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Sanjana Malviya 04/17/2012

The Role of Ovarian Steroids in the Glucocorticoid Receptor System

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An abstract of
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Abstract

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One of the most common clinical findings in patients with major depressive disorder (MDD) is hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, the system in the body that regulates the stress response. It has been suggested that alterations in the glucocorticoid receptor (GR)-mediated feedback prolongs activation of the HPA axis, leading to the dysfunction observed in MDD. Additionally, the risk for developing MDD is heightened by several risk factors, namely gender, genetics and early life stress. Previous studies from our lab showed a sexually dimorphic change in the molecular regulation of GR activity in females following early life stress, which could mediate their heightened risk to HPA axis dysfunction. The purpose of this project was to determine whether steroid hormones mediate the altered adaptation of the GR chaperone system during stress in hippocampal neurons. *Methods*: First, we examined whether GR translocation was altered in the hippocampi of female rats that had a history of chronic adolescent stress. Next, we determined the extent to which serum ovarian steroid levels, stage of estrous, and uterine weights predicted expression of *Gr* and two co-regulators: *Fkbp5* and *Ppid*. Finally, we assessed the impact of corticosterone (cort), estradiol (E2), and progesterone (P4) treatments on the expression of these genes *in vitro* in HT-22 hippocampal neurons. *Results*: Compared to control female animals, females with a history of chronic adolescent stress displayed an attenuated increase in GR translocation that was induced by a forced swim test. The amount of GR, however, did not change due to chronic stress, suggesting a difference in the molecular regulation of GR. Uterine weights predicted expression of *Fkbp5* and *Ppid* but did not predict expression of *Gr*. Additionally, treatment of HT-22 cells with increasing doses of cort increased the expression of *Fkbp5,* an effect that was potentiated by E2. Exposure of HT-22 cells to increasing doses of E2 decreased the expression of *Ppid*. Collectively, these results suggest that the expression of GR co-regulators is directly influenced by exposure to gonadal steroids, and provides a basis for the attenuated GR translocation that we observed in females with a history of chronic stress.

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Introduction

Major Depression

The World Health Organization projects Major Depressive Disorder (MDD) to be the leading cause of global disease by the year 2020 (Mathers et al., 2001). Depression is also a major risk factor that underlies the risk of suicide and the development of cardiovascular diseases (Nemeroff, 2008). According to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), MDD is characterized by a combination of affective symptoms such as as depressed mood, reduced energy, weight loss, and anhedonia, which persist for longer than two weeks (American Psychiatric Association, 2000). Major depression has a high frequency of comorbidity with other psychiatric illnesses including anxiety disorders, eating disorders, substance abuse disorders, and bipolar disorder (Kupfer & Frank, 2003). Physiologically, depression is associated with morphological changes in limbic and prefrontal cortical brain structures, diminished neurochemical transmission, and dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, the mammalian system that regulates the body's response to stress (Nestler et al., 2002)

At this time, 6.2% of the U.S. population has suffered from MDD symptoms in the past twelve months (Kessler et al., 2005). Despite the high prevalence of this mood disorder, the mechanisms that lead to the physiological changes that accompany MDD have not been fully elucidated and warrant further study.

The HPA axis

Dysfunction of the HPA axis is common among depressed patients, though it remains unclear whether this dysfunction is a cause or consequence of the disease (Nestler et al., 2002).

The HPA axis normally functions to regulate the stress response and primarily involves the hypothalamus, anterior pituitary gland and adrenal glands (Figure 1). The presence of a stressor activates the parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus. The hypothalamus receives input from the prefrontal cortex, limbic structures including the amygdala and hippocampus, as well as serotonergic and possibly dopaminergic neurons, which may modulate its function in response to stress (Nemeroff, 2008). Activation of the PVN causes the release of corticotropin releasing factor (CRF) into the hypothalamo-hypophyseal portal system (McCormick & Mathews, 2007). CRF in turn binds to G-protein-coupled-CRF receptors in the anterior pituitary gland, which initiates a signaling cascade that leads to the release of adrenocorticotrophic hormone (ACTH) into the blood stream. ACTH then binds to melanocortin type II receptors in the adrenal cortex, triggering a similar signaling cascade. This cascade converts cholesterol to the intermediate pregnenolone and subsequently to glucocorticoids (GCs), a class of steroid hormones that are secreted into the bloodstream and mobilize the body's response to a stressor (Smith & Vale, 2006). The principal GC in humans is cortisol, whereas in rodents it is corticosterone (cort). Cort travels through the bloodstream either bound to corticosterone binding globulin (CBG) or in an unbound form, which is considered to be biologically active (McCormick & Mathews, 2007).

Cort triggers several downstream effects in peripheral tissues by acting on glucocorticoid receptors (GRs), which stimulate mobilization of energy (proteolysis, lipolysis, glycogenolysis), immunosuppression, inflammatory suppression and reproductive suppression. Collectively, these physiological changes assist the organism in coping with the increased demands on the body in the presence of a stressor (Sapolsky et al., 2000). Activation of GRs in the anterior pituitary gland and hypothalamus reduces the synthesis and secretion of ACTH and CRF. In the

central nervous system (CNS), the role of cort is to regulate energy needs during the stress response, influence cognitive and memory processes, and control the feedback inhibition of the HPA axis as a mechanism to restore the body back to homeostasis (Garrido, 2011). The latter function is performed by cort binding to GRs in a variety of brain structures, which then exert inhibitory control on the PVN, reducing the synthesis and secretion of CRF. In this way, cort negatively feeds back on the HPA axis to reduce its activity after the cessation of a stressor.

Normally, the HPA axis is negatively regulated by GRs located in the hippocampus, prefrontal cortex, and pituitary gland, which function to restore homeostasis through negative feedback (Nemeroff, 2008). Specifically, a rise in the plasma concentration of cort triggers GR translocation into the nucleus of cells in these structures, which in turn inhibits further release of CRF. The hippocampus is an important structure of interest in this feedback loop because of its role in cognitive learning (Garrido, 2011). Normal physiological cort levels are thought to promote HPA function and promote cognitive abilities (Nestler et al., 2002). Impairment of this feedback mechanism, however, causes elevated plasma cort, which is associated with hippocampal damage including loss of dendritic branching in CA3 pyramidal neurons and reduced granule cell birth in the dentate gyrus. This damage reduces the ability of the hippocampus to inhibit the HPA axis, thus prolonging further elevations of cort (Garrido, 2011). The focus on the hippocampus in this project is not only due to the high abundance of GRs in this region, but also because of its role on the feedback inhibition of the HPA axis as a whole.

HPA axis dysfunction in depression

One of the most common clinical observations, occurring in 50% of patients with MDD, is hyperactivity of the HPA axis (Pariante & Miller, 2001). Specifically, patients display elevated

cort levels in the urine, plasma, and cerebrospinal fluid (Gold *et al.*, 1988). Depressed patients also perform differently in the dexamethasone suppression test (DST). This clinical test involves the administration of oral treatments of the synthetic glucocorticoid dexamethasone (DEX), which should lead to a suppression of the endogenous glucocorticoids via negative feedback. Depressed patients, however, fail to suppress plasma cort levels in depressed patients (Pariante and Miller, 2001). In addition, post mortem studies of depressed patients reveal elevated CRF in the PVN and low levels of CRF receptors on the anterior pituitary gland. These low levels of CRF receptors in the pituitary are presumably due to receptor down-regulation from elevated CRF. This result is supported by the finding that depressed patients have blunted ACTH responses to intravenous administration of CRF (Gold et al., 1988; Nemeroff, 1996). In these cases, two mechanisms with respect to the HPA axis are believed to be at work—increased drive of the HPA axis and reduced feedback inhibition by GRs.

The evidence of impaired negative feedback in depression has led to a hypothesis that depressed patients have either a reduction in the *function* or the *amount* of GR available, which might underlie their resistance to circulating glucocorticoids. Of these possibilities, several investigators report reductions in total GRs in depressed patients in peripheral cells (Calfa et al., 2003; Gormley et al., 1985; Yehuda et al., 1993). The methods and interpretations of these studies, however, have been called into question (Pariante & Miller, 2001; Pariante, 2004). Specifically, many of the studies reporting a reduction in the numbers of GRs in blood cells of depressed patients measured GR levels only in the cytoplasm; a reduction in the cytoplasmic numbers of GR would show altered GR *translocation*, but would not necessarily reflect differences in total GR. Studies of whole-cell extracts reflect no differences in GR numbers (Wassef *et al.*, 1990; Rupprecht *et al.*, 1991; Maguire *et al.*, 1997). On the other hand, altered

GR levels have been reported in the brain. In a post mortem study, Webster et al. (2002) reported reduced GR mRNA levels in hippocampi of depressed suicide victims compared to healthy controls, but it is important to note that this reduction could be a consequence of general hippocampal neuron death in these patients**.** In contrast to the above report, Lopez et al. (1998) failed to find differences in GR expression in the hippocampi of suicide victims who suffered from depression. Furthermore, although the same group found stress-induced alterations in GR in some brain regions, they reported no difference in GR mRNA in the hippocampi of rats who exposed to chronic unpredictable stress, which has been shown to predispose animals to depressive-like behavior, compared to non-stressed controls (Lopez et al., 1998). Our group has further investigated GR expression in the hippocampus in suicide completers and, using more sensitive methods of detection, found no differences in GR expression compared to controls (Bourke CH, personal communication). Conversely, genetically engineered mice that have a 50% reduction in GR expression are more likely to display depressive-like behavior after exposure to stressors, suggesting that a reduction in GR number could predispose an organism to the development of depression or depressive-like behaviors (Ridder et al., 2005). From a therapeutic perspective, many antidepressants act through up-regulation of GR protein and mRNA in the hippocampus and hypothalamus (Pariante & Miller, 2001).

Studies of GR function in depressed patients have focused on cellular localization of GR outside of the CNS (Pariante, 2004). Euthymic patients, as mentioned above, typically suppress cortisol secretion in response to the synthetic glucocorticoid, DEX. After DEX treatment, these individuals also show reduced GR levels in the cytoplasm of their leukocytes, an indication of GR activation. Conversely, patients who fail to suppress cortisol in response to DEX do not have a reduction in cytoplasmic GR (Wassef et al., 1990; Yehuda, 2002). Several studies of GR

function also measure downstream cellular effects that are regulated by the GR. For example, lymphocyte proliferation is a cellular function that is normally reduced by activated GR. In depressed patients, however, treatment with DEX fails to reduce lymphocyte proliferation compared to normal patients (Rupprecht et al., 1991; Wodarz et al., 1992). This has been replicated *in vitro* using cultured lymphocytes from depressed patients (Calfa et al., 2003). Taken together, these findings suggest a GR resistance to glucocorticoids in depressed patients, a more complex finding than simply alterations in receptor density. Subtle changes in the molecular regulation of GR activity may account for the predisposition to stress-induced HPA axis dysfunction in depressed individuals, which is explored in this project.

GR autoregulation: focus on molecular mediators

Actions of glucocorticoids in target tissues are mediated by type I and type II receptors, known respectively as mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). MRs have a 10-fold higher affinity for cort than GRs and are mostly occupied under basal physiological levels of cort, suggesting that they function in maintaining HPA axis tone during circadian fluctuations of cort. In contrast, GRs have a lower affinity for cort, and are thus considered to regulate HPA axis activity during stress-induced releases of cort (Garrido, 2011). The "classic" mechanism of cort involves its binding to cytosolic receptors (either GR or MR) and subsequent translocation into the nucleus. Here, activated receptors influence gene transcription directly by binding to response elements on the DNA, or indirectly via interactions with other proteins, namely transcription factors (Pariante & Miller, 2001). It is also important to note that cort has recently been postulated to bind to a membrane receptor, triggering a rapid G protein-coupled signaling cascade instead of affecting transcription (Tasker & Malcher-Lopes,

2005). This mechanism was not examined in this project. Several chaperones and co-regulators that make up the GR complex mediate the "classic" activity of GR, acting to tune its sensitivity and regulate its translocation to the nucleus upon ligand binding. This process is detailed below.

Cytoplasm: In general, the GR is a ligand-activated protein that translocates from the cytoplasm to the nucleus after ligand binding (Grad & Picard, 2007). Thus, the process of GR signaling begins in the cytoplasm. After synthesis of the GR peptide, GR enters a complex of proteins, namely heat shock proteins (HSP) and their co-regulators which are responsible for GR folding, maturation, and trafficking into the nucleus (Binder, 2009). GR is first folded into a low-affinity conformation by the chaperone protein HSP70. The process of maturation involves the conversion of GR to a high-affinity state and is mediated by HSP90 and p23. The mature GR, therefore, resides in the cell cytoplasm associated with HSP90, P23, and immunophilin co-chaperone proteins (Figure 2). These immunophilin co-chaperones, namely FKBP5 and PPID, compete for binding to the same specific domain on the HSP90 protein, and act to regulate GR sensitivity to circulating glucocorticoids (Grad & Picard, 2007).

Hormone receptor localization studies show that the immunophilin FKBP5 is a normal component of the mature GR complex, lowering its affinity for cort and tuning GR sensitivity. Binding of GR to cort triggers the exchange of FKBP5 for FKBP4, which recruits the motor protein dynein to help the complex move along the cytoskeleton into the nucleus (Figure 2) (Davies *et al.*, 2002; Wochnik *et al.*, 2005). Ppid, an immunophilin also known as Cyp40, also binds to these TRP domains and is believed to further facilitate GR translocation (Pariante, 2004; Grad & Picard, 2007). The process of GR modulation and translocation is dynamic; it is even possible that different proportions of these proteins are bound at different GRs within the same

cell. However, FKBP5 is present in 55% of GR complexes, making it the most common co-chaperone (Pariante, 2004).

Nucleus: It is unknown whether HSP90 is still present in the GR complex when the GR translocates (Grad & Picard, 2007). Once it is in the nucleus, however, GR dissociates from the HSP90/p23/immunophilin complex, which allows it to form a dimer with another unit of GR in a homodimer, or another unit of MR in a heterodimer (Figure 2) (Gaestel, 2006). Subsequently, it can mediate gene expression directly, by binding to glucocorticoid response elements on the DNA, or indirectly by association with other transcription factors to induce or repress transcription of genes via crosstalk mechanisms. GR activates a multitude of genes in a variety of tissues, and one of its functions has been shown to increase expression of *Fkbp5* in an ultra-short feedback loop that negatively regulates further GR translocation (Binder, 2009). Given that FKBP5 suppresses translocation of GRs and has a higher affinity for the GR than FKBP4, increased expression of FKBP5 would lead to decreased translocation of GRs and thereby reduced negative feedback on the HPA axis.

Dissociation and Degradation: It is unknown when cort dissociates from the receptor and when the dimers separate. Once the receptor is unoccupied, chaperones HSP70, HSP90 and p23 help the unoccupied GR dissociate from the chromatin. Upon leaving the nucleus, GR is in a monomeric form in this hetero-complex of chaperones (Pratt et al., 2006). This complex can either be recycled back to its ligand-receptive state or degraded. This seems to be dependent on HSP70 and the "CHIP" proteins, which ubiquitylate the GR, targeting it for proteasome degradation (Grad & Picard, 2007; Pratt et al., 2006). Recent evidence, however, suggests that GRs can be degraded independently of this mechanism.

Risk factors for the development of depression

Genetics: Genetic polymorphisms of products involved at multiple levels of the stress response have been implicated in a higher risk for depression. For example, two polymorphisms of *NR3C1*, the gene that encodes GR, were implicated in increasing the risk of developing an episode of major depression (Rossum, et al., 2006). Interestingly, these polymorphisms led to altered sensitivity of the GR (Rossum et al., 2006). In addition, functional polymorphisms of FKBP5 that lead to a higher gene expression have repeatedly been associated with a higher risk of developing mood disorders (Binder 2009). Polymorphisms of CRF have been associated with mood disorders as well, but this is beyond the scope of the current project.

MDD is estimated to be 30-40% heritable, but twin control studies suggest that the risk extends beyond genetics alone (Heim & Binder, 2012; Kendler et al., 2000). In fact, susceptibility to depression seems to be determined by a gene x environment interaction (Caspi & Moffitt, 2006; Krishnan & Nestler, 2008). Data from our lab show that chronic stress during adolescence can lead to an increase in *FKBP5* expression as well as an increase in depressive behavior (Bourke et al., unpublished). Recent genetic-knockout studies show that mice that lack *Fkbp5* are more resistant to the development of stress-induced depressive behavior (Hartman et al., 2012; O'Leary et al., 2011). These results are supported by human epidemiological studies showing that exposure to adverse events interacts with *Fkbp5* polymorphisms to predict the incidence of depression (Zimmerman et al., 2011).

Developmental Stage: Early life stress and trauma have repeatedly been associated with the occurrence of depression, especially during certain windows of development. A correlation was found between the level of childhood adversities and the development of mental disorders in adulthood (Edwards et al., 2003**)**, and a four-fold increase in the risk of developing depression

was associated with individuals who suffered from multiple adverse experiences during childhood (Felitti et al., 1998). Early life stress paradigms have been used in animal models as well, and seem to suggest a causal link between the exposure to early adverse experience and the development of depressive behavior later in life (Lupien et al., 2009). Additionally, within early life, the negative effects of chronic stress seem to be heightened during critical periods when the brain undergoes maturation and morphological change. During adolescence, for example, the HPA axis develops in parallel with limbic structures and the prefrontal cortex (Lupien et al., 2009; Vazquez & Akil, 1993). Rats that were exposed to juvenile stress display reduced exploratory behavior and inferior avoidance learning during their adulthood (Tsoory & Richter-Levin, 2006). The susceptibility to stress during development may be mediated by permanent or semi-permanent changes in the HPA axis. For example, adolescent rats have a prolonged release of cort in response to stress, and do not habituate to stressors compared to adults (Romeo & McEwen, 2006; Vazquez & Akil, 1993). The gene x environment interaction that increases the susceptibility for mood disorders has been associated with developmental stage as well. For example, Binder (2008) showed that polymorphisms of *Fkbp5* interacted with childhood trauma to predict PTSD symptoms. The interaction between *Fkbp5* polymorphisms and the experience of physical child abuse was also associated with the incidence of adult depression (Appel et al., 2011). Childhood trauma and *Fkbp5* polymorphisms have been shown to predict suicide attempts (Brent et al., 2010; Roy et al., 2010). Importantly, the polymorphisms of *Fkbp5* that were implicated in all these studies were associated with higher induction of gene product.

Morphological changes resulting from adult chronic stress have been shown to be somewhat reversible after the cessation of the stressor. In adult rodents, chronic stress causes a reduction in the density of dendrites on CA3 hippocampal neurons, an effect that was not

detected until several weeks of exposure to the stressor and was reversed ten days after termination of chronic stress (Conrad, 2008).

Sex Differences: Epidemiological studies reflect a 2-fold higher rate of depression among women than men (RC Kessler, 2003). Animal studies suggest that these differences first appear during adolescence, with females displaying more depressive behavior after exposure to chronic stress than do males (Bourke & Neigh, 2011; Bourke et al., under review). This period is marked by a rapid increase in circulating gonadal hormones, which have an influence on the morphology of the brain and a profound impact on the HPA axis on several levels. In particular, gonadal hormones during adolescence can affect CRF mRNA expression, HPA axis development, and hippocampal structure (Heim & Binder, 2012; Bourke *et al*, under review).

On a molecular level, gonadal steroids influence the activity of both the GR itself and its co-regulators (Bourke et al, 2012; Bourke et al., under review). For example, estradiol (E2) has been shown to reduce GR action, and progesterone (P4) competes with cort for GR binding (Kontula *et al.*, 1983; Zhang *et al.*, 2009). Additionally, gonadal hormones influence GR co-regulators. In breast cancer cells, E2 has been shown to regulate the expression of *Ppid,* whose gene-product is believed to facilitate GR translocation (Kumar et al., 2001). *Fkbp5* has hormone response elements for androgens and progestins, as well as glucocorticoids (Hubler & Scammell, 2004; Ratajczak et al., 2003). This crosstalk in regulation of these co-regulators by gonadal steroids suggests a molecular mechanism by which gonadal steroids can modulate GR sensitivity. This hypothesis is supported by human studies as well. Recent evidence from pregnant women with a history of mood disorders shows elevations in the expression of *Fkbp5, Ppid,* and *Nr3C1*. However, pregnant women showing depressive symptoms showed an attenuated increase in the expression of these genes as well as a reduction in GR sensitivity.

Given the long-term elevated levels of gonadal steroids during pregnancy, this suggests the influence of gonadal steroids in regulation of these genes (Katz et al., 2012). Taken together, these studies suggest a role of sex steroids in the modulation of the GR system. Several studies have focused on GR regulation *in vitro* but they have focused mainly on cells in the periphery, and thus cannot be applied to the feedback of the HPA axis as a whole. However, further study is needed to elucidate the mechanisms by which sex steroids influence the effects of cort activation on GR regulation in CNS structures.

Current Study

Recently, members of our lab found sex-differences in depressive behavior and the expression of GR co-chaperones in rats with a history of chronic adolescent stress (Bourke and Neigh 2011; Bourke et al., under review). Females with a history of chronic adolescent stress displayed increased depressive-like behavior, a finding that was not observed in males. Females with a history of chronic adolescent stress also displayed a prolonged cort release in response to an acute stressor, suggesting an impaired negative feedback of the GR (Bourke et al., under review) After exposure to an acute stressor, females with a history of chronic adolescent stress displayed elevated expression of *Ppid* and *Fkbp5* compared to females without such a history (Figure 3), along with prolonged release of E2. Collectively, these findings suggest that altered ovarian steroids may mediate the sex-specific alterations of the GR system during stress. This data set indicates sex differences in behavior, hormone release, and expression of GR co-regulators in the female hippocampus as a result of chronic adolescent stress. However, these data do not demonstrate whether chronic stress alters functional GR translocation in a sex

dependent manner, and further do not demonstrate whether ovarian steroids directly influence the expression of GR co-chaperones.

From these results, we hypothesized that these sex differences were mediated by gonadal steroids. The purpose of the current project is to address whether gonadal steroids underlie translocation and regulation of co-chaperones of the GR. To investigate these questions, we first examined the effects of chronic adolescent stress on GR translocation in the rat hippocampus. Further, we examined whether natural hormonal variation during estrous can affect gene expression of *Gr, Fkbp5,* and *Ppid* in the female rat hippocampus. Finally, we examined whether the administration of ovarian steroid hormones would change expression of these genes *in vitro* in HT-22 mouse hippocampal neurons.

Methods

Experiment 1: assessment of GR translocation in chronically stressed rats

Animals: All animal experiments were carried out according to the National Institute of Health's guide for the care and use of Laboratory Animals (National Academy of Sciences) . The stress paradigm is described below and is further detailed in Bourke and Neigh (2011). Animal work, including weaning, stress, and tissue collection was completed prior to the beginning of my Honors thesis work. For clarity, these protocols are described below.

Sixty-one Wistar rats (Charles River, Raleigh, NC) were housed in polycarbonate cages on a 14:10 reverse light: dark cycle in an IACUC approved animal facility that was controlled for temperature (20-23 °C) and humidity (60%). Rats received food (Purina Mills, Richmond, IN) and water *ad libitum* throughout the study. Three days after birth, litters were culled to four male and four female pups. At postnatal day (PND) 23, rats were weaned and divided into groups. No more than two pups per litter were assigned to a group to prevent litter effects (Holson and Pearce 1992). Rats were pair-housed in same-sex groups and weighed once per week.

In total, there were 30 males and 31 females. Rats were divided by gender and then into the following groups: rats that did not receive stress (NS), rats that received acute stress only (AS), rats that received chronic stress only (CS), and rats that received both chronic and acute stress (CAS) (Table 1).

Chronic Mixed-Modality Adolescent Stress: The chronic mixed modality stress protocol was performed as previously described in Bourke and Neigh (2011) to induce chronic stress during adolescence. Rats were weaned on PND 23 and exposed to chronic stress from PND 37 to PND 49. Rats were stressed during the light cycle, during which time nocturnal

animals are at the nadir of their circadian cycle of cort levels. This was performed in order to maximally increase cort after stress.

In an initial study, rats were divided by gender and either pair-housed or single-housed, and subsequently tested on behavioral endpoints. This revealed that individual housing was a potent stressor (Bourke & Neigh, 2011). Therefore, based on previous studies in this lab, we defined chronic adolescent stress as single-housing beginning on PND 37 and randomly alternating daily exposure to social defeat or restraint from PND 37-49 (Bourke & Neigh, 2011). Social defeat involved the placement of the experimental rat in the same cage as an adult sex-matched Long Evans rat (Charles River, Wilmington, MA), which would act aggressively towards it (Bourke and Neigh, 2011). Female Long Evans rats were ovariectomized to prevent changes in behavior that might occur due to stage of estrous. The rats were initially separated by a wire-mesh barrier. In the first round of social defeat, the barrier was removed after five minutes and rats were allowed to interact for five minutes or until the experimental rat was pinned by the aggressor five times, at which point the barrier was replaced. In the second round of social defeat, the rats were allowed to interact for five minutes or until the experimental rat was pinned three times. All other rounds of social defeat included either pin by the aggressor or five minutes of total exposure. After the screens were replaced, the two rats were separated for another thirty minutes after which the experimental rat was returned to its cage. Restraint stress involved the physical restraint of animals for 60 minutes in acrylic rat restraints (BrainTree Scientific, Braintree, MA), which inhibited the rat's movement from head to tail (Bourke and Neigh 2011). Each rat in the CS and CAS groups received six exposures to social restraint and social defeat.

The order of the presentation of the stressor was randomized. This was performed in order to prevent them from acclimating to the experience (Bourke & Neigh, 2011).

Acute Stress: Forced Swim Test: On PND 53, rats in the CAS and AS groups were subjected to the forced swim test, which has been validated as both an acute stressor and a measure of depressive-like behavior (Porsolt et al., 1979). The test involved placing them in clear, acrylic tanks (60 cm high x 22 cm in diameter) filled with 45 cm of 30°C water for five minutes during the light cycle, and measuring floating behavior, which is believed to be an index of hopelessness. Previously, we have previously shown that chronically stressed animals show increased floating behavior and thus more depressive-like behavior when subjected to this test (Bourke & Neigh, 2011). Therefore, in this paradigm, we use the forced swim test to test whether a history of chronic stress altered abilities to respond to this acute stressor.

Tissue Fractionation Either within two minutes of removal from the colony on PND 53 (NS and CS groups) or immediately following the 5 min forced swim stress (AS and CAS groups), rats were rapidly decapitated, brains were removed, and tissue was stored at -80°C. Frozen hippocampi were dissected on dry ice with reference to the Paxinos brain atlas (Paxinos and Watson, 1994). Frozen brains were cut using an RNAse free razor. Brains were kept cold to prevent protein degradation. Frozen hippocampi (approximately 100 mg) were homogenized on ice in 1 mL ice cold homogenization buffer (50 mM Tris, pH= 7.2, 1 mM EDTA, 6 mM $MgCl_2$, 10% sucrose and 1:1000 Protease inhibitor cocktail; Sigma Aldrich, St. Louis, MO) using a pre-cooled hand-held 2 mL Kontes Tenbroek tissue grinder (Fisher Scientific, Pittsburgh, PA) on ice.

Homogenate was spun in a pre-cooled (4°C) centrifuge at 2000 x *g* for 5 minutes. The supernatant was separated for further cytosolic purification, and the pellet was used as the nuclear extract. The cytosolic fraction was spun at 105,000 x g in a pre-cooled (4°C) high-speed ultracentrifuge for 30 minutes, and the supernatant was kept as the purified cytosolic extract. The nuclear pellet was re-suspended twice in 0.5 mL of homogenization buffer and spun at 2,000

x *g* at 4°C for 5 minutes. After each spin, the supernatant was discarded and the pellet was kept for the subsequent wash. The pellet was re-suspended in 0.5 mL of nuclear buffer (50 mM Tris (ph=7.2), 1mM EDTA, 6 mM MgCl2, 10% sucrose, 0.5 M NaCl, and 1:1000 Protease Inhibitor), and incubated on ice for 1 hour. Finally, the homogenate was spun in a pre-cooled centrifuge (4°C) for 10 minutes at 8000 x *g*, and the supernatant was kept as the purified nuclear extract.

Concentrations of proteins in each sub-cellular extract were assessed using the BCA assay kit (Pierce Biotechnology, Rockford, IL). This protocol involves the addition of a fixed amount of cell lysate to a colorimetric reagent, which produces a color change that is proportional to the amount of protein in the lysate. These color changes are analyzed in reference to a standard curve, which consists of known concentrations of a reference protein provided with the kit (Pierce Biotechnology, Rockford, IL). Final concentrations were detected using a spectrophotometer (Biotek, Winooski, VT).

Western Blots to assess GR translocation: Western blots were performed to determine the relative amount of GR in each tissue fraction. For each blot, 10 µg of protein was loaded into each lane of a 10% Bis-Tris gel (Invitrogen, Carlsbad, CA). A protein standard ladder (Invitrogen, Carlsbad, CA) was loaded into the first lane of each blot to use as standard by which to gauge protein size. Proteins were then separated by gel electrophoresis at 120 V and 500 mAmp. This method separates proteins in each lane according to molecular weight, with lower molecular weight proteins traveling a longer distance through the gel. Next, the gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) and blocked overnight at 4°C in a solution containing 7.5% skim milk diluted in 1X Tris-Buffered Saline with 0.1% Tween-20 (TBST). Blocking solution binds to all the areas on the gel where

proteins have not attached, minimizing the non-specific binding of the primary antibody in the subsequent step. This reduces the noise on the final image.

Blots were incubated with gentle rotation at room temperature (23°C) for 2 hours with one of the following primary antibodies: GR M-20 (1:2500), GAPDH FL-335 (1:50,000), or NF-YA H-209 (1:2500) (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with goat anti-rabbit IgG- HRP (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour, and were visualized with the SuperSignal ECL kit (Pierce Biotechnology, Rockford, IL). Protein density was analyzed with AIS 6.0 Imaging Software (Imaging Research, St. Catharine's, Canada), and GR translocation was reported as (density of nuclear GR)/ (density of cytosolic GR+ density of nuclear GR).

GAPDH was used as a loading control for cytosolic extracts, and NFYA was used as a loading control for nuclear extracts. These loading controls are proteins that are expressed evenly in their respective cellular compartments, and are used as standards to ensure equal loading of protein in each lane of the blot (Abcam, Cambridge, MA).

Experiment 2: Expression of *Gr*, *Fkbp5*, *and Ppid* in various stages of the estrous cycle

Animals: For this study, 25 adult female Wistar (Charles River, Raleigh, NC) were housed in polycarbonate cages on a 14:10 reverse light: dark cycle in an IACUC-approved facility that was controlled for temperature (20-23°C) and humidity (60%). Rats received food (Purina Mills, Richmond, IN) *ad libitum* and water throughout the study. Adult female rats were monitored for stage of estrous cycle with vaginal swabbing for four days prior to decapitation. Vaginal smears were obtained by the placement of a sterile swab (Fischer Scientific) in saline,

and then gently swabbing the vaginal lumen. The swabs were then spread on labeled sterile glass slides and visualized using an Olympus Microscope (40x)**.**

On the last day of swabbing, animals were selected based on their stage of estrous, and were rapidly decapitated. Their brains were stored at -80°C. Following decapitation, uteri were collected and weighed, and trunk blood was collected in BD Vacutainer EDTA Collection tubes (BD, Franklin Lakes, NJ). Blood was spun down at 1800 relative centrifugal force (rcf), and the supernatant, or serum fraction, was used for assessment of hormone concentrations.

Hormone assays: Serum P4 was assayed with the Progesterone EIA kit (Enzo Life Sciences, Farmingdale, NY). This kit has an intra-assay variability of 5.4% and an inter-assay variability of 8.3%, and the sensitivity was 8.6 pg/mL. Serum estradiol levels were assayed using the Estradiol EIA kit, which has an intra-assay variability of 5.3%, an inter-assay variability of 4.7%, and a sensitivity of 10 pg/mL (Cayman Chemical Company, Ann Arbor, MI). Samples were run in duplicate for all endocrine analyses. Hormone levels were compared with swabbing data to confirm the correct identification of the estrous cycle stage.

These kits work through competitive binding between a hormone present in a sample and a known concentration of a hormone tracer to a limited amount of antiserum. The concentration of tracer is held constant, while the amount of hormone present in each sample varies. Thus, the amount of tracer that binds to the antiserum is inversely proportional to the concentration of hormone in the well. A colorimetric reagent that absorbs at a certain wavelength was added to each well to determine the amount of bound tracer, and the intensity of the color was determined using a spectrophotometer (Biotek, Winooski, VT) The colorimetric reagent in the progesterone EIA kit absorbed at 405 nm, and the colorimetric reagent in the estradiol EIA kit absorbed at 412

nm. The intensities of samples were compared to the intensities from a standard curve in which the hormone concentrations are known, and thus the amounts of hormone were quantified.

Real Time Polymerase Chain Reaction (RT-PCR): Frozen hippocampi were homogenized using the Tissue Lyser II (Qiagen, Valencia, CA), and RT-PCR was performed to quantify gene expression of *Fkbp5*, *Ppid*, and *Gr*. This technique involves extraction of RNA, conversion of RNA to cDNA using reverse transcriptase, and the quantification of the amount of the mRNA of interest that was present in the sample using a Polymerase Chain Reaction (PCR). This procedure is described in more detail below.

RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA), and RNA was quantified using the Nanodrop ND-1000 (Thermo Scientific, Waltham, MA). RNA was diluted to 1 µg in 10 µL nuclease free H_2O, and was reverse transcribed using the high capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). The amount of cDNA per sample was quantified using the PicoGreen assay (Invitrogen, Carlsbad, CA), and cDNA was normalized to a concentration of 1 µg/ µL. *Tfrc* was selected as the housekeeping reference gene based on the fact that there is ubiquitous expression at a constant concentration in the hippocampus (Boda et al 2009). This gene encodes the transferrin receptor, which is known to be involved in iron processing in cells. Primers for *Nr3c1, Ppid, Fkbp5,* and *Tfrc* were purchased from Assays on Demand (Applied Biosystems, Foster City, CA).

Samples were run in triplicate, and 4.5 ng of cDNA from each sample was loaded into each triplicate well of the plate along with TaqMan Gene Expression Master mix (Applied Biosystems, Foster City, CA) and the appropriate concentration of primer. TaqMan Gene expression master mix contains AmpliTaq Gold DNA polymerase, dNTPs, the ROX reference dye, and buffers that optimized the PCR reaction (Applied Biosystems, Foster City, CA).

AmpliTaq Gold DNA polymerase is active only at high temperatures. Primers contained the FAM reporter dye on their 5' end. When the primer anneals to the cDNA, the reporter is released and the computer quantifies the increase in fluorescence units, plotting it against the cycle number of the reaction. These fluorescence units are compared to the fluorescence units released by the ROX passive reference dye. The computer quantifies the threshold value, or the level at which the fluorescence is higher than the background from the ROX passive reference dye. RT-PCR quantifies the amount of cDNA template in the exponential phase of growth, and this threshold occurred at the point of inflection of this curve. The PCR cycle at which this threshold occurs is called the "cycle threshold" or the C_t value.

For each sample, the change in C_t value (ΔC_t) was computed via the following formula:

$$\mathbf{C_t\ (target\ gene) - C_t\ (housekeeping\ gene) = \Delta C_t}$$

Experiment 3: Expression of *Gr*, *Fkbp5*, *and Ppid* after exposure to hormones in vitro

Cell line: An HT-22 immortalized cell line derived from mouse hippocampi was obtained from the Salk Institute (La Jolla, CA). Cells were cultured in media made of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) under standard conditions (37 °C, 5% CO_2).

Hormone preparations: Prior to each hormone exposure, cells were seeded at a density of 6.2 $x10^4$ into six-well plates using 0.05% Trypsin-EDTA, and allowed to grow for 24 hours. Media was then replaced with phenol red-free growth media containing 10% charcoal-stripped fetal bovine serum for 24 hours. This medium has minimal hormones, and thus will not confound the effects of exposing cells to hormones. After this period, cells were exposed to various hormone concentrations (Table 2) for a 24 hour period. In total, eight wells (n=8) were used for each hormone condition, taken from four separate passages of HT-22 cells. Hormone

concentrations used in this study were determined based on peak serum hormone levels observed previously in our lab (Bourke *et al,* under review).

The corticosterone solution was prepared by dissolving corticosterone (Sigma Aldrich, St. Louis, MO) in EtOH to make a 29 mM solution (10 mg/mL). Corticosterone was further diluted 1:200 in sterile PBS to make 0.145 mM (50,000 ng/mL) stock solution. The estradiol solution was prepared by dissolving β-estradiol (Sigma Aldrich, St. Louis, MO) in EtOH to make 3.7 mM (1 mg/mL) solution. The estradiol solution was diluted 1:100,000 in sterile PBS to make a 37 nM stock solution (10 ng/mL). The progesterone solution was prepared by dissolving Progesterone (Sigma Aldrich, St. Louis, MO) in ETOH to make a 3.2 mM (1 mg/mL) solution. The progesterone solution was diluted 1:200 in sterile PBS to make 0.016 mMl (5000 ng/mL) stock solution. For baseline conditions, liquid EtOH was diluted 1:200 in sterile PBS to make vehicle solution, and control samples were treated with the same amount of this vehicle solution. These stock solutions were added to stripped media to make the appropriate concentrations of hormones, which are summarized in Table 2.

RT-PCR: After 24 hours of hormone exposure, cells were pelleted using 0.05% Trypsin-EDTA. RNA was extracted and RT-PCR was performed as described above, quantifying the expression of *Nr3c1, Ppid,* and *Fkbp5*. *Tfrc* was used as the housekeeping reference gene as described above, and ΔC_t values were calculated in the same manner. Additionally, ΔC_t were normalized to baseline conditions in the following formula:

$$\mathbf{\Delta\Delta C_t = \Delta C_t\,(Treatment) - \Delta C_t\,(Baseline)}$$

Changes in gene expression are reported as fold change compared to baseline, which was calculated using the following formula:

$$\mathbf{Fold\ change = 2^{-\Delta\Delta Ct}}$$

Statistical Analyses

GraphPad Prism 4.0 and SPSS were used for all statistical analyses in this study, and an alpha value was set to 0.05 for all tests.

The data from experiment 1 were analyzed by first obtaining protein density of blots using the AIS 6.0 Imaging Software (Imaging Research, St. Catharine's, Canada). GR translocation is reported as (density of nuclear GR)/ (density of cytosolic GR+ density of nuclear GR). A Two-way ANOVA was used to test for effects of chronic stress x acute stress on GR translocation, conducted separately for males and females. Bonferroni post hoc tests were used to assess the individual group differences.

In experiment 2, One-way ANOVAs were conducted to test whether gene expression varied significantly according to stage of estrous. Pearson's correlations were also conducted to assess whether gene expression was correlated with serum ovarian hormones or uterine weights.

In experiment 3, Two-way ANOVAs were conducted to analyze whether P4 and cort or E2 and cort had an effect on gene expression. If a main effect was found, a Tukey's post hoc test was conducted. If an interaction effect was found, a simple main effect test was conducted in order to determine the level at which the hormones interacted.

Results

Experiment 1: Chronic stress attenuates GR translocation in the female hippocampus

Rats were exposed to an acute stressor (forced swim) to induce GR translocation. Two-way ANOVAs were conducted to examine whether a history of chronic stress and the experience of acute stress impacted the total GR protein content in the hippocampus according to sex. In males, there was no effect of either chronic stress ($F_{1,22}$=0.20; p>.05) or swim stress ($F_{1,22}$=0.02, p>0.05) on total GR content (Figure 4a). Similarly, in females, there was no effect of either chronic stress ($F_{1,21}$=0.41, p>0.05) or swim stress ($F_{1,21}$=0.04, p>0.05) on total GR content (Figure 4b). Additionally there were no interaction effects between chronic stress and acute stress for either gender (p>0.05).

Two-way ANOVA analysis was to test whether chronic adolescent stress and acute stress affected GR translocation. In males, neither chronic stress ($F_{1,21}$=0.14 p<0.05; Figure 4c), nor acute stress ($F_{1,21}$=2.03, p>0.05; Figure 4c) significantly affected GR translocation. In females, exposure to the acute stress (forced swim) significantly increased GR translocation ($F_{1,21}$=5.211, p>0.05; Figure 4d). *A priori* post hoc tests revealed that in females with a history of chronic stress, GR translocation did not differ with exposure to the acute swim stress (t_8=1.601 p>0.05; Figure 4d). This data was interpreted to imply that a history of chronic stress prevented the acute stress-induced increase in GR translocation in female rats. Therefore, GR translocation is attenuated in females with a history of chronic stress.

GAPDH was present only in the cytosolic fraction, while NF-YA was present only in the nuclear fraction, suggesting that the tissue fractionation was pure. Equal amounts of protein were loaded into each lane, because GAPDH and NF-YA concentrations were constant (p>0.05).

Experiment 2: Gene expression of *Fkbp5* and *Ppid* positively correlated with uterine weights

In total, four females (n=4) were in diestrus 1, five females (n=5) were in diestrus 2, four females (n=4) were in estrus, and twelve animals (n=12) were in proestrus. Two animals from the proestrus phase were omitted from this study because of insufficient cDNA synthesis from their mRNA, so in total 10 animals were used in the analysis for proestrus. (n=10).

A one-way ANOVA was performed to analyze the relationship between the stage of the estrous cycle and gene expression. The mean fold change of *Gr* did not vary across the estrous cycle ($F_{3, 19}$=0.192, p>0.05; Figure 5a). Similarly, the fold change *Fkbp5* and *Ppid* did not vary by stage of estrous ($F_{3,19}$= 0.169, p>0.05, Figure 5b) and ($F_{3,19}$=0.205, p>0.05; Figure 5c).

A Pearson product moment correlation analysis was performed to assess whether there were significant associations between serum gonadal hormones and gene expression, and between uterine weights and gene expression. Neither progesterone nor estradiol levels correlated with gene expression of our target genes. These results are summarized in Table 3.

Overall, uterine weights positively correlated with the fold change of *Fkbp5* (R_{21}=0. 569, p<0.01; Figure 6b) and *Ppid*, (R_{21}= 0.512, p<0.05; Figure 6c). The r^2 values indicated that approximately 26% of the variance in *Ppid* expression and 32% of the variance in *Fkbp5* expression can be predicted from uterine weight. However, uterine weights did not significantly correlate with the fold change of *Nr3c1* (R_{21}=0.366, p=0.086; Figure 6a).

Experiment 3: Steroid hormone treatments altered the expression of *Fkbp5* and *Ppid*

Two-way ANOVAs were conducted to analyze the effect of E2 and/ or cort or the effects of P4 and/ or cort on the fold change of gene expression. For the baseline condition, n=12. For all other conditions, n=7 or 8; some wells were removed as they did not amplify during PCR.

Gr*:* There were no main effects for either cort ($F_{3,88}$=0.3728, p>0.05; Figure 7a), or E2 ($F_{2,88}$=0.4791, p>0.05; Figure 7a) on *Gr* expression. There was no significant interaction between E2 and cort. Additionally, there were no main effects for either P4 ($F_{2,88}$=0.281, p>0.05; Figure 7b) or cort ($F_{3,88}$=0.295, p>0.05; Figure 7b). There was no interaction between P4 and cort.

Fkbp5*:* The two-way ANOVA that assessed the effects of E2 and cort on *Fkbp5* fold change showed a main effect of cort ($F_{3, 87}$= 87.344, p<0.001; Figure 8a), but no main effect of E2 ($F_{2, 87}$ =0.601, p>0.05; Figure 8a). Tukey's post-hoc tests revealed that cort significantly increased *Fkbp5* expression at concentrations of 400 ng/mL and 800 ng/mL (p<0.001). There was also a significant interaction between E2 and cort on *Fkbp5* expression, ($F_{6,87}$=2.286, p=0.043). Analysis of the simple main effects showed that E2 significantly increased *Fkbp5* expression when cort was held at 400 ng/mL (p=0.003), but not when cort was at 0 ng/mL (p=0.821), 50 ng/mL (p=0.935), or 800 ng/mL (p=0.339). In the test of cort and P4, cort had a main effect ($F_{3,87}$)=88.900, p<0.001; Figure 8b), but P4 did not ($F_{2,87}$=0.677, p>0.05; Figure 8b). Tukey's post-hoc tests showed that cort significantly increased *Fkbp5* expression at concentrations of 400 ng/mL and 800 ng/mL (p<0.001). There was no significant interaction.

Ppid*:* There was a main effect of E2 on *Ppid* expression ($F_{2,85}$=4.904, p=0.01; Figure 9a), but not cort ($F_{3,85}$=1.052, p>0.05; Figure 9a). Tukey's post-hoc tests showed that 20 pg/mL of E2 significantly decreased the fold change of *Ppid* compared to 0 pg/mL (p<0.05).There was no significant interaction between cort and E2. The ANOVA of P4 and cort showed again that cort did not significantly change *Ppid* expression ($F_{3, 85}$ =1.498, p>0.05; Figure 9b). Additionally, there was no main effect of P4 ($F_{2,85}$=0.081, p>0.05; Figure 9b), and there was no significant interaction between cort and P4.

Discussion

The risk for developing stress-related disorders, namely depression, is almost twice as high in females compared to males (Kendler, 2004). The HPA axis has been implicated in the sex-specific effects of these disorders. Recently, it has been suggested that these sex differences occur through alterations in GR-mediated negative feedback of this system (Bourke et al., under review). Several studies have suggested a role of gonadal steroids in the regulation of GR but these mechanisms have not been fully elucidated.

Previously, data from our lab demonstrated that females with a history of chronic adolescent stress displayed decreased sucrose consumption and passive behavior during the forced swim test, two indices of depressive like behavior (Bourke & Neigh, 2011). Also, compared to female controls, chronically stressed females display a prolonged elevation of cort and E2 along with an elevated baseline level of P4 during the forced swim test (Bourke et al., under review**)**. Finally, these females displayed elevated *Gr*, *Fkbp5,* and *Ppid* expression in the hippocampus after an acute stressor compared to female controls, and *Ppid* levels correlated with serum E2 levels (Bourke et al., under review**).** Collectively, these data suggest that altered steroid hormones observed in females could mediate the altered adaptation of the glucocorticoid chaperone system during stress, a hypothesis that was explored in the current project. The purpose of this project was to examine the influence of steroid hormones on GR translocation and the GR co-chaperone system in the hippocampus.

First, we tested whether GR translocation was altered in females with a history of chronic adolescent stress. Next, we determined the extent to which gonadal steroids, stage of estrous, and uterine weights predicted expression of *Gr* and its co-regulators *Fkbp5* and *Ppid.* Finally, we assessed the impact of corticosterone in combination with either E2 or P4 on the expression of

Gr, *Fkbp5*, and *Ppid in vitro* in HT-22 mouse hippocampal neurons. This project was novel because most studies of GR regulation *in vitro* have focused on peripheral cells, not hippocampal cells, which are involved in the negative feedback of the HPA axis. HT-22 neurons express receptors for cort, E2, and P4, and thus were a suitable cell line in which to examine the mechanistic effects of these hormones.

Chronic stress attenuates the acute stress-induced increase in GR translocation

Previous work demonstrated that female rats exposed to chronic adolescent stress exhibited more depressive-like behaviors and an exaggerated HPA axis response (Bourke & Neigh, 2011). One explanation for this finding is altered negative feedback on the HPA axis, which could be mediated by alterations in the availability of GR. Data from this project show that GR protein expression did not change as a result of chronic adolescent stress (Figure 4a, 4b). This suggested that an alternative mechanism may account for the altered function of the HPA axis. Though the amount of GR did not significantly change, the translocation of the receptor, and thereby the efficacy of the receptor, could be altered. To this end, although all female rats exhibited an increase in GR translocation following exposure to an acute stressor during adulthood (Figure 4d), female rats with a history of chronic stress had an attenuated increase in GR translocation (Figure 4d). Taken together, these data suggest GR resistance to circulating glucocorticoids and impaired GR negative feedback in chronically stressed females. This impaired negative feedback is consistent with data from Mizoguchi et al. (2003), who report that chronically stressed rats have an attenuated ability to suppress cort levels in response to a systemic injection of dexamethasone. These data are also consistent with previous studies that

report altered GR function in depressed human patients (Maguire et al., 1997; Rupprecht et al., 1991; Wassef et al., 1990).

Collectively, alterations in GR translocation in combination with elevations of gonadal steroids that we observed in chronically stressed females suggest alterations in the molecular regulation of GR, a finding that is supported by earlier evidence of elevated expression of molecular chaperones (Figure 3a, b, c**)**. We thus hypothesized that gonadal steroids directly regulate expression of *Gr* and its co-regulators.

Expression of Fkbp5 and Ppid are predicted by uterine weights

Given that estrous cycle stage can predict stress reactivity (Baker et al., 2006), we determined whether expression of *Gr*, *Fkbp5*, or *Ppid* was predicted by estrous cycle stage. We found that gene expression did not differ significantly by stage of estrous (Figure 5) and that serum gonadal steroids at the time of decapitation did not predict gene expression (Table 3). Uterine weights did not predict expression of *Gr* (Figure 6a), but did predict expression of *Fkbp5* (Figure 6b) and *Ppid* (Figure 6c). Figure 6b and c demonstrate that higher uterine weight predicts higher expression of *Fkbp5* and *Ppid.* Heavy uterine weights reflect an increase in cell proliferation in the uterus, which is stimulated by E2 (Groothuis et al., 2007). In studies of ovariectomized female rats, E2 does not stimulate mitosis until 24 hours after administration. Indeed, proliferation of uterine epithelial cells seems to require long-term accumulation of high concentrations of E2, which down-regulates mitosis inhibitors and thus leads to increased cell proliferation (Lan and Katzenellenbogen 1976; Barton 1998; Groothuius et al 2007). This requirement of a long term accumulation of E2 in the uterus suggests that higher uterine weights

may not reflect serum E2 levels at the time of decapitation, but instead could reflect E2 levels accumulated over time (Groothius et al 2007; (Anderson et al., 1975).

In contrast, gene expression did not vary by stage of estrous cycle as documented by either vaginal lavage or serum hormone concentrations. Given the positive correlation between uterine weights and gene expression of *Fkbp5* and *Ppid*, the lack of variation according to estrous stage was unexpected. Uterine weights are typically the heaviest during the proestrus stage, in which the serum E2 levels and P4 levels are at their highest points, and lightest during diestrus, when levels are at their lowest (Hood & Parker, 2008). However, stage of estrous determined from vaginal lavage is approximately one-half day behind the stage determined by weight of the uterus (Hood & Parker, 2008). In the rat, the estrous cycle stages vary in duration, with proestrus lasting between 8 and 12 hours, and estrus lasting between 8 and 15 hours. Collectively, diestrus 1 and 2 comprise 60 hours of the estrous cycle (Hood & Parker, 2008). Because vaginal lavages were only performed once per day, it was not possible to translate the data to align with the uterine weights.

We can determine from this study that a positive correlation exists between uterine weights and expression of *Ppid* and *Fkbp5*. Though these data are confounded by the lack of correlation of serum gonadal steroids and gene expression, and lack of variation across stage of estrous, the time delays between hormone release and influence on the uterus provides a basis for this incongruity. If uterine weights are indeed the heaviest during proestrus, the data would indicate a correlation between uterine E2 levels and hippocampal expression of GR co-chaperones. This finding is consistent with our previous results from *in vivo* animals, which demonstrate a correlation between serum E2 and expression of PPID in the hippocampus. Therefore, these data suggest regulation of co-chaperones by gonadal steroids.

Treatment of HT-22 cells with steroid hormones does not change expression of Gr

Next, we determined whether steroid manipulation directly altered expression of *Gr* and its co-regulators *in vitro*. We found that treatment of HT-22 cells with increasing doses of cort, in conjunction with increasing doses of E2 (Figure 7a) or P4 (Figure 7b) did not change the expression of *Gr*. This suggests that neither cort nor gonadal steroids regulate the expression of *Gr* at the transcriptional level.

Many studies present evidence of down-regulation of GR in the presence of cort in multiple peripheral cell lines and tissue (Burnstein et al., 1991; Denton et al., 1993; Silva et al., 1994). Down-regulation reflects the reduction in *Gr* expression after exposure to cort. Herman and Spencer (1998) demonstrate the necessity of activation of the mineralocorticoid receptor (MR) in the down-regulation of *Gr* in the hippocampus. This study involved the administration of cort to adrenalectomized rats, which decreased the expression of *Gr*. Upon subsequent administration of a selective MR antagonist, *Gr* expression increased. As mentioned earlier, MR is the type I glucocorticoid receptor that has a higher affinity for circulating cort, and is occupied at basal levels of cort. In our pilot studies with the HT-22 cell model, we did not determine the expression of *Mr*, and some studies suggest that HT-22 cells do not express it (Rozeboom, 2008). If this is the case, a lack of MR expression in HT-22 cells could underlie why treatments with cort may not result in the down-regulation of GR. Additionally, our *in vivo* studies demonstrated that rats exposed to stress did not have altered *Gr* expression in the hippocampus compared to non-stressed controls (Figure 3). Data from our HT-22 cells confirm this finding, reflecting that cort may not regulate Gr expression in hippocampal neurons.

Treatment of HT-22 cells with increasing doses of cort increases gene expression of Fkbp5

Treatment of HT-22 cells with increasing doses of corticosterone increased the expression of *Fkbp5* (Figure 8a and 8b). This main effect of corticosterone coincides with previous reports of an up-regulation of *Fkbp5* expression in the hippocampus and hypothalamus following glucocorticoid exposure both *in vitro* in hippocampal cell lines and *in vivo* as a result of stress (Lee et al., 2010; Scharf et al., 2011). This up-regulation of *Fkbp5* after exposure to glucocorticoids is believed to be part of an ultra-short feedback loop whereby GR regulates its own sensitivity (Binder, 2009). Specifically, an increase in circulating cort results in increased *FKbp5* expression, which prevents prolonged GR translocation. Mechanistically, corticosterone has been shown to exert epigenetic effects on the expression of *Fkbp5*—prolonged exposure to cort reduced DNA methylation of the *Fkbp5* gene in the hippocampus both *in vivo* and *in vitro* (Lee et al., 2010; Lee et al., 2011). DNA methylation is associated with gene silencing, and thus the cort-induced decrease in DNA methylation results in higher expression of *Fkbp5*. Thus corticosterone could affect *Fkbp5* expression by chromatin remodeling instead of direct binding to the DNA.

At a cort concentration of 400 ng/mL, there is an interaction effect in which E2 concentrations of 20 pg/mL and 40 pg/mL further increase the expression of *Fkbp5* compared to cort at 400 ng/mL alone (Figure 8a). *Fkbp5* does not have an Estrogen Response element (Hubler & Scammell, 2004), but E2 can nonetheless modulate gene expression through indirect interaction with transcription factors that regulate *Fkbp5* expression.

Conversely, the administration of P4 did not have an effect on the expression of *Fkbp5*. This finding is somewhat perplexing given the fact that *Fkbp5* has a response element where the glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR) can each

bind. Induction of *Fkbp5* by progestins, however, has been shown only in human carcinoma cell lines and human breast cancer cells (Hubler & Scammell, 2004; U *et al.*, 2004)**,** and perhaps is not regulated in the same way in the brain. Furthermore, in the hypothalamus and the uterus, some studies show that PRs may require E2 "priming" in order for P4 to successfully bind to its own receptor (Al-Khouri & Greenstein, 1985; Etgen, 1985; Maclusky & McEwen, 1978). Therefore, future studies will focus on the simultaneous variation of P4 and E2 to further elucidate whether P4 has a regulatory effect.

Treatment of HT-22 cells with increasing doses of E2 decreases gene expression of Ppid

Treatment of HT-22 cells with 20 pg/mL E2 reduces the expression of *Ppid* (Figure 9a), but neither corticosterone nor P4 affected expression of *Ppid* (Figure 9a and 9b). *Ppid,* also known as *Cyp40,* is a major immunophilin present in approximately 10% of all GR complexes and the most common in estrogen receptor complexes. It is believed to facilitate the nuclear translocation of GRs through recruiting the motor protein dynein to the steroid receptor complex. For estrogen receptors, which are already localized in the nucleus, cyp40 is thought to facilitate the conformational change once it binds to estrogen (Kumar et al, 2001).

In the current study, we found a main effect of E2 in the expression of *Ppid*. Specifically, 20 pg/mL of E2 reduced the expression of *Ppid* at cort concentrations of 0, 50, and 800 ng/mL (Figure 9a**)**. These data do not necessarily agree with hippocampal *in vivo* data from our previous study, in which *Ppid* levels positively correlated with E2 levels in females with a history of chronic adolescent stress. Also, the uterine weights of non-stressed adult females positively correlated with *Ppid* expression in Experiment 2. Though these results are dissimilar, it is important to note the differences between an *in vitro* and *in vivo* model. The expression of *Ppid*

could be mediated by other transcription factors that were activated in either model—we have no information about other processes occurring in the cells at the time. Further, although the expression of *Ppid* is prevalent in steroid receptor complexes, its regulation has not been well characterized. For example, Wang et al. (2004) reported that estrogen exposure in rat testis gonocytes does not change the expression profile of *Ppid*. On the other hand, Kumar et al. (2001) demonstrated that treatment of breast cancer cells with increasing concentrations of estrogen does induce *Ppid* expression, an effect which is reduced by the estrogen receptor antagonist. Thus it seems that *Ppid* is differentially regulated by *Ppid* in different cell types. Furthermore, regulation of *Ppid* transcription has never been examined in the brain. Mechanistically, it makes sense that *Ppid* expression would be reduced in the presence of E2 as a regulatory mechanism to prevent too much steroid receptor activity. Therefore, from the current results, we can conclude only that *Ppid* is responsive to E2 treatments *in vitro* in HT-22 cells.

Implications

Collectively, these results suggest a causal link between concentrations of serum gonadal hormones and previously observed changes in the GR signaling system (Bourke et al., under review). The results of this study provide a basis for the attenuated GR translocation that we observed in females with a history of chronic adolescent stress. Chronic stress in females resulted in a sustained increase in both cort and E2 after exposure to an acute stressor (Bourke et al., under review). The present study suggests that this combination of hormones could lead to an up-regulation of *Fkbp5*, and the elevation in E2 could lead to a down-regulation of *Ppid*. This would reduce GR sensitivity to circulating glucocorticoids, and could reduce GR translocation in a stress response.

These results lend support to the hypothesis that female adaptations to chronic stress are mediated by interactions among gonadal steroids and the GR system. Female rats react to physical and psychological stressors with a more pronounced cort release response than males (Handa et al., 1994), and estradiol has been shown to increase basal levels of cort and to exaggerate the cort response to stressors (Burgess & Handa, 1992). Estradiol also prolongs the ACTH release in response to a stressor (Burgess & Handa, 1992), suggesting that it impairs GR negative feedback. Handa et al. (1994) suggest that this estrogen-mediated inhibition of GR function could reflect an evolutionary strategy to prevent the deleterious actions of elevated glucocorticoids on reproduction.

Limitations and future directions

The current study had several limitations. First, I based my choice of hormone concentrations based on previous observations of serum hormone levels in rats during stress. However, it is difficult to know how much of these steroid hormones actually pass the blood brain barrier, and thus know what physiological levels of hormones that the hippocampus is exposed to. This problem is difficult to address given the difficulty of measuring hormone concentrations in the brain. A more complete study would have looked at the combination of varying cort, E2 and P4 administration simultaneously, in order to mimic *in vivo* conditions in females. Further, I examined gene expression in these cells at only one time point (24 hours), but it is unknown at what precise time point gene expression is affected by these hormones. Future studies will thus quantify gene expression at both earlier and later time points to create both dose-dependent and time-dependent curves.

Although the current data set reflects that gonadal steroids directly influence the expression of GR co-chaperones, it does not describe the ultimate effect of steroidal manipulations on GR translocation. Originally, GR translocation was supposed to be addressed in this project, but it was omitted due to time constraints and experimental difficulties. Future studies will look at this endpoint via western blotting or confocal imaging. It might also be interesting to note downstream effects of steroid treatments by receptor-binding to DNA response elements using CHIP sequencing, or looking at the expression of immediate early genes that are induced by GR. This could provide valuable information on the impact of gonadal steroids on GR function, not just GR translocation.

Additionally, the interpretations that can be made from studies with immortalized cell lines are limited. The HT-22 immortalized cells that were used in this project have transformed growth properties and therefore can grow indefinitely in the proper conditions. These altered growth properties often create a phenotype different from what is observed *in vivo* (Dyer, 2011). The main advantage of this model over an *in vivo* model, however, is the relative ease with which experimental variables can be manipulated under controlled conditions. The ideal experimental model would use both *in vivo* and *in vitro* models in conjunction with each other. The current data set is an *in vitro* study of our earlier *in vivo* reports—females exposed to chronic adolescent stress displayed elevated expression of *Ppid* and *Fkbp5* after exposure to an acute stressor. Collectively, this project demonstrates that gonadal steroids do have the ability to exert mechanistic influence over the regulation of these genes. To further investigate the influence of gonadal steroids on stress-induced co-chaperone expression, a relevant future study would focus on the forced-swim induced co-chaperone expression and GR translocation in ovariectomized females with a history of chronic adolescent stress. Since ovariectomized females would not

naturally secrete ovarian steroids, such an experiment could provide information about whether gonadal steroids are necessary for the expression of these genes *in vivo*.

Despite these limitations, the current data set establishes that ovarian steroids have the ability to differentially regulate the stress response in neuronal cells. These findings may explain the biological mechanisms that underlie the higher occurrence of depression among females. Understanding these mechanisms is important given the clinical association of depression with events in the female reproductive cycle. For example, pre-menstruation, the postpartum phase, and menopause are associated both with significant hormone changes and depressive changes in females (Weissman & Klerman, 1977). The studies included in this thesis build on previous work conducted in our laboratory and provide additional insight into the role of gonadal steroids in modulation of the GR system. Importantly, they also provide a framework for future studies on the role of sex in the etiology of depression.

The interaction between psychosocial influences in the female susceptibility to stress

The above studies address the physiological and molecular basis for the role of sex in major depressive disorder**.** However, depression is a complex condition, resulting not only from biological factors, but also from psychosocial influences, particularly the experience of stress. Therefore, it is also necessary to investigate the sources of stress in a social context that may put females at risk. Several studies have suggested that environmental disparities between genders are involved in the higher prevalence of depressive symptoms among women. For example, women have a higher likelihood of living in poverty compared to men (Belle & Doucet, 2003). This finding is interesting given the fact that individuals living in poverty experience significantly more stress than those in higher social classes, making socioeconomic status one of

the best predictors of depression (Chonody & Siebert, 2008). For women, a low socioeconomic status creates problems in balancing life between work and family (Belle & Doucet, 2003). Wage disparities between men and women leave have also been associated with the risk of depression (England et al., 2007). Indeed, Chen et al. (2005) found that women that live in conditions of high economic autonomy have lower scores on depression scales compared with women experiencing low economic autonomy. Women are also more likely to perceive diminished control over their environments, given the fact that they are charged with caring for children and ailing family members, domestic work of the home, and managing careers in many cases (Astbury, 2001; Crosby, 1982).

Chonody and Siebert (2008) argue that these examples are merely signs of greater problems within the social structure. They outline differential gender roles and distribution of power among genders within society and suggest that these factors increase the female risk for depression. In their model, the division of labor and power by gender interact with one another to create risk factors that negatively impact women's health. The gender difference in labor distribution refers to differences in the job distribution and segregation of the economic market, which can result in unequal pay and the concept of "women's work." The division of power between genders refers to a correlation between the male gender and authority, which can "subordinate" women, preventing them from participating in the upper levels of society. According to Chonody and Siebert (2008), these two factors interact with each other to create feelings of passivity and dependence that are common among women, thereby putting them at risk for developing mood disorders.

The vulnerability to the negative aspects of stress could also be attributed to the different way that women *perceive* stress. For instance, women are more prone to "ruminative" thinking,

which causes them to repeatedly focus their attention on symptoms of distress and the possible consequences. This manner of thinking has been associated with longer and more severe episodes of depression (Hoeksema et al., 1999). Women also tend to experience stress from family-related events, whereas men tend to experience stress related to work and finances (Matud, 2004). Males and females differ in their mechanisms for coping with stress. Matud (2004) showed that women are more prone to use emotional avoidance as a coping mechanism, whereas men were more likely to use "resolution"-based coping aimed at finding concrete solutions to their problems.

These gender differences in coping strategies can perhaps be attributed to larger evolutionary mechanisms. The role of the stress-response system is most commonly associated with the mobilization of bodily resources to help an organism cope with a stressor. This "fight or flight" model is crucial to the survival of the organism (Cannon 1932). Taylor et al. (2000) recently proposed the "tend and befriend" hypothesis, suggesting that females tend to affiliate with social groups and exhibit protective behaviors towards their offspring in order to maximize survival of their offspring. Perhaps females respond to stressors with a higher affective response because they are hardwired to care for their offspring.

A significant body of work in the laboratory has described relationships between hormonal influences and precursors of stress and depression. Whether such relationships exist between physiological influences and individual psychosocial aspects of the stress-depression interaction remains unknown. It is valuable, however, to continue pursuing answers to these questions both on a biological level and on a psychosocial level in order to create effective therapies in the future.

Tables and Figures

Table 1. Animal Groups; Experiment 1

Gender	Type of Stress		
		No Acute	**Acute**
Males	**No Chronic Stress**	No chronic, no acute (NS)	No chronic, acute (AS)
	Chronic Stress	Chronic, no acute (CS)	Chronic, acute (CAS)
Females	**No Chronic Stress**	No chronic, no acute (NS)	No chronic, acute (AS)
	Chronic stress	Chronic, no acute (CS)	Chronic, acute (CAS)

Table 2. Concentrations of steroid hormones exposed to HT-22 cells; Experiment 3

Dose		E2 (pg/mL)		
		0	20	40
Cort (ng/mL)	0	0/0	0/20	0/40
	50	50/0	50/20	50/40
	400	400/0	400/20	400/40
	800	800/0	800/20	800/40

Dose		P4 (ng/mL)		
		0	10	30
Cort (ng/mL)	0	0/0	0/10	0/30
	50	50/0	50/10	50/30
	400	400/0	400/10	400/30
	800	800/0	800/10	800/30

Table 3. Pearson correlation results of serum hormones and gene expression; Experiment 2

Hormone	Gene	Statistic
Progesterone	*Ppid*	r(21)= -0.048, p>.05
	Fkbp5	r(21)= 0.151, p>.05
	Nr3c1	r(21)= -0.055, p>.05
Estradiol	*Ppid*	r(21)= -0.084, p>.05
	Fkbp5	r(21)= -.159, p>.05
	Nr3c1	r(21)= -0.103, p>.05

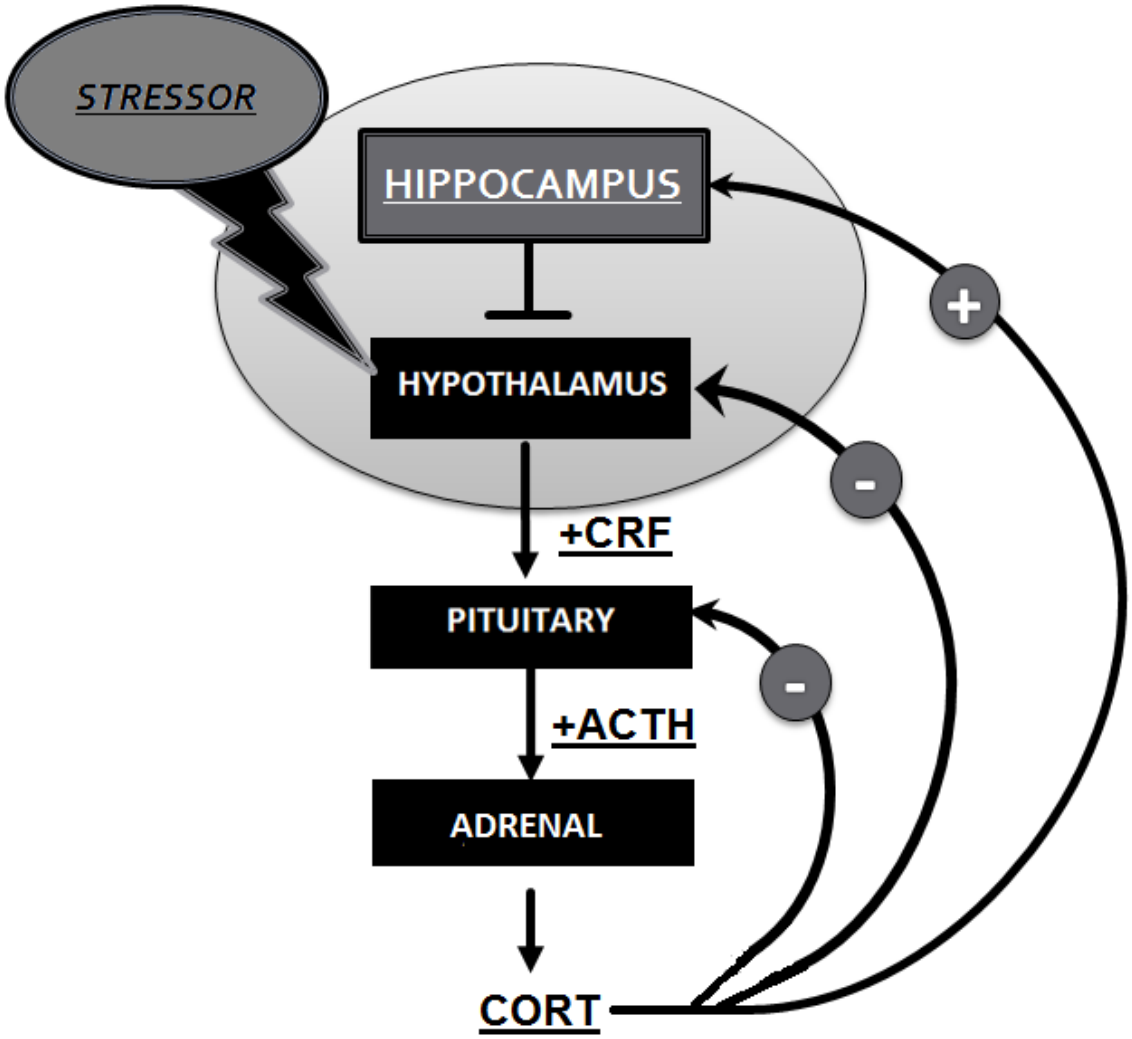

Figure 1. Mechanism of the Hypothalamo-Pituitary-Adrenal Axis:
The HPA axis is the system in the body that regulates the stress response. In the presence of a stressor, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing factor (CRF), which binds to receptors in the anterior pituitary gland and triggers the release of adrenocorticotropic hormone (ACTH). This hormone travels through the blood and binds to receptors in the adrenal cortex, triggering the release of glucocorticoids (cort). Cort triggers several downstream effects in the body that help the organism cope with the stressor, such as energy mobilization and immunosuppression. In the CNS, cort inhibits the synthesis and secretion of CRF and ACTH. Additionally, activation of GRs in the hippocampal neurons leads to inhibition of the hypothalamus. Together, these two mechanisms comprise a negative feedback loop that shuts off the HPA axis to restore homeostasis.

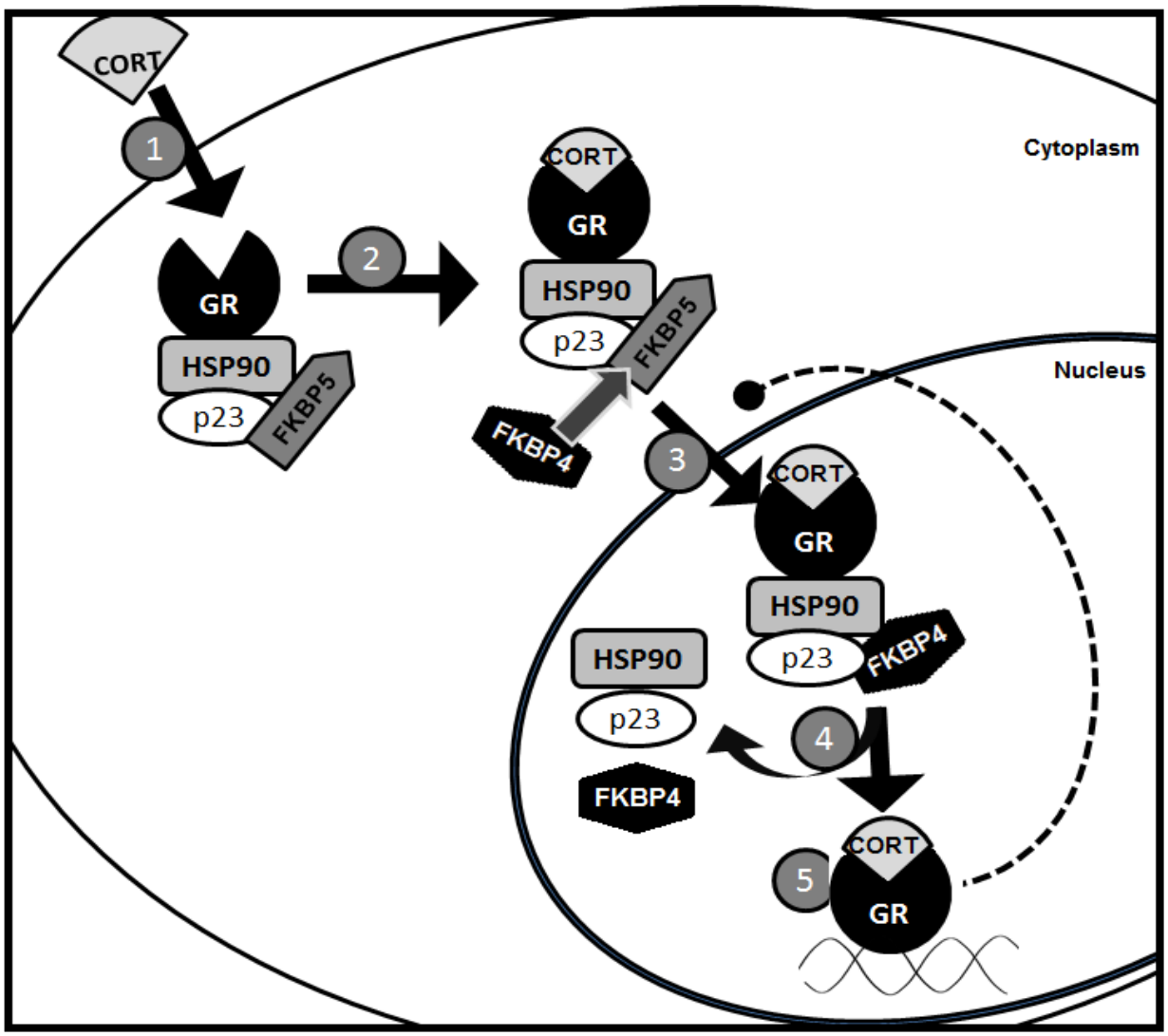

Figure 2: Hypothesized Mechanism of GR signaling with Fkbp5 and Fkbp4 co-regulators. GR is a ligand-activated transcription factor that resides in the cytoplasm in a network of chaperones and co-regulators. (1) As a steroid hormone, circulating cort diffuses through the plasma membrane and binds to GR. Association of the GR complex with FKBP5 reduces GR binding affinity for cort and inhibits its translocation. (2) Upon successful binding, FKBP5 is displaced by FKBP4. (3) FKBP4 facilitates GR translocation using the cytoskeletal system. (4) Upon entry into the nucleus, ligand-activated GR dissociates from its chaperone complex and dimerizes with another GR or MR (omitted for simplicity) (5) GR mediates gene transcription by binding to GR response elements on the DNA or by interacting with transcription factors. One of its functions is to increase the expression of *Fkbp5*, thereby reducing further GR translocation in an ultra-short feedback loop (Grad & Picard 2007).

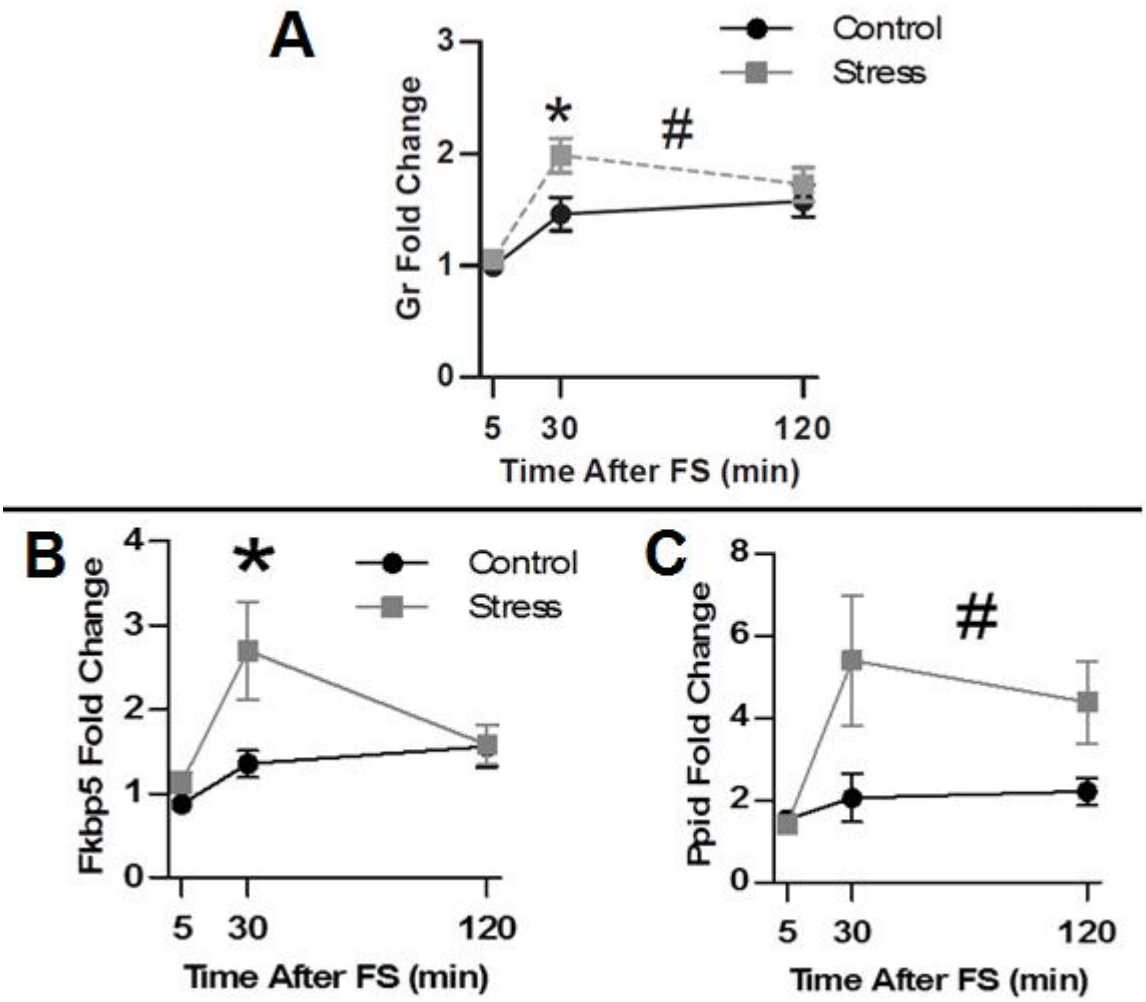

Figure 3: Expression of GR and co-chaperones in the hippocampi of chronically stressed females
Data presented in this figure were not collected during this honors project, and are described here for clarity. These figures aim to show the increased induction of gene expression of *Gr* and its co-chaperones after chronically stressed animals were exposed to a forced swim test that we found in previous studies in our lab. Overall, females with a history of chronic adolescent stress expressed higher amounts of *Gr* 30 minutes after the cessation of the acute stressor (A). Additionally, chronically stressed animals expressed more *Fkbp5* at the same time point (B), and expressed more Ppid as well. # indicates significance due to chronic stress, and * indicates a significant *Bonferroni* post-hoc test ($p<0.05$). These data are summarized in Bourke *et al* (under review).

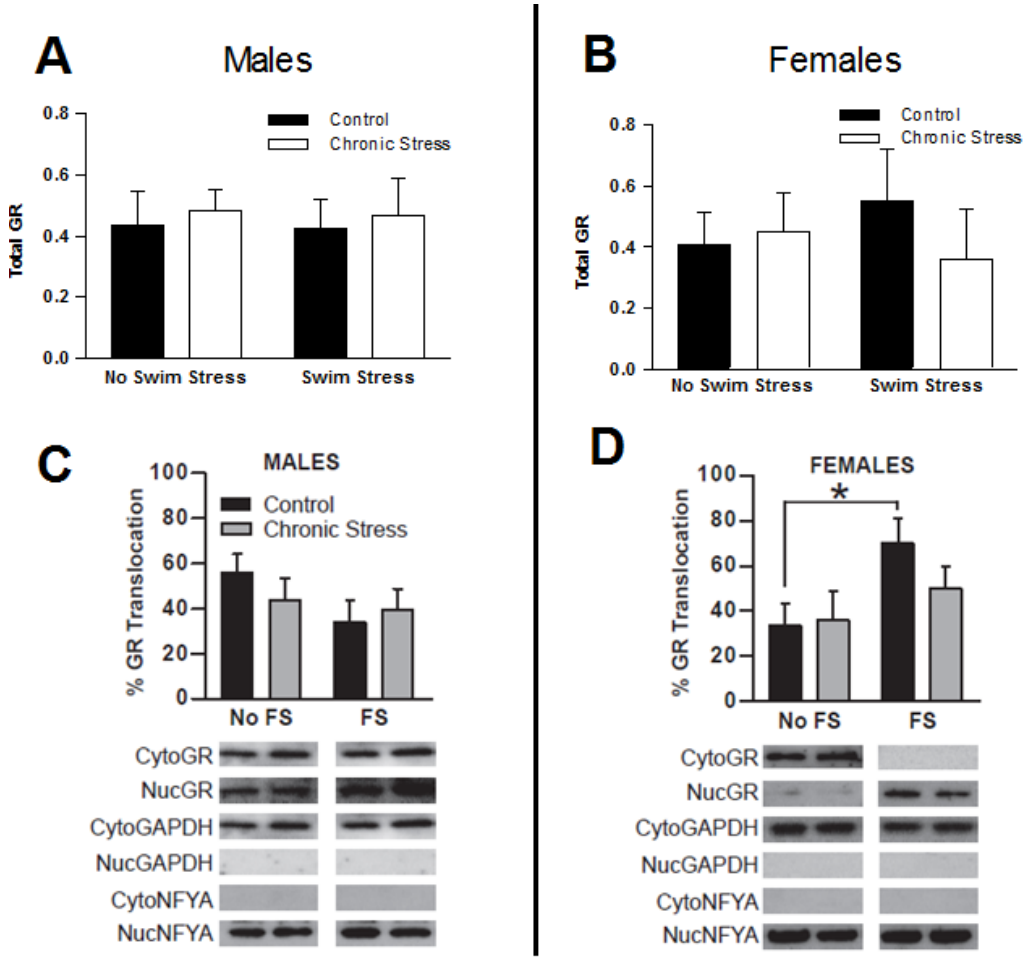

Figure 4: Glucocorticoid receptor protein expression (a,b) and receptor translocation (c,d) (experiment 1). Animals were exposed to a five minute forced swim test to induce GR translocation. Subcellular fractionation of hippocampal tissue and subsequent western blotting were then carried out to determine the percentage of the total GR in the nucleus (amount of GR in the nucleus/ amount of GR in the nucleus+ amount in the cytoplasm). In males, neither acute nor chronic stress altered the total GR content and the GR translocation (a, c). In females, neither acute nor chronic stress altered the total GR content (b). However, females who were exposed to acute stress all exhibited a significant increase in GR translocation relative to baseline (d). This effect was attenuated in females who experienced a history of chronic adolescent stress (d). N=6-7, alpha was set to 0.05, and data are presented as mean+/- SEM.

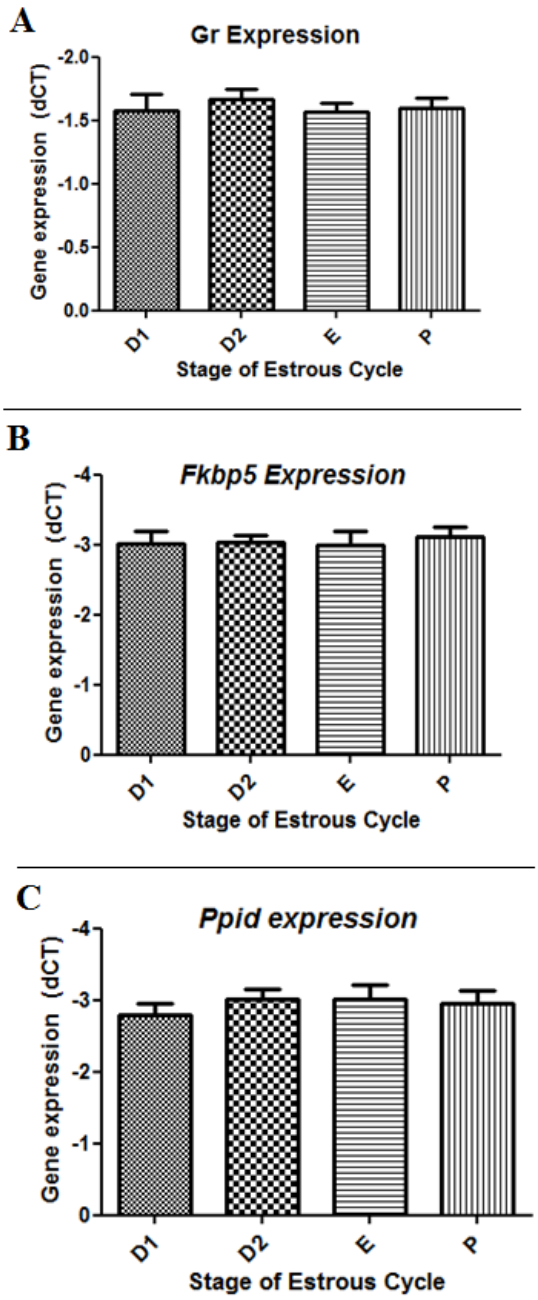

Figure 5: Relationship between stage of estrous and expression of *Gr*, *Fkbp5*, and *Ppid* (Experiment 2). Adult female rats received vaginal lavage for four days prior to decapitation. Serum trunk blood was used in conjunction with vaginal lavage in order to confirm stage of estrous. After decapitation, RT-PCR was conducted to quantify gene expression of these target genes in the hippocampi. Average expression did not significantly vary by stage of estrous ($p>0.05$). For each group, N=4-10, and data are presented as mean +/-SEM.

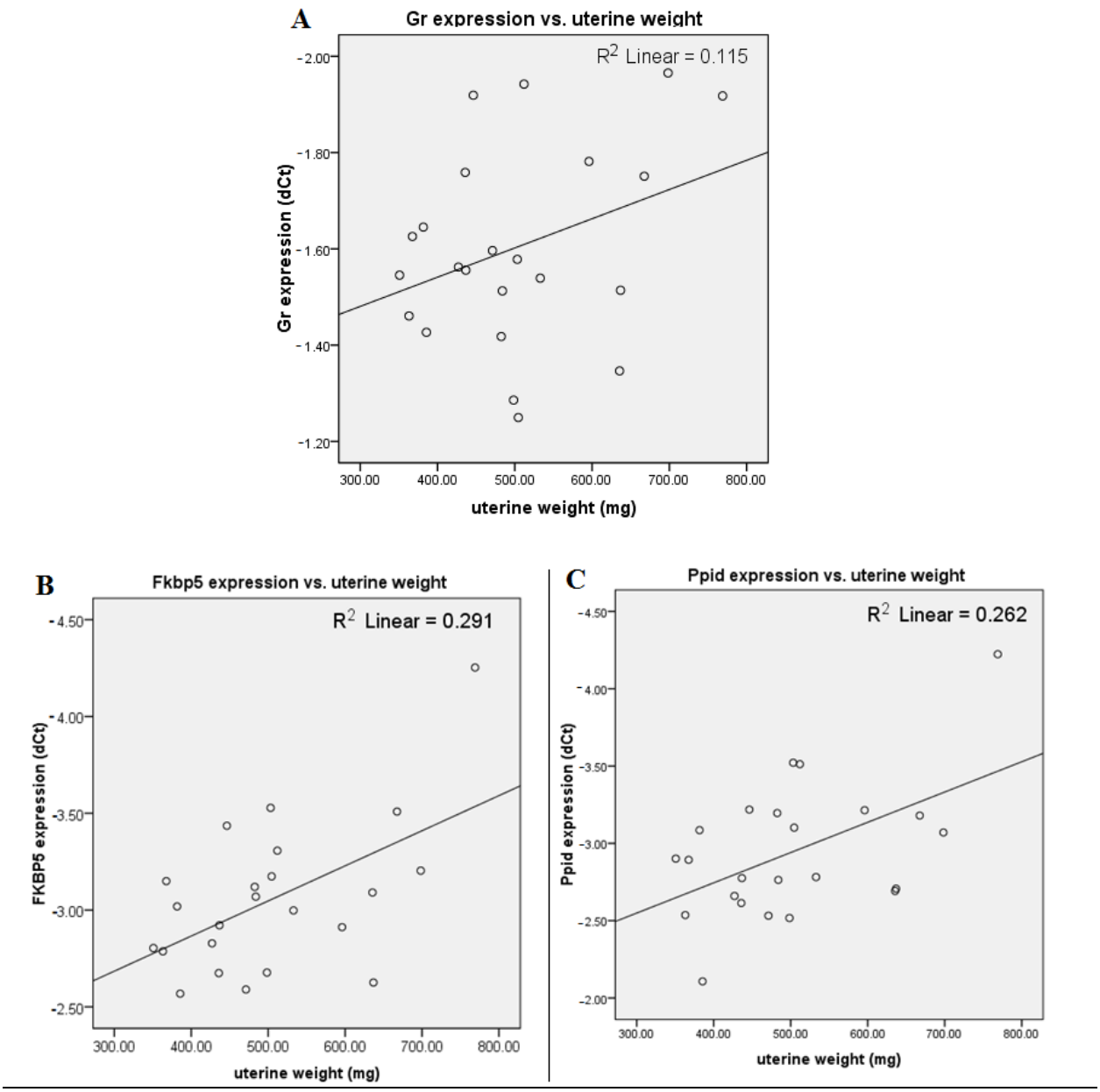

Figure 6: Pearson regression analyses between expression of *Gr*, *Fkbp5*, and *Ppid* vs. uterine weight (experiment 2).Analyses were performed to test whether the dCt value of these genes correlated with uterine weights. Adult female rats in various stages of their estrous cycle were decapitated, their uteri were weighed, and gene expression in the hippocampus was analyzed using RT-PCR. Pearson's correlations were conducted to test whether uterine weight predicted gene expression. Expression of *Gr* was not predicted by uterine weight (A), but expression of *Fkbp5* (B) and *Ppid* (C) correlated positively with uterine weight ($p<0.01$, $p<0.05$, respectively). For this group, N=23, and alpha was set to 0.05.

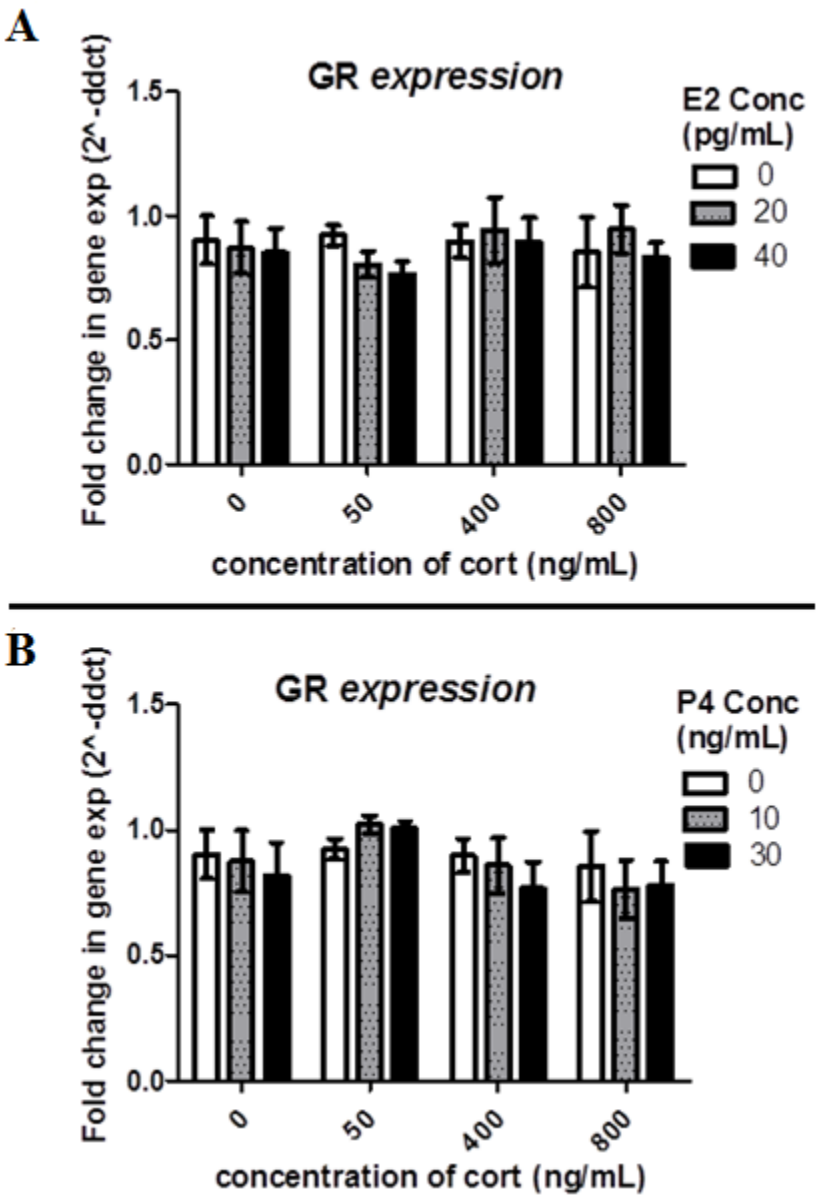

Figure 7: Expression of *Gr* in HT-22 cells after 24-hour exposure to steroid hormone treatments (experiment 3). Two-way ANOVAs were conducted to determine whether cort and E2 (A) or cort and P4 (B) were associated with the expression of *Gr*. Overall, neither of these hormones had a significant effect, and did not interact with expression of *Gr* ($p>0.05$). Data are reported as mean +/-SEM, and N=7-8 for all conditions.

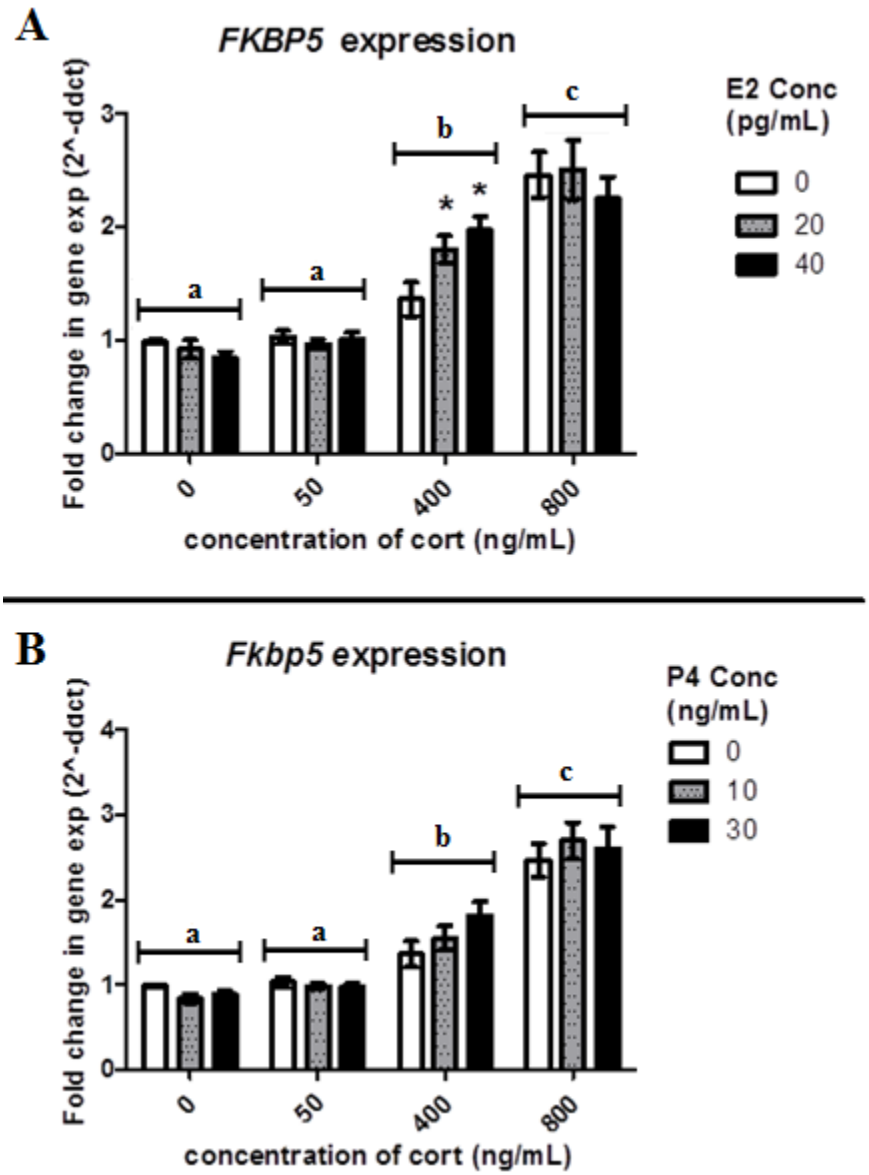

Figure 8: Expression of *Fkbp5* in HT-22 cells after 24-hour exposure to steroid hormone treatments (experiment 3). Two-way ANOVAS were conducted to determine whether cort and E2 (A) or cort and P4 (B) were associated with the expression of *Fkbp5*. Overall, there was a main effect of cort. Concentrations of 400 ng/mL and 800 ng/mL significantly increased the expression of *Fkbp5* compared to concentrations of 0 ng/mL and 50 ng/mL ($p<0.05$; A, B). Additionally, there was an interaction between cort and E2 when cort was held at 400 ng/mL. At this concentration, higher doses of E2 increased *Fkbp5* expression (A). No main effect of E2was found. No main effects or interactions were found for P4 (B). Data are reported as mean +/- SEM, and N=7-8 for all conditions.

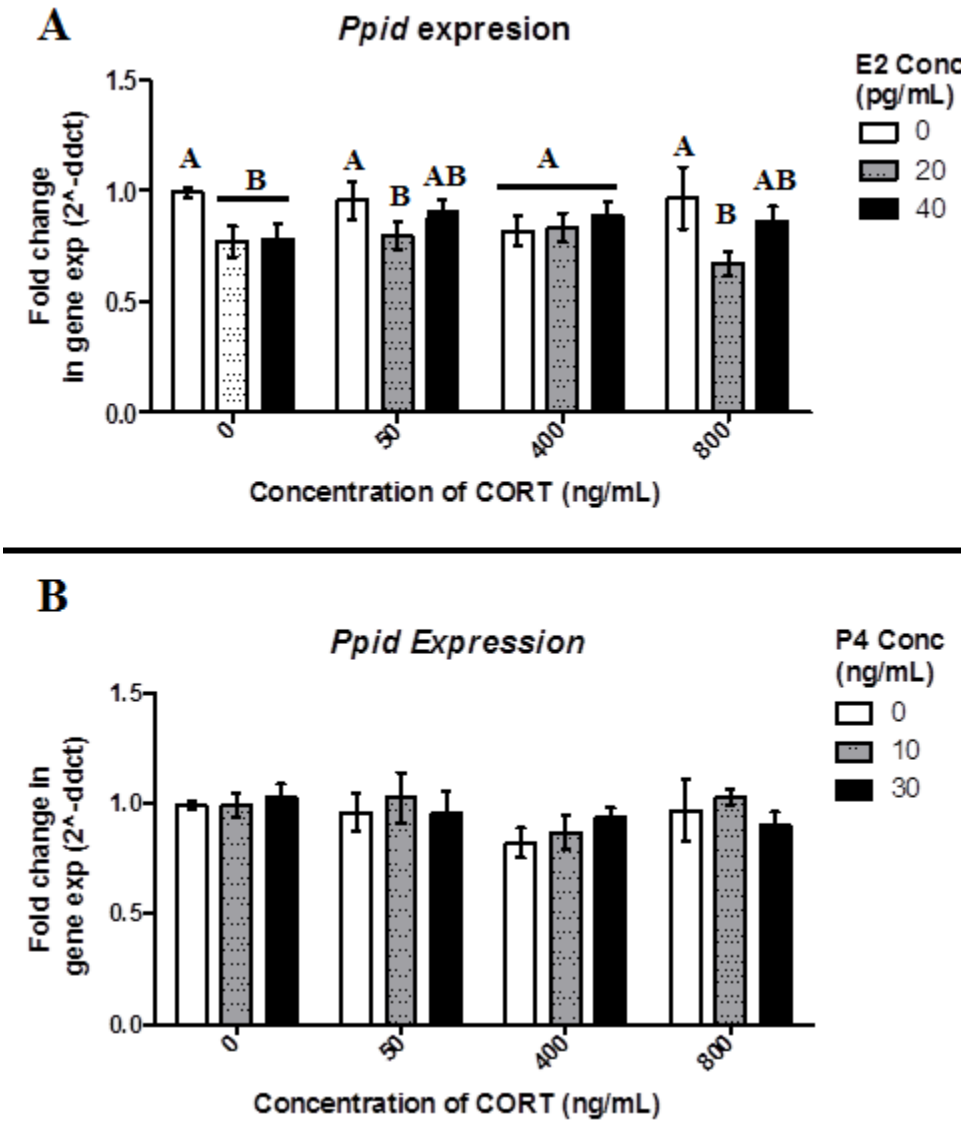

Figure 9: Expression of *Ppid* in HT-22 cells after 24-hour exposure to steroid hormone treatments (experiment 3). Two-way ANOVAs were conducted to determine whether cort and E2 (A) or cort and P4 (B) were associated with the expression of *Ppid.* Overall, there was a main effect of estradiol; a 20 pg/mL concentration of E2 significantly reduced the expression of *Ppid* at baseline, 50 ng/mL and 800 ng/mL of cort (A). There were no main or interaction effects of cort (A, B), nor were there effects of P4 (B). Data are reported as mean +/- SEM, and N=7-8 for all conditions.

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