*	*Included*	*Included*	*8*
2 (Middle)	*Included*	*Included*	*Included*	*--*	*Included*	*-- **	*Included*	*Included*	*Included*	*Included*	*8*
3 (Middle)	*Included*	*Included*	*Included*	*--*	*Included*	*Included*	*Included*	*Included*	*Included*	*--*	*8*
4 (Sub)	*Included*	*Included*	*Included*	*--*	*Included*	*-- **	*Included*	*Included*	*Included*	*--*	*7*
5 (Sub)	*Included*	*--*	*Included*	*--*	*Included*	*Included*	*--*	*-- **	*Included*	*--*	*5*
Total	*5*	*4*	*5*	*0*	*5*	*2*	*4*	*4*	*5*	*2*	*36*

Table 2.2. Effects of Treatment Week on Female Rhesus Monkey Serum E2 and Behavior. Significant main and interaction effects of E2 Dose ([1]), Observation Week([2]), Dose by Week([3]), on serum E2 concentration, proceptive and receptive sexual behavior are listed in the table below. Significant changes in values based on post hoc analysis are shown between Observation Week (* = difference from Week one $p < 0.05$) and E2 Dose ([a] = difference from 0*μg/kg/day;* [b] = difference from 2*μg/kg/day* $p < 0.05$; [c] = difference from 4*μg/kg/day* $p < 0.05$.) All data are raw averages of behavior per 30 minute observation session or serum E2 concentration taken during observational week 1 and week 3.

	Serum E2 (pg/mL)[1,2]		***Proceptive Behavior (/30min)[1,3]***		***Receptive Behavior (/30min)[1]***	
E2 Dose (μg/kg/day)	***Week 1***	***Week 3***	***Week 1***	***Week 3***	***Week 1***	***Week 3***
0	-----	-----	0.35 ± 0.10	0.16 ± 0.50 *	0.44 ± 0.17	0.53 ± 0.20
2	73.97 ± 9.66	13.84 ± 2.98 *	0.16 ± 0.04	0.35 ± 0.10	0.35 ± 0.14	0.19 ± 0.12
4	127.79 ± 13.46 [b]	38.86 ± 6.15 * [b]	0.57 ± 0.13	1.62 ± 0.51 [a]	2.03 ± 0.57	1.05 ± 0.30 *
8	202.38 ± 19.72 [b,c]	78.15 ± 12.68 * [b,c]	0.52 ± 0.25	0.58 ± 0.16	1.07 ± 0.36	0.91 ± 0.20

Table 2.3. Effects of E2 on Female Rhesus Monkey Behavior. Main effect of Rank ([1]) on fe affiliation given, female-aggression received and given, female-submission given, and self-directed anxious behaviors are listed in the table below. Significant changes in valued based post hoc analysis are shown between Rank categories (* = difference from Alpha $p < 0.05$; difference from Middle-Ranking Female $p < 0.05$) and E2 Dose ([a] = difference from 0*μg/kg* $p< 0.05$; [b] = difference from 2*μg/kg/day* $p < 0.05$; [c] = difference from 4*μg/kg/day* $p < 0.05$.) data are raw averages of behavior frequency during six 30-minute behavioral observations.

BEHAVIOR	*E2 Dose (μg/kg/day)*	*Alpha*	*Middle*	*Subordi*
Affiliation Toward Females[1]	0	3.44 ± 0.88	3.24 ± 0.61	1.36 ± 0.7
	2	3.09 ± 0.78	2.72 ± 0.54	1.31 ± 0.6
	4	2.72 ± 0.88	3.12 ± 0.61	1.46 ± 0.7
	8	2.52 ± 0.67	2.93 ± 0.47	1.25 ± 0.5
Aggression Toward Females[1]	0	3.41 ± 1.90	3.78 ± 1.31	1.29 ± 1.5
	2	3.63 ± 1.04	2.17 ± 0.72	0.47 ± 0.8
	4	2.17 ± 1.15	2.59 ± 0.79	1.17 ± 0.9
	8	5.27 ± 1.62	1.69 ± 1.11	1.13 ± 1.2
Aggression From Females[1]	0	0.02 ± 1.45	1.51 ± 1.00	5.68 ± 1.1
	2	0.00 ± 0.79	1.30 ± 0.54	3.01 ± 0.6
	4	0.02 ± 1.24	1.01 ± 0.86	4.50 ± 0.9
	8	0.00 ± 1.37	2.29 ± 0.95 * [c]	4.38 ± 1.0
Submission Toward Females[1]	0	0.02 ± 2.44	6.84 ± 1.68 *	8.75 ± 1.9
	2	0.00 ± 1.59	4.11 ± 1.10 * [a]	7.39 ± 1.2
	4	0.00 ± 2.02	4.30 ± 1.40 *	8.99 ± 1.6
	8	0.02 ± 2.27	6.32 ± 1.57 * [b c]	9.54 ± 1.8
Anxiety-like Behavior	0	5.14 ± 1.29	5.76 ± 0.89	3.74 ± 1.0
	2	4.12 ± 1.50	7.00 ± 1.04	3.92 ± 1.1
	4	3.28 ± 1.01	6.53 ± 0.69 *	4.22 ± 0.8
	8	4.97 ± 1.11	4.79 ± 0.77	4.04 ± 0.8

Figure 2.1. Serum concentrations of E2 following low (2µg/kg/day), medium (4µg/kg/day), and high (8µg/kg/day) doses delivered using E2 sustained pellets low. Post-Hoc tests show significant dose differences in serum samples across all doses. Data are presented as average frequencies ± standard error. (a) > (b) > (c), $p<.05$. For sample time point 1, the high dose was greater than the medium dose only at the trend level ($p=.066$).

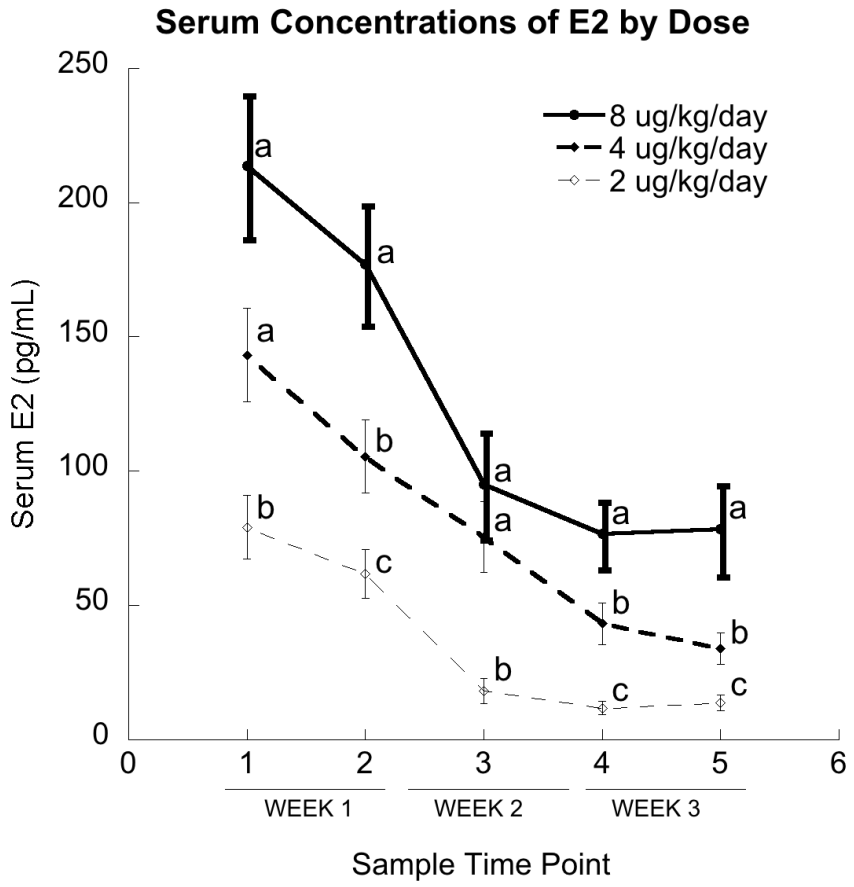


Figure 2.2. Interaction between Rank and Dose on sexual behavior and toward males. Both (A) proceptive and (B) receptive behavior showed a main effect of Rank, a main effect of Dose, and an interaction effect between Rank and Dose. Post-hoc analysis showed activational effects only in Alpha females. Data are presented as average frequencies ± standard error.

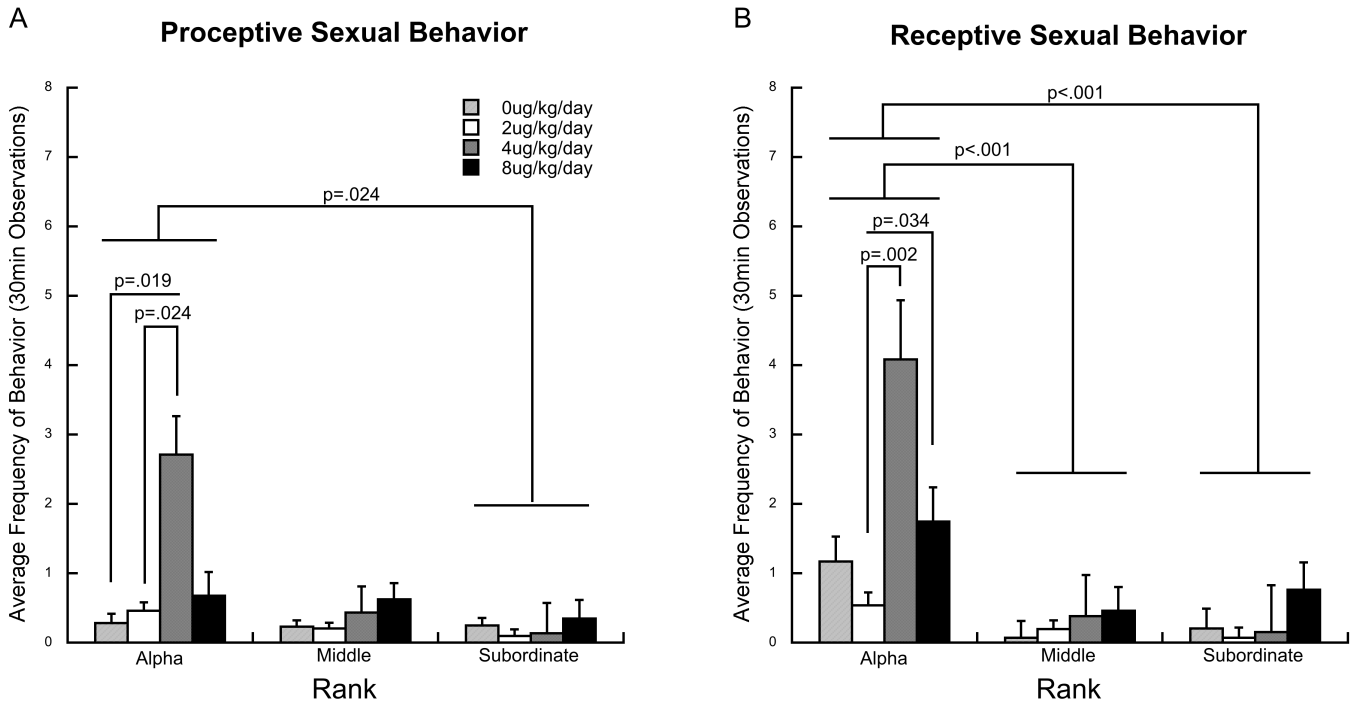


Figure 2.3. Initiation of male-directed affiliation showed a main effect of Rank, but no mair effect of Dose or interaction effect between Rank and Dose. Post-hoc analysis showed activational effects of only in Alpha females. Data are presented as average frequencies ± standard error.

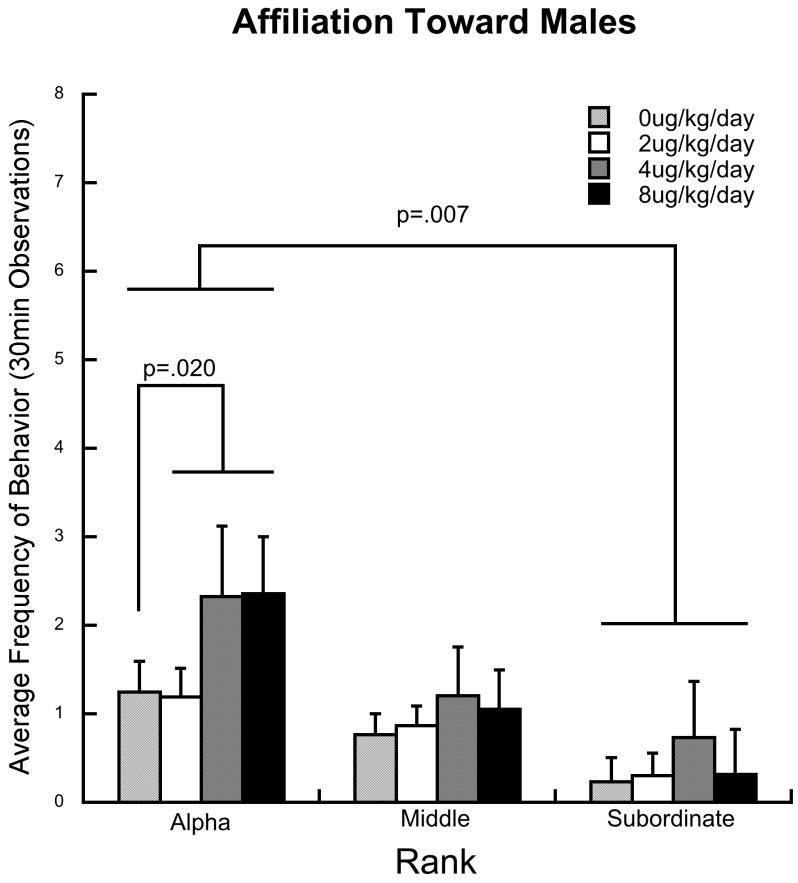


Figure 2.4. Aggressive behavior, separated into Contact Aggression and Non-Contact Aggression, show significantly different responses to E2 Dose. Post-Hoc tests show significant dose differences in (A) the Alpha Females, and (B) Alpha and Middle-Ranking Females. Data are presented as average frequencies ± standard error. (a) > (b), $p<.05$.

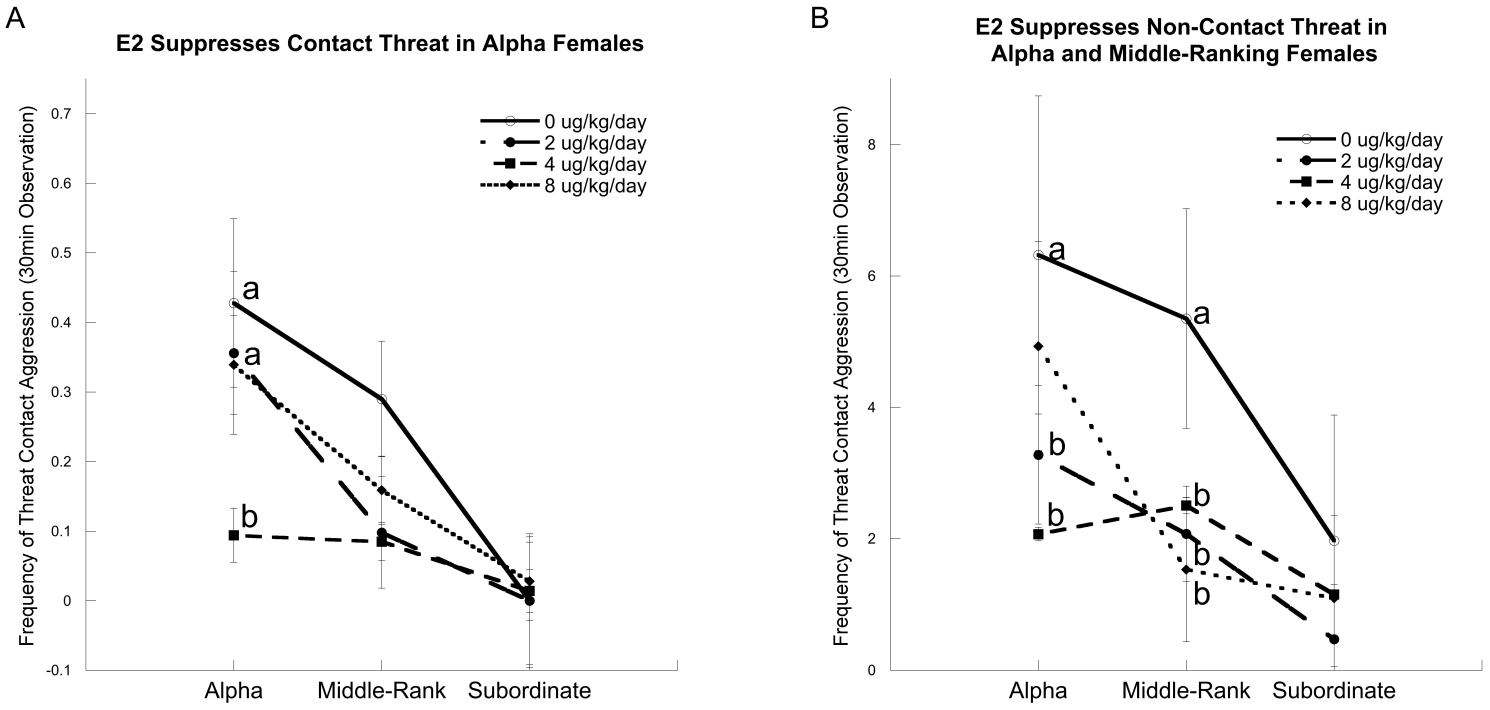
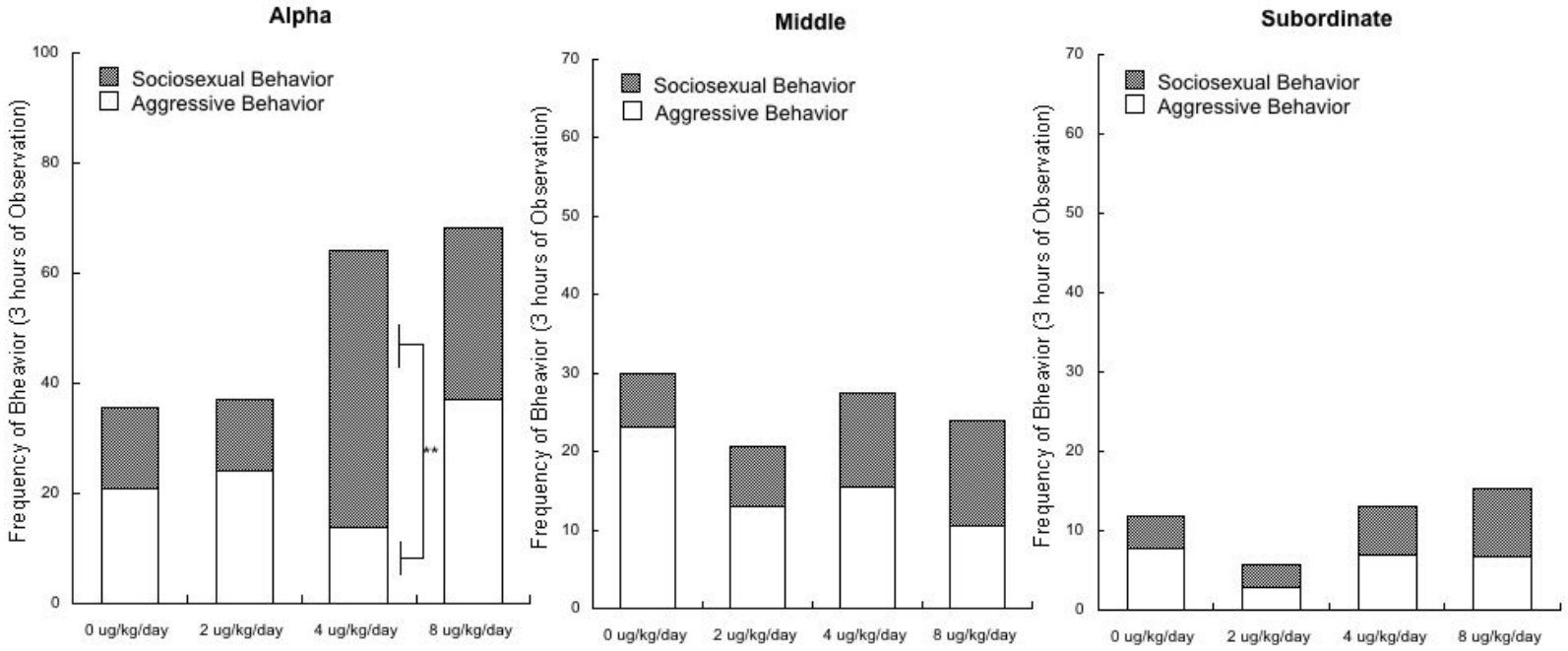


Figure 2.5. Sociosexual behavior, defined as the combination of Proceptive, Receptive and Male-Affiliation behavior was compared to total Aggression in a rmANOVA using dose (0,2,4,8µg/kg/day) as a within subject variable and rank as a between subject variable. We found a significant main effect of dose ($F_{3,90}$ =3.50, p=0.033) and rank ($F_{2,30}$ =13.59, p<.001) but no main effect of behavior type ($F_{1,30}$ =1.90, p=0.178). However, there was an interaction between dose and behavior ($F_{3,90}$ =4.56, p=0.005). ** = p<0.01.



Chapter 3: Social subordination alters estradiol-induced changes in cortico-limbic brain volumes in adult female rhesus monkeys.

3.1 Abstract

Women are twice as likely as males to develop stress-related psychopathology and the experience of stressful life events is the best predictor for its development among women. The rodent literature suggests that exposure to chronic stress can result in brain structural changes, including alterations in neuronal morphology in corticolimbic brain regions. However these changes are sexually dimorphic, and appear to be dependent on the ovarian hormone, 17β estradiol (E2) which can also independently affect neuronal plasticity. In some studies, exposure to stressors appears to modify E2's trophic effect on neuronal morphology, however the translation of these data to human is limited by both the stressor used (e.g. restraint stress) and reduced brain homology, particularly within the prefrontal cortex. To address these limitations, we utilized a well-validated and ethologically relevant rhesus monkey model of social subordination stress used to study the adverse effects of psychosocial stress in women. To date, no studies have been conducted examining the effects of social subordination and E2 on regional brain volumes in adult rhesus monkeys. The current experiments aim to examine these effects in regions known to be affected by both stress and E2, including cortical volumes of frontal, prefrontal, and cingulate gray and white matter and subcortical hippocampal and amygdala volumes. Our results show that E2 treatment decreased frontal cortex gray matter volume. Although no main effects of social subordination were found in total, cortical, or subcortical brain volumes, gray matter volume in the cingulate cortex varied significantly by E2, dependent on social status. Thus, the data demonstrate dichotomous effects of E2, such that cingulate gray matter volumes decreased in subordinates and

increased in dominant females following a month of E2 treatment. Together these data suggest that a background of chronic stress imposed by a history of social subordination can reverse the effects of E2 on female brain structure.

3.2 Introduction

Chronic psychosocial stress results in adverse health outcomes and increased susceptibility to psychopathology, including mood and anxiety disorders (Hammen, 2005a; Cohen et al., 2007). Women are two to three times more vulnerable to develop these disorders than men (Kessler, 2003; Seeman, 1997), and the experience of stressful life events often precipitates disease development (Kendler et al., 1993). One possibility for these sex-differences is an interaction between chronic stress and ovarian hormone signaling (Dorn and Chrousos, 1997; Pinkerton et al., 2010), such that 17β-estradiol (E2) may render women more susceptible to the negative effects of stress hormones, such as glucocorticoids (GCs) (Seeman, 1997). Corticolimbic regions involved in socioemotional processing, including the prefrontal cortex (PFC), amygdala, and hippocampus, express high levels both GCs and estrogen receptors (Sousa et al., 1989; Morimoto et al., 1996; Shughrue et al., 1997; Sánchez et al., 2000), making them sensitive to effects of stress and E2. A better understanding of how chronic stress interacts with E2 to modify these structures may provide insight into susceptibility for mood disorders in adult women and may help inform treatments for the development or reoccurrence of these disorders, reducing the overall disease burden.

Prolonged unpredictable and uncontrollable stress has been shown to have damaging effects on rodent corticolimbic brain regions including the PFC, amygdala, and hippocampus (Joels et al., 2007). In the hippocampus, chronic stress or prolonged treatment with glucocorticoids results in reduced neuronal dendritic complexity (e.g. arborization, length, spine density) specifically within the CA3 regions and the dentate gyrus (Watanabe et al., 1992; Magarinos and McEwen, 1995b; Magarinos et al., 1998; McEwen, 1999). Chronic restraint stress also decreases dendritic arborization in the mPFC (Shansky and Morrison, 2009; Radley et al., 2013) while it has opposite effects on the amygdala, increasing neuronal dendritic arborization (Rosenkranz et al., 2010). These structural alterations are associated with increased fear and anxiety-like behavior. However, the majority of these studies have focused on stress-induced changes in males (Conrad et al., 1999; Gameiro et al., 2006) and may not be directly translatable to females.

The effects of chronic stress are, indeed, strikingly different in females, and may be a result of its modification of E2's effects on the brain. As opposed to males, chronic restraint stress in ovariectomized female rats receiving E2 replacement results in increased apical dendritic length (Garrett and Wellman, 2009) and spine density in medial (m)PFC neurons projecting to the basolateral amygdala (BLA) (Shansky et al., 2010). These structural effects are seen even in the absence of stress, as mPFC pyramidal neurons show dendritic retraction following ovariectomy (Wallace et al., 2006) and growth following E2 (Shansky et al., 2010). Together, these data suggest that chronic stress may potentiate E2's neurotrophic effects, increasing dendritic complexity, at least

in rodent mPFC. Stress and E2 have a different interaction effect in the hippocampus, where studies in ovariectomized females with E2 replacement suggest a sparing of CA1 and CA3 atrophy during stress by E2 and facilitation of hippocampal-dependent tasks (e.g. Morris water maze) (McLaughlin et al., 2010). This is inconsistent with a large body of literature supporting E2-dependent increases in spine density and synapse formation in CA1, but not CA3, pyramidal neurons within the hippocampus (Gould et al., 1990; Woolley et al., 1990a; Woolley and McEwen, 1992), suggesting that chronic stress modifies E2 trophic effects in the rodent hippocampus. In the amygdala, although data show stress increases dendritic complexity in the BLA in males (Rosenkranz et al., 2010), and E2 decreases BLA spine density during proestrous (Rubinow et al., 2009), the interaction of stress and E2 have not been examined (Farrell et al., 2013). Overall, the literature suggests that E2's neurotrophic effects may be modified by exposure to chronic stress in certain neuronal populations, at least in rodents.

Experimental manipulation of exposure to chronic stress are not possible in humans, and data from individuals with stress-related psychopathology cannot dissociate the effects of chronic stress in adulthood from either early life stress or disease related alterations (Tottenham and Sheridan, 2010). The most consistent finding has been the reduction of hippocampal volumes associated with both major depressive disorder and post-traumatic stress disorder (PTSD) (Campbell et al., 2004; Videbech and Ravnkilde, 2004; Karl et al., 2006a; Tottenham and Sheridan, 2010). However, the structural brain changes in the amygdala and PFC reported in stress-related disorders are equivocal. Some studies suggest reductions in amygdala volume (Tottenham and Sheridan, 2010; Bellani et al.,

2011), as well as reduced gray matter volume in the mPFC, including the anterior cingulate cortex (ACC) (Bora et al., 2012) and more specifically the subgenual ACC (Drevets et al., 1997). However, other studies report increased amygdala volumes (Tottenham and Sheridan, 2010; Bellani et al., 2011; Kuo et al., 2012) or no psychopathology related alteration in either the amygdala (Bellani et al., 2011) or subgenual ACC (Brambilla et al., 2002). Our understanding gets further complicated when the effects of sex and gonadal hormones are considered. Studies of mood and anxiety disorders suggest sex differences in volumetric changes, although in women none have isolated effects specific to E2 (Lorenzetti et al., 2009). For example, in a sample of patients with major depressive disorder, males showed greater volume loss in the ACC compared to females, and females, but not males, showed reductions in amygdala volume (Hastings et al., 2004). These data suggest that, apart from sexually dimorphic differences, E2 may have complex and opposite, region-specific, effects in the female brain, being either protective (less ACC volume loss under stress in comparison to males) or detrimental (increased amygdala volume loss), and together with the rodent literature suggests an interaction between E2 and chronic stress within corticolimbic regions commonly associated with stress-induced psychopathology.

In summary, human studies on adult individuals exposed to chronic stress are confounded by both early life stress experience and comorbid clinical features of the diseases (e.g. PTSD, depression). They also lack experimental control as experimental manipulations that involve chronic stress present prohibitive ethical challenges in humans. Furthermore, to determine effects of E2, experimental manipulation of endogenous hormones is

necessary. Female rodent experimental paradigms of chronic stress, on the other hand, often lack social stressor features common to stress-related psychopathology in women and have reduced construct validity (Palanza, 2001). To address these limitations in the human and rodent literature, we chose to use an adult female rhesus monkey model to investigate the specific impact of chronic psychosocial stress and its interactions with E2 on the volumes of corticolimbic regions, including PFC, amygdala and hippocampus. Social subordination in adult female macaques is a well-defined and highly translational paradigm used to examine the consequences of chronic exposure to psychosocial stress in women (Michopoulos et al., 2012a). Furthermore, the use of ovariectomized monkeys with and without E2 replacement allows us to conduct a prospective, cross-over treatment design to control for exposure to E2 in combination with social status.

The goal of the current study is to identify how the effects of social subordination interact with E2 to change corticolimbic and brain volumes in the adult female brain. Specifically we examine the structural impact of both factors on the hippocampus, amygdala, and PFC, frontal, and cingulate cortices. We hypothesize that chronic subordination will result in reduced hippocampal and PFC, frontal, and cingulate volumes, but in increased amygdala volume, similar to the alterations seen in stress-related pathology and chronic stress in rodents. Additionally, we hypothesize that E2 replacement will increase cortical, and hippocampal volumes, and decrease amygdala volumes as suggested by the rodent literature. Finally, we hypothesize that E2 replacement in combination with chronic stress will be protective in cortical and hippocampal brain regions, but detrimental in the amygdala. Understanding whether social subordination attenuates or exacerbates E2

structural alterations in corticolimbic regions, can increase our understanding of the potential role and mechanisms of this gonadal steroid on increased vulnerability to stress-related disorders such as anxiety and depression in adult women, in comparison to men.

3.3 Methods

3.3.1 Subjects

Subjects were 20 ovariectomized adult female rhesus monkeys (*Macaca mulatta*), socially-housed and maintained at the Yerkes National Primate Research Center (YNPRC) Field Station. Subjects were either the most dominant (DOM, Rank 1, N=10) or the most subordinate (SUB, Rank 4 or 5, N=10) females in each of ten groups of 4-5 females housed with one resident adult male. Subjects were housed in indoor - outdoor enclosures that measured 20 X 15 X 8 feet each. Animals were fed a standard commercial low-fat high-fiber monkey chow diet (Ralston Purina Company, St. Louis MO) ad libitum supplemented daily with seasonal fruits and vegetables. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) 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 groups containing the subjects had been established as previously described (Jarrell et al., 2008). Briefly, middle ranking, ovariectomized females were removed from their natal breeding groups (>100 animals each) and introduced to one another to form new smaller groups of five unrelated, unfamiliar females and one adult male each.

Females were added sequentially, and dominance hierarchies were established based largely on order of introduction, with the final female often assuming the lowest rank. Since group formation, subjects have been included in several studies in which all females received periodic replacement therapy with E2 lasting approximately 2 to 4 weeks and/or progesterone for similar durations (Jarrell et al., 2008; Michopoulos et al., 2009; Michopoulos et al., 2011; Asher et al., 2012; Reding et al., 2012). During the course of data collection for this study, two DOM females were unable to complete both treatment conditions, and were therefore excluded from the analysis, bringing the sample size to 18 females. In the final sample, animals were 14 (± 2) years of age with no significant age difference between groups (DOM=14±2; SUB=14±3; $F_{1,16}$=.02, p=.90,η^2<.01). Furthermore, all females were ovariectomized 6 (± 2) years prior to the start of the study with no significant difference between DOM and SUB females (DOM= 5 ± 2; SUB=7 ± 2; $F_{1,16}$=2.54, p=.13,η^2=.14). Finally, there was no difference in the age of ovariectomy between DOM (8.1 ± 2.3 years) and SUB females (6.4 ± 4.1 years, $F_{1,16}$=1.14, p=.30, η^2<.07)

3.3.2 Social Subordination

Rhesus monkeys social groups are structured in linear dominance hierarchies that are maintained by both contact and non-contact aggressive behavior from more DOM females to more SUB females (Bernstein, 1970; Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984; Michopoulos et al., 2012a). Thus, frequency of aggressive, affiliative, and submissive behaviors are rank dependent, with SUB females receiving the most aggression, often random and unpredictable, from more DOM animals

as well as exhibiting the highest rates of submissive behaviors directed toward more DOM animals to attenuate the aggression (Sapolsky, 1995; Silk, 2002). Previous studies show that SUB females have increased behavioral, metabolic, and neuroendocrine markers of a chronic stress phenotype in comparison to the highest-ranking DOM females providing a well-validated translational model to further explore the consequences of exposure to chronic stressors in women (Michopoulos et al., 2012a). For our study, we chose to only examine females at the extreme ranks, assuming this would reflect maximum differences in subordination-induced effects.

3.3.3 Estradiol Treatment

All females were studied during an E2 replacement and a control, no E2 replacement condition. The order of treatment was counterbalanced across female ranks. In the E2 condition, treatment was administered via E2-filled continuous-release Silastic® capsules placed between the scapulae under anesthesia, to yield serum levels between 90-100 pg/mL, which correspond to physiological levels typical of the mid- to late follicular phase in this species (Wilson et al., 1982) for an average of 31 (± 3) days. There were no significant differences between DOM and SUB females in duration of time females were treated with E2 at the time of the scan (DOM = 33 ± 12 days; SUB= 33 ± 11 days; $F_{1,16}<.001$, $p=.99$, $\eta^2<.01$). During the no-treatment (control) condition, the interval of time between the previous E2 treatment and the scan (mean of 43 (± 13) days) did not vary significantly based on status (DOM = 43 ± 14 days; SUB= 44 ± 12 days; $F_{1,16}=.07$, $p=.78$, $\eta^2< .01$).

3.3.4 Neuroimaging Protocol

Structural MR Image Acquisition. All females were scanned twice: during the E2 treatment and the control condition. Subjects were transported from their social group to the YNPRC MRI Center the day before the scans. All scans were acquired on a 3T Siemens Magnetom TRIO system (Siemens Med. Sol., Malvern, PA, USA) and using an 8-channel phase array coil. Two structural scans (T1- and T2-weighted MRI) were conducted during each session. The T1-MRI scan was acquired using a 3-dimensional (3D) magnetization-prepared rapid gradient-echo (3D-MPRAGE) parallel imaging sequence (TR/TE/TI=3000/3.52ms/950ms, voxel-size=0.5mm^3, isotropic, 6 averages). A T2-weighted MR 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 aid with delineation of regions of interest (ROIs) by improving the contrast of grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) borders (Rapisarda et al., 1983; Knickmeyer et al., 2010) All animals were scanned supine in the same orientation, achieved by placement and immobilization of the head in a custom-made head holder via ear bars and a mouth piece. A vitamin E capsule was taped on the right temple to mark the right side of the brain. Scans were acquired under isoflurane anesthesia (1-1.2% to effect, inhalation), following initial induction with Telazol (5mg/kg, i.m.). Animals were fitted with an oximeter, ECG, rectal thermistor and blood pressure monitor for physiological monitoring, an i.v catheter to administer dextrose/NaCl (0.45%) to maintain normal hydration and an MRI-compatible heating pad. Upon completion of the scans and full recovery from anesthesia, each female was returned to their social group.

MRI Data Processing and Analysis. Structural data was analyzed using an automatic atlas-based segmentation program, AutoSeg (version 2.6.2), an open-source software pipeline developed at the Neuro Image Research and Analysis Laboratories of the University of North Carolina at Chapel Hill (Wang et al., 2014). AutoSeg is used to automatically parcellate brain tissue (GM, WM, CSF), cortical lobes, and subcortical structures in the rhesus macaque as described previously (Knickmeyer et al., 2010). Briefly, the subjects' structural MR images were registered to a T1-MRI rhesus atlas image made from 18 rhesus macaques (10 males and 8 females) and then automatically parcellated based on the subject's T1- and T2-weighted scans into cortical lobes and subcortical regions, as well as automatically segmented into GM, WM, and CSF as previously published (Styner et al., 2007). For this study, automatic parcellations and segmentations of lobar volumes (GM and WM) corresponding to the prefrontal, frontal, and cingulate cortices (Figure 3.1 A,B), as well as the subcortical hippocampus and amygdala ROIs were manually adjusted to ensure accurate neuroanatomical delineation by two raters who remained blind to experimental groups and using published anatomical criteria for the macaque brain (Paxinos et al., 2000; Saleem and Logothetis, 2006). The hippocampus was defined with the horn of the lateral ventricle as the dorsal and lateral boundary and the white matter separating the hippocampus from the entorhinal cortex used as the ventral border (Rosene and Hoesen, 1987). The hippocampus was further divided into an anterior and a posterior portion, with the boundary delineated after the last coronal slice to include the uncus (Willard et al., 2011). The amygdala was defined following additional anatomical landmarks published for the macaque (Price et al., 1987;

Amaral and Bassett, 1989) with the hippocampus as the posterior boundary, and the beginning of the periamygdaloid cortex as the anterior boundary, the CSF as ventral border, white matter as ventrolateral boundary, and when this was not available due to low contrast, the rhinal fissure defined the ventromedial border. Intra and inter-rater reliability of volumes were assessed using intraclass correlation coefficients (ICCs). Using a subset of five subjects, two raters (K.M.R, C.L.F) manually adjusted the GM/WM and hippocampus segmentations with the following inter-rater reliability ICCs: GM (r=.99), WM (r=.99), ICV(r=.99), and hippocampus (r=.67). Intra-rater reliability using the same subset of five subjects was assessed for both raters with the following ICCs: GM (r=.99 ; r=.93), WM (r=.99 ; r=.99), ICV (r=.99 ; r=.99), hippocampus (r=.74; r=.71), and amygdala (ICC r=.55).

3.3.5 Statistical Analysis

Total brain volume, or intracranial volume (ICV), was calculated for each individual at both treatment conditions by adding total GM, WM, and CSF volumes. In order to control for potential differences in total ICV, all ROI volumes (cortical and subcortical) were adjusted for ICV volumes (e.g. GM/ICV) within each condition. ICV-adjusted ROI volumes were analyzed using repeated measures analysis of variance (RMANOVA) to test for between subject effects of social status (DOM; SUB), and the within subject effects of E2 treatment (E2; Control) and hemisphere (right; left). Additionally, for each lobe (frontal, prefrontal, and cingulate) GM/WM ratios were also calculated and analyzed using rmANOVA. Finally, unadjusted raw data were analyzed using the same

RMANOVA models. Results are summarized as unadjusted ICV, GM, WM, and subcortical volumes ($mm^3 \pm$ S.E.).

3.4 Results

3.4.1 Total Brain Volume

There was no significant effect of status ($F_{1,16}$=.05, p=.83, η^2<.01), E2 treatment ($F_{1,16}$=.42, p=.52, η^2=.03), or a status by E2 treatment interaction ($F_{1,16}$=.29, p=.60, η^2=.02), on total brain volume (Table 3.1).

3.4.2 Prefrontal Cortex

Gray Matter. There were no effects of status ($F_{1,16}$ = .91, p=.35, η^2=.05), treatment ($F_{1,16}$ = .63, p=.44, η^2=.04), or treatment by status ($F_{1,16}$ = 1.52, p=.24,η^2=.09). There was however, a main effect of hemisphere ($F_{1,16}$ = 9.07, p=.008, η^2=.36), with greater GM volume in the left hemisphere. There were no other interaction effects (status by hemisphere ($F_{1,16}$ = .05, p=.83, η^2=.00), treatment by hemisphere ($F_{1,16}$ = .01, p=.94, η^2<.01), or status by treatment by hemisphere, ($F_{1,16}$ = 2.42, p=.14, η^2=.13)). Commensurate results were seen using unadjusted data, and raw data are listed in Table 3.1.

White Matter. There was no effect of status ($F_{1,16}$ = .11, p=.74, η^2=.01), treatment ($F_{1,16}$ = 1.75, p=.21, η^2=.10), or a status by treatment interaction ($F_{1,16}$ = 1.23, p=.28, η^2=.07) in WM volumes. No main effects of hemisphere ($F_{1,16}$ = 2.34, p=.15, η^2=.13), or interaction

effects between status and hemisphere ($F_{1,16} = 2.57$, p=.13, η^2=.14), treatment and hemisphere ($F_{1,16} = 2.34$, p=.15, η^2=.13), or three-way interactions between status, treatment, and hemisphere ($F_{1,16} = .36$, p=.56, η^2=.02) were detected. Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

Gray Matter/ White Matter Ratio. No effect of status ($F_{1,16} = .19$, p=.67, η^2=.01), treatment ($F_{1,16} = .11$, p=.74, η^2=.01), or a status by treatment interaction ($F_{1,16} <.01$, p=.98, η^2<.01) was detected in GM/WM ratios. However, there was a significant hemispheric lateralization effect in GM/WM ratio ($F_{1,16} = 62.71$, p<.001, η^2=.80) with increased GM/WM ratios (GM>WM) in the left than in the right hemisphere (Right= 2.30 ± .05; Left= 2.38 ± .04). Furthermore, there was an interaction effect between status and hemisphere in the prefrontal cortex ($F_{1,16} = 7.81$, p=.013, η^2=.33). Post hoc analysis revealed that the GM/WM ratios were bigger in the left than in the right hemisphere in both DOMs (p=.001; Right= 2.29 ± .07; Left= 2.35 ± .07) and SUBs (p<.001; Right=2.30 ± .06; Left= 2.42 ± .06), but no difference between SUBs and DOMs in the left hemisphere (p=.43) or right hemisphere (p=.92). There were no treatment by hemisphere ($F_{1,16} = 2.41$, p=.14, η^2=.13), or status by treatment by hemisphere effects ($F_{1,16} = .10$, p=.76, η^2<.01). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.2.

3.4.3 Frontal Cortex

Gray Matter. There was a main effect of treatment on GM volume in the frontal cortex ($F_{1,16} = 11.19$, p=.004, η^2=.41, Figure 3.1 C,D). E2 replacement reduced GM volume

compared to the control condition (noE2), but there were no effects of status ($F_{1,16}$ = 1.77, p=.20, η^2=.10) or status by treatment interaction effect ($F_{1,16}$ <.001, p>.99, η^2<.01). A significant effect of hemisphere was detected on GM volume ($F_{1,16}$ = 6.94, p=.018, η^2=.30), with larger right than left volumes. No other interaction effects were found (status by hemisphere ($F_{1,16}$ = 1.36, p=.26, η^2=.08), treatment by hemisphere ($F_{1,16}$ = .28, p=.61, η^2=.02), or status by treatment by hemisphere ($F_{1,16}$ = .35, p=.56, η^2=.02)). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

White Matter. There was no effect of status ($F_{1,16}$ =.04, p=.84, η^2<.01), treatment ($F_{1,16}$ = .50, p=.49, η^2=.03), or a status by treatment interaction ($F_{1,16}$ = .82, p=.38, η^2=.05) in WM volume. No main effects of hemisphere ($F_{1,16}$ = 3.44, p=.08, η^2=.18), status by hemisphere ($F_{1,16}$ = .22, p=.64, η^2=.01), treatment by hemisphere ($F_{1,16}$ = 1.77, p=.20, η^2=.10), or status by treatment by hemisphere were detected ($F_{1,16}$ = .28, p=.61, η^2=.02), either. Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

Gray Matter/ White Matter Ratio. There was no main effect of status ($F_{1,16}$ = .95, p=.34, η^2=.06), treatment ($F_{1,16}$ = .89, p=.36, η^2=.05), or status by treatment interaction ($F_{1,16}$ = .33, p=.58, η^2=.02) in GM/WM ratios. No main effect of hemisphere ($F_{1,16}$ = .08, p=.79, η^2=.01), or status by hemisphere ($F_{1,16}$ = 2.57, p=.13, η^2=.14), treatment by hemisphere ($F_{1,16}$ = .30, p=.60, η^2=.02), or status by treatment by hemisphere interaction effects ($F_{1,16}$

<.01, p=.98, η^2=<.01) were detected, either. Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.2.

3.4.4 Cingulate Cortex

Gray Matter. Although no main effects of status ($F_{1,16}$ <.001, p=.97, η^2<.001) or treatment ($F_{1,16}$ = .27, p=.61, η^2=.02) were found, there was a significant status by treatment by hemisphere interaction ($F_{1,16}$ = 5.24, p=.036, η^2=.25) on cingulate cortex GM volume. As shown in Figure 3.1 (E,F) GM volumes in the right hemisphere were differentially affected by status and treatment, as DOMs had greater GM volume in the E2 condition compared to control treatment (p=.012) and SUBs had decreased GM volume in during the E2 condition compared to the control treatment (p=.010). Post hoc tests also showed there was no significant status difference between right hemisphere volumes during the no treatment condition (p=.28). Supporting this three-way interaction, there was also a main effects of hemisphere ($F_{1,16}$ = 9.35, p=.008, η^2=.37) and a interaction effect of status by treatment ($F_{1,16}$ = 8.65, p=.010, η^2=.35). There were no other significant interaction effects (status by hemisphere ($F_{1,16}$ <.01, p=.95, η^2<.01), or treatment by hemisphere ($F_{1,16}$ = .59, p=.46, η^2=.04). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 1.

White Matter. There was no main effects of status ($F_{1,16}$ = .17, p=.68, η^2=.01), treatment ($F_{1,16}$ = .22, p=.64, η^2=.01), or hemisphere (($F_{1,16}$ = 1.85, p=.19, η^2=.10), or any interaction effects (status by treatment ($F_{1,16}$ = .82, p=.38, η^2=.05); status by hemisphere ($F_{1,16}$ = .89, p=.36, η^2=.05), treatment by hemisphere ($F_{1,16}$ = 1.08, p=.32, η^2=.06), or

status by treatment by hemisphere ($F_{1,16}$ = 3.09, p=.10, η^2=.17)) on WM volume in the cingulate cortex. Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

Gray Matter/ White Matter Ratio. There was no main effect of status ($F_{1,16}$ = .14, p=.72, η^2=.01), treatment ($F_{1,16}$ = .28, p=.61, η^2=.02), or a status by treatment interaction ($F_{1,16}$ <.01, p=.96, η^2<.01) on GM/WM ratios. However, there was a significant hemisphere effect ($F_{1,16}$ = 12.82, p=.002, η^2=.45), with increased GM/WM ratios (GM>WM) in the right than left hemisphere (Right= 3.97 ± .08; Left=3.64 ± .07). No interaction effects were detected (status by hemisphere ($F_{1,16}$ = .39, p=.59, η^2=.02), treatment by hemisphere ($F_{1,16}$ = 1.76, p=.20, η^2=.10), or status by treatment by hemisphere ($F_{1,16}$ =.10 ., p=.76, η^2=.01). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.2.

3.4.5 Hippocampus

No main or interaction effects were observed in the hippocampus. There was no effect of status ($F_{1,16}$ =2.51, p=.13, η2=.14), treatment ($F_{1,16}$ =.17, p=.69, η2=.01), or a status by treatment interaction ($F_{1,16}$=.79, p=.39, η2=.05) on total hippocampal volumes. Furthermore, when divided into anterior and posterior hippocampus, no effects of status (anterior: $F_{1,16}$ =1.23, p=.28, η2=.07; posterior: $F_{1,16}$ =.84, p=.37, η2=.05), treatment (anterior: $F_{1,16}$ =.48, p=.50, η2=.03; posterior: $F_{1,16}$ =2.44, p=.14, η2=.13), or status by treatment effects were detected on these volumes (anterior: $F_{1,16}$ =3.20, p=.09, η2=.17; posterior: $F_{1,16}$ =.07, p=.79, η2=.01). There were no effects of hemisphere (total: $F_{1,16}$

=.34, p=.57, η2=.02; anterior: $F_{1,16}$ =.02, p=.89, η2<.01; posterior: $F_{1,16}$ =.36, p=.58, η2=.02), or status by hemisphere (total, $F_{1,16}$ =1.13, p=.30, η2=.07; anterior,$F_{1,16}$ =3.51, p=.08, η2=.18; posterior, $F_{1,16}$ =.36, p=.56, η2=.02), treatment by hemisphere (total: $F_{1,16}$ =1.17, p=.30, η2=.07; anterior, $F_{1,16}$ =.01, p=.91, η2<.01; posterior, $F_{1,16}$ =2.07, p=.17, η2=.12), or status by treatment by hemisphere interaction effects (total, $F_{1,16}$ =.04, p=.84, η2<.01; anterior, $F_{1,16}$ =.57, p=.46, η2=.03; posterior, $F_{1,16}$ =.91, p=.36, η2=.05). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

3.4.6 Amygdala

Although no main effects of status ($F_{1,16}$ =2.54, p=.13, η2=.14) or treatment ($F_{1,16}$ =1,28, p=.27, η2=.07) were found, there was a main effect of hemisphere ($F_{1,16}$ =30.53, p<.001, η2=.66), such that left amygdala volumes were larger than the right ones. No interaction effects were detected for this structure (status by treatment ($F_{1,16}$ =.58, p=.46, η2=.04), status by hemisphere ($F_{1,16}$ =.03, p=.87, η2<.01), treatment by hemisphere ($F_{1,16}$ =1.54, p=.23, η2=.09), or status by treatment by hemisphere ($F_{1,16}$ =1.58, p=.23, η2=.09)). Commensurate results were seen using unadjusted data, and all raw data are listed in Table 3.1.

3.5 Discussion

Our findings suggest that both E2 and social subordination cause specific, but not widespread, structural changes in the adult female brain. In particular, E2 treatment of

ovariectomized adult female macaques for just a month caused significant decreases in frontal cortex gray matter volume. Furthermore, subordinate status modified the effects of E2 in the cingulate cortex, which were opposite depending on rank, increasing gray matter volume in DOMs, but decreasing it in SUBs. These data suggest that a background of chronic stress imposed by social subordination can reverse the effects of E2 on brain structure.

The lack of social status effects on brain volume, independent of treatment, may seem contradictory to both the rodent, nonhuman primate and human stress literature (Uno et al., 1994; Sanchez et al., 1998; Coe et al., 2003; Joels et al., 2007; Spinelli et al., 2009; Tottenham and Sheridan, 2010; Pryce et al., 2011). Although there is always a possibility that chronic stress, or exposure to stress hormones, does not affect total or regional brain volumes (Leverenz et al., 1999), we do not think that our findings support that interpretation. Indeed, social subordination effects have been previously reported in young adult male rhesus monkeys brain structure, and suggest that higher social status was associated with increased gray matter density in the rostral PFC (Sallet et al., 2011). Although social subordination could be a different experience of chronic stress for males and females in this species (Abbott et al., 2003; Sapolsky, 2005), an alternative explanation is that although the female macaques in our studies show signs of stress (Michopoulos et al., 2012a; Michopoulos et al., 2012b)that result in brain stress-related changes, these changes may have been too subtle to detect with the current methodology. In the studies done by Sallet et al. (2011), reductions in frontal gray matter density were identified in small clusters using deformation based morphometry (DBM), as opposed to

the regional volumes studied here. Additionally, the majority of gross regional alterations in rhesus monkey brain volumes associated with stress have been detected mainly when the animals were exposed to stress during development when brain organization is still underway, including prenatal and early-life exposure (Uno et al., 1994; Sanchez et al., 1998; Coe et al., 2003; Spinelli et al., 2009; Pryce et al., 2011). In these studies, chronic stress or glucocorticoids have been associated with decreased prefrontal cortex (Sanchez et al., 1998), frontal cortex, cingulate cortex (Spinelli et al., 2009), and hippocampus volumes (Uno et al., 1994; Coe et al., 2003). In our current study our goal was to examine the specific structural effects of social subordination imposed during adulthood, actually controlling for exposure to early life stress by using adult females that were born and raised into middle ranking matrilines within their natal groups (Jarrell et al., 2008). Because we have evidence that juvenile female subordinate rhesus monkeys show significant differences in regional brain volumes, including a larger amygdala, compared with more dominant females (Godfrey et al., 2013), the effect of subordination on regional brain volume my be limited to the developing brain.

Another critical finding in our data is the modifying effect of social subordination on E2's effects on cingulate gray matter volume, as E2 reduced cortical gray matter volume in subordinate but increased it in dominant females, in the right hemisphere. Reductions in cingulate cortical volume have been linked with post traumatic stress disorder (PTSD) (Karl et al., 2006b; Eckart et al., 2011), and reductions in subgenual cingulate volume are characteristic alterations in individuals with familial major depressive disorder (Drevets et al., 2008b). These decreases, however, are not always detected (Hamani et al., 2011),

as some studies show no difference in subgenual volume in bipolar or unipolar depression (Brambilla et al., 2002). The cingulate GM volume represented in our study includes areas 23, 24, 25 (subgenual), and 32 as described in a rhesus monkey stereotaxic atlas (Saleem and Logothetis, 2006), and therefore our findings suggest that E2 can result in deleterious effects on the broader cingulate cortex dependent on social subordination. Data on normally cycling women without psychopathology have also shown a negative correlation between circulating E2 and anterior cingulate volumes, although stress history was not measured (De Bondt et al., 2013). It is possible that the modification of E2-induced changes in cingulate volumes by social subordination explains some of the disparities in the literature regarding the effects on cingulate volume in clinical populations with stress-related disorders. Findings from our lab in this same model of social subordination show alterations in cingulate resting-state functional connectivity (FC) in SUB females (increased FC with other brain regions), suggesting a functional correlate to the volumetric reduction (Reding et al., Chapter 4).

The data from the present study also suggest that E2 treatment reduces frontal GM volumes, and that this effect is independent of social status. This finding was contrary to our hypotheses, as rodent data show increases in frontal volumes following one week of E2 replacement (Shansky et al., 2010). Notably, the effects we detected in the current study were measured following one month of E2 treatment at mid-follicular cycle levels, and thus E2 levels are comparable to both naturally cycling women and post menopausal women on estrogen replacement therapy (ERT). However, in the literature on post-menopausal women, the effects of E2 are thought to be moderated by age and/or timing

of treatment following menopause, among other factors (Wnuk et al., 2012). Thus, younger postmenopausal women who initiate E2 replacement therapy within an average of one year following menopause show increased frontal volume following ERT (Boccardi et al., 2006). However, older postmenopausal women who participated in the MRI component of the Women's Health Initiative Memory Study (WHIS-MRI) and began treatment with conjugated equine estrogen no sooner that 65 years of age actually showed decreased frontal volumes (Resnick et al., 2009). Although all animals in the present study were ovariectomized for ~ 6 years prior to the scans, having had intermittent exposure to E2 during that time, there were no social status differences in age at ovariectomy or time from ovariectomy. However, the average age of the females at the time of the scans was 14 years, comparable to middle age premenopausal women (Walker, 1995). Thus, it is possible that the effects of E2 on regional brain volumes would be different in younger adult females or on females that had been ovariectomized more recently.

Although E2 was administered in a physiologically relevant dose, females were treated chronically for approximately one month, which does not mimic naturalistic alteration across the menstrual cycle but is comparable to short-term ERT. The effects of chronic versus acute E2 treatment on neuronal morphology have not been systematically tested. For example, data on CA1 spine density within the hippocampus suggest that E2's structural effects are seen 24 hours following acute E2 treatment and persist for up to 9 days (Gould et al., 1990; Woolley and McEwen, 1993). However, chronic E2 administration (approximately 5 weeks) also resulted in increased CA1 dendritic

arborization and spine density (McLaughlin et al., 2010). Furthermore, seven to ten months of either cyclic (1/week) versus chronic E2 treatment, also suggest comparable positive effects on hippocampal-dependent spatial memory performance despite differences in treatment regimen (Gibbs, 2000). Less is known about the effect of chronic or acute E2 treatment in rodent PFC and amygdala dendritic morphology, however, the data presented in the introduction varied from one week (Shansky et al., 2010) to approximately two weeks (Garrett et al., 2009), which exceeds the typical rodent estrus cycle of four to five days (Becker et al., 2005). In general E2's effects on neural morphology in adulthood, and the effect of treatment duration, appear to function in a region specific manner (Woolley and Cohen, 2002) and more data is needed to determine it's effects within brain regions.

Hemispheric lateralization effects (main and interaction) were also detected in several regions, including larger right than left frontal and cingulate cortices GM volumes, and the opposite, larger left than right PFC GM and amygdala volumes. The cingulate cortex also showed increased GM/WM ratios (GM>WM) in the right, but not left, hemisphere while the prefrontal cortex showed increased ratios in the left, but not right, hemisphere, suggesting that these lateralization effects were primarily driven by GM differences. Studies on primate brain lateralization suggest that humans have greater frontal GM volumes in the left hemisphere, and comparative phylogeny of monkey species show the reverse pattern (Smaers et al., 2011), and this same trend is seen in rhesus monkeys (Falk et al., 1990). Other studies on rhesus monkeys, however, suggest no specific cerebral or amygdalar lateralization but larger hippocampal volumes in the left hemisphere, at least

in female infants/juveniles (0-28 months) (Payne et al., 2010). The functional significance of these hemispheric asymmetries is unknown, although there is some data suggesting no correlation with handedness preferences (Hopkins and Rilling, 2000).

One limitation of our studies is the lack of information about the cellular mechanisms that account for volumetric differences (i.e. are they reflecting changes in dendritic length and/or branching, or neuronal or glial density or size?). Rodent studies comparing reductions in ACC and hippocampal volumes obtained using sMRI with postmortem histological analyses from the same animals suggest that volume loss is associated with reduced density of synaptic spines, dendritic length, but not total number or surface area of neurons or glia (Kassem et al., 2013). However, post-mortem data from individuals diagnosed with major depression suggest that reduced subgenual ACC volumes are due to a reduction of the number of glia (Ongur et al., 1998), while volume reductions of PFC regions, including the dlPFC and the OFC, are associated with reduced cell volumes of both neurons and glia (Rajkowska, 2000). Further studies are needed to address the underlying cellular and molecular mechanisms of the volumetric changes reported in our study and to understand the neurobiological mechanisms underlying stress-related changes in adult brain structure, but our data suggest that particular attention needs to be paid to hormonal state in future neuroimaging analyses on brain regional volumes.

In conclusion, social subordination and E2 treatment in adult female rhesus monkeys both induce changes in cortical GM volumes. This study presents novel data supporting the plasticity of the adult female primate brain in response to social experience and

changing hormonal environments. Our data suggest that exposure to chronic stress that is imposed by social subordination alters subsequent structural effects of E2 on specific cortical regions. Importantly, social status effects on brain volumes are not detected without concomitant treatment with E2, suggesting that social subordination has profound, yet dichotomous, effects on E2. Importantly, the E2- and status-related structural changes reported here are striking, as they are visible following only one month of E2 treatment in ovariectomized females, and are found despite periods of time without endogenous ovarian hormones. Together these results underscore the plasticity of the female primate brain and shed light on effects of social subordination and E2 treatment on it, providing insights into possible mechanism of adaptation to the social and hormonal environments that may exacerbate risk of developing stress-related psychopathology.

Table 3.1. Regional brain volumes. Total intracranial volume (ICV), cortical and subcortical regional volumes are listed by hemisphere, status, and treatment. (^{a,b}) Main effect of E2 treatment, a's > b's, $p<.05$. (^{c,d}) Main effect of hemisphere c's > d's (^{e,f}) Interaction of status and treatment, such that within each status e's > f's, $p<.05$. (*) Interaction of status, treatment, and hemisphere, post hocs were significant only in the right hemisphere, $p<.05$. Data are summarized by unadjusted volumes ($mm^3 \pm$ S.E.).

Table 3.1. Continued.

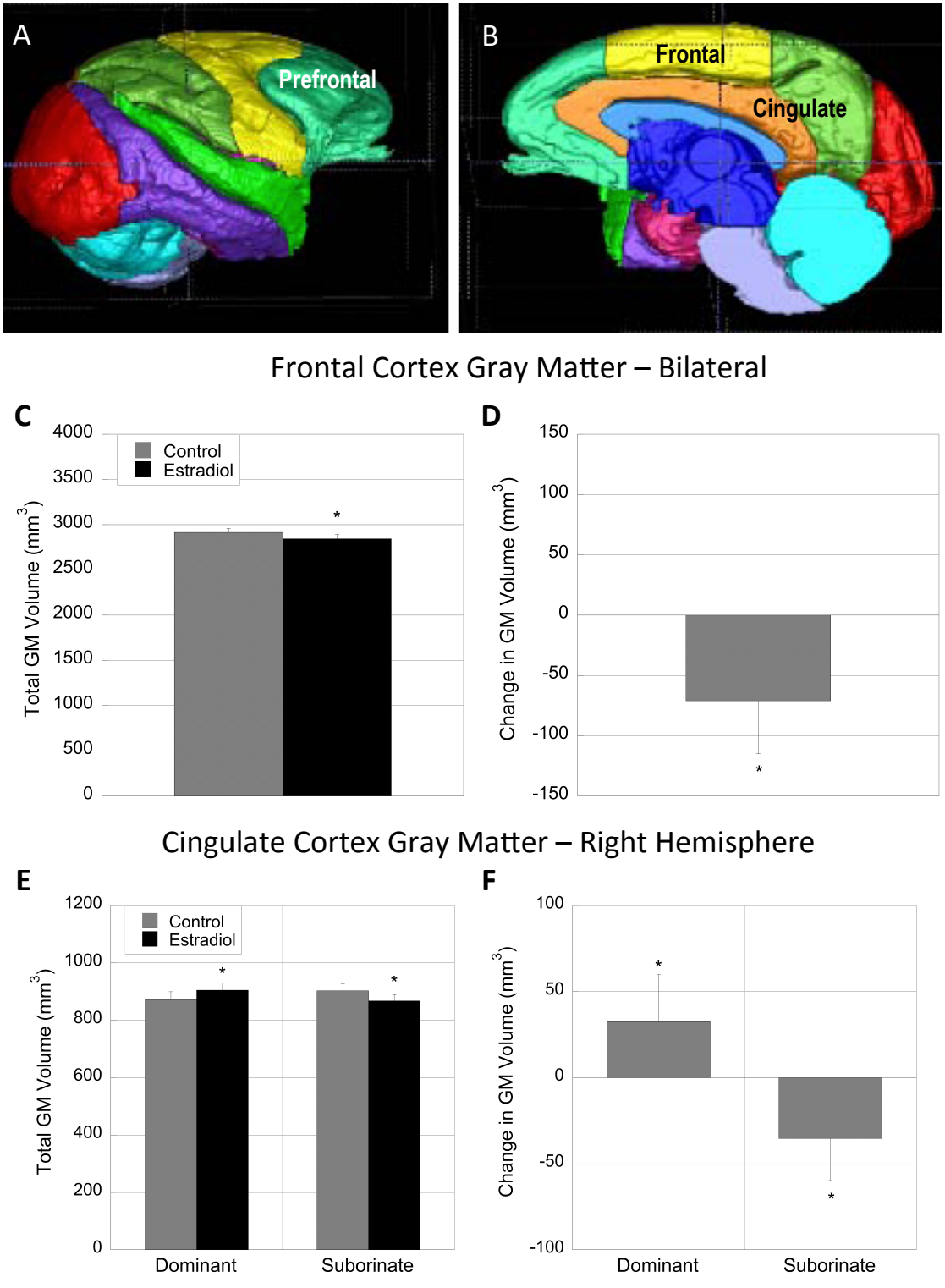
Region	Hemisphere	Dominant		Subordinate	
		Control	Estradiol	Control	Estradiol
Total					
ICV	NA	89449.7 ± 2062.3	88987.8 ± 2030.6	88641.1 ± 1844.6	88595.9 ± 1816.3
Prefrontal					
Gray Matter	R	2463.4 ± 87.4 [d]	2501.5 ± 98.2 [d]	2557.6 ± 78.2 [d]	2539.7 ± 87.9 [d]
	L	2539.8 ± 94.8 [c]	2555.8 ± 100.4 [c]	2604.9 ± 84.8 [c]	2604.4 ± 89.8 [c]
White Matter	R	1076.5 ± 37.6	1101.3 ± 38.1	1105.7 ± 33.7	1110.3 ± 34.0
	L	1088.4 ± 38.6	1093.6 ± 41.1	1079.1 ± 34.6	1075.5 ± 36.8
Frontal					
Gray Matter	R	2980.3 ± 64.1 [a,c]	2914.4 ± 70.7 [b,c]	2902.1 ± 57.3 [a,c]	2838.4 ± 63.2 [b,c]
	L	2959.3 ± 72.1 [a,d]	2868.9 ± 74.2 [b,d]	2821.8 ± 64.5 [a,d]	2758.0 ± 66.4 [b,d]
White Matter	R	1645.3 ± 43.7	1628.6 ± 53.8	1617.5 ± 39.1	1626.5 ± 48.2
	L	1612.9 ± 54.1	1561.4 ± 40.0	1599.3 ± 48.4	1592.5 ± 35.8
Cingulate					
Gray Matter	R	872.6 ± 27.3 [c, f,*]	905.2 ± 24.4 [c,e,*]	903.3 ± 24.5 [c,e,*]	868 ± 21.8 [c,f,*]
	L	843.9 ± 30.2 [d,f]	838.5 ± 32.6 [d,e]	842.9 ± 27 [d,e]	828.1 ± 29.2 [d,f]
White Matter	R	220.9 ± 8.4	231.0 ± 11.7	230.3 ± 7.5	220.3 ± 10.4
	L	233.0 ± 8.4	239.2 ± 9.7	225.0 ± 7.5	228.9 ± 8.7
Hippocampus					
All	R	433.0 ± 10.8	439.1 ± 10.1	455.2 ± 9.6	445.3 ± 9.0
	L	435.2 ± 13.2	433.2 ± 12.9	463.1 ± 11.8	447.9 ± 11.5
Posterior	R	222.0 ± 8.2	217.4 ± 7.4	230.6 ± 7.3	225.3 ± 6.7
	L	231.7 ± 8.4	215.8 ± 7.3	231.1 ± 7.5	223.8 ± 6.5
Anterior	R	210.9 ± 8.6	221.7 ± 8.3	224.6 ± 7.7	220.0 ± 7.5
	L	203.5 ± 9.7	217.3 ± 11.2	232.0 ± 8.7	224.1 ± 10
Amygdala					
All	R	323.7 ± 10.1 [d]	331.2 ± 12.5 [d]	309.6 ± 9.1 [d]	302.9 ± 11.2 [d]
	L	345.9 ± 10.5 [c]	352.9 ± 11.8 [c]	321.8 ± 9.4 [c]	331.8 ± 10.6 [c]

Table 3.2. Gray matter/ white matter ratios, adjusted for ICV and listed by hemisphere, status, and treatment. Data are reported as ratios of adjusted GM to WM volume ± SEM ([a,b]) Main effect of hemisphere a's > b's, $p<.05$. ([c,d]) Interaction of status and hemisphere, such that within each status c's > d's, $p<.05$. Data are summarized by unadjusted volumes (mm^3 ± S.E.).

Region	Hemisphere	Dominant		Subordinate	
		Control	Estradiol	Control	Estradiol
Prefrontal					
	R	2.30 ± 0.07 [b,d]	2.28 ± 0.07 [b,d]	2.32 ± 0.06 [b,d]	2.29 ± 0.07 [b,d]
	L	2.35 ± 0.06 [a,c]	2.35 ± 0.07 [a,c]	2.41 ± 0.06 [a,c]	2.42 ± 0.06 [a,c]
Frontal					
	R	1.82 ± 0.04	1.80 ± 0.07	1.8 ± 0.04	1.75 ± 0.06
	L	1.84 ± 0.05	1.84 ± 0.05	1.77 ± 0.04	1.74 ± 0.05
Cingulate					
	R	3.96 ± 0.13 [a]	3.97 ± 0.16 [a]	3.95 ± 0.11 [a]	3.98 ± 0.14 [a]
	L	3.64 ± 0.12 [b]	3.55 ± 0.14 [b]	3.76 ± 0.11 [b]	3.62 ± 0.13 [b]

Figure 3.1. Brain images depict the GM segmentation of the (A) prefrontal (teal), (E frontal (yellow), and cingulate (orange) cortices. WM volumes correspond to total volume within each subdivision. (C) Main effect of E2 treatment on bilateral frontal volume. (D) Graphical representation of the change in frontal GM volume from the control condition to the E2 condition, negative values represent GM volume loss. (E Interaction of status, treatment, and hemisphere in cingulate cortex GM. (F) Graphic representation of the change in cingulate GM volume from the control condition to t condition, negative values represent GM volume loss. Data are reported as unadjust mean volume changes (mm^3) ± SEM, (*) significant difference from control conditi $p<.05$. [Brain images adapted from Knickermeyer et al., 2010]

Figure 3.1. Continued.



Chapter 4: Effects of social subordination and estradiol on resting-state prefrontal and amygdala functional connectivity in adult female rhesus monkeys.

4.1 Abstract

Women are twice as likely as males to develop stress-related psychopathology, such as anxiety and mood disorders, and stressful life events are the best predictors for development of depression among women. This suggests that ovarian hormones may render women more susceptible to the deleterious effects of chronic stress. Medial prefrontal cortex (mPFC) – amygdala circuits are critical for emotional regulation, and altered structural connectivity of this circuit in female rodents following both exposure to a chronic stressor and the ovarian hormone estradiol (E2) suggest that functional connectivity (FC) may also be altered. In this study, we performed a resting-state functional magnetic resonance imaging (rs-fMRI) analysis in ovariectomized rhesus monkeys (*Macaca mulatta*) in the absence of E2 and following ~ 4 weeks of E2 treatment to test the hypothesis that social subordination, an established chronic social stressor in macaque females, would alter FC between the amygdala and the mPFC (areas 10, 24, and 32), and that this effect would be exacerbated following E2 treatment. Results show main effects of E2 and social status on mPFC - amygdala FC but no interaction, such that FC was increased between area 10 and the amygdala in subordinate (SUB) females and decreased during the E2 condition, independent of status. In addition whole brain voxel-wise analysis revealed more global effects of social status, with SUBs showing decreased negative FC between the amygdala and reward regions and increased positive FC between the mPFC and the supplemental motor area (SMA), posterior cingulate cortex (pCC), and the superior temporal sulcus (STS). E2 replacement increased negative FC between the mPFC and the nucleus of the solitary tract (NTS) but

decreased negative FC between the mPFC and the cerebellum. Our data show functional plasticity of the adult brain in response to both social subordination and E2, and suggest that neural adaptations to chronic stress resulting from social subordination may impact systems involved in reward and vigilance, while E2 appears to modulate brain circuitry regulating executive control of behavior as well as homeostasis.

4.2 Introduction

Chronic psychosocial stress results in adverse health outcomes and increased susceptibility to mood and anxiety disorders (Hammen, 2005a; Cohen et al., 2007). Women are at a significantly higher risk to develop stress-related psychopathology compared to men, suggesting an interaction between stressor sensitivity and ovarian hormone signaling (Dorn and Chrousos, 1997; Pinkerton et al., 2010). Clinical neuroimaging studies in mood and anxiety disorders implicate alterations in brain circuits involved in socioemotional behavior and stress regulation, converging within the amygdala – medial prefrontal cortex (mPFC) pathway (Liotti and Mayberg, 2001; Drevets et al., 2008a; Sheline et al., 2010; Hahn et al., 2011; Hamani et al., 2011; Hughes and Shin, 2011). A better understanding of the neurobiological effects of chronic stress and their interaction with those of the ovarian hormone estradiol (E2) on amygdala – mPFC circuits will provide critical insight into the underpinnings of increased susceptibility of women to the deleterious effects of stress on health outcomes.

Evidence from rodent models suggests that chronic stress and exposure to E2 produce structural changes in the amygdala and mPFC, including fibers connecting both regions (McLaughlin et al., 2009). Stress hormones, in particular glucocorticoids (GC), and E2 can, indeed, have structural effects via genomic and non-genomic mechanisms in these regions (McEwen, 2002) given that both GC and estrogen receptors are expressed in the amygdala and PFC (Sousa et al., 1989; Morimoto et al., 1996; Shughrue et al., 1997). Behaviorally, the mPFC plays a significant role in gating amygdala responses to threat or salient emotional stimuli, therefore regulating behavioral, neuroendocrine, and autonomic responses to stressors, and modulating fear learning and extinction processes (Quirk and Gehlert, 2003; Quirk and Beer, 2006; Sotres-Bayon and Quirk, 2010). Therefore, disruptions of this pathway caused by chronic stress may lead to a dysregulation of these adaptive functions, resulting in maladaptive socioemotional behavior (Joels et al., 2007; Arnsten, 2009; Kim et al., 2011b). Specifically, in male rodents, chronic restraint stress decreases dendritic arborization in the mPFC (Shansky and Morrison, 2009; Radley et al., 2013) while increasing amygdala dendritic arborization (Rosenkranz et al., 2010), structural alterations associated with increased fear and anxiety-like behavior (Conrad et al., 1999; Gameiro et al., 2006). Furthermore, stress increases both mPFC (Jackson and Moghaddam, 2006) and amygdala excitability (Rosenkranz et al., 2010), while also reducing amygdala – mPFC long-term potentiation (Maroun and Richter-Levin, 2003; Joels et al., 2007) and synchronization of electrophysiological activity between these regions, suggestive of impaired top-down inhibitory control of the amygdala by the mPFC (Lee et al., 2011). Females, however, show a different mPFC response to chronic stress, which seems E2-dependent. Notably, chronic restraint stress in ovariectomized

female rats with E2 replacement results in increased apical dendritic length (Garrett and Wellman, 2009) and spine density in mPFC neurons projecting to the basolateral amygdala (BLA) (Shansky et al., 2010). Interestingly, E2 alone can also increase density of dendritic spines in the mPFC (Shansky et al., 2010) and amygdala (Walf and Frye, 2006). These data suggest that chronic stress and E2 increase dendritic fields and synaptic density in the mPFC pathway connecting with the amygdala in females, but not males, at least in rodents. However, whether these structural modifications result in enhanced structural and functional connectivity between the two regions is unclear. Taken altogether, this evidence from rodent models suggests that E2 may modulate the structural effects of stress on the mPFC-amygdala pathway involved in emotional and stress regulation.

A number of studies show that the mPFC-amygdala circuits are also critical in emotional regulation, fear learning, and detection of social and emotional salient stimuli in humans (see Kim et al., 2011b for a review). In healthy controls, resting-state functional magnetic resonance imaging (rs-fMRI) data shows increased functional connectivity (FC) between the amygdala and mPFC following acute stress (van Marle et al., 2010; Veer et al., 2011). The effects of chronic stress on this circuit, however, have not been examined. Data suggest that increased mPFC – amygdala FC is associated with enhanced top-down control of amygdala activation (Hare et al., 2008). Increased structural connectivity (Kim and Whalen, 2009) and FC (Kim et al., 2011a) in this pathway is also associated with lower levels of trait anxiety in health individuals. Conversely, individuals with stress-induced psychopathology show a marked decrease in mPFC-amygdala FC (Hahn et al.,

2011; Prater et al., 2013; Stevens et al., 2013). One possibility is that chronic stress leads to alterations in FC (less top-down control of mPFC over amygdala) that results in poor emotional regulation. Given the sex-differences in these regions reported in the rodent literature and the effects of stress and E2 on both structure and function in females, the increased incidence of stress-related psychopathology in women may be in part due to the interaction of chronic stress and E2 on the FC of the mPFC-amygdala pathway.

Modeling the effects of chronic stress in humans presents prohibitive ethical challenges, and observational studies on individuals exposed to chronic stress are lacking in experimental control. Although the foundation of our knowledge of the effects of stress and E2 on the mPFC – amygdala pathway is largely based on rodent studies, given the issues of homology between the rodent and primate mPFC in the literature, such that the rodent mPFC cortex is more homologous with the primate premotor cortex and anterior cingulate cortex (ACC) (Preuss, 1995), we chose to use a non-human primate model of chronic social stress to investigate the specific impact of chronic stress and E2 on mPFC-amygdala FC. The female monkey model of social subordination stress is a well-defined and highly translational paradigm used to examine the consequences of chronic exposure to stress in women. Furthermore, use of ovariectomized monkeys allow us to conduct a prospective, cross-over treatment design to control for exposure to E2 in combination with stress. For this analysis we focused on mPFC regions defined in the rhesus monkey with relevance to the human literature on emotional regulation (Quirk and Beer, 2006) including the medial frontal pole (area 10) and the ACC (areas 24, 32) (Carmichael and Price, 1994, 1995; Ghashghaei et al., 2007; Barbas, 2013).

Social subordination in nonhuman primates is a potent psychosocial stressor (Sapolsky, 1995) that for female rhesus monkeys results in stress-related phenotypes, providing a translational model to explore the consequences of chronic stress exposure in women (Michopoulos et al., 2012a). Socially subordinate females show stress-induced impairment of HPA axis function (Shively et al., 1997; Shively, 1998; Jarrell et al., 2008; Michopoulos et al., 2012b), increased adrenal gland size (Shively and Kaplan, 1984; Adams et al., 1985), compromised ovarian function (Adams et al., 1985; Shively et al., 1997; Kaplan and Manuck, 2008) and reduced monoamine signaling (Grant et al., 1998; Shively, 1998; Kaplan et al., 2002; Michopoulos et al., 2012a). Moreover, social subordination impairs E2-induced prosocial and sexual behavior (Reding et al., 2012), exacerbates E2-induced attenuation of glucocorticoid negative feedback (Wilson et al., 2005), and potentiate E2 negative feedback inhibition of luteinizing hormone secretion (Michopoulos et al., 2009). Together, these data show that social subordination in adult female rhesus monkeys modifies several effects of E2 action on behavior and physiology and, thus, provides a significant opportunity to identify changes in neurobiology, including within PFC-amygdala circuits, that may help identify potential mechanisms for the increased susceptibility of women to mood and anxiety disorders.

Using this female rhesus macaque model of chronic subordination stress, in combination with rs-fMRI to measure *in vivo* intrinsic FC, the goal of the current study is to understand the neurobiological embedding (McEwen, 2012) of chronic social stress on primate mPFC-amygdala connectivity and its modulation by E2. We hypothesize that

social subordination will decrease mPFC-amygdala FC in adult female rhesus monkeys, and that this effect will be exacerbated when combined with E2 replacement. Furthermore, we hypothesize that social subordination and E2 will also decrease FC of these two regions with other brain structures. These data will serve to bridge the gap between rodent and human data to understand the interaction effects of chronic social stress and E2 on the adult female brain.

4.3 Methods

4.3.1 Subjects

Subjects were 20 ovariectomized adult female rhesus monkeys (*Macaca mulatta*), socially-housed and maintained at the Yerkes National Primate Research Center (YNPRC) Field Station. Subjects were either the most dominant (DOM, Rank 1, N=10) or the most subordinate (SUB, Rank 4 or 5, N=10) females in each of ten groups of 4-5 females housed with one resident adult male. Animals were 14 (± 2) years of age with no significant age difference between groups (DOM=13±2; SUB=14±3; $F_{1,11}$=.147, p=.71,η^2=.01). Subjects were housed in indoor - outdoor enclosures that measured 20 X 15 X 8 feet each. Animals were fed a standard commercial low-fat high-fiber monkey chow diet (Ralston Purina Company, St. Louis MO) *ad libitum* supplemented daily with seasonal fruits and vegetables. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) 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."

Rhesus monkeys social groups are structured in linear dominance hierarchies that are maintained by both contact and non-contact aggressive behavior from more DOM females to more SUB females (Bernstein, 1970; Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984; Michopoulos et al., 2012a). Thus, frequency of aggressive, affiliative, and submissive behavior are rank dependent, with SUB females receiving the most aggression, often random and unpredictable, from more DOM animals as well as exhibiting the highest rates of submissive behaviors directed toward more DOM animals to attenuate the aggression (Sapolsky, 1995; Silk, 2002). In this study social groups were well established having been formed experimentally as described previously (Jarrell et al., 2008). Briefly, middle ranking, ovariectomized females were removed from natal social breeding groups and introduced to one another to form new smaller groups of five unrelated, unfamiliar females each. Females were added sequentially, and dominance hierarchies were established based on largely order of introduction, with the final female often assuming the lowest rank. Since group formation, subjects have been included in several studies in which they received periodic replacement therapy with E2 and or progesterone (Jarrell et al., 2008; Michopoulos et al., 2009; Michopoulos et al., 2011; Asher et al., 2012; Reding et al., 2012). During the course of data collection for this study, two DOM females were unable to complete both treatment conditions, and were therefore excluded from the analysis, bringing the sample size to 18 females. An additional five females were removed from the analysis due to artifacts present in the MRI data, making the final sample size for these studies N=13 females (6 DOM, 7 SUB).

4.3.2 Estradiol Treatment

All females were studied under two experimental conditions: an E2 replacement and no-treatment control condition, which were presented in a counterbalanced order. All females were ovariectomized 6 (± 2) years prior to the start of the study with no significant age difference between groups (DOM= 5 ± 2; SUB=7 ± 3; $F_{1,11}$=1.511, p=.25,η^2=.12). For the E2 treatment condition, females were implanted with subcutaneous E2-filled continuous-release Silastic® capsules placed between the scapulae under anesthesia, to yield serum levels between 90-100 pg/mL corresponding to physiological levels typical of the mid- to late follicular phase in this species (Wilson et al., 1982). E2 treatment was administered for an average of 31 (± 3) days prior to the neuroimaging scans with no significant differences between groups (DOM = 28 ± 8 days; SUB= 33 ± 12 days; $F_{1,11}$=.944, p=.35, η^2=.08). During the control condition, females had received no E2 treatment for at least 43 (± 13) days prior to the neuroimaging scans with no significant differences based on status (DOM = 44 ± 15 days; SUB= 41 ± 12 days; $F_{1,11}$=.151, p=.71, η^2= .01).

4.3.3 Behavioral Data

To verify social status ranks as well as provide a behavioral phenotype of each subject, behavioral data were collected during the first week of each treatment condition. Due to the physical alterations associated with E2 treatment (e.g. reddening and or swelling of skin on face, genitals, and buttocks), observers were not blind to treatment. Observations were conducted for 30 min at 14:00 hr on three separate days to provide information on

social behavior within the dominance hierarchy of each group. Data was recorded in an actor – behavior – recipient format using the "HandObs" program developed by the Center for Behavioral Neuroscience, Atlanta, Georgia (Graves and Wallen, 2006). Inter-observer reliability exceeded 90%. Behavior was coded using a well-established rhesus monkey ethogram (Altmann, 1974) with modifications (Pope et al., 1987; Jarrell et al., 2008) and was analyzed as frequency within the following behavioral categories of (1) agonistic behavior directed toward females (attacks, chases, or threats with and without contact), (2) agonistic behavior received from females, (3) submissive behavior directed toward females (withdraws or grimaces), (4) submissive behavior received from females, (5) anxiety-like behavior (yawns, body-shakes, pacing, or self-directed scratching and bodily exploration), (6) affiliative behavior directed toward females (initiated proximity or grooming), (7) affiliative behavior received from females, (8) affiliative behavior toward males, (9) affiliation received from males, (10) proceptive sexual behavior directed toward males (e.g. female hindquarter presentation, handslap, standup, threataway, or crouch behavior), and (11) receptive sexual behavior toward males (e.g. received hiptouch or mount from male).

Behavioral data were normalized using a logarithmic transformation and analyzed with a repeated-measures analysis of variance (rmANOVA) using E2 dose (E2, Control) as the repeated, within, subjects variable, and social status (rank: SUB, DOM) as the between subjects variable. Bonferroni corrections for an $\alpha=.05$ for the multiple comparisons (33) would require $p<.002$; however, due to the small sample size in this study, we set our

significant level at $p<.05$ and provide effect sizes (η^2). Results are reported using mean raw behavioral data and standard error of the mean (SEM).

4.3.4 fMRI Data Acquisition and Analysis

Imaging Protocol. All females underwent two neuroimaging sessions following either the E2 treatment or the control condition. Subjects were transported from their social group to the YNPRC Imaging Center the day before the scans. All scans were acquired on a 3T Siemens Magnetom TRIO system (Siemens Med. Sol., Malvern, PA, USA) using an 8-channel phase array coil. Scans were conducted during a single session, which included T1- and T2-weighted MRI scan for registration purposes, and 4 x15 minute rs-fMRI (T2*-weighted) scans to measure temporal changes in blood-oxygen-level dependent (BOLD) signal change. All animals were scanned supine in the same orientation, achieved by placement and immobilization of the head in a custom-made head holder via ear bars and a mouthpiece. Scans were acquired under isoflurane anesthesia (1-1.2% to effect, inhalation, kept to a minimum to minimize effects of anesthesia on BOLD signal), following initial induction with telazol (5mg/kg, i.m.). Patterns of coherent BOLD fluctuations seen within this range of isoflurane are similar to those seen in awake and behaving monkeys, including sensory, motor, and cognitive-task related systems (Vincent et al., 2007). Animals were fitted with an oximeter, ECG, rectal thermistor and blood pressure monitor for physiological monitoring, an i.v. catheter to administer dextrose/NaCl (0.45%) and maintain normal hydration, and an MRI-compatible heating pad. Upon completion of the scans and full recovery from anesthesia, each female was returned to their social group.

fMRI acquisition and preprocessing. Whole-brain rs-fMRI data was acquired using a T2*-weighted gradient-echo echo-planar imaging (EPI) sequence with the following scan parameters: 400 volumes, TR/TE = 2060/25ms, voxel size =1.5mm^3 (isotropic). Four rs-fMRI scans (15min each) were acquired and the first two volumes of each were removed to allow for scanner equilibrium resulting in a total of 1592 concatenated volumes. The high-resolution anatomical scans were acquired using a T1-weighted 3-dimensional (3D) magnetization-prepared rapid gradient-echo (3D-MPRAGE) parallel imaging sequence (128 coronal slices, TR/TE=3000/3.52ms, voxel-size=0.5mm^3 -isotropic) and a T2-weighted scan (50 coronal slices, TR/TE=7900/125ms, TE=125ms, voxel-size=0.5mm x 0.5mm x 1mm).

All raw rs-fMRI data was preprocessed using the FMRIB Software Library (FSL) fMRI Expert Analysis Tool (FEAT), including slice-time correction, rigid body correction for head motion, unwarping of field map distortions, and rigid-body co-registration of the rs-fMRI volumes with the high resolution T2- and then with the T1-weighted structural images. T1-weighted structural images were transformed using nonlinear registration to conform to a T1-weighted image that was an average of 18 rhesus monkeys, 10 males and 8 females (Styner et al., 2007), which was nonlinearly registered onto the widely-used macaque F99 atlas, freely available as part of the CARET software package (http://brainvis.wustl.edu/wiki/index.php/Main_Page). Thus, the registration parameters obtained from each step allowed raw rs-fMRI images to be transformed into F99 space, combining motion correction, fieldmap unwarping, and atlas transformation in one

interpolation step. Several additional steps were also taken to prepare the data for connectivity analyses (Fox et al., 2005), including temporal bandpass filtering (0.009 Hz < f < 0.08 Hz), spatial smoothing (3mm full-width at half-maximum), and regression of nuisance signals. Nuisance signals consisted of the whole-brain signal and the six parameters related to rigid-body motion correction. All subjects were monitored continuously for stable vitals and to confirm effective sedation during scanning at the standardized isofluorane levels (1-1.2%). Although no detectable motion was observed for any subject, motion censoring was nonetheless performed according to procedures recommended in prior work (Fair et al., 2012; Power et al., 2012; Iyer et al., 2013).

4.3.5 Functional Connectivity Analysis

Region of Interest (ROI) FC Analysis. All mPFC ROIs, including 10, 24ab, and 32, were defined based on the Lewis and Van Essen (2000) anatomical parcellations provided in the CARET software (Van Essen et al., 2001) and mapped onto the cortical surface of the F99 rhesus monkey atlas (Van Essen and Dierker, 2007). The right and left amygdala BOLD rs-fMRI signal time courses were separately correlated with each of the mPFC ROIs (10, 24ab, 32). BOLD signal time courses for the right and left amygdala, along with those in each of the mPFC regions were extracted and pair-wise correlation analysis between regions yielded values (Pearson's r-values) indicative of FC between two regions (Friston, 2011). Correlation values were then Fisher z-transformed and analyzed in a rmANOVA using E2 treatment as the within subject variable (E2, Control), and status as the between subjects variable (DOM, SUB). Although Bonferroni corrections for an α=.05 for the multiple comparisons (total: 36) would require a $p<.001$ level, due to

the small sample size in this study, we set our significant level at $p<.05$, and provide effect sizes (η^2) information. All raw r-values ± SEM are reported.

Voxel-wise FC Analysis. In an exploratory effort to examine effects outside of the PFC-amygdala circuits of interest, we performed a voxel-wise analysis of amygdala and PFC FC across the whole brain using the FIDL software package (http://www.nil.wustl.edu/~fidl/) developed at the Neuro-Imaging Laboratory at Washington University, St. Louis. Whole brain correlation maps were obtained using the bilateral amygdalae and PFC ROIs defined above as seeds, including areas 10, 24ab, and 32. Whole-brain correlation maps were analyzed in separate rmANOVAs using E2 treatment as the within subject variable, and status as the between subjects variable. Clusters of voxels with statistically significant main or interaction effects of E2 and status were anatomically identified using published rhesus monkey brain atlases (Paxinos et al., 2000; Saleem and Logothetis, 2006), and cortical regions were also identified following the Lewis and Van Essen F99 cortical maps, when possible. To minimize both type I and type II errors in our small sample size, we applied a cluster correction by setting a cluster threshold of $p<.001$ ($|Z|> 3.0$) and a minimum of 5 contiguous significant voxels (Lieberman and Cunningham, 2009), only clusters with $r>.1$ are reported.

4.4 Results

4.4.1 Behavioral Data

SUBs showed significantly higher rates of submissive behaviors (Table 4.1; $F_{1,11}$= 99.44, p<.001, η2=.90), and received more aggression ($F_{1,11}$= 14.53, p=.003, η^2=.57), and affiliative behaviors from other animals compared with DOMs ($F_{1,11}$= 14.03, p=.003, η2=.56). DOMs showed significantly more male-directed affiliative behavior ($F_{1,11}$= 5.38, p=.041, η2=.33), increased aggression towards other animals in the group ($F_{1,11}$= 36.08, p<.000, η2=.77) and received more submissive behaviors from other animals than SUBs ($F_{1,11}$= 48.25, p<.001, η2=.81). E2 increased receptive sexual behavior ($F_{1,11}$= 5.43, p=.040, η2=.33). No further main or interaction effects of status and treatment were detected.

4.4.2 ROI Functional Connectivity Analysis

The results of the ROI FC analysis between the amygdala and each of the prefrontal ROIs (Table 4.2), shows a main effect of status on left amygdala FC with left area 10 ($F_{1,11}$=6.146, p=.03, η^2=.36), such that FC between these two regions was greater in SUB females compared to DOM females. A main effect of E2 treatment was also detected on right amygdala FC with right area 10 ($F_{1,11}$=5.180, p=.04, η^2=.32), which was higher in the control condition than during E2 treatment. As noted in Table 2, no other significant main or interaction effects were detected.

4.4.3 Voxel-wise Functional Connectivity Analysis

Amygdala. A voxel-wise analysis of left amygdala FC yielded two significant clusters of temporally correlated voxels with main effect of status and an interaction effect between

status and treatment, but no effects were detected for the right amygdala (Table 4.3). DOM females had increased negative FC compared to SUB females between the amygdala and one cluster (N= 9 voxels, Figure 4.1A) located in the right ventral pallidum (VP) that extended rostrally and bilaterally into the ventral striatum and caudally into the globus pallidus (Figure 1A). A separate cluster (N= 7 voxels, Figure 4.1B) in the left visual area 2 (V2) showed an E2 treatment x status interaction effect, such that DOM females on E2 replacement had the greatest negative FC to the left amygdala compared to SUB females on E2 and both DOM and SUB females in the control condition. No main effects of E2 treatment were detected.

Medial Prefrontal Cortex. The mPFC voxel-wise analysis detected multiple clusters of temporally correlated voxels that showed significant status, treatment, and status by treatment interaction across the brain (Table 4.3). Main effects of status were found in area 24ab and 32 FC, but not in area 10. A main effect of E2 treatment was observed in area 10 and 24ab, and an interaction of treatment by status was detected in area 32. These findings are described in detail below.

Status main effects. mPFC regions 24ab and 32 showed bilateral status effects with SUB females showing higher positive FC with all the cortical and subcortical significant clusters of correlated voxels detailed in the next sentences, as compared to DOM females. Thus, right 24ab FC showed significant status effects with five clusters (Figure 4.2A), all showing greater FC in SUB than DOM females including the right posterior cingulate cortex (pCC) including 23, right 24d, left supplemental motor area (SMA) including 6m,

right visual area 1, and right visual area 2. Left 24ab FC showed a main effect of status in four clusters (Figure 4.2B), all of which also had greater FC in SUB than DOM females, including right pCC, right SMA, left SMA, and left V2. Right 32 FC showed significant main effects of status in one cluster (Figure 4.2C) with increases FC to the left SMA in SUB compared to DOM females. Left 32 FC showed a significant main effect of status in two clusters (Figure 4.2D) with increased FC in SUB compared to DOM females to right superior temporal sulcus (STS) specifically within the temporal parietal occipital (TPOr) subdivision of the upper bank of the STS and left SMA. In SUB female, FC from 24ab and 32 seed-regions (N=4) was significantly greater with the bilateral SMA and FC from bilateral 24ab seed regions (N=2) was significantly greater with the pCC than in DOMs, as shown in the overlap map represented in Figure 4.2D.

E2 treatment main effects. Voxel-wise FC analysis with area 10 and 24 yielded three clusters of correlated voxels with main effects of treatment. From right area 10 females with no E2 treatment (Control condition) had increased negative FC with the right lateral cerebellum (Figure 4.3A), specifically the right Crus II division of Lobule VIIa, than after E2 treatment. Similarly, in left area 10 females had increased negative FC (Figure 4.3B) with an overlapping region of the right lateral cerebellum in the control than in the E2 treatment condition. Right 24ab had greater negative FC in the E2 condition (Figure 4.3C) with a brainstem region identified as the nucleus of solitary tract (NTS).

Status x E2 treatment interaction effects. An interaction effect of E2 treatment and status was seen in area 32 FC with a cluster of voxels located within the NTS (Figure 4.3D).

Thus, FC in the control condition was similar, while in SUBs E2 treatment resulted in greater negative FC and in DOMs E2 decreased negative FC. No other significant interaction effects were observed.

4.4. Discussion

In this study, we examined the effects of social subordination and E2 on FC between the amygdala and the mPFC, including area 10, 24, and 32, in female rhesus monkeys. Our ROI analysis of amygdala – mPFC connectivity showed increased FC between the left amygdala and area 10 in SUB females independent of treatment, and decreased FC between right amygdala and area 10 following E2 treatment, independent of social status. Our exploratory voxel-wise analysis suggests that the effects of social subordination and E2 are broader, though, affecting connectivity of the amygdala and mPFC not only with each other, but also with other brain regions. The broader effects of social subordination included increased negative FC between the amygdala and the VP, considered part the reward processing system (Haber and Knutson, 2009), in DOM compared to SUB females, and increased positive FC between the mPFC and regions involved in action-monitoring (SMA; Nelissen et al., 2011; Bonini et al., 2014) and social perception (STS, pCC; Freiwald et al., 2009; Mars et al., 2013) in SUBs compared to DOMs. The whole brain analysis also suggests that E2 replacement increased negative FC between the mPFC and regions important in the maintenance of autonomic function and metabolic homeostasis (NTS; van der Kooy et al., 1984) but decreased mPFC negative FC within the executive behavioral cerebrocerebellar pathway (O'Reilly et al., 2010). Altogether,

our findings suggest that the adult female brain is very plastic, showing neural adaptations to social subordination in systems involved in reward and vigilance, potentially setting the stage for increased susceptibility to stress-related disorders. In addition, 4 weeks of E2 replacement affects brain circuitry involved in executive control of behavior as well as autonomic and homeostatic regulation, particularly in females exposed to social subordination.

Our behavioral data are consistent with status-related differences in agonistic behavior in DOM and SUB rhesus monkey females previously reported, as SUB females received higher amounts of aggression and displayed higher amounts of submissive behaviors compared to DOMs (Shively and Kaplan, 1991; Sapolsky, 1995; Shively et al., 1997; Michopoulos et al., 2009; Michopoulos et al., 2012a; Michopoulos et al., 2012b; Reding et al., 2012). However, our data also show higher frequency of social affiliation in SUBs, which is different from previous data (Kikusui et al., 2006; Michopoulos et al., 2012a; Reding et al., 2012). We also failed to find an effect of status on E2's activational effects on reproductive behaviors, which previous data suggest are attenuated by in SUBs {Reding et al., 2012}. However, the different findings could be due in part to the timing of behavioral data collection, which was done during both the rhesus monkey breeding (August – April) and nonbreeding (May – September) seasons, with females being less responsive to the activational effects of E2 on sexual behavior during the latter (Pope et al., 1987). Additionally, absence of an effect of E2 on female sexual behavior may have been the product of a smaller sample size in the current study, as DOMs, but not SUBs,

did indeed show increased sexual activity following E2, although these effects were not significant.

Our findings did not support the hypothesis that chronic social subordination status decreases mPFC-amygdala connectivity. Our data actually shows higher left amygdala – area 10 FC in SUBs compared to DOMs, independent of E2 condition, as well as lower left amygdala - area 10 FC in E2 treated animals, compared to the control condition, regardless of status. Our initial hypothesis was based in part on the literature on human psychopathology that suggests decreased in FC in the amygdala - mPFC in stress-related disorders, including anxiety and major depression (Hahn et al., 2011; Prater et al., 2013; Stevens et al., 2013), as the rodent literature did not measure functional alterations in addition to the structural ones reported (Garrett and Wellman, 2009; Shansky et al., 2010). The possibility that the increased mPFC-amygdala FC detected in SUB in our study is due to increased – mPFC-amygdala structural connectivity would be supported by previous evidence from our group in a separate cohort of juvenile females in which SUBs did, indeed, show increased structural integrity of mPFC tracts likely connecting with temporal limbic regions such as the amygdala, and positively correlated with the higher submissive and fear behavior exhibited by SUBs in comparison to DOMs (Howell et al, 2014). In humans, increased FC between the amygdala and area 10 has also been associated with increased salivary cortisol levels, suggesting that the increased FC positively correlates with hypothalamic pituitary adrenal (HPA) axis activation (Veer et al., 2012). Although not measured in the current study, this data is consistent with prior evidence that SUB females show both hypercortisolemia and impaired glucocorticoid

negative feedback (Shively et al., 1997; Michopoulos et al., 2012b). However, prior data also suggest that E2 increases escape from glucocorticoid negative feedback, particularly in SUB females (Wilson et al., 2003; Wilson et al., 2005), and this is inconsistent with our current finding that E2 decrease amygdala – area 10 FC if one assumes increased FC between these regions is associated with impaired HPA function.

In the whole-brain voxel-wise analysis, amygdala FC to reward-processing regions was affected by social status. Thus, DOMs had greater negative FC, or anticorrelations, between the amygdala and regions in the reward circuit, including the ventral pallidum (VP), ventral striatum, and globus pallidus, while SUB females showed very low connectivity between these regions. These differences between DOM and SUB females suggest reduced amygdalo-striatal connectivity likely associated with the chronic stress imposed by social subordination. It has to be noted that many of these effects were the result of differences in negative correlations of rs-fMRI signal, which is more strongly observable after the inclusion of whole-brain or global signal regression as used here. There is considerable disagreement on the appropriateness of whole brain regression (or global signal regression) for resting-state functional connectivity processing (Cole et al., 2010). While there is strong evidence that the brain does indeed have a global signal (Scholvinck et al., 2010), and controlling for it does improve specificity of the regional functional connectivity signal (Fox et al., 2009), the debate continues on the appropriateness of its use (Fox et al., 2009; Murphy et al., 2009; Weissenbacher et al., 2009). Interpretation of negative FC is also debated, and some data suggest it represents “anti-correlations”, such that two regions are coupled but that the increase of BOLD

signal in one region is correlated with a decreased in another (Buckner et al., 2013). Others, however, suggest that negative FC is should not be interpreted as it is an artifact of the global signal regression, and thus may not represent biological data(Saad et al., 2012). Given that the physiological source of the negative functional correlations (or anticorrelations) is not known (i.e. Is it a result of inhibitory pathways or is it an artifact of the global signal regression correction?) and the interpretation of negative FC is an ongoing point of contention in the field, throughout this discussion we present all correlations, negative and positive, but limit our functional interpretations of negative FC to the mere report of those group differences rather than directionality of these differences.

Structurally, we know that there is dense monosynaptic connectivity between the amygdala and the ventral striatum (Fudge et al., 2002), which connects to the VP topographically and overlaps with input from area 25 and 32 (Haber et al., 1995). The VP is thought to be associated with both hedonic reward (e.g. liking), as well as motivation (e.g. wanting), and is considered the 'final common pathway' for both aspects of reward-based behavior (Smith et al., 2009). Behaviorally, this amygdalo-striatal circuit is important in forming stimulus-reward associations important during both fear and reward learning (Cador et al., 1989; Everitt et al., 1991; Haber and Knutson, 2010; Paz and Pare, 2013). Results from a number of studies suggest that stress can potentiate fear learning while attenuating reward-based learning and motivation (Conrad et al., 1999; Willner, 2005; Kleen et al., 2006; Farrell et al., 2013). Although the amygdala plays a central role in the regulation of these behaviors (Falls et al., 1992; Fendt and Fanselow, 1999),

increasing evidence suggests that dopamine neurotransmission in the ventral striatum and pallidum is critical for motivation (Berridge, 2007) as well as fear learning and extinction (Martinez et al., 2008; Raczka et al., 2011; Pohlack et al., 2012; Rodriguez-Romaguera et al., 2012). Previous reports show reduced expression of dopaminergic receptors (D2) in the basal ganglia of SUB females, including both the ventral striatum and VP (Grant et al., 1998; Morgan et al., 2002), associated with increased self-administration of cocaine (Morgan et al., 2002). This is consistent with the status-related differences in amygdalo-striatal FC found in our study. Our data, therefore, supports the view that the FC difference between DOM and SUB females is indicative of attenuated amygdala–VP connectivity in social subordination. These changes may be potentially related to some of the behavioral alterations in social function, including decreased social motivation or alternatively, increased isolation (Shively et al., 1997), as well as reward processing including increased food intake of a highly palatable calorically dense diet, in SUBs compared to DOMs (Michopoulos et al., 2012c).

Social subordination status increased cortico-cortical FC locally, within the mPFC, and across long-range monosynaptic and polysynaptic connections with parietal, temporal, and occipital cortices. Thus, in SUB females, the mPFC was more positively correlated with activity in clusters located in the SMA, pCC, and STS, and more negatively correlated to both V1 and V2, than in DOMs. This pattern of increased local and long-range PFC FC is consistent with a recent publication by our group showing that SUB juvenile females had increased local and long-range PFC structural connectivity (Howell et al, 2014). Recent data parcellating the connectivity of the PFC in rhesus monkeys has

showed a pattern of local and long-rang connectivity consistent with our findings (Hutchison and Everling, 2014), suggesting that the differences in our study are in FC circuits that parallel previously reported PFC networks. The overlap FC of these two regions (24 and 32) is likely indicative of their similar neuroanatomical function, and both regions are considered part of the ACC (Barbas, 2013).

In human neuroimaging data, increased ACC-SMA activation is associated with behavioral inhibition (van Gaal et al., 2010). The ACC specializes in error detection and subsequent behavioral adjustment (Magno et al., 2009; Wittfoth et al., 2009; Nee et al., 2011; Amiez et al., 2012) while the SMA is involved in performance monitoring independent of the ACC (Bonini et al., 2014). This includes higher order motor function, bimanual goal directed movement (Kermadi et al., 1997; Kazennikov et al., 1999), and motor representations of both hands and face (Morecraft et al., 2004). Therefore, increased ACC–SMA activity in SUBs could facilitate appropriate submissive behavioral responses in order to attenuate/prevent the aggressive behaviors they receive from more dominant animals in the group and limit exposure to escalation of aggression (Maestripieri and Wallen, 1997; Silk, 2002). Our behavioral data show that SUBs did, indeed, receive consistently high levels of aggression from higher ranking females in their group and showed higher levels of submissive behavior, consistent with the literature (Bernstein et al., 1974; Michopoulos et al., 2012a; Reding et al., 2012). Our FC findings, in combination with and consistent with structural connectivity data showing increased white matter tract integrity along the dorsal medial wall of the PFC of juvenile SUB females, which includes motor, premotor, and SMA areas (Howell et al., 2013),

suggest that chronic social subordination increases both structural and functional PFC connectivity with these cortical regions.

Further support for this potential relationship between increased FC and increased monitoring of social environment (e.g. vigilance) and subsequent motor output is provided by the additional findings of increased ACC connectivity to both the STS and pCC. Both 32 and 24 have direct connectivity to the middle upper bank of the STS (Barbas et al., 1999), which in turn receives connections from unimodal sensory areas including visual, auditory, and somatosensory cortices (Barnes and Pandya, 1992). In rhesus monkeys, the STS is highly responsive to faces including both individual recognition and orientation (Tsao et al., 2008; Freiwald et al., 2009), which may be important in interpreting social behavior in the rhesus hierarchy. Previous studies of social dominance showed increased gray matter volume in the STS as well as greater STS–rostral PFC connectivity in animals living in larger, more complex, social groups (Sallet et al., 2011), at least in males. Rs-fMRI studies of the rhesus monkey STS suggest that this region is similar in connectivity to that of the human temporal-parietal-junction (TPJ), commonly implicated in 'theory of mind' processing in humans and also shows FC to both the ACC and pCC (Mars et al., 2013). The pCC, however, does not have direct structural connectivity to the ACC, and is anatomically connected to regions of the dorsolateral PFC, including area 46 and 9, the parahippocampus and somatosensory regions and is also considered integral in transducing perceptions of the environment to actions (Kobayashi and Amaral, 2003). Therefore, stronger ACC FC to the STS and pCC in SUB compared to DOM females suggests increased FC within a social perception

pathway that could support the production of appropriate behavioral responses to more dominant members of the social hierarchy to prevent aggression. Thus, these findings could be interpreted as a potentially adaptive mechanism.

The effects of E2 were seen in more phylogenetically conserved regions of the central nervous system including the cerebellum and the NTS, involved in motor/behavioral/emotional control and regulation of autonomic and HPA axis activity, respectively. Our data show that E2 reduces negative FC between area 10 and the cerebellum and increases FC between both areas 24 and 32 and NTS. These treatment effects are supported by extensive E2 receptor expression in both the cerebellum and the NTS in rodents (Shughrue et al., 1997) and rhesus monkeys (Pau et al., 1998). The lateral cerebellum, specifically the lobule VIIa crus II region identified in our analysis, is polysynaptically connected with area 9 and 46 in the rhesus monkey PFC (Ramnani, 2006), and thought to be involved in executive control of voluntary action and behavioral inhibition (Ide and Li, 2011; Stoodley, 2012; Brunamonti et al., 2014). Resting-state data in humans has suggested broader PFC FC with the crus II region (O'Reilly et al., 2010), although this is the first study reporting FC with area 10. Neuroimaging data on postmenopausal women has shown that E2 replacement therapy increases cerebellar volume in tandem with increased executive and linguistic function (Boccardi et al., 2006; Ghidoni et al., 2006). E2 similarly increases in executive function in ovariectomized monkeys (Voytko et al., 2009). However, evidence of increased executive function in postmenopausal women following ERT is not consistent (Henderson and Popat, 2011) and positive effects of E2 on executive function may be attenuated by individual-

differences such as exposure to early life stress (Shanmugan and Epperson, 2014). Overall, our data suggest that E2's attenuation of area 10 – cerebellar negative FC may be linked with increased cognitive function, and highlights the need for further investigation of these pathways in regards to cognition.

Our data also showed that E2 treatment increases negative FC between both areas 24 and 32 and the NTS, which modulates HPA axis, maintains metabolic homeostasis, and acts as a relay station for ascending visceral, gastrointestinal, and somatic information to the PFC, bed nucleus of the stria terminalis (BNST), and central nucleus of the amygdala (CeA) (van der Kooy et al., 1984; Rinaman, 2010). NTS input to the paraventricular nucleus (PVN) appears to increase HPA axis activity, seen by increased c-Fos early response gene expression after a stressor (van der Kooy et al., 1984), and is modulated by input from the mPFC (Herman et al., 2003). As previously described, E2 potentiates HPA axis response in rhesus monkeys (Wilson et al., 2003; Wilson et al., 2005) and our data suggest that negative FC between the mPFC and NTS may contribute to these changes. However, activation of the NTS also affects other systems, and mPFC – NTS FC has been associated with cortical control of cardiovascular function (Cechetto, 2014). The NTS is also important in the integration of both cognitive and visceral input in modulating meal size (Schwartz, 2006). Data from our group suggest that E2 replacement to ovariectomized monkeys may reduce overall food intake and alter meal parameters (Johnson et al., 2013), although these effects are dependent on palatability of diet and diet history (Michopoulos et al., 2012c). Together, these data suggest that E2 may influence

basic homeostatic functions regulated by the NTS and of relevance for the social subordination model studied here.

Study limitations: The small sample size and lack of corrections for multiple comparisons in both our ROI and voxel-wise analysis limits the generalizability of our results and suggests caution in their interpretation. Due to the small sample size, we did not use more conservative methods of multiple corrections such as family wise error (FWE) or false discovery rate (FDR), and therefore our data is more susceptible to false positives, or Type I errors (Lieberman and Cunningham, 2009). In order to maximize our analytical power, but not be so stringent as to increase false negative, or Type II errors, we limited our ROI analysis to *a priori* regions of interest and cluster corrected voxel-wise results above a conservative threshold of 5 contiguous voxels with $p<.001$ (Lieberman and Cunningham, 2009). Although there is a possibility that some of our voxel-wise findings are false positives, as cluster correction, at least in humans, is typically limited clusters of more than ten voxels (opposed to five in our study) with a p-value threshold of $p<.005$, however this is balanced by an increase in our p-value threshold to $p<.001$ (Lieberman and Cunningham, 2009). In the ROI analysis, the current findings show a moderate to large effect size ($\eta^2>.3$), suggesting that although our sample size was limited, differences between groups were robust. The best assurance for limiting both Type I and Type II errors is replication. Future analyses would benefit from larger samples and the addition of whole brain data driven approaches including network analysis and graph theory to provide a global picture of connectivity and network structure outside of amygdala and PFC circuits (van den Heuvel and Hulshoff Pol, 2010). Furthermore, the relatively small

size of our anatomical amygdala seeds forced us to examine FC using a time course of neural activation that encompassed multiple subnuclei with distinctly different connectivity patterns (e.g. lateral, basolateral, central and medial nuclei). Moreover, as the temporal pole is susceptible to EPI signal-loss, our amygdala seed included portions with a very low signal-to-noise ratio further diluting the derived time course. Averaging across these disparate voxels can obscure important changes in FC specific to each subregion, and future studies will benefit from further parcellation of the amygdala using a higher magnetic field scanner to increase neuroanatomical resolution.

In conclusion, social subordination and increased serum concentrations of E2 in adult female rhesus monkeys both induce changes in FC between amygdala and mPFC, but also have broader effects on the connectivity of these two regions with other brain structures. This study presents novel data supporting the plasticity of the adult brain to respond to changing social or hormonal environments. Our data suggest that neural adaptations to chronic social subordination are biologically embedded in brain circuits, and may result from concomitant behavioral adaptations including reduced reward-processing and increased behavioral monitoring in SUB females exposed to high levels of chronic unpredictable stress. Furthermore, despite periods of time without endogenous ovarian hormones, ovariectomized females showed significant changes in amygdala – PFC circuits as well as PFC connectivity in response to tonic E2 replacement that were modified by the chronic stress imposed by social subordination. Together these results shed light on the role of social subordination and E2 treatment on the plasticity of emotional circuits of the brain and provide insight into possible mechanism of adaptation

to these environments that may exacerbate risk of developing stress-related psychopathological disorders in women.

Table 4.1. Behavioral Data. A rmANOVA analysis of behavior showed main effects of status and a main effect of treatment, but no interaction effects. (*p<.05)

	Aggressive Given	Aggressive Received	Submissive Given	Submissive Received	Anxiety-Like Behavior	Affiliative Given (Female)	Affiliative Received (Female)	Affiliative Given (Male)	Affiliative Received (Male)	Proceptive Sexual Behavior	Receptive Sexual Behavior
Main Effect of Status											
Dominant	10.25±2.48*	0.17±4.74*	26.29±5.11*	42.75±7.86*	14.58±3.45	5.50±1.70	1.92±4.21*	3.92±1.51*	2.50±1.57	3.67±1.59	3.33±1.65
Subordinate	0.00±2.30*	15.71±4.39*	0.08±5.52*	0.14±7.28*	13.00±3.19	3.00±1.57	14.64±3.89*	0.57±1.40*	2.79±1.45	1.57±1.47	0.14±1.53
Main Effect of Treatment											
Control	5.42±2.83	6.79±2.83	15.23±5.79	20.81±6.75	12.62±3.44	4.61±1.48	8.21±3.32	1.49±0.70	2.66±1.40	1.92±1.10	0.67±.61*
E2	4.83±1.25	9.10±4.59	11.14±3.28	22.08±6.58	14.96±1.99	3.89±1.13	8.35±2.73	3.00±1.39	5.42±2.83	3.32±1.84	2.81±1.67*
Interaction of Status and Treatment											
Dominant Control	10.83±4.15	0.00±4.16	0.17±8.50	41.33±9.91	11.67±5.05	5.50±2.18	2.00±4.86	2.83±1.03	2.83±1.28	2.83±1.63	1.33±0.90
Dominant E2	9.67±1.83	0.33±6.74	0.00±4.82	44.17±9.66	17.50±2.92	5.50±1.66	1.83±4.01	5.00±2.05	2.17±2.05	4.50±2.70	5.33±2.45
Subordinate Control	0.00±3.85	13.57±3.85	30.27±7.87	0.29±9.17	13.51±4.68	3.71±2.02	14.43±4.50	0.14±0.95	2.43±1.19	1.00±1.49	0.00±0.83
Subordinate E2	0.00±1.70	17.86±6.24	22.29±4.46	0.00±8.95	12.43±2.71	2.29±1.53	14.86±3.71	1.00±1.89	3.14±1.90	2.14±2.50	0.29±2.27

Table 4.2. Amygdala ROI FC analysis. Connectivity to mPFC regions 10m, 32, and analyzed in a rmANOVA with Status (Alpha, Subordinate) as a between subjects fa and Treatment (Control, Estradiol (E2)) as a within subjects factor. All p-value, eta-squared, and Pearson's r-values (estimated marginal means) are listed. No correctio were made for multiple comparisons. (**) p<.05

ROI Analysis	p-value	η^2	r-values			
Amygdala						
Main Effect of Status			***Alpha***	***Sub***		
Right Amygdala						
R 10	.81	,01	.019±.02	.013±.02		
L 10	.36	.08	.033±.02	.004±.02		
R 24ab	.63	.02	-.011±.02	-.022±.02		
L 24ab	.32	.09	-.029±.03	.009±.03		
R 32	.78	.01	.026±.03	.036±.02		
L 32	.62	.02	.043±.02	.027±.02		
Left Amygdala						
R 10	.80	.01	.011±.03	.020±.02		
L 10	.03**	.36	-.010±.02	.05±.02		
R 24ab	.76	.01	-.003±.02	-.033±.02		
L 24ab	.33	.09	.026±.02	.000±.02		
R 32	.76	.01	.020±.02	.032±.02		
L 32	.32	.09	.019±.03	.056±.03		
Main Effect of Treatment			***Control***	***Estradiol***		
Right Amygdala						
R 10	.04**	.32	.038±.02	-.006±.01		
L 10	.22	.13	.041±.02	-.004±.02		
R 24ab	.69	.02	-.009±.02	-.024±.02		
L 24ab	.77	.01	-.018±.04	-.003±.03		
R 32	.93	.00	.033±.02	.030±.03		
L 32	.98	.00	.034±.02	.035±.03		
Left Amygdala						
R 10	.06	.28	.048±.03	-.017±.02		
L 10	.97	.00	.022±.03	.020±.03		
R 24ab	.75	.01	-.013±.02	-.023±.02		
L 24ab	.47	.05	-.009±.03	.035±.04		
R 32	.54	.04	.040±.03	.012±.03		
L 32	.71	.01	.026±.03	.049±.04		
Interaction Effect of Status x Treatment			***Alpha Control***	***Alpha E2***	***Sub Control***	***Sub E2***
Right Amygdala						
R 10	.28	.11	.052±.03	-.014±.02	.023±.03	.002±.01
L 10	.70	.01	.049±.03	.017±.03	.033±.03	-.026±.02
R 24ab	.93	.00	-.005±.03	-.016±.03	-.013±.03	-.031±.03
L 24ab	.44	.06	-.057±.05	-.002±.04	.021±.05	-.003±.04
R 32	.89	.00	.026±.03	.027±.04	.040±.02	.033±.04
L 32	.53	.04	.032±.02	.054±.04	.037±.02	.016±.04
Left Amygdala						
R 10	.51	.04	.054±.04	-.032±.03	.042±.03	-.002±.03
L 10	.43	.06	-.029±.04	.008±.04	.072±.04	.031±.03
R 24ab	.29	.10	-.015±.03	.009±.03	-.011±.02	-.055±.03
L 24ab	.60	.03	-.013±.04	.064±.05	-.006±.04	.006±.05
R 32	.54	.03	.021±.04	.020±.04	.059±.04	.005±.04
L 32	.32	.09	-.024±.05	.062±.06	.076±.04	.036±.05

Table 4.3. Voxel-wise FC analysis. Seed regions were placed in the right and left Amygdala and right and left mPFC areas 10m, 32, 24ab. Data was analyzed in a rmANOVA with Status (Alpha, Subordinate) as a between subjects factor and Treatment (Control, Estradiol (E2)) as a within subjects factor. Only significant clusters are listed ($p < .005$, cluster size thresholded at >5 voxels per cluster).

Table 4.3 Continued

Clusters	LVE Region	Seed Hemisphere	Cluster Size	z-score	r-values			
Amygdala								
Main Effect of Status						***Alpha***	***Sub***	
R Ventral Pallidum		L	9	3.20		-.124±.03	.016±.01	
					Alpha	**Alpha**	***Sub***	***Sub***
Main Effect of Status x Treatment					**Control**	**E2**	***Control***	***E2***
L Visual Area 2	V2d	L	7	3.79	-.006±.02	-.112±.02	-.018±.01	-.001±.01
Area 10								
Main Effect of Treatment						***Control***	***E2***	
R Cerebellum (Cru II)		R	5	3.20		-.112±.02	-.025±.02	
R Cerebellum (Cru II)		L	11	3.55		-.125±.02	-.041±.01	
Area 24ab								
Main Effect of Status						***Alpha***	***Sub***	
R Area 24d	24d	R	7	3.36		.558±.01	.730±.02	
R Posterior Cingulate Cortex	23	R	6	3.36		.102±.01	.285±.02	
R Posterior Cingulate Cortex	23	L	8	3.31		.068±.04	.219±.02	
L Supplemental Motor Area	6m	R	13	3.71		.068±.02	.292±.03	
R Supplemental Motor Area	6m	L	10	3.67		.086±.07	.324±.03	
L Supplemental Motor Area	6m	L	7	3.14		.012±.07	.181±.02	
R Visual Area 1	V1	R	9	3.69		-.010±.01	-.102±.01	
R Visual Area 2	V2d	R	6	3.28		-.007±.01	-.105±.02	
L Visual Area 2	V2v	L	5	4.08		.028±.05	-.102±.02	
Main Effect of Treatment						***Control***	***E2***	
R Nucleus of the Solitary Tract		R	6	3.40		-.104±.03	-.203±.04	
Area 32								
Main Effect of Status						***Alpha***	***Sub***	
R Superior Temporal Sulcus	TPOr	L	7	3.20		.039±.06	.157±.02	
L Supplemental Motor Area	6m	R	12	3.72		.031±.01	.186±.03	
L Supplemental Motor Area	6m	L	7	3.50		.076±.05	.265±.03	
					Alpha	**Alpha**	***Sub***	***Sub***
Main Effect of Status x Treatment					**Control**	**E2**	***Control***	***E2***
R Nucleus of the Solitary Tract		L	9	3.58	-.123±.04	-.078±.03	-.126±.03	-.266±.03

Figure 4.1. Amygdala functional connectivity (FC) based on the voxel-wise analysi

Main effect of status on left amygdala seed connectivity with cluster located in VP.

Interaction effect of status and treatment on left amygdala seed connectivity with V2

Data are shown as z-scores, FC (r-values) listed in Table 2. Abbreviations – VP: ver

pallidum; V2: visual area 2.

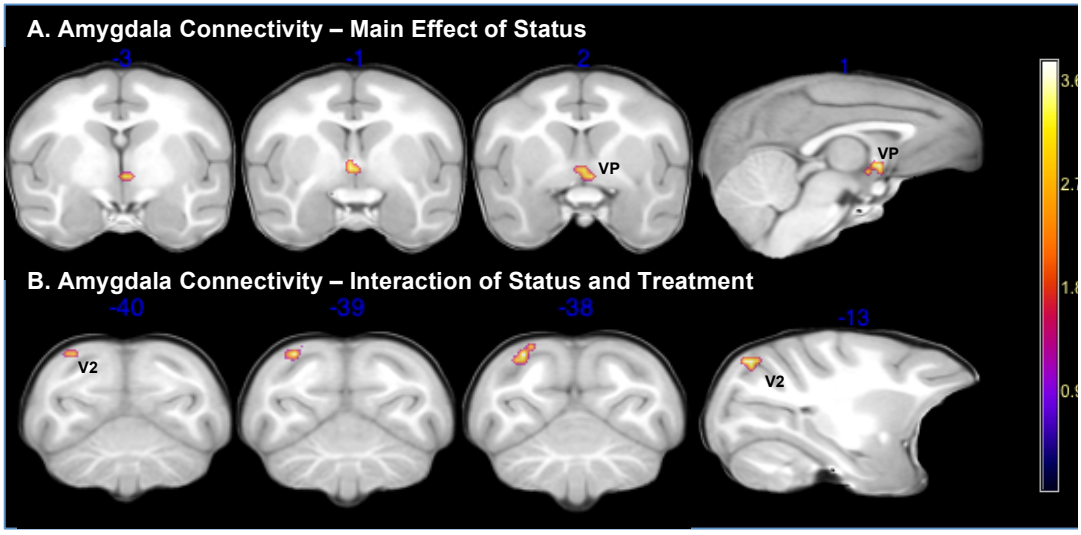


Figure 4.2. PFC functional FC based on the voxel-wise analysis. Main effect of status on (A) right BA24ab, with increased FC in SUBs to R V1/V2, R pCC, L SMA, and R 24d; (B) left BA24ab, with increased FC in SUBs to L V2, R pCC, and R/L SMA; (C) right BA32, with increased FC in SUBs to L SMA; (D) left BA 32, with increased FC in SUBs R/L SMA and STS. Data are shown as z-scores, FC (r-values) listed in Table 2. (E) Overlap analysis of these PFC regions shows increased FC in SUBs to the bilateral SMA from all 4 seeds, and increased FC in SUBs to the pCC from 2 seeds. Abbreviations – pCC: posterior cingulate cortex; SMA: supplemental motor cortex; STS: superior temporal sulcus; V1: visual area 1; V2: visual area 2.

Figure 4.2. Continued

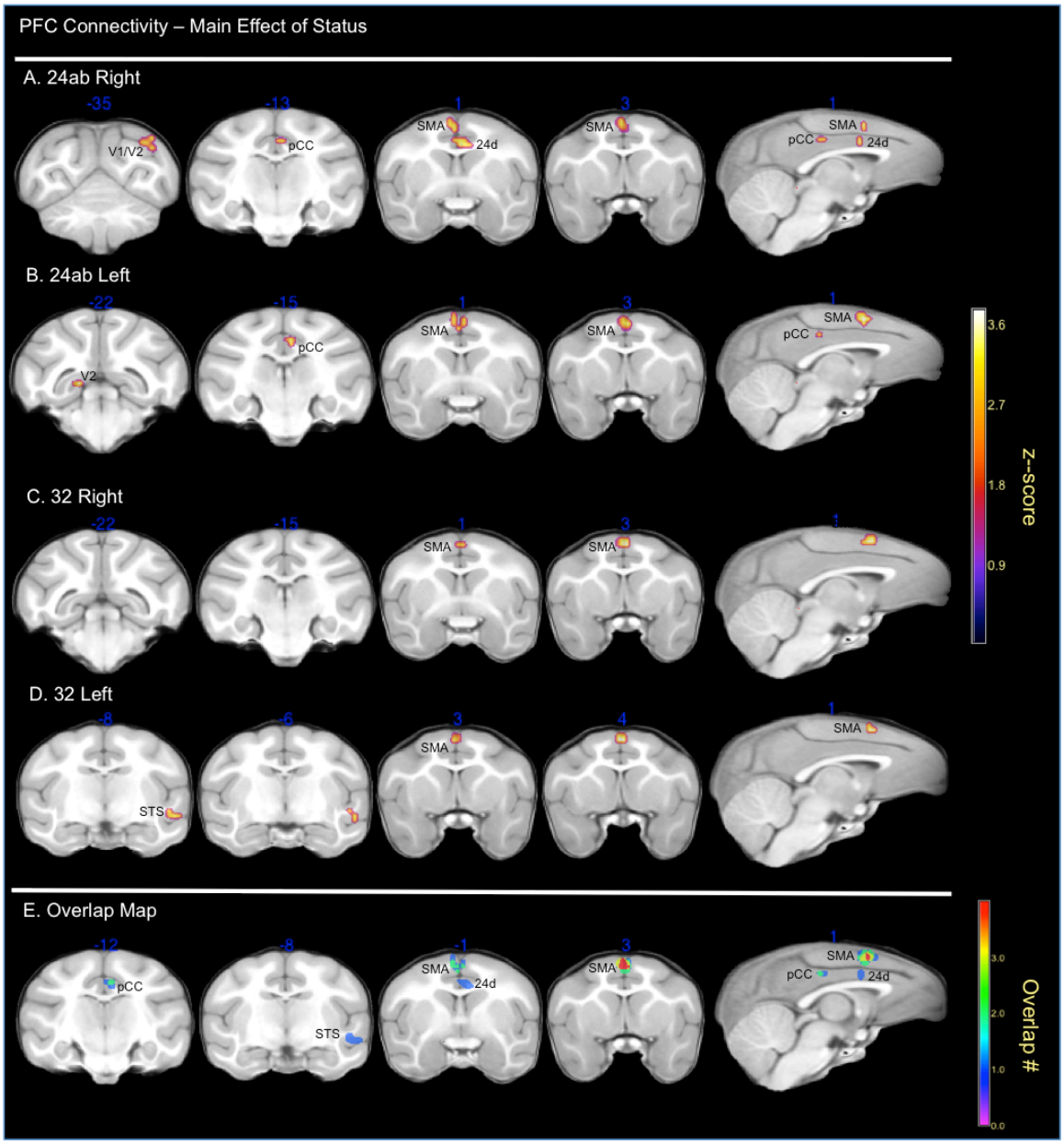
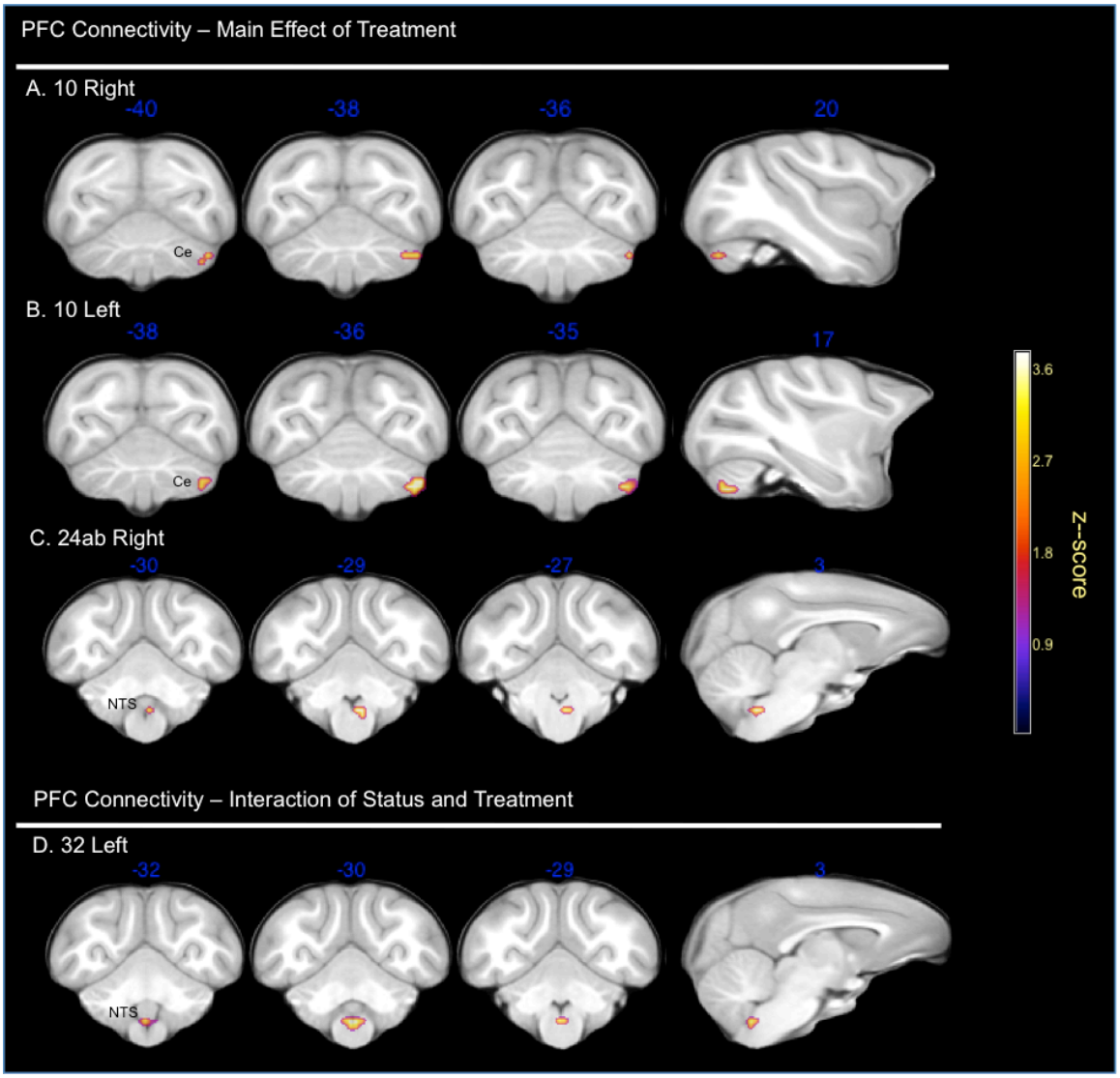


Figure 4.3. PFC functional FC based on the voxel-wise analysis. Main effect of treatment on (A) right BA10, with increased negative FC in controls to R Ce; (B) left BA10, with increased FC in controls to R Ce; (C) right BA24ab, with increased FC in E2 condition to R NTS. Interaction effect of status by treatment on (D) left BA 32, with increased FC in SUBs on E2 to R NTS. Data are shown as z-scores, FC (r-values) listed in Table 2. Abbreviations – Ce: cerebellum, NTS: nucleus of the solitary tract.



Chapter 5: Summary and Conclusion

5.1 Summary of results

5.1.1 Chapter two

In chapter two, I tested the hypothesis that chronic social subordination diminishes the behavioral efficacy of E2 on a range of socio-emotional behaviors in female rhesus monkeys. The data show that social subordination inhibits the expression of E2-mediated increases in sexual and male-affiliative behaviors, and that these effects of social subordination may not be overcome by higher doses of E2. Data also suggest that decreased female–female aggression in both alpha and middle-ranking females following E2 treatment may be influenced by social and environmental factors, and not directly related to treatment. Finally, the data do not support the hypothesis that E2 attenuates anxiety-like behaviors as measured in our study (i.e. via observations of animals' behavior in their social environments); using paradigms that enhance stress reactivity seem necessary to elucidate the complex interaction between social subordination, E2 and anxiety-like behavior. Overall, future studies are needed that determine what biological signals (e.g. CRF, CGs) mediate the attenuating effects of social subordination on the behavioral effects of E2.

5.1.2 Chapter three

In chapter three, I tested the hypothesis that exposure to a chronic social subordination interacts with E2 to change corticolimbic brain regions structure measured as regional volumes. Our findings suggest that both one month of E2 replacement and prolonged social subordination can cause structural changes in the adult female brain. In particular,

E2 treatment of ovariectomized adult female macaques caused significant decreases in frontal cortex gray matter volume. Social status further modified the effects of E2 in the cingulate cortex, increasing gray matter volume in dominant females and decreasing it in subordinate females. These data suggest that a background of chronic stress can reverse the effects of E2 on brain structure. Overall, future studies are needed that determine what associated changes in neuromorphology may mediate theses dichotomous effects of E2 on brain volume.

5.1.3 Chapter four

In chapter four, I tested the hypothesis that exposure to chronic social subordination interacts with E2 to alter FC in amygdala – PFC circuits, as well as FC of these regions across the whole brain. Our ROI analysis of amygdala – mPFC connectivity showed increased FC between the left amygdala and mPFC in subordinate females independent of E2 treatment. In contrast, decreased FC between right amygdala and mPFC was observed following E2 treatment, independent of social status. Our exploratory voxel-wise analysis suggests that the effects of social subordination and E2 are widespread, affecting connectivity of the amygdala and mPFC not only with each other, but also with other brain regions. The voxel wise analyses shows that dominant social status is associated with increased negative FC between the amygdala and the VP, considered part the reward system, compared to subordinate females. In addition, increased positive FC between the mPFC and regions involved in action-monitoring as well as social perception was observed in subordinate compared to dominant females. With respect to the main effect of E2, the whole brain analysis suggests that E2 replacement increased negative FC

between the mPFC and regions important in the maintenance of autonomic function and metabolic homeostasis (e.g. NTS) but decreased mPFC negative FC within the executive behavioral cerebrocerebellar pathway. Altogether, our findings suggest that the adult female brain is very plastic, showing neural adaptations to social subordination in systems involved in reward and vigilance, potentially setting the stage for increased susceptibility to stress-related disorders. In addition, 4 weeks of E2 replacement affects brain circuitry involved in executive control of behavior as well as autonomic and homeostatic regulation, particularly in females exposed to social subordination.

5.2 Integration of findings

Using the social subordination model of chronic stress in adult female rhesus monkeys, my data suggest that social subordination attenuates E2's activational effects on socioemotional behavior and this may be due to alterations in the neurobiological effects of E2 on brain structure and function. Initially, the overarching hypothesis was that social subordination would dampen or attenuate E2's activational effects on brain and behavior, such that higher levels of E2 would be necessary to stimulate E2-induced changes. However, it appears that these alterations are not based on simple attenuation of effects, but a modification of E2's effects on the brain, which sometimes are opposite in subordinates compared to dominants, and which then results in an attenuation of behavior.

Social subordination is a potent and naturalistic social stressor in female rhesus macaques. Subordinate females, with or without E2 treatment, show significant differences in social behavior compared to dominant females, including reduced social affiliation and increased submissive behavior including physical withdrawal from dominant females and behavioral gestures of submission (e.g. lipsmack, grimace). They also experience a greater frequency of aggressive behavior from other females, including direct physical aggression and threats of aggression. Submissive behaviors present an adaptation to social subordination that limits escalation of further aggression. As matrilineal hierarchies are naturally formed and maintained by agonist interactions, these behaviors express species typical adaptations to the macaque social environment. In the current studies, we manipulated the social environment in such a way as to artificially enhance the negative consequences of social subordination, including reduction of the enclosure space and removal of an individual from their natal kin groups thus separating them from familial social support.

My data suggest that behavioral adaptations to social subordination are reflected in the brain, and that the expression of behavior and/or the response to the social environment could be mediated by alterations in FC between brain regions that control socioemotional behavior. Alterations in amygdala – VP, amygdala – mPFC, mPFC – STS, mPFC – SMA in subordinate females all support this hypothesis, as the FC between these regions is associated with evaluation of rewards, emotional regulation, observation of social behavior, and regulation of goal-directed motor behavior. Although these changes in FC appear to be independent of changes in regional brain volumes, the underlying

neurobiological substrate is likely altered at a cellular level in support of these functional changes. Alterations in FC may in fact help prime the female to act optimally, i.e., cope, within her environment, or alternatively, reflect a history of neural coactivity between these regions in response to the environment. Future analysis using a rank reversal paradigm, whereby dominants take on a subordinate social status, and longitudinal analysis of FC and behavior during this transition would ultimately show both the plasticity of the brain in response to the social environment.

Within this social environment, E2 was unable to induce increased reproductive and prosocial behaviors in socially subordinate females, even at higher doses. This is contrary to the rodent literature that suggests that increased E2 can overcome exposure to restraint stress (White and Uphouse, 2004; Walf and Frye, 2005a). However, it is likely that both the characteristics of this physical stressor and the limited duration of 'chronic stress' (e.g. 5 – 20 minutes) may produce different physiological effects than the theorized psychosocial stress associated with social subordination. In the current data it is difficult to conclude that the attenuation of E2's effects on behavior was associated with the underlying alterations in corticolimbic FC, as E2 did not significantly affect these pathways. Data did show that social subordination modified E2's effects on mPFC – NTS FC, suggesting that limbic regulation of both autonomic and metabolic function may be altered in subordinate females following E2 treatment. This may underlie greater alterations in stress reactivity, and thus greater behavioral inhibition independent of alterations in FC. However, alterations in corticolimbic FC based on status may still be associated attenuation of behavioral in subordinates during E2 treatment. For example,

changes in FC in subordinate females independent of E2 may be associated with alterations in other brain circuits that subsequently displace socio-sexual behavior when E2 is present. Further analysis using whole-brain measures of FC such as network analysis using graph-theory, may help identify changes in overall network parameters that may be altered following social subordination and E2 treatment.

A critical finding in these studies is the interaction of E2 and social subordination on regional brain volumes. Specifically, socially subordinate females showed a marked reduction in GM volume within the cingulate cortex following E2 treatment. Importantly, the cingulate cortex includes the brain regions that showed status effects in FC, including the ACC and pCC. Together these data suggest that underlying difference in neural connectivity may be further altered following E2 treatment, due to changes in region brain morphology (e.g. neuron/glia ratio, dendritic arborization, etc.). Further examination of smaller focal effects on GM volume, such as using deformation based morphometry (VBM) to measure voxel-wise changes instead of large region of interest alteration in brain volume (Sallet et al., 2011), might show subtler alterations caused by both social subordination and E2 treatment.

5.3 Conclusions and future directions

Exposure to stressors can be a precipitating factor in the development of psychopathology. The accumulation of stressful events across the lifespan and exposure to chronic psychosocial stressors can render an individual more susceptible to the

development of mood and anxiety disorders. Women are two to three times as likely to develop stress-related psychopathology than men and history of exposure to stressful life events is the best predictor for disease development. The mechanisms of this susceptibility, however, are far from being resolved. Evidence from these studies suggests that the adaptation to prolonged exposure to a chronic social stressor may impact the function of corticolimbic circuits, and that the effects of circulating E2's on the stressed female brain are altered by this history. Together, these data suggest that chronic stress modifies E2's effects on the adult female brain.

Often we think of the adaptation to prolonged exposure to stress as being maladaptive, especially in the context of modern society, where stressors are increasingly more psychosocial and can't be escaped by a 'fight or flight' response. However, in rhesus monkey societies, social subordination is part of their natural social structure and biology, and presumably, the neurobiological and behavioral alterations seen in response to this experience should be considered adaptive.

In 1963, Nikolass Tinbergen published a paper outlining four questions, or four levels or analysis that can inform and direct biological research (Bateson and Laland, 2013). These questions are 'what is the current utility?', 'how did it develop?', 'how did it evolve?', and 'how does it work?'. I believe that these questions are of the upmost importance to understanding and framing the interpretation of results of the experiments within this dissertation as well as guiding future analyses.

The current utility of subordinate animals blocking E2's activational effect on social and sexual behavior may be to direct reproductive strategy or even to reduce risk of physical harm from group members. Exposure to stress hormones, including CRF or GCs, may serve to reduce HPG axis function and thus reduce sexual behavior and fertility. As not all females show reproductive compromise as a consequence of chronic stress, the simultaneously reduction of reproductive behaviors in response to E2 may serve as a secondary mechanism to modify reproductive strategies. Furthermore, reducing sexual or prosocial motivation, may also serve to limit aggression often directed toward females forming male consort relationships. Alternatively, increasing vigilance and awareness of the social environment to protect against aggression may also shift a female's motivation from engaging in sexual behavior to behaviors that minimize the likelihood of aggression from more dominant group mates. Overall the utility is to optimize current behaviors to adapt to the physical and social environment. Future studies would benefit from the evaluation of E2's activational effects in subordinate females during various social scenarios to differentiate the behavioral inhibition versus lack of motivation. For example, in the current experiments, all females were exposed to E2 simultaneously. Administration of E2 to only the most subordinate female may reduce female-female competition, and thus increase the activational effects of e2 on these prosocial behaviors, by reducing the risk of aggression from more dominant animals.

It is likely that the influence of chronic stress on E2's effects may be greater during critical organizational periods while the brain is still maturing, such as prenatal, postnatal, or pubertal phases. The current experiments do not address the developmental aspects of

the interaction between stress and the organizational effects of E2. Our data showed a lack of structural effects on corticolimbic brain regions associated with social subordination imposed during adulthood. However, social subordination did modify E2's effects on cingulate cortex GM volumes. E2 production during puberty combined with a developmental history of social subordination may show significantly greater effects on brain structure, and these effects may in turn alter a female's future response to both stress and E2 during adulthood. Data is currently being collected to address these questions by examining the effect social subordination during the pubertal transition in both hormonally suppressed females (i.e. with delayed puberty onset) and control, naturally pubertal, females. Preliminary results suggest that E2 and social subordination during development act to alter volumes of corticolimbic brain regions including increased amygdala volumes in socially subordinate versus socially dominant control females (Godfrey et al., 2013). Suppression of E2 using the GnRH analog, depot Lupron, also resulted in increased frontal GM volume across the pubertal transition, suggesting that similar to our data, E2 acts to reduce frontal GM volume.

The question of evolutionary origin is a theoretical analysis of the adaptive value of a behavior or a trait in light of the phylogeny of that behavior. The importance of this question for the data presented within this dissertation is in the choice of animal model used to identify adaptions to chronic stress and the use of ethologically relevant stressors. Rhesus monkeys have adapted to their social environments, and therefore have evolved mechanisms, both psychological and biological to respond to their status within a social dominance hierarchy. Although humans have markedly more complex social system, the

phylogenetic distance, and thus shared evolutionary history, between us is much smaller than that between the rodent and humans. By understanding the adaptations of rhesus monkeys to their social environment, we can begin to better understand our own behavioral and neurobiological adaptations to chronic social stress and pressures. The ability to manipulate the life history, social environment, and hormonal profile further accentuates the usefulness of the rhesus monkey model in elucidating the effects of stress and E2 on female brain and behavior.

Since Phoenix et al.'s (1959) paper on the organizational and activational effects of gonadal hormones, the study of behavioral neuroendocrinology has investigated the myriad mechanisms by which E2 structures behavior (Wallen, 2009). However, the mechanisms of E2's actions are far from resolved, and the more experiments that are conducted, the more genomic or non-genomic effects of E2 are discovered. Discovery of the modification of the effects of E2 following a history of stressor exposure are gradually emerging (Pierce et al., 2008; Garrett and Wellman, 2009; Michopoulos et al., 2009; Shansky et al., 2010; Papargiris et al., 2011b; Asher et al., 2013; Michopoulos et al., 2014). The data within this dissertation serve to add to this literature, and suggest that E2 can have different structural effects on the adult female brain dependent on its social rank/status. These data demand further attention, and follow up studies looking at changes in neuronal morphology and function would significantly increase our knowledge. The identification of changes in estrogen, GC, and CRF receptor expression, as well as in dendritic morphology, and anatomical connectivity will significantly add to the neuroimaging data presented here. The findings reported in this dissertation would

suggest focusing further analysis on the mPFC, specifically within the ACC, which was sensitive to both FC changes following social subordination, and status-dependent structural changes following E2.

In conclusion, the studies within this dissertation found that stress attenuates E2's effect on behavior (Chapter 2) and modifies E2's effects on brain structure (Chapter 3) and function (Chapter 4). Together they represent the first experiments identifying the effects of social subordination status and E2 on the adult female primate brain and behavior. The experiments serve to bridge the gap between the rodent literature, which is often difficult to translate to the human conditions and based on non-ethologically relevant models, with those of human adaptations to socially adverse environments that often lead to psychopathology. These data serve to fill critical gaps in our knowledge of how neurobehavioral adaptation to psychosocial stress can increase susceptibility to the development of mood and anxiety disorders in women.

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